Editors

Allan Granoff, *St. Jude Children's Research Hospital, Memphis, USA*

Robert G. Webster, *St. Jude Children's Research Hospital, Memphis, USA*

Editorial Advisory Board

Bernard Moss, *NIAID, NIH, LVD, Bldg 4, Room 229, 9000 Rockville Pike, Bethesda, MD 20892-0455, USA*

A Nomoto, *Department of Microbiology, The Institute of Medical Science, The University of Tokyo, 4-6-1, Shirokanedai, Mionato-ku, Tokyo, 108-8639, Japan*

E Norrby, *MTC, Karolinska Institutet, PO Box 280, S-171 77, Stockholm, Sweden*

MBA Oldstone, *Department of Neuropharmacology, Scripps Clinic and Research Foundation, La Jolla, CA 92037, USA*

B Roizman, *Marjorie B Kovler Viral Oncology Laboratories, University of Chicago, Chicago, IL 60637, USA*

Margarita Salas, *Centro de Biologica Molecular (CSIC-VAM), Universidad Autonoma de Madrid, Canto Blanco, 28049 Madrid, Spain*

PK Vogt, *Department of Molecular & Experimental Medicine, SBR-7, The Scripps Research Institute, 10666 N Torrey Pines Road, La Jolla, CA 92037, USA*

RR Wagner, *Department of Microbiology, University of Virginia School of Medicine, Charlottesville, VA 22908, USA*

G Wertz, *University of Alabama School of Medicine, Microbiology-BBRD 17/366, BBRB 17/366, 845 19th Street South, Birmingham, AL 35294, USA*

Milton Zaitlin, *Cornell University, 334 Plant Science Building, Ithaca, New York, 14853-4203, USA*

PREFACE

The *Encyclopedia of Virology*, now in its second edition, continues to assemble the basic and practical aspects of virology in a concise form, providing a rapidly accessible synopsis of each subject for the use of both professional and interested lay readers. The number of subjects (including animal, insect, plant, and bacterial viruses) and the number of illustrations have been significantly increased, and the contents have been revised and updated. Although it is not possible to cover all members of taxonomic families or genera, important examples of each are discussed by acknowledged experts in those fields. Articles that address general subjects related to specific viruses or viral diseases help to synthesize the relevant biomedical and economic issues. Thus, the encyclopedia addresses a large number of topics that are of interest to experimentalists, clinical virologists, and students, as well as to scientists and educators in related fields. The format of the book readily lends itself both to casual reading of articles on general subjects and to use as a primary reference source. As the Foreword to this edition, we are pleased to present two articles that describe 100 years of research on Tobacco Mosaic Virus and Foot and Mouth Disease Virus. Studies of these two viruses have contributed many conceptual and practical advances to the field of virology.

Although the report of the Seventh International Committee on Taxonomy of Viruses (ICTV) will not be published until the summer of 1999, we were provided preliminary information about prospective revisions of the scheme of virus classification. The editing and production deadlines prevented the comprehensive incorporation of these changes into the encyclopedia and we include a table listing the changes in taxa which follows the Table of Contents. This information was provided by Claude M. Fauquet who is the author of the *Taxonomy, Classification and Nomenclature of Viruses* entry and is also a member of our Advisory and ICTV Committees.

To accommodate the anticipated broad readership of the encyclopedia, we have provided a comprehensive Table of Contents that allows readers to locate any topic quickly. First, all entries are listed alphabetically by title. In the titles, common virus names are followed by the taxonomic family of the virus or viruses addressed. When viruses have not been assigned to a family, the genus is specified in the text. After the list of titles is an alphabetized list of general topics, followed by a list of all virus entries alphabetized by taxa.

The encyclopedia is presented in three volumes. Volume 1 covers *Adenoviruses* to *Hepatitis E Virus*, Volume 2 covers *Herpes Simplex Viruses* to *Polyomaviruses*, and Volume 3 covers *Pomoviruses* to *Zoonoses*. A complete Table of Contents appears at the beginning of each volume. When multiple viruses or subjects are addressed in a single entry, each is listed in the Table of Contents. Our aim is to allow the reader to locate quickly any topic that does not have its own separate entry by referring to the Table of Contents or the Index. The Guide to Use of the encyclopedia provides detailed information about locating subjects of interest. We have also included, as an Appendix, the complete list of all virus names from the Seventh ICTV meeting report, kindly provided by Claude M. Fauquet.

The individual articles are written by researchers who are widely acknowledged as experts in their fields. Because each entry is complete in itself, some overlap is inevitable. Redundancy has been minimized where possible, but some repetition is needed to ensure that readers can locate information without repeated reference to the index. Because the size of entries had to be limited, the information supplied is not exhaustive. Our aim was to provide the most relevant information in as concise a manner as possible. Readers should consider each entry an introduction to the specific topic addressed; the updated reading list at the end of each provides ample direction for more detailed study. Further, each entry contains a 'See Also' list that directs readers to related sections in the encyclopedia.

New developments will obviously continue to emerge in the rapidly advancing field of virology, but we expect the fundamental information offered by the encyclopedia to remain valid. Contributors were asked to

project their fields into the future and to anticipate progress that can be expected at the beginning of the new millenium. Our colleagues in the subspecialties of virology have consistently found the *Encyclopedia of Virology* a valuable source of quick information and a useful teaching aid. Our goal has been to maintain and to improve that usefulness.

Allan Granoff
Robert G. Webster

ACKNOWLEDGMENTS

Like the first edition, the second edition of the *Encyclopedia of Virology* represents the work of many people. Its success rests most significantly on the expertise of the individual contributors, who are preeminent in their fields. We thank each of them for their willingness to take part in this endeavor and for the quality of their contributions. Our task as editors was eased enormously by the help of our outstanding editorial advisory board, which identified potential contributors and provided advice, especially in the areas of the insect, plant, and bacterial viruses, as the project progressed. We are pleased to acknowledge Ms. Valerie Audino at St. Jude Children's Research Hospital who provided expert editorial assistance in the production stage of publication. Special thanks are also due to Dr. Martha Howe, who was particularly helpful in identifying prospective authors for the bacteriophage sections and to Dr. Milton Zaitlin for his help in identifying appropriate authors for plant virus entries. We greatly appreciate Dr. Claude M. Fauquet's extensive contribution to the updated taxonomic organization of viruses and to his providing the ICTV's virion icons for use in the book. We also wish to acknowledge Lorraine Parry's contributions in bringing the second edition of the *Encyclopedia of Virology* to completion. As associate editor at Academic Press, she had the considerable task of seeing that this work was published on schedule.

FOREWORD: ONE HUNDRED YEARS OF FOOT-AND-MOUTH DISEASE

Foot-and-mouth disease (FMD) is a highly contagious disease of cattle, goats, pigs and sheep which often leads to great losses in productivity. Consequently, as an understanding of infectious diseases began to emerge during the last two decades of the nineteenth century, stemming from the pioneering work of Pasteur and Koch, FMD became the subject of intensive study in Germany because of the many outbreaks that were occurring there. The problem was so great that it led to the offer in 1893 by the Prussian Ministry of Agriculture of a prize of 3000 Reichmarks for the person who "identified and, if possible, isolated the contagious matter causing foot-and-mouth disease and demonstrated its effectiveness by means of decisive experiments on animals". Ten applicants worked unsuccessfully on the problem until 1895. A year later a motion was submitted to the Reichstag "to request the Imperial Chancellor to take account of the need for the immediate establishment of experimental institutions for the thorough investigation of FMD on behalf of the Empire and in the individual Federal States". This led to the allotment of 55,000 Reichmarks and to the establishment of a commission for research into the disease. Loeffler, who was Professor in the Institute of Hygiene at the University of Greifswald, started experiments on the disease in 1897, working with Frösch in Berlin, and in their third Report to the Prussian Minister of Culture in 1898 they described their landmark studies which established the causal agent as a virus. This was an historic event because it was the first demonstration that an animal disease could be caused by a virus and, remarkably, was made in the same year as Beijerink's description of the nature of tobacco mosaic virus. Loeffler continued the work at Greifswald but the highly contagious nature of the disease resulted in pressure from farmers in the area to stop the studies until they could be done in a safe place. This led eventually to the move in 1909 to Insel Riems, a small island in the Baltic Sea just north of Greifswald.

Clearly vaccination against the disease was the major target and methods to provide effective vaccines dominated research for the first 60 years of this century. One of the problems was the expense and difficulties associated with working with the natural hosts and led to the search for a susceptible laboratory animal. This search did not succeed until 1920 when Waldmann and Pape produced the disease in guinea pigs. This animal has been particularly useful in assessing the potency of experimental vaccines because the immunized animal can be challenged for its ability to resist infection. It was not until Skinner's work in 1951 that the suckling baby mouse was added to the repertoire of experimental animals that could be used for FMD research. The baby mouse was used extensively for the titration of the virus in the days before tissue culture cells became generally available. Moreover, it was the species used extensively by Skinner in attempts to produce strains of the virus which would be useful as attenuated vaccines.

A major step in the study of the disease and its control by vaccination took place in the early 1920s when Vallée and Carré in France and Waldmann in Germany showed that the virus occurs as multiple serotypes. The two groups discovered three serotypes now known as O, A and C. The nomenclature caused some confusion until it was resolved by discussion at the Office International des Epizooties in Paris. Vallée and Carré had first recognized the occurrence of two serotypes and named them after their place of origin, O for the Department of Oise in France and A for Allemagne. Their observation was confirmed by Waldmann and Trautwein in Germany who called them A and B. The latter group then discovered a third serotype which they called C. It was eventually decided to name them Vallée O, Vallée A and Waldmann C, which have now been reduced to O, A and C. The antigenic differences were such that an animal that had been infected with a virus of one serotype was still susceptible to the other serotypes. Clearly this was to have considerable importance in the control of the disease by vaccination and the problem was exacerbated by the demonstration of sub-types

within the major serotypes in the late 1920s, a problem which can cause havoc in the control of the disease because antigenic variants continue to emerge.

To add to the problem, four further serotypes were identified later by the Pirbright triumvirate of Galloway, Brooksby and Henderson, three in the Southern African Territories (SAT1, SAT2 and SAT3) in the 1940s and Asia 1 in 1954. Interestingly, no other major serotypes have been identified in the past 45 years. Nevertheless the wide antigenic spectrum within each serotype and the new variants which are constantly emerging means that vaccines have to correspond closely to these emerging variants if they are to be effective in controlling outbreaks. Many devastating outbreaks in vaccinated animals provide evidence of the importance of this issue.

Consequently the composition of vaccines remains important in FMD. The first experimental vaccines were prepared in 1925, using formaldehyde-inactivated vesicular fluid from infected calves. However, large-scale field trials did not take place until 1948 when Rosenbusch and his colleagues successfully vaccinated more than two million cattle in Argentina. It was clear, however, that the necessary large-scale production of the virus would require its *in vitro* cultivation. Work in the early 1930s by Hecke in Germany and the Maitlands in England had shown that this could be achieved. It was Frenkel who showed in 1947 that *in vitro* cultivation of the virus on a large scale could be achieved by using surviving bovine tongue epithelial fragments. This was a major step in the provision of vaccines for the control of the disease and it was Holland, where Frenkel worked, which introduced the first comprehensive vaccination programme. Germany and France soon followed and these vaccination programmes have led to the eradication of the disease from Western Europe.

There have been minor hiccups on the way. One of the problems was the uncertainty surrounding the use of formaldehyde as the inactivant. This reagent had been used by Glenny and Ramon to detoxify diphtheria and tetanus toxins and it was probably the influence of Ramon on the French workers Vallée, Carré and Rinjard which persuaded them to use formaldehyde in their experimental vaccine studies in 1925. However, the suspicion that formaldehyde-inactivated vaccines were not innocuous, first expressed by Moosbrugger, led to studies with alternative reagents. From these studies, first N-acetylethyleneimine and then ethyleneimine were introduced and the latter compound is now used exclusively for the preparation of FMD vaccines.

The comprehensive vaccination programmes introduced have gradually led to the control of the disease and its elimination from many countries. Western Europe and several countries in South America which formerly had tens of thousands of outbreaks each year are now free from the disease.

The development of molecular techniques during the past 50 years has enabled us to study the virus and its nucleic acid and proteins in great detail. The structure of the virus at a resolution of 2.8 Å has been described and its mode of replication is known in the most intimate detail. From these fundamental studies have emerged two important practical issues. The first is the demonstration that protective immunity can be achieved by the inoculation of a 20-mer peptide corresponding to the highly immunogenic epitope located on the surface of the virus particle. Moreover, the important antigenic variation of the virus is almost certainly related to the amino acid sequence in the surface epitope. The second practical issue is the ability to identify animals that have been infected with the virus. Many of these animals become persistently infected for as long as three years and are consequently potential sources of further outbreaks of the disease. These animals can be identified by the presence in their sera of antibodies against non-structural proteins which are not present in naive or vaccinated animals. This information has emerged from the finding that certain of the non-structural proteins are not secreted from the cells in which the virus is grown and are consequently not included in the medium from which the vaccine is prepared.

Consequently, eradication of the disease could be accomplished worldwide because the scientific knowledge and means are available. It would be an appropriate ending to a century of FMD research if this goal were placed high on the agenda of the Food and Agricultural Organisation.

Fred Brown
Department of Agriculture,
Agricultural Research Service,
Greenport, New York 11944-0848, USA.

FOREWORD: TMV – 100 YEARS OF CONTRIBUTIONS TO VIROLOGY

Tobacco mosaic virus (TMV) has played a prominent role in the development of the concept of viruses as pathological agents and in unraveling the composition and structure of these unique agents. It also is the virus with which many concepts and phenomena unique to plant virology have been discovered. The mosaic disease which afflicted tobacco was known in Europe since tobacco was introduced there in the 17th century. It causes light and dark green areas on infected plants, accompanied by considerable stunting and loss of yield. The first person to transmit it experimentally was Adolph Mayer who in 1886 reported that when he took juice from diseased plants, it was infectious to healthy plants; he also gave the disease its name. The capacity to transmit the disease experimentally facilitated the studies which ultimately resulted in depicting the new class of infectious agents, the viruses.

Attribution for the discovery of the virus concept is in dispute, ascribed by some to Dmitrii Ivanowski in 1892, but by others, to Martinus Beijerinck in 1898. The basis for the dispute lies in the interpretation of the results both workers obtained when tobacco sap was passed through a porcelain, bacteria-retaining filter; the agent had retained its infectivity. Ivanowski found it difficult to accept that the agent was something new, even after he was aware of Beijerinck's conclusions. Ivanowski was concerned that the filter might have had a crack, or that some small spore of the bacterial causal organism had passed through. Beijerinck, on the other hand (1898) concluded that he had a unique pathogen, which he termed a *'contagium vivum fluidum'* – a contagious living fluid. Animal virologists will also argue that early work on foot-and-mouth disease also should be considered for attribution for the virus concept (see foreword by Fred Brown in this volume).

In the years before the agent was purified and characterized, several findings were made using TMV that had important ramifications for plant virology. In 1929, McKinney discovered the phenomenon of 'cross protection', i.e., that infection of a plant with a mild strain of a virus would protect the plant from disease when inoculated subsequently with a severe form of that virus, but would not protect it against unrelated viruses. This phenomenon was used for many years to determine virus relationships, and in a few cases it was used to protect crops against disease. Additionally, in 1929 Holmes developed a quantitative bioassay for the virus using host plants that developed necrotic local lesions in response to infection, thereby potentiating experiments where virus quantification was important.

Prior to the ultimate isolation of the infectious agent in the 1930s, a number of studies gave clues to its nature and pointed the direction for its isolation. By injecting sap from infected plants into rabbits, Purdy (1929) demonstrated that the agent was immunogenic; others found that the infectious agent could be precipitated from tobacco sap with protein precipitants, both findings hinting at a proteinaceous agent. Further, birefringence experiments suggested it was elongate. Purification was being actively pursued in the United States by several groups, and by scientists in Australia and England. Wendell Stanley, then at the Rockefeller Institute in Princeton, New Jersey, was the first to publish that he had purified the virus (1935), and he received the principal recognition, most notably the Nobel Prize in 1946. However, he incorrectly thought the virus to be composed of only protein, missing the fact that it contained both phosphorus and carbohydrate. Bawden and Pirie, who were one step behind Stanley in the purification, set the record straight in 1936, by showing that the virus contained RNA.

TMV was the first virus seen in the electron microscope (1939) and in 1941 it was the first to have its structure revealed by X-ray crystallographic analysis. Further investigations in the 1950s revealed the position of the RNA in the particle, and defined its structure.

The 1950s saw an intense rivalry between workers in Tübingen, Germany (Schramm, Melchers, Gierer, Wittmann and others) and the group in Berkeley directed by Fraenkel-Conrat at the Virus Laboratory established by Wendell Stanley. Many of the findings outlined here were made almost simultaneously in both laboratories, although partisans might not agree with such attribution. The work was greatly facilitated because TMV, unlike any other virus known at the time, was easy to purify, and because of the large quantities which could be obtained. (In my own laboratory we isolated 60 grams of highly purified virus for one of our studies.) Also, TMV – at least the common 'strain' – is very stable, and will maintain its infectivity for decades at refrigerated temperatures if a little chloroform is added to inhibit growth of microorganisms.

In the 1950s both groups discovered that the RNA of the virus was infectious, that the virus could be reconstituted from isolated coat protein and RNA, determined the sequence of amino acids in the coat protein, and found that chemical agents could mutate the virus. The latter studies were particularly meaningful in the confirmation of the universality of the genetic code, in that directed mutational changes induced in the RNA led to predictable changes in the viral coat protein. Additionally, mixed reconstitution experiments, utilizing proteins from one strain and RNA from another, demonstrated conclusively that the RNA was indeed the genetic material of the virus, as some had questioned that the infectivity of 'naked' RNA was really a reflection of a small amount of a protein contaminant. These studies demonstrated conclusively that the coat protein was coded for by the RNA component of the mixed reconstituted virus.

In 1969 Takebe and Otsuki published a paper which revolutionized plant virology when they demonstrated that cell wall-free protoplasts isolated from tobacco leaves could be infected by TMV and that TMV would replicate in those cells. Thus, plant virologists now had a system to enable single cycle analysis of virus replication, previously not possible in inoculated leaf tissues involving only a few initial infections, followed by subsequent sequential replication in surrounding layers of cells.

In 1970 my colleague V. Hari and I ascertained that TMV-infected tissues contained at least four viral proteins. Subsequent events showed these to be the 126-kDa and 183-kDa proteins of the replicase complex, the 30-kDa movement protein, and the 17.6-kDa coat protein. There is also an open reading frame for a 54-kDa protein within the read-through portion of the 126-kDa replicase protein, coincident with sequences in the 183-kDa protein. This protein has not been detected in diseased tissues, however.

Plant viruses are now known to potentiate their passage from cell-to-cell in their hosts. The 30-kDa protein of TMV was shown to be involved in this process, deduced from studies with a temperature sensitive mutant of the related tomato mosaic virus, which was defective in cell-to-cell movement. In 1987 the movement protein was shown to modify the size exclusion limit of plant plasmodesmata to allow for cell-to-cell movement, by some elegant microinjection studies from the laboratories of William Lucas and Roger Beachy. Movement proteins have now been found in most plant viruses in which they have been sought, and have been shown to have RNA binding properties.

The concept that translation of some proteins in many RNA viruses is controlled by the production of subgenomic mRNAs was first demonstrated with TMV. Virus-infected tissues were shown to contain small, virus related RNAs, and *in vitro* translation demonstrated their mRNA capacities.

Other phenomena of particular significance to plant virology were the detailed analysis of how the virus assembles *in vivo*, and the phenomenon of co-translational disassembly in which it was demonstrated that cytoplasmic ribosomes remove the coat protein from the virion, thus exposing the RNA, which is also being translated during the process to synthesize the replicase subunits. TMV was the first plant virus to be completely sequenced (1982), and was the first virus to be shown to have at least one ubiquitinated subunit in the virion. In 1996, Barbara Baker reported the first isolation of a plant gene conferring resistance to a virus. This was the *N gene*, utilized in the aforementioned bioassay developed by Holmes in 1929.

The important discoveries in which transformation of plants with viral sequences to induce resistance or tolerance to disease were all pioneered with TMV. The widely-adopted coat protein-mediated protection concept was developed in 1983–84 in the laboratory of Roger Beachy, then at Washington University, in collaboration with scientists at the Monsanto Company who had developed technology for plant transformation. They found that the gene for the TMV coat protein, when transformed into tobacco plants gave significant delay of symptom development, and in some cases led to complete resistance. This concept has now been widely applied to at least 30 different viruses, representing at least 15 genera. Squash plants have been marketed which are resistant to several viruses, and currently there are plantings of papaya trees in Hawaii resistant to the devastating papaya ringspot virus.

The use of replicase genes for resistance was also first shown with TMV in my laboratory in work started in 1988. We found that a portion of the 183-kDa replicase gene gave near immunity to TMV disease. This

phenomenon, known as 'replicase-mediated resistance' has also found application with a number of other viruses in other genera. Potato plants with the replicase gene of potato leaf roll virus are in commercial production.

TMV movement protein was also the first to be used in plant transformation experiments, seeking resistance. The laboratories of Beachy, Allan Dodds, and Josef Atabekov were pioneers in this technology. Interestingly, transformation with a defective movement protein from TMV was able to provide some measure of resistance to TMV, other tobamoviruses, and to several unrelated viruses.

A recently-published anthology on TMV contains reprints of the original papers with commentaries on the discoveries described above (Scholthof, K-B.G., Shaw, J.G., and M. Zaitlin (1999) *Tobacco Mosaic Virus: One Hundred Years of Contributions to Virology*. American Phytopathological Society Press, St. Paul, Minnesota, USA).

Milton Zaitlin
Department of Plant Pathology,
Cornell University, Ithaca, New York 14853, USA

Notes on the Subject Index

Entries

1. Bacteriophages are listed after the main index entry 'Bacteriophage', unless there are cross-references to other locations. ϕ is alphabetized as 'phi'. ψ is alphabetized as 'psi'.
2. Readers are advised to seek not only the genus, but also the group name e.g. *Baculovirus* and *Baculoviruses*.
3. US spelling is used.

Abbreviations

Abbreviations used in index subentries are as follows:

CMV	Cytomegalovirus
EBV	Epstein-Barr virus
FIV	Feline immunodeficiency virus
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HSV	Herpes simplex virus
IRES	Internal ribosome entry site
LCMV	Lymphocytic choriomeningitis virus
LDV	Lactate dehydrogenase-elevating virus
ORF	Open reading frame
RSV	Respiratory syncytial virus
SIV	Simian immunodeficiency virus
VSV	Vesicular stomatitis virus
VZV	Varicella zoster virus

sion over more than 40 weeks. It was demonstrated that, following liver gene transfer, transcriptional initiation was only from the liver-specific promoter, and that splicing of the primary transcript and processing of RNA and protein were normal. Gene transfer with increasing doses of this vector resulted in very high and stable α_1-antitrypsin serum levels that so far are unparalleled by gene transfer with any other tested combination of strong viral promoters and the α_1-antitrypsin cDNA. Significantly, the high serum α_1-antitrypsin levels that would be considered supraphysiological in humans were not accompanied by any significant hepatotoxicity. Gene transfer with the same dose of a first-generation vector carrying an α_1-antitrypsin expression cassette led to hepatic damage that was morphologically characterized by liver cell necroses and infiltration by granulocytes and was accompanied by significantly elevated liver enzymes in the serum. Improved expression and decreased toxicity with high-capacity adenoviral vectors were confirmed with another vector that carried the murine leptin cDNA under the control of a viral promoter. Taken together, these results indicate that high-capacity adenoviral vectors will be useful for functional studies on gene transfer and likely also for somatic gene therapy because of their improved safety and expression profiles. Their increased capacity for foreign DNA is a significant advantage over first and second generation adenoviral vectors.

See also: **Adenoviruses (*Adenoviridae*): General features; Latency; Persistent viral infection; Vectors: Animal viruses.**

Further Reading

Doerfler W (1991) Abortive infection and malignant transformation by adenoviruses: integration of viral DNA and control of viral gene expression by specific patterns of DNA methylation. *Adv. Virus Res.* 39: 89.

Doerfler W (1996) A new concept in (adenoviral) oncogenesis: integration of foreign DNA and its consequences. *BBA Rev. Cancer* 1288: F79.

Doerfler W and Böhm P (eds) (1995) The molecular repertoire of adenoviruses. *Curr. Top. Microbiol. Immunol.* 199/I–III.

Lucher LA (1995) Abortive adenovirus infection and host range determinants. *Curr. Top. Microbiol. Immunol.* 199/I: 119.

Schiedner G, Morral N, Parks RJ *et al* (1998) Genomic DNA transfer with a high-capacity adenovirus vector results in improved *in vivo* gene expression and decreased toxicity. *Nature Genet.* 18: 180.

Schröer J, Hölker I and Doerfler W (1997) Adenovirus type 12 DNA firmly associates with mammalian chromosomes early after virus infection or after DNA transfer by the addition of DNA to the cell culture medium. *J. Virol.* 71: 7923.

van der Vliet PC (1995) Adenovirus DNA replication. *Curr. Top. Microbiol. Immunol.* 199/II: 1.

Animal Viruses

William C Russell, School of Biomedical Sciences, University of St Andrews, St Andrews, Fife, Scotland

Mária Benkö, Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary

History

Members of the adenovirus family (*Adenoviridae*) can be recognized in virtually every class of vertebrate animals (**Table 1**). However, not all of the family members contain the same common antigen and initially two genera have been established each of them being characterized by a common (or group) antigen. The genus *Mastadenovirus* comprises viruses isolated from mammals (humans, monkey, cattle, pig, sheep, horse, dog, goat, tree shrew, deer, whale, mouse, etc.) whereas the *Aviadenovirus* genus contains viruses isolated from avian species (chicken, turkey, goose, pheasant, duck, etc.). The organization of the genome, though normally rather conserved within a genus, differs considerably between the two genera. Recently, strong evidence (based on genomic organization and phylogenetic analysis; **Fig. 1**) supported the establishment of a third genus incorporating several bovine adenoviruses (BAVs), the ovine adenovirus isolate 287 (OAV287) and the egg drop syndrome (EDS) virus which (lacking the genus-specific common antigens) had been recorded as 'atypical' members of the *Mastadenovirus* or *Aviadenovirus* genera. The proposed name of this possible third genus is *Atadenovirus* (referring to the characteristic AT-rich genome rather than the host animal taxon). The candidate BAV serotypes (4 to 8) of this proposed genus do not fit clearly into the *Mastadenovirus* genus and are presently separated as subgroup 2 from the subgroup 1 BAVs which obviously belong to the mastadenoviruses. A number of adenoviruses isolated from (or only detected in) lower vertebrate hosts (fish, frog, snake, chameleon, crocodile, etc.)

Table 1 Adenovirus infections detected in animal species

	Types	*Affected organs*	*Evidence*	*DNA sequence*
Fish				
Cod	?	Epidermis	EM	–
Dab	?	Epidermis	EM	–
Amphibians				
Leopard frog	frog 1	Kidney	Isolated	Partial
Common frog	?	Liver	Isolated	–
Reptiles				
Corn snake	?	Liver, kidney, spleen	Isolated	?
Royal python	?	?	Isolated	–
Boa constrictor	?	Liver	Isolated	–
Jackson's chameleon	?	Trachea, esophagus	EM	–
Nile crocodile	?	Liver, intestine	EM	–
Birds				
Chicken	FAV-1	Egg (CELO)	Isolated	Full
	FAV-2–12	Trachea, liver	Isolated	Partial (3)
Quail	FAV-1	Bronchial epithelium	Isolated	–
Guinea fowl	FAV-1	Lung, pancreas, spleen	Isolated	–
Pigeon	FAV-2	Liver, intestine	Isolated	–
Crow	?	Healthy	Serology	–
Goose	Goose 1–3	Liver, intestine	Isolated	–
Turkey	turkey 1–3	Respiratory	Isolated	–
	HEV	Intestine, spleen, kidney	Isolated	Partial
Pheasant	MSDV	Spleen	Isolated	–
Duck	duck 1	Egg (EDS)	Isolated	Full
Muscovy duck	duck 2	Liver	Isolated	–
Wild duck, coot, grebe	duck 1	Healthy	Serology	–
Herring gull	?	Bursa of Fabricius	EM	–
Common murre	?	Kidney	EM	–
Cockatiel	?	Liver	EM	–
Rose-ringed parakeet	?	Bronchial epithelium	EM	–
Amazon parrot		Liver, intestine	EM	–
Emu	?	Intestine	EM	–
Ostrich	FAVs	Trachea, lung, intestine, pancreas, kidney	Isolated	–
American kestrel	?	Liver	EM	–
Mammals				
Platypus	?	?	EM	–
Brush tail possum	?	Intestine	EM, serology	–
Mouse	MAV-1–2	Lung, intestine, kidney	Isolated	Partial
Wild meadow vole	?	?	Serology	–
Syrian hamster	MAV-1?	Intestine, liver	EM, serology	–
Ground squirrel	?	Kidney	Isolated	–
Guinea pig	?	Lung	EM, PCR	Partial
Rabbit	?	Intestine	Isolated	–
Dog	CAV-1	Liver, CNS	Isolated	Full
	CAV-2	Resp	Isolated	Full
Fox	CAV-1	CNS	Isolated	–
Black bear	CAV-1	Kidney, liver	Isolated	–
Black panther	?	Liver	LM	–
Cat	?	Resp, intestine	Serology, PCR	Partial
California sea lion	CAV?	Liver	EM	–

Table 1 Continued

	Types	*Affected organs*	*Evidence*	*DNA sequence*
Sei whale	?	Intestine	Isolated	–
Horse	EAV-1–2	Resp, intestine	Isolated	Partial (2)
Swine	PAV-1–5	Lung, intestine, CNS	Isolated	Partial (5)
Cattle	BAV-1–10	Lung, intestine	Isolated	Partial (8)
Sheep	OAV-1–6	Liver, intestine, kidney,	Isolated	Partial (2)
	BAV-2	Resp	Isolated	
	OAV287		Isolated	Full
Goat	caprine 1–2	Intestine, CNS	Isolated	–
Llama	?	Lung, liver	Isolated	–
Red deer	?	Lung	EM	–
Fallow deer	BAV-6	Lung	Isolated	–
Mule deer	BAV?	Lung, blood vessels	Isolated	–
African buffalo	BAVs	Healthy	Isolated	–
Caribou	BAVs	Healthy	Serology	–
Tupaia (tree shrew)	TAV-1–2	Resp, intestine, kidney	Isolated	Partial
Monkeys (African green, baboon, macaque, owl, rhesus, squirrel, vervet)	SAV1–20	Conjunctiva, resp, lung, intestine, pancreas, kidney	Isolated	Partial (12)
Chimpanzee	SAV-21–27	Resp, intestine, kidney	Isolated	Partial (6)

Abbreviations. BAV, bovine adenovirus; CAV, canine adenovirus; CELO, chicken embryo lethal orphan; EAV, equine adenovirus; EDS, egg drop syndrome; FAV, fowl adenovirus; HEV, hemorrhagic enteritis virus; MAV, murine adenovirus; MSDV, marble spleen disease virus; OAV, ovine adenovirus; OAV287, OAV isolate 287; PAV, porcine adenovirus; SAV, simian adenovirus; TAV, tupaia adenovirus; CNS, central nervous system; intestine, gastrointestinal tract; resp, respiratory organs; EM, electron microscopy; LM, light microscopy; PCR, polymerase chain reaction; partial, partial sequences (can be very short; in parentheses the number of sequenced serotypes); full, the complete genome is sequenced.

have as yet no genus attribution – mainly because of the lack of comparative data.

Properties of the Virion

The virion has an icosahedral capsid of diameter 75–90 nm and a core containing the viral DNA and basic proteins. The capsid has 252 capsomers; 240 of them are at the faces and on the edges of the icosahedral capsid, surrounded by six others and hence called hexons. The remaining 12 capsomers are located at the apices and, being on the axes of fivefold symmetry, are surrounded by five hexons and termed pentons. The proximal hexons are further defined as peripentonal hexons. These peripentonal hexons and pentons can be detached from the remainder of the capsid by a variety of treatments and it appears that the remaining 180 hexons are cemented together in 20 groups of nine via another structural component, polypeptide IX in mastadenoviruses. However, in the aviadenoviruses and in the members of the proposed third genus, protein IX is missing. The pentons are characteristic of the *Adenoviridae* and have a dumbbell shape with a (penton) base and a protruding knobbed fiber. The fiber is of variable length, the largest so far described being visualized on an equine adenovirus of length 50 nm. Several bovine adenoviruses also have considerably longer fibers than human adenoviruses and sometimes these are bent. Most of the aviadenoviruses have two fibers normally of very different sizes (varying from 8.5 to 47 nm) attached to the same penton base. High-resolution x-ray crystallography of the hexon and image reconstruction from cryoelectron micrographs of human adenoviruses have provided a detailed understanding of virion structure and all studies of animal adenovirus so far completed have indicated that they will in general conform to these same structural parameters. Thus it has been demonstrated that avian adenoviruses possess a terminal protein covalently linked to the 5′ termini of the virus DNA and that animal virus hexons possess a similar spectrum of type, subgroup and group specificity already noted in the human viruses. In the studied members of the *Aviadenovirus* and the proposed third genus, however, besides the above-mentioned protein IX, a structural component protein V is also missing. Moreover, in the OAV287 and in the EDS virus (candidate members of the third

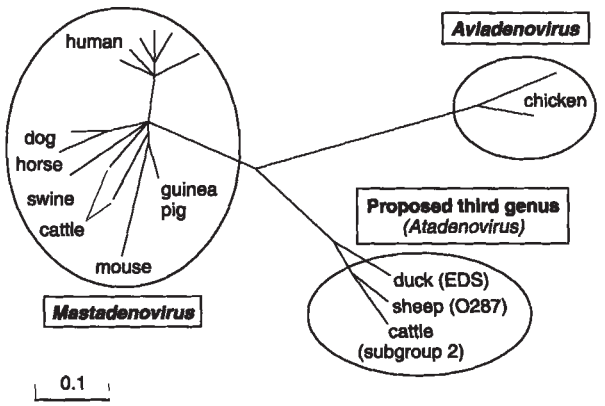


Figure 1 Genetic distance between adenoviruses isolated from different animal species resulting from the phylogenetic analysis of 23 known hexon gene amino acid sequences. The unrooted phylogenetic tree was generated by distance matrix analysis: PROTDIST and FITCH of the PHYLIP v3.5c program package of J. Felsenstein (*http://evolution.genetics.washington.edu/phylip.html*). The length of the branches indicates the phylogenetic distance between the different viruses, the scale bar represents 10% mutations. The virus types composing the two existing and third (proposed) genera are circled. The original sequence alignment and the alignment edited for the calculations are available at *http://www.vmri.hu/~harrach*. From the hexon gene of the guinea-pig adenovirus only a short sequence was available and therefore only preliminary conclusions could be drawn for this virus type. The tree was visualized using the TreeView program of R. Page (*http://taxonomy.zoology.gla.ac.uk/rod/treeview.html*) and the Windows Metafile was edited in Microsoft Word 7.0 for demonstrating the discussed new (three cluster) grouping of the adenoviruses of different hosts. Similar clustering was obtained by the analysis of protease gene (either amino acid or nucleic acid) sequences.

genus), the occurrence of new, homologous open reading frames (ORFs) encoding putative structural proteins termed 28K or 24K, respectively, were observed. In OAV287, the presence of the 28K protein in the virion was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

Properties of the Genome

Adenoviruses have a linear double-stranded DNA genome with inverted terminal repeats (ITRs) of varying length and all animal adenoviruses studied have conserved this feature. Aviadenoviruses appear to have about 17% of their total weight as DNA and a molecular weight of $28–30 \times 10^6$ which is slightly greater than that of human adenoviruses. Compared to the long genome of aviadenoviruses exceeding 44 kb, mastadenoviruses have a genome length of 31–36 kb, and the DNA of the members of the proposed third genus is shorter in the range 28–33 kb. There is very limited base homology between the genomes of viruses of the different genera. Within a genus however, as a rule, more significant base homologies have been detected among the DNAs of different serotypes of the same host species. Cattle, sheep and chicken seem to be exceptions in this respect, since these animal species can apparently be infected with unrelated adenoviruses from different genera (no DNA homology could be detected by DNA hybridization between bovine adenoviruses belonging to the *Mastadenovirus* genus or to the proposed new genus).

Besides the four entirely sequenced human adenoviruses (types 2, 5, 12 and 40), there are five animal adenoviruses with completely sequenced genomes: canine adenovirus types 1 and 2 (mastadenoviruses), fowl adenovirus 1 (aviadenovirus), and OAV287 and EDS virus (proposed third genus). Many further partial sequences are available (or to be published soon) from different hosts, such as ovine adenovirus type 3, porcine adenovirus types 1 to 5, BAV-1, 2, 3 and 10, mouse adenovirus type 1, equine adenovirus types 1 and 2, tree shrew (tupaia) adenovirus, fowl adenovirus types 8 and 10, turkey hemorrhagic enteritis virus, and BAV-4, 6, 7 (candidate members of the proposed new genus) etc. (see **Table 1**).

The data in hand in relation to regulatory events and the sequence data available for the animal adenoviruses suggest that genome organization of the mastadenoviruses should be generally similar to that already discerned for the human adenoviruses. The only real differences between the members of the *Mastadenovirus* genus can be seen in the content of the E3 region, which has a different number and entity of genes even in the different types of human adenoviruses. To date, the shortest E3 region was found in the murine adenovirus (MAV) type 1 and BAV-10 (only one but a different gene in each), whereas the longest was described in the subgenus B human adenoviruses (eight E3 genes in type 35, and possibly nine in type 3). The aviadenoviruses and the members of the proposed new genus, however, have strikingly different genomic organization especially at the two ends of their genomes mainly concerning the early regions. Although the genes of the structural proteins (apart from the missing proteins V and IX) and their location and organization in the middle part of the genome is very similar to that of the mastadenoviruses, apparently there is no recognizable E1A region in the aviadenoviruses and in the candidate members of the proposed genus. Similarly, the region contained between the pVIII and fiber genes (at the conventional location of the E3 region) has no similarity to the E3 of mastadenoviruses. Furthermore, in the aviadenoviruses, no E1B region has been identified. The corresponding region in the members

of the candidate new genus is also very divergent from the mastadenoviruses and needs further characterization. There are several additional ORFs found on the right hand end of the genome, which are homologous only among the members of the aviadenoviruses or proposed new genus, respectively.

Apparently, most human and chimpanzee adenoviruses encode two RNA polymerase III-transcribed low-molecular-weight 'virus-associated' (VA) RNAs which are important in efficient translation of late virus mRNAs. In the twelve monkey adenoviruses which have been examined, however, there is only one VA RNA. The avian adenoviruses also code for a single VA RNA but this is much shorter (90 compared to 160 nucleotides (nt)) and does not exhibit sequence homology with the human VA RNA species; nevertheless, its predicted secondary structure is similar. The location of the VA RNA in aviadenoviruses (CELO) at the far right hand part of the genome is also different from that in mastadenoviruses. In the EDS virus a VA RNA similar to that of CELO virus and located at the far right end of the genome has been described.

Properties of Virus Proteins

Most of the proteins encoded by human adenovirus prototype strains have been well-characterized and, as mentioned, the animal adenoviruses of the *Mastadenovirus* genus seem to be similar. The aviadenoviruses and the proposed new genus show differences in the number of the structural proteins: the lack of protein V and IX, and the existence of several new, yet uncharacterized proteins (28K in OAV287, or 24K in EDS virus). Most avian adenoviruses also have an additional fiber protein (see earlier) coded by a second gene, but a similar phenomenon also occurs in human adenovirus types 40 and 41. The most distinctive feature of the CELO virus polypeptide composition appears to be the increase in the amount of core polypeptides present and it has been suggested that these may be needed to neutralize the negative charge of the DNA which is 30% larger than human adenovirus DNA.

Physical Properties

Virions have a molecular weight of $\sim 170 \times 10^6$–185×10^6, sedimentation value ~560S and a density in CsCl of 1.32–1.35 g/cm^{-3}. As noted earlier, aviadenoviruses appear to have larger genomes and this is reflected in the physical properties of the virions. Virus infectivity is resistant to lipid solvents and is relatively acid stable.

Replication

In the animal adenoviruses (so far examined) and which belong to the *Mastadenovirus* genus the genome organization appears to be broadly similar to that of the human adenoviruses. The early regions E1A and E1B are similarly located at the left hand end of the genome and E3 at around 80 map units. All three of these transcription units are transcribed in the rightward direction and a region corresponding to E4 transcribed (as in the human viruses) in the leftward direction has also been detected. In the avian adenoviruses and the members of the new proposed genus, however, an E1A region could not be identified. Moreover, in OAV287, BAV-6 and EDS a supposedly nonessential region tentatively called 'E3' (because it was proven to be deletable in OAV287) is located after the E4 region and is transcribed in the leftward direction. The role of this region in the replication is not known and neither is the role of the unidentified open reading frames of the aviadenoviruses at the right-hand end of their genome. Analogous findings to human adenoviruses with respect to the mechanisms of virus DNA replication have been established with a number of animal virus systems (e.g. avian and murine adenoviruses). Thus all the sequences of the ITRs from animal adenoviruses contain sequences at nucleotides 8–15, consistent with the termini being the origins of DNA replication. Other aspects of the molecular biology of the animal mastadenoviruses have so far indicated that they appear to conform with that already established in much more detail for the human adenoviruses, while the lack of the E1A region in the avian and candidate third genus adenoviruses raises interesting questions about the early events in their replication.

Host Range and Virus Propagation

Animal adenoviruses generally can be readily propagated in a range of epithelial cells derived from tissues of their own species, although some adenoviruses can also replicate in other tissue cell lines. There is a wide variation in their ability to replicate in cells from other species, e.g. ovine adenoviruses can be propagated in bovine and porcine cells as well as ovine, but murine adenoviruses are more restricted to mouse cells, preferably mouse embryo cells. Aviadenoviruses can only replicate in avian cells, doing best in cells from the homologous species. Although the animal adenoviruses appear to be specific to their original host, there are some reports on isolation of bovine adenoviruses from sheep, buffalo, and deer. On the other hand, restriction enzyme fragmentation, DNA hybridization and limited sequencing suggested that

BAV-9 clearly belongs to subgenus C of human adenoviruses. These observations leave open the possibility that the isolation of BAV-9 could be an artifact (laboratory contamination). On the other hand group C human adenoviruses can perhaps establish infection in cattle. Nevertheless, HAV-5 is also capable of replication in bovine cell cultures or even in cell lines with similar efficiency as in human cells. Similarly to human adenovirus types 40 and 41, the hemorrhagic enteritis virus of turkey can be propagated in live animals or in transformed cell lines only. BAV serotypes 4 to 8 and 10 are also restricted to primary or low-passage number bovine testicular or endothelial cells.

Genetics and Evolution

Complete sequence data are available from five animal adenovirus genomes, and numerous partial sequences have been determined recently (**Table 1**). Sequence data along with restriction enzyme patterns have been used in some cases (e.g. bovine and avian adenoviruses) for the confirmation of groupings based on serological results. More importantly, however, these sequence data also make possible the examination of the phylogenetic relationships among adenoviruses. Initially, the ITRs present at the termini of the virus DNA were used for comparisons, whereas nowadays the DNA and predicted amino acid sequences of selected viral proteins available from many different adenovirus types provide useful data for comparative taxonomy. For example, on the basis of serological crossreactivity and similarities in restriction enzyme cleavage patterns, several simian adenoviruses could previously be allocated into four (A, B, C, E) out of the six known human subgenera. Comparison and phylogenetic analysis of the VA RNAs of human and simian adenoviruses further defined their close relationship and possible common origin. Phylogenetic analyses based on reasonably large data sets comprising those possibly most restricted to their host species (sequences of the hexon or the protease genes are most appropriate) consistently resulted in the obvious separation of three clusters corresponding to the two accepted, and the third proposed genera (**Fig. 1**). The subgenus classification of HAVs, and the general similarity of adenoviruses isolated from the same host was also confirmed.

Several interesting aberrations could be observed among adenoviruses isolated from cattle or sheep. BAVs apparently belong to two different genera and the isolate OAV287 is definitely more closely related to subgroup 2 BAVs (the proposed new genus) than to OAV-3. On the other hand, OAV-3 and BAV-2 are clustered together, whereas other subgroup 1 BAVs (BAV-1 and -3) are further from BAV-2. Although as its name indicates, BAV-2 was first isolated from cattle, it has in fact also been isolated from sheep with an equal or higher frequency, and might therefore genuinely be an ovine adenovirus. Another questionable virus concerning its host and evolutionary origin is BAV-10 which was originally described as a member of subgroup 2 BAVs. Its genome analysis and the phylogenetic calculation clarified however that BAV-10 is a typical mastadenovirus, although distantly related to subgroup 1 BAVs.

An interesting example of the use of sequencing and phylogenetic calculations is a guinea-pig adenovirus which has never been isolated, yet partial amplification of its genome by polymerase chain reaction (PCR) was successful. The amplified partial hexon sequence could then be included into phylogenetic calculations, and the obtained distance (branch length) data confirmed that the virus is not closely related to any other known adenoviruses and will likely be a new type (**Fig. 1**).

Serologic Relationships and Variability

Typing of mammalian animal adenoviruses on the basis of neutralization has recognized at least 57 different types distributed as follows: simian (27), bovine (10), ovine (6), swine (5), equine (2), canine (2), caprine (1), murine (2), tree shrew (2). All are mastadenoviruses with the exception of BAV types 4 to 8 and OAV287 (showing one-way crossneutralization with BAV-7). Among the 21 avian adenoviruses there are fowl (12), turkey (3), goose (3), pheasant (1) and duck (2) serotypes (duck adenovirus type 1 being the EDS virus belonging to the proposed new genus). In addition to these type-specific antigens, there are group-specific antigens characteristic of the three genera as well as a variety of subgroup antigens which indicate more diverse antigenic relationships between the types. Panels of monoclonal antibodies have been produced which can further define these antigenic relationships, e.g. between the fowl serotypes. Most of these different antigenic sites appear to be associated with hexons, although some have also been ascribed to the fiber components (see earlier). Hemagglutination with rodent and monkey erythrocytes has proved to be a useful laboratory tool to establish serological groupings and inhibition and enhancement of hemagglutination can be used to make further serological refinements.

Epizootiology, Transmission and Tissue Tropism

The animal adenoviruses appear to be widely dispersed in their respective host species mostly in an

asymptomatic manner. Similarly to human adenoviruses, persistent infection might be established and the virus can be excreted to the environment from the respiratory and gastrointestinal tracts. Vertical (transplacental) spreading in cattle and swine, as well as egg transmission in poultry have also been described. There are several reports on fulminating diseases attributed to adenovirus infection (over a thousand Californian mule deer died of a disseminated hemorrhagic disease). In other cases of fatal hemorrhagic enteritis in cattle or epizootic lethal pneumonia in guinea pigs, low contagiousness and morbidity, but acute course and very high mortality (up to 100%) were observed. A very wide spectrum of tissue tropism among the animal adenoviruses is evident, e.g. porcine adenovirus type 3 shows a tropism for columnar epithelial cells whereas bovine adenoviruses prefer capillary endothelial cells; however, it is not clear how far tissue tropism relates to pathogenesis and disease. Nevertheless, it is known that for example canine adenovirus types 1 and 2 cause distinct pathology. The two murine adenovirus serotypes (MAV-1 and -2) also have very divergent pathogenic effects. Moreover, recent studies demonstrated that different inbred or outbred, but fully immunocompetent, murine strains show different susceptibility to MAV-1 and according to the presence of genetically determined cellular receptors the infection also has different manifestations.

There is some evidence that an adenovirus which is asymptomatic in its natural host can produce disease in another species. Thus epidemics of reduced laying and soft-shell or shell-less eggs in some flocks of chickens could be attributed to a duck adenovirus (EDS). Aviadenoviruses appear to require avian cells to replicate although they can abortively infect mammalian cells and under the appropriate conditions can transform them.

Pathogenicity and Clinical Features of Infection

Most adenoviruses can be isolated from tissues obtained from healthy animals, but isolations have been associated with respiratory, gastrointestinal and conjunctival diseases, particularly in intensively reared herds and flocks. There is also some evidence for disease etiology in natural infections in the wild. In the case of canine adenoviruses (CAV) the two serotypes, CAV-1 and CAV-2 do seem to have differing tropisms associated with disease patterns. Thus CAV-1 is the agent responsible for infectious canine hepatitis (Rubarth's disease) and along with CAV-2 contributes some of the viruses constituting the 'kennel cough' syndrome. CAV-1 also appears to induce transient corneal opacity and may also cause encephalopathy. Epizootic infections with CAV-1 in foxes, bears, wolves, coyotes and skunks have also been reported. CAV-2 on the other hand appears to be confined to infections of the canine respiratory tract. Recently, an adenovirus was isolated in connection with the deaths of over a thousand mule deer in California. The virus seemed to be a subgroup 2 BAV. It is also notable that every isolate of BAV-10 has so far originated from diseased and subsequently dead animals showing intestinal hemorrhages. Retrospective *in situ* DNA hybridization of tissue sections from similar cases suggested that not only BAV-10 but the subgroup 2 BAVs may also be responsible for economic losses.

There is evidence that, as in humans, pre-existing adenovirus infection in animals can also develop into generalized disease in immunocompromised hosts. Disseminated adenovirus infection affecting the vascular endothelium was detected by electron microscopy in a cat that had a concomitant feline leukemia virus infection. The origin of the adenovirus infection has not been determined. Clinical manifestation of adenovirus infection was also described in horses with combined immunodeficiency syndrome and in a simian immunodeficiency virus (SIV)-infected rhesus monkey.

Avian adenoviruses have been associated with a wide range of disease patterns, e.g. haemorrhagic enteritis in turkey, bronchitis in quail, 'marble spleen' disease in pheasant, inclusion body hepatitis, pulmonary congestion and edema. Recent pathogenesis studies on turkey's hemorrhagic enteritis suggested that HEV has a tropism for the B lymphocytes and macrophages and that intestinal lesions are not induced by local cytopathic viral replication, but rather are immune mediated.

Several animal models have been used for the study of human adenovirus pathogenicity. Cotton rats, rabbits and chinchilla could successfully be infected with HAV-5 or HAV-1, and reproducible pneumonia, ocular infection and otitis media could be established, respectively.

Pathology and Histopathology

Animal adenoviruses infect susceptible cells and elaborate similar gross pathology to that seen with human adenoviruses, e.g. early rounding of cells and aggregation followed by the later appearance of characteristic basophilic nuclear inclusions. Examination by electron microscopy can also reveal crystalline assays of virus particles in the case of aviadenoviruses. 'Marble spleen disease' of chickens and pheasants induced by avian adenoviruses can be accompanied by

gross lesions including pulmonary congestion, splenomegaly, hepatomegaly and congestion of egg follicles. Microscopic lesions include multifocal pneumonia and edema and reticuloendothelial cell hyperplasia of the spleen with concurrent white-pulp neurosis and lymphocyte depletion. Many of the hyperplastic cells contain the basic intranuclear inclusions characteristic of adenovirus infection. However, there are conflicting reports on the nature of the etiological agents relating to pathologies seen in avian species and it has been suggested that concurrent infections with other microorganisms may play a synergistic role in tandem with adenovirus infection.

Immune Response, Prevention and Disease Control

Serological analyses of animal species have indicated that there is widespread natural infection demonstrated by the presence of antibody against the group antigens. In studies with murine adenoviruses a protective role for T cells was demonstrated but in other systems there have been very few definitive investigations of the nature of the immune responses to infection. On the other hand, vaccination programs have been successfully developed with bovine, ovine, canine, equine and avian adenoviruses. Live attenuated, inactivated and *ts* viruses have all been used in these programs. Inactivated combined bovine adenovirus vaccines exist consisting of one subgroup 1 and one subgroup 2 serotype, thereby conferring immunity to BAVs belonging to two different genera. Recently, there has been great interest in using animal adenoviruses themselves as viral vectors to express and deliver foreign antigens in cattle, sheep, swine, dog, poultry, etc. For the insertion of foreign genes, generally the E3 region is replaced. This region has been or is now being characterized in a number of animal mastadenoviruses (BAV-2, BAV-3, etc.). In addition a nonessential, deletable, putative E3 region has been identified in the OAV287 (a member of the proposed new genus) and a gene expression vector has already been developed from it and also from BAV-3.

See also: **Adenoviruses (*Adenoviridae*): General features, Malignant transformation and oncology, Molecular biology; Vectors: Animal viruses.**

Further Reading

Harrach B and Benkö M (1998) Phylogenetic analysis of adenovirus sequences. Proof of the necessity of establishing a third genus within the *Adenoviridae* family. In: Wold WSM (ed.) *Adenovirus Methods and Protocols* (*Methods in Molecular Medicine*, Vol. 21), pp. 309–339. Totowa, NJ: Humana Press Inc.

Kraft V (1994) Adenoviruses. In: Osterhaus ADME (ed.) *Virus Infections of Vertebrates 5: Virus Infections of Rodents and Lagomorphs*, Chapter 24. Amsterdam: Elsevier Science.

Mautner V (1989) *Adenoviridae*. In: Porterfield JS (ed.) *Andrewes' Viruses of Vertebrates*, p. 249. London: Baillière Tindall.

McCracken RM and Adair BM (1993) Avian adenoviruses. In: McFerran JB and McNulty MS (eds) *Virus Infections of Vertebrates* 4: *Virus Infections of Birds*, Chapter 7. Amsterdam: Elsevier Science.

Russell WC, Adrian T, Bartha A *et al* (1995) Family *Adenoviridae*. In: Murphy FA, Fauquet CM, Bishop DHL *et al* (eds) *Virus Taxonomy – Classification and Nomenclature of Viruses*, Sixth Report of the International Committee on Taxonomy of Viruses, p. 128. Wien, New York: Springer-Verlag.

Studdert MJ (1996) Equine adenovirus infections. In: Studdert MJ (ed.) *Virus Infections of Vertebrates 6: Virus Infections of Equines*, Chapter 6. Amsterdam: Elsevier Science.

Malignant Transformation and Oncology

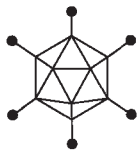
Margaret P. Quinlan, Department of Microbiology and Immunology, University of Tennessee, Memphis, Tennessee, USA

Introduction

Adenoviruses (Ad) are double-stranded DNA viruses that cause a number of acute respiratory infections, in addition to some other types of infections, depending upon the cell type infected. They came to prominence in 1962 because they were shown to cause tumors in rodents. From this, they were considered DNA tumor viruses. The various serotypes have been classified into subgroups, which exhibit different degrees of oncogenic potential in rodent systems (**Table 1**). Subsequently, it was determined that induction of tumors is an alternative path followed when a productive lytic cycle cannot be completed. Adenovirus transformation has not been reported for humans, which are permissive for the genus Mastadenovirus of the adenoviridae family. Ad normally infects quiescent epithelial cells, but requires the host DNA synthetic machinery for its own replication, thus the virus has evolved mechanisms to reactivate quiescent cells into the cell cycle. It has been through the identification of these viral genes, which are

A

ADENOVIRUSES (*ADENOVIRIDAE*)



Contents

General Features

Göran Wadell, University of Umeå, Umeå, Sweden

History

Characteristic outbreaks of epidemic keratoconjunctivitis occurred among factory workers in Germany during the nineteenth century. In 1953, Wallace Rowe and colleagues explanted surgically removed adenoids from 53 children in Washington DC in order to evaluate different tissues for growth of polio viruses. After 4 weeks, 33 of the cultures showed a slowly progressive cytopathic effect. The agents isolated from these cultures were named the adenoid-degenerating (AD) agent. In 1980, it was demonstrated that the enteric adenoviruses that were associated with infantile diarrhea differed from all other human adenovirus by being fastidious and could be assigned to a separate subgenus designated F.

The observation by Trentin (1962) that adenovirus 12 (Ad12) of subgenus A induced sarcomas in all inoculated newborn hamsters introduced human adenoviruses as a model of viral oncogenesis. It was subsequently demonstrated that only the early (E1) gene of the adenovirus genome was required for *in vitro* transformation of normal human cells. Furthermore, the E1a region could cooperate with the *Ras* oncogene in the transformation process. Mukai demonstrated (1980) that Ad12 could induce retinoblastomas in newborn baboons. On the molecular level, Harlow (1988) found that E1a gene products interacted with the retinoblastoma protein. Furthermore the E1b gene products interacted with the p53 protein thus providing a means for inactivation of these oncogene suppressor proteins.

Taxonomy and Classification

Adenoviruses have been isolated from every species of the placental mammals, marsupials, birds and amphibians studied. The adenovirus family has two genera: *Mastadenovirus* and *Aviadenovirus*. There is no shared family-specific antigen. The mastadenoviruses share a genus-specific antigen (bovine adenovirus serotypes 4–8 are an exception). However, there is no common antigen among the aviadenoviruses.

The classification of adenoviruses has evolved from schemes based on biological properties to a reproducible and discriminating system based on genomic differences. Human adenoviruses were originally classified into four groups based on different hemagglutination properties with rat and rhesus monkey erythrocytes. In 1967, it was suggested that human adenoviruses should be divided into subgenera on the basis of high, weak and no oncogenesis in newborn hamsters.

The polypeptides of the virion represent a major portion of the products of the viral genome. The structural internal polypeptides were expected to be conserved and offered a means of classifying human adenoviruses. The 47 human adenovirus serotypes are divided into six subgenera by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of polypeptides. This method was compatible with the previous method. Furthermore, Ad4 and enteric adenoviruses, Ad40 and Ad41, were recognized as two newly identified subgenera E and F, respectively (**Table 1**).

The conclusive classification of viruses should be based on nucleotide sequence differences between the

Table 1 Properties of human adenovirus serotypes of subgenera A–F

Subgenus	Serotype	DNA: Homology (%) Intrageneric	DNA: Homology (%) Intergeneric	G + C (%)	Number of SmaI[a] fragments	Apparent molecular mass of the major internal polypeptides (kD): V	VI	VII	Hemagglutination pattern[b]	Length of fibers (nm)	Oncogenicity in newborn hamsters	Tropism symptoms
A	12, 18, 31	48–69	8–20	48	4–5	51.0–51.5 46.5–48.5[c]	25.5–26.0	18	IV	28–31	High (tumors in most animals in 4 months)	Cryptic enteric infection
B:1 B:2	3, 7, 16, 21 14,[d] 11, 34, 35	89–94	9–20	51	8–10	53.5–54.5	24	18	I	9–11	Weak (tumors in few animals in 4–18 months)	Respiratory disease Persistent infections of the kidney
C	1, 2, 5, 6	99–100	10–16	58	10–12	48.5	24	18.5	III	23–31	Nil	Respiratory disease persists in lymphoid tissue
D	8, 9, 10, 13, 15, 17, 19, 20, 22 to 30, 32, 33, 36, 37, 38, 39, 42 to 47[e]	94–99	4–17	58	14–18	50.0–50.5[f]	23.2	18.2	II	12–13	Nil	Keratoconjunctivitis
E	4		4–23	58	16–19	48	24.5	18	III	17	Nil	Conjunctivitis Respiratory disease
F	40, 41	62–69	15–22	52	9–12	46.0–48.5	25.5	17.5	IV	28–33	Nil	Infantile diarrhea

Modified from Wadell (1984).

[a] The restricted DNA fragments were analyzed on 0.8–1.2% agarose slab gels. DNA fragments smaller than 400 bp were not resolved.

[b] I, Complete agglutination of monkey erythrocytes; II, complete agglutination of rat erythrocytes; III, partial agglutination of rat erythrocytes (fewer receptors); IV, agglutination of rat erythrocytes discernible only after addition of heterotypic antisera.

[c] Polypeptide V of Ad31 was a single band of 48 kD.

[d] Members of subgenus B are divided into two clusters of DNA homology based on pronounced differences in DNA restriction sites.

[e] Only polypeptide analysis and/or DNA restriction analysis has been performed with Ad32–Ad39 and Ad42–Ad47.

[f] Polypeptides V and VI of Ad8 showed apparent molecular mass of 45 and 22 kD, respectively. Polypeptide V of Ad30 showed an apparent molecular mass of 48.5 kD.

genomes of the different viruses. The DNA homology of adenoviruses has been studied by hybridization techniques, DNA restriction analysis and nucleotide sequencing. These techniques confirm the assignment of the 47 human adenovirus serotypes into six subgenera.

Members belonging to the genus *Mastadenovirus* share common epitopes on the hexons. Subgenus is defined by the DNA homology of more than 50% between members within a subgenus and less than 20% between members of different subgenera.

The serotype is defined by quantitative neutralization with hyperimmune sera. The ratio of homologous to heterologous neutralization titer must be greater than 16. The designation recombinant should be used only when the two parent genomes have been identified. An evolutionary variant is one in which genetic alteration was generated via insertion or intragenomic recombination in progeny of the same strain. A genomic cluster is a group of genome types that are significantly more closely related to each other than to any other genome type. The genome type denotes a distinct viral entity within a genomic cluster identified by DNA restriction analysis. Strain corresponds to the progeny of each wild-type isolate.

Geographic and Seasonal Distribution

Ad8 causing epidemic keratoconjunctivitis is common in East Asia and rare in Europe and the United States. Respiratory infections show two peaks in mid-winter and late summer. The first predominantly among small children and the latter among school children. Newly enlisted military recruits are infected by the end of the third week after their recruitment. Enteric adenoviruses causing infantile diarrhea show no seasonality. Keratoconjunctivitis in Japan is presented as epidemic outbreaks during late summer and early fall. Eye infections are particularly prone to appear in hot and dry climates or in densely populated areas. Within each serotype certain genome types show an appearance characteristic for regions and continents.

Host Range and Virus Propagation

Adenoviruses are highly species specific. Human adenoviruses may infect chimpanzee. The adenoviruses belonging to subgenus C can cause symptoms after infection of rodents. Ad2 and Ad5 produce pneumonia in cotton rats or hamsters. Ad5 can cause hepatitis in mice. Several animal adenovirus infections may serve as models for human adenoviruses.

Genetics

Changes occur by accumulation of point mutations in the adenovirus DNA. There is no evidence that the mutation rate of adenoviruses differs from that of the host chromosome. The subgenera serve as recombination barriers. Experimentally, recombinants can only be created within each subgenus.

Evolution

As adenoviruses are detected in all host species studied, they are considered to be phylogenetically old. Members of the six subgenera of the human adenoviruses display characteristically different tropisms, i.e. they will replicate in different organs. They have also evolved so far from each other that subgenera serve as recombination barriers. That adenoviruses display an unusually high host species specificity speaks in favor of the notion that adenoviruses have evolved with their host species. It should therefore be possible to find correlates of different subgenera of human adenoviruses also among adenoviruses of other host species.

Serological Relationships and Variability

The single capsid is composed of 240 hexon capsomers and 12 vertex capsomers. From the latter 12 fibers project. Genus-specific epitopes reside in the basal part of the hexon. The serotype is defined by neutralization of infectious activity or by hemagglutination inhibition. The epitopes capable of inducing type-specific antibodies are localized on the external portion on the hexons and on the most distal knob of the fiber. Furthermore, the hexon carries epitopes which are the structural correlates of a diverse cross-reactivity. The vertex capsomer contains intrasubgenus-specific epitopes and subgenus-specific epitopes. The fibers contain subgenus-specific, intrasubgenus-specific and type-specific epitopes. Serological intermediates are found with neutralizing hexon epitopes of one serotype, and hemagglutination-inhibiting epitopes of the fiber of other serotypes. Monoclonal antibodies or extensively absorbed hyperimmune sera have to be used to obtain type-specific reagents to be used for typing of adenoviruses by enzyme-linked immunosorbent assay (ELISA) techniques.

The serological cross-reactivity of Ad4, the only member of subgenus E may have evolutionary implications. Hexons of Ad4 cross-neutralize hexons of Ad16 (subgenus B); the fibers of Ad4 share epitopes of fibers of subgenus C members; hexons of Ad4 share external epitopes with the hexons of Ad40 (subgenus F); and the early antigens of Ad4 share epitopes with

subgenera of all other adenoviruses to an extent that is not noted for other human adenoviruses.

Epidemiology

Among the isolates reported to the World Health Organization (WHO) register during a 10-year period, adenovirus accounted for 13% of the reported isolations providing evidence that adenovirus infection is second only to influenza A which accounts for 28% of the reported isolates. The human adenoviruses represent different organ tropisms leading to different diseases and are therefore presented according to subgenus.

Subgenus A

Ad12, Ad18 and Ad31 are rarely isolated because they are difficult to cultivate. However, infections are relatively common. The prevalence of neutralizing antibodies demonstrated that Ad31 and Ad18 were second to members of subgenus C. The majority of the isolates were obtained from infants and 60% of these children had gastrointestinal disease. It is still difficult to evaluate their role as a cause of diarrhea.

Subgenus B:1

Ad3 and Ad7 account for 13 and 20% of all adenovirus isolates reported to WHO. Both Ad3 and Ad7 show epidemic appearance with 4–5 years intervals. Three different epidemic patterns of Ad7 infection have been identified. The first pattern represents outbreaks mainly during the winter season among infants and infections can be severe. The second pattern consists of epidemic outbreaks of respiratory disease among school children. Moderate to severe infections with fever for 7 days are usually seen but fatal outcome is rare. Outbreaks among newly enlisted military recruits constitute the third epidemic pattern. These are caused by Ad4, Ad7, Ad14 and Ad21. The etiology of the syndrome may be sought in the fact of gathering of young men with lowered immunity sleeping in crowded quarters after hard exercise. The frequency of reported Ad7 isolated in Japan and the antibody prevalence were highly discordant. Adenovirus-specific antibodies were detected in 50% of the adult sera with only 2% of adenovirus strains isolated in Japan typed as Ad7. By contrast, 52% of isolates were typed as Ad3. In other parts of the world, Ad7 is the predominant adenovirus respiratory pathogen. DNA restriction analysis has revealed the occurrence of different genome types within each serotype. In Europe a shift from genome type Ad7c to Ad7b occurred in 1969 and the analogous shift took place in Australia 1975. Ad7b dominates in the United States whereas Ad7c now dominates in Africa. Ad7d is the predominating genome type in China. Ad7e is unique to Brazil and Ad7h responsible for a recently recognized severe outbreak of respiratory illness among small children in Argentina, Chile and Uruguay. Ad21 has been isolated from military recruits at the major basic training centers in the US army as a cause of severe respiratory disease.

Subgenus B:2

Ad14 causes outbreaks among military recruits. Ad11, Ad34 and Ad35 cause persistent infections of kidneys and are the most common adenoviruses isolated from the urine of AIDS patients or bone marrow transplant recipients.

Subgenus C

The members Ad1, Ad2 and Ad5 are regarded as endemic. Infants are infected by older siblings shedding the virus after recrudescent infections for up to 2 years. By the age of 4 years at least 50% of the children have antibodies against Ad1 or Ad2. By the end of the grammar school years all children will have had several adenovirus infections. Subgenus C members account for 50% of all adenovirus strains reported to the WHO.

Subgenus D

Contains 28 serotypes, i.e. more than half of all recognized human adenoviruses, Ad8, Ad19 and Ad37 are causes of epidemic keratoconjunctivitis in particular in dry climates or in densely populated areas.

Subgenus E

Ad4 is rarely isolated from children in Europe or the US. Two distinct genome types, Ad4 and Ad4a, have been identified. Ad4 is responsible for epidemic outbreaks of respiratory disease among military recruits and the Ad4a genome type is second only to Ad8 as a cause of adenovirus-associated eye disease in Japan.

Subgenus F

The enteric adenoviruses Ad40 and Ad41 are the only members of subgenus F. They are second only to rotaviruses as a cause of infantile diarrhea and in contrast to rotaviruses they cause diarrhea in children throughout the year. Enteric adenoviruses cannot be found in stools of healthy controls using the polymerase chain reaction (PCR) technique. Ad40 or Ad41 have been isolated in stool specimens from Africa, Asia, Europe, Latin America and North America. Fifty percent of 6–8 year old children in Asia and Europe have neutralizing antibodies to Ad41. At the

end of the 1970s Ad40 dominated in Europe. Ad41 is the predominant type for 1992.

Transmission and Tissue Tropism

Adenoviruses of humans can be spread by aerosols. Transmission is particularly effective when the population density of sensitive individuals is high such as several young children of the same age group or newly enlisted recruits that sleep in the same dormitory. During early childhood, infection by the oral route, as the consequence of shedding into stools after recrudescence, also occurs. Similarly, transmission by this route may result in the characteristic swimming pool outbreaks of Ad3 and Ad7. Infections of the eye can be transmitted through direct contact as revealed by outbreaks from ophthalmologists' office via tonometers as the common source of infection.

Airborne transmission has been experimentally demonstrated with the avian FAV1 virus. Among fowl, vertical transmission may also occur.

Tissue tropism varies with the subgenera. Subgenus A cause cryptic infections of the gut where the host cell has not yet been identified. Subgenus B viruses cause respiratory infections or infections of the urinary tract. Members of subgenus C cause respiratory infections but have also been demonstrated to replicate in lymphocytes. The subgenus D viruses can infect the conjunctiva and cornea of the eye. Subgenus E virus can infect both the eye and respiratory tract whereas subgenus F viruses infect enterocytes. This information might indicate that different adenoviruses utilize different host cell receptors. These have not been identified.

Pathogenicity

Among the 47 adenovirus serotypes, several differ in pathogenicity. Ad7 is the most severe cause of respiratory infections. Among the 28 serotypes of subgenus D that have predilection for the eye only one serotype, Ad8, is responsible for most severe outbreaks of keratoconjunctivitis. Even within a particular serotype, there are differences. The Ad19 prototype has rarely been isolated during the last three decades whereas genome type Ad19a has been responsible for numerous outbreaks of keratoconjunctivitis since 1973. Even after infection with the same genome type, it is obvious that infants or young animals display more severe or fatal symptoms that are not apparent after infection of older individuals. Infections with mouse adenovirus will kill suckling mice, whereas adult mice display viruria for 24 months. Canine Ad1 displays 80% mortality in experimentally infected fox pups that are less than 6 months old. The adenoviral genes responsible for the pathogenicity have not been identified. However, it is obvious that deletion of the entire E3 region can effect pathogenicity.

Clinical Features of Infection

Respiratory disease in children

Serotypes 1, 2 and 5 are endemic whereas serotypes 3 and 7 cause epidemic outbreaks of adenopharyngoconjunctival fever. The pharyngeal conjunctival fever appears after a mean incubation period of 6 days. The initial conjunctivitis is followed by high fever and sore throat. One half of the susceptible population shows asymptomatic infection. Vomiting, diarrhea and meningeal signs are found and temperature returns to normal after 7–8 days. Symptoms are usually more severe in children but sequelae are rare.

Lower respiratory tract infections with symptoms as bronchiolitis and pneumonia appear after an incubation of 8 days. Symptoms appear as cough, dyspnea, inspiratory and expiratory wheezing with fever of 7–8 days. Among hospitalized patients after infection with Ad3, Ad4, Ad7 and Ad21, significant chronic lung damage as impaired lung function or bronchiectasis occur. Unusually severe forms are seen among children below the age of 2 years, in particular amongst North American Indians, Polynesians and children in Latin America.

Epidemic keratoconjunctivitis (EKC)

After 1–7 days incubation, symptoms in the form of pain, photofobia, lacrimation and corneal erosion and edema appear. The duration is characteristically 4–6 weeks and may result in scarring caused by Ad8, Ad19 and Ad37 transmitted via contact in particular to eyes damaged by foreign bodies or extensive sunlight exposure. Ad37 can also be sexually transmitted.

Diarrhea

After an incubation period of 8 days, infection with enteric adenoviruses, Ad40 and Ad41, results in diarrhea, vomiting, moderately elevated temperature and dehydration in less than one-fifth of patients. The diarrhea is not as acute as rotavirus-induced diarrhea but the duration is significantly longer, 9–12 days. Appearance of sequelae has not been sufficiently evaluated.

Other symptoms

Acute hemorrhagic cystitis among young boys, caused by Ad11 and Ad21, is common in Japan but not so frequent in Western countries. Intussusception, meningoencephalitis, pericarditis, rhabdomyolysis,

polyarthritis and Steven Johnson like exanthemas may be seen after adenovirus infection.

In immunocompromised hosts, hepatitis may be seen after transplantation and acute hemorrhagic cystitis after kidney transplantation; in severe combined immunodeficiency, hepatitis and pneumonia usually with a poor prognosis can be caused by Ad5 or Ad31.

Pathology and Histopathology

The property of adenoviruses to shut off the expression of the host mRNA and the unregulated synthesis of viral proteins is incompatible with normal cell function. The structural proteins form characteristic intranuclear inclusions that are the pathological hallmarks of adenovirus infection. In the upper respiratory tract, ciliar abnormalities and microtubular aberrations may cause a defective mucociliar clearance. In adenovirus pneumonia, a necrotizing destruction of the broncheal epithelium occurs with damage of the broncheal gland. The intranuclear inclusion bodies are refractile like ground glass. Adenoviruses of subgenera B and C produce large amounts of free vertex capsomers with an associated toxin-like activity, but the viral gene products that are responsible for the adenovirus-induced symptoms in the host are not defined. Some of the infrequent symptoms of adenovirus infection seen in man mirror adenoviral disease in animals. Diarrhea as a function of infection of endothelial cells in the gut is seen in bullocks. Hemorrhagic enteritis is also seen in turkeys. Encephalitis resulting from infection of endothelial cells with perivascular cuffing is seen in foxes. Hepatitis is seen in dogs, sheep, Nile crocodiles, boa constrictors and domestic fowl. Cauda equina nervitis is seen in immunodeficient foals. Porcine adenoviruses can also infect nervous tissue.

Immune Response

Human antibodies directed against epitopes on the external towers of the hexons and the knobs of the fiber may induce neutralizing antibodies. Antigenic extinction experiments have revealed that 1 ng hexon and 100 ng fibers or 10^7 virions are needed to elicit a primary immune response. A cell mediated immune response is required for resolution of adenovirus infections. Adenovirus infections are seen in individuals devoid of adequate cellular immunity such as neonates or children with congenital cell disorders. Some bone marrow transplantation recipients experience severe or even fatal adenovirus infections. Mouse Ad1 causes duodenal lesions in adult nude mice but heterozygotes are not susceptible. The immune response against adenoviruses is modulated by gene products of the E1a and E3 region. The E1b/19K protein prevents lysis of human cells by tumor necrosis factors (TNFs) whereas the E3/14.7K protein protects transfected cells from TNF-induced cytolysis. On the other hand, E1A products render infected cells sensitive to lysis by TNF. The E3/gp19K protein downregulates MHC1 antigens. The promoter of E3/gp19K is driven by NF Kβ factor. This consequently provides a molecular mechanism for the observed evasion of the immune elimination of adenovirus infected lymphocytes.

E1a-induced tumors can be eradicated by E1a-specific cytotoxic T lymphocytes. Expression of Ad2E3/19K protein inhibits lysis of the cytotoxic T lymphocytes by blocking the cell surface expression of MHC1 that consequently also inhibits the expression of the immunogenic E1A antigen. Five additional gene products are expressed from the E3 region with unknown function.

Prevention and Control

No antivirals have been conclusively demonstrated to have a clinical effect on adenovirus infections. In epidemic keratoconjunctivitis topical steroids may delay the appearance of corneal filtrates and also delay the final corneal healing. Their use is consequently not justified.

Enteric coated vaccines for oral administration protect against respiratory illness caused by Ad4, Ad7 and Ad21. So far these vaccines are widely but exclusively used in military populations. The extent of the attenuation of these vaccine strains has not been documented and they have been demonstrated to spread to household contacts. It is consequently not recommended to extend their use to young children.

Adenoviruses can serve as vaccine vectors. Foreign genes have been introduced to the E1 region. In this case, complementation by an immortalized host cell transfected by the E1 region is mandatory. Alternatively, foreign genes are inserted in the E3 region which is dispensible for growth *in vivo*. However, the effect of the elimination of the immunomodulating gene products of this region is incompletely known. Several vaccines against adenovirus infection among animals have been applied. Live attenuated canine Ad2 can be used for protection against the hepatitis caused by canine Ad1. There is also a vaccine against hemorrhagic enteric virus in turkeys and an inactivated vaccine protecting against equine adenoviruses.

Future Perspectives

The increasing awareness of the impact of adenovirus infection among children living in crowded conditions

urges an evaluation of the possibility to develop protective vaccines that are effective also in young children. The extended application of bone marrow, liver and kidney transplantation and improved care of immune-compromised patients demands introduction of rapid methods for the detection and typing of adenovirus infections. Subsequently development of immunoprofylaxis in the form of safer vaccines to be used before transplantation may become justified. Epidemiological surveillance is required to detect re-emerging and previously unrecognized genome types as has been observed with Ad7, Ad19, Ad21 and Ad41.

See also: **Adenoviruses (*Adenoviridae*): Molecular biology; Immune response: General features; Transplantation and virus infections; Vaccines and immune response.**

Further Reading

Doerfler W (ed.) (1983 and 1984) The molecular biology of adenoviruses 1, 2 and 3. *Curr. Top. Microbiol. Immunol.* vols 109, 110, 111.

Ginsberg HS (ed.) (1984) *The Adenoviruses*. New York: Plenum Press.

Horwitz MS (1990) Adenoviruses. In: Fields BN *et al.* (eds) *Virology*, 2nd edn. p. 1723. New York: Raven Press.

Mautner V (1989) Adenoviridae. In: Porterfield JS (ed.) *Andrewes' Viruses of Vertebrates*, p. 249. London: Baillière Tindall.

Smith GM (1998) Adenovirus-mediated gene transfer to treat neurologic disease. *Archives of Neurology* 55: 1061–4.

Wadell G (1990) Adenoviruses. In: Zuckerman AE, Banatvala JE and Pattison JR (eds) *Principles and Practices of Clinical Virology*, 3rd edn. Academic Press.

[This article is reproduced from the 1st edn (1994).]

Molecular Biology

Jörg Schröer, Institute of Genetics, University of Cologne, Koeln, Germany

Stefan Kochanek, Center for Molecular Medicine, University of Cologne, Koeln, Germany

Marianne Hösel, Institute of Genetics, University of Cologne, Koeln, Germany

Walter Doerfler, Institute of Genetics and Center for Molecular Medicine, University of Cologne, Koeln, Germany

Introduction

For a long time, adenoviruses have served as excellent tools for detailed studies on basic molecular mechanisms in mammalian cells, on the mechanism of viral oncogenesis and on gene transfer in mammalian organisms. In this brief article, it will not be possible to cover all of the recent progress accomplished in adenovirus research in a sensible way. We have therefore chosen to summarize current research on the following topics:

- The fate of viral DNA in mammalian cells.
- The abortive infection of hamster cells with human adenovirus type 12 (Ad12).
- Viral DNA integration and oncogenesis.
- A third generation adenovirus gene transfer vector.

References to more comprehensive surveys on ongoing adenovirus research have been listed under Further Reading.

Fate of Viral DNA in Mammalian Cells

The fate of foreign DNA in mammalian cells is of general biological interest. Foreign DNA can integrate into the genomes of mammalian cells, and this process plays major roles in viral oncogenesis and in the generation of transgenic organisms and will be important in evolving regimens for human somatic gene therapy. At least in cell culture, foreign DNA can be taken up by mammalian cells under appropriate conditions or in a stabilized conformation, e.g. in a complex with basic proteins.

Human adenovirus type 12 (Ad12) infects human cells productively and leads to viral DNA replication, whereas infection of Syrian hamster cells remains abortive with total blocks in viral DNA replication and late viral gene transcription. In abortively Ad12-infected BHK21 cells, the viral DNA can be integrated into the cellular genome early after infection. Viral oncogenesis by Ad12 has been demonstrated in

gross lesions including pulmonary congestion, splenomegaly, hepatomegaly and congestion of egg follicles. Microscopic lesions include multifocal pneumonia and edema and reticuloendothelial cell hyperplasia of the spleen with concurrent white-pulp neurosis and lymphocyte depletion. Many of the hyperplastic cells contain the basic intranuclear inclusions characteristic of adenovirus infection. However, there are conflicting reports on the nature of the etiological agents relating to pathologies seen in avian species and it has been suggested that concurrent infections with other microorganisms may play a synergistic role in tandem with adenovirus infection.

Immune Response, Prevention and Disease Control

Serological analyses of animal species have indicated that there is widespread natural infection demonstrated by the presence of antibody against the group antigens. In studies with murine adenoviruses a protective role for T cells was demonstrated but in other systems there have been very few definitive investigations of the nature of the immune responses to infection. On the other hand, vaccination programs have been successfully developed with bovine, ovine, canine, equine and avian adenoviruses. Live attenuated, inactivated and *ts* viruses have all been used in these programs. Inactivated combined bovine adenovirus vaccines exist consisting of one subgroup 1 and one subgroup 2 serotype, thereby conferring immunity to BAVs belonging to two different genera. Recently, there has been great interest in using animal adenoviruses themselves as viral vectors to express and deliver foreign antigens in cattle, sheep, swine, dog, poultry, etc. For the insertion of foreign genes, generally the E3 region is replaced. This region has been or is now being characterized in a number of animal mastadenoviruses (BAV-2, BAV-3, etc.). In addition a nonessential, deletable, putative E3 region has been identified in the OAV287 (a member of the proposed new genus) and a gene expression vector has already been developed from it and also from BAV-3.

See also: **Adenoviruses (*Adenoviridae*): General features, Malignant transformation and oncology, Molecular biology; Vectors: Animal viruses.**

Further Reading

Harrach B and Benkö M (1998) Phylogenetic analysis of adenovirus sequences. Proof of the necessity of establishing a third genus within the *Adenoviridae* family. In: Wold WSM (ed.) *Adenovirus Methods and Protocols* (*Methods in Molecular Medicine*, Vol. 21), pp. 309–339. Totowa, NJ: Humana Press Inc.

Kraft V (1994) Adenoviruses. In: Osterhaus ADME (ed.) *Virus Infections of Vertebrates 5: Virus Infections of Rodents and Lagomorphs*, Chapter 24. Amsterdam: Elsevier Science.

Mautner V (1989) *Adenoviridae*. In: Porterfield JS (ed.) *Andrewes' Viruses of Vertebrates*, p. 249. London: Baillière Tindall.

McCracken RM and Adair BM (1993) Avian adenoviruses. In: McFerran JB and McNulty MS (eds) *Virus Infections of Vertebrates* 4: *Virus Infections of Birds*, Chapter 7. Amsterdam: Elsevier Science.

Russell WC, Adrian T, Bartha A *et al* (1995) Family *Adenoviridae*. In: Murphy FA, Fauquet CM, Bishop DHL *et al* (eds) *Virus Taxonomy – Classification and Nomenclature of Viruses*, Sixth Report of the International Committee on Taxonomy of Viruses, p. 128. Wien, New York: Springer-Verlag.

Studdert MJ (1996) Equine adenovirus infections. In: Studdert MJ (ed.) *Virus Infections of Vertebrates 6: Virus Infections of Equines*, Chapter 6. Amsterdam: Elsevier Science.

Malignant Transformation and Oncology

Margaret P. Quinlan, Department of Microbiology and Immunology, University of Tennessee, Memphis, Tennessee, USA

Introduction

Adenoviruses (Ad) are double-stranded DNA viruses that cause a number of acute respiratory infections, in addition to some other types of infections, depending upon the cell type infected. They came to prominence in 1962 because they were shown to cause tumors in rodents. From this, they were considered DNA tumor viruses. The various serotypes have been classified into subgroups, which exhibit different degrees of oncogenic potential in rodent systems (**Table 1**). Subsequently, it was determined that induction of tumors is an alternative path followed when a productive lytic cycle cannot be completed. Adenovirus transformation has not been reported for humans, which are permissive for the genus Mastadenovirus of the adenoviridae family. Ad normally infects quiescent epithelial cells, but requires the host DNA synthetic machinery for its own replication, thus the virus has evolved mechanisms to reactivate quiescent cells into the cell cycle. It has been through the identification of these viral genes, which are

Table 1 Tumorigenic potential of adenovirus serotypes

Subgroup	Serotype	Oncogenic potential Tumors in animals	Transformation in vitro
A	12, 18, 31	High	+
B	3, 7, 11, 14, 16, 21, 34, 35	Moderate	+
C	1, 2, 5, 6	Low to none	+
D	8–10, 13, 15, 17, 19, 20, 22–30, 32, 33, 36–39	Low to none	+
E	4	Low to none	+
F	40, 41	Unknown	?

referred to as viral oncogenes, and the mechanisms by which their gene products accomplish this essential goal that several mammalian cell cycle regulators have been identified and characterized and contributed to our understanding of them and tumor suppressors (especially pRB and p53), which are often the targets of Ad, and other DNA tumor virus oncoproteins. These viral genes, in the absence of the rest of the viral genome, are able to effect growth stimulation and transformation of a variety of primary and established cells. These are properties that Ad oncoproteins share with other DNA tumor virus transforming proteins. The major oncogenes encoded by Ads are E1A and E1B, each of which encodes multiple mRNAs. However, recently, additional viral genes that exhibit transforming potential have been identified in Ad9 (subgroup D). These are *E4ORF1* and *E4ORF6*. Interestingly, Ad has two proteins, E1B55K and E4ORF6, that bind to the p53 tumor suppressor and promote transformation by modulating its transcription functions.

E4

The E4 region of Ad9, of subgroup D, exclusively elicits estrogen-dependent mammary tumors, predominantly fibroadenomas, in rats, but also transforms CREF cells in culture. E4 from Ad subgroups A (Ad12) or C (Ad5) does not. E4 encodes several transcripts, two of which have been implicated in tumorigenic transformation. Open reading frame (ORF) 1 encodes a novel, cytoplasmic transforming protein, three regions of which are required. The C-terminus contains a PDZ domain and interacts with the PDZ domain of hDlg/SAP97, a mammalian homologue of the Drosophila discs large tumor suppressor, which also binds APC, another tumor suppressor. E4ORF6 encodes a protein with similarities to Ad E1B (see below). It interacts with the C-terminus of p53 and prevents p53 binding to $TAF_{II}31$, a component of TFIID of the basal transcription complex (BTC), thus blocking p53 transcriptional activation and repression activities. E4ORF6 also blocks the induction of p53-mediated apoptosis. The levels of p53 protein are also diminished. ORF6 promotes focus formation in cooperation with E1A and E1B and accelerates *in vivo* tumor formation of E1A + E1B transformed cells.

E1B

E1B cannot transform primary cells on its own, but cooperates with E1A, an immortalizing oncogene, to do so. E1B encodes two oncoproteins, 55K and 19K. E1B19K is functionally similar to the proto-oncogene *bcl*-2, which was originally isolated from a follicular lymphoma. There is little similarity in the primary sequence, but there are three short regions of homology in the central region of the protein. This same region is necessary to cotransform with E1A and for its antiapoptosis activity. 19K contributes to oncogenesis by preventing p53-mediated apoptosis, similar to *bcl*-2 and *bcl*-xL. It interacts with bax, bak and bik, *bcl*-2 family members which promote apoptosis. E1B19K alleviates p53 repression, but does not affect p53 activation. E1B19K also negatively interferes with the activation of NFkB.

E1B55K extends the lifespan of normal mammalian cells in culture. It associates with E4ORF6. 55K binds p53 without displacing it from DNA, and thus serves as a direct repressor targeted to p53-responsive genes. Transcriptional repression is regulated by phosphorylation at the C-terminus. E1B55K mutants defective in p53 binding are unable to cooperate with E1A.

E1A

E1A encodes two nuclear, multifunctional phosphoproteins, of 289 and 243 amino acids, derived from 13S and 12S transcripts, respectively, that can affect the proliferative state of cells. Either polypeptide can

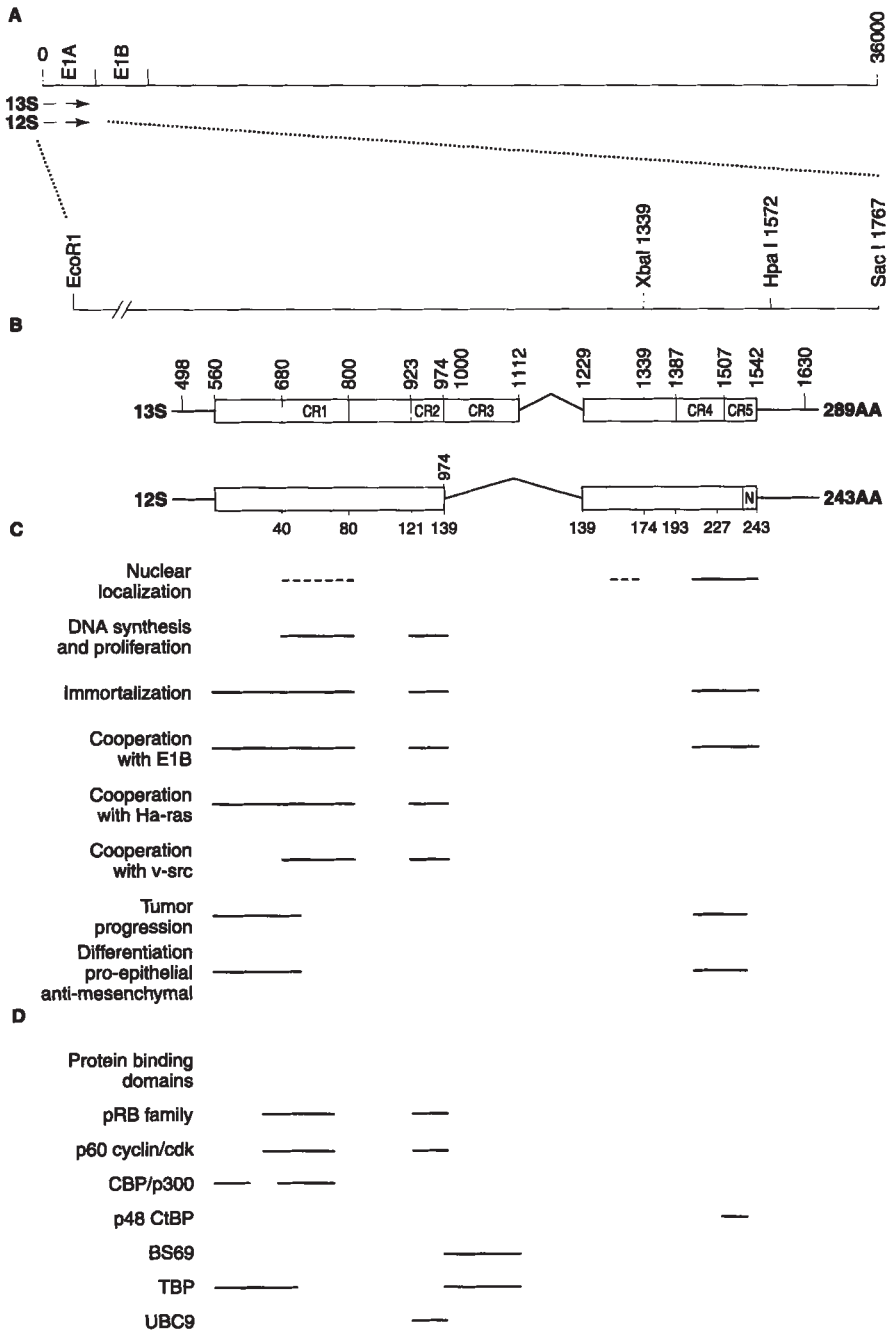


Figure 1 Structures and functional regions of the major adenovirus type 5 E1A gene products. (**A**) Localization of the E1 transforming region in the adenovirus genome. (**B**) Expanded view of the E1A region. The numbers above refer to nucleotide numbers. The top line indicates salient restriction enzyme cleavage sites. Below are the 13S and 12S transcripts. The numbers below the 12S transcript indicate amino acid positions in the 243 aa protein. The boxes represent exons, the solid lines represent non-coding regions and the carets indicate the introns. CR1, CR2, CR3, CR4, CR5 indicate the regions conserved among the different serotypes. (**C**) Functional regions of E1A. The functions or phenotypes are indicated on the left. The region of the protein required for that function is delineated with a solid line. (**D**) Cellular polypeptide binding regions of E1A are indicated by a solid line at the right of the name of each protein.

immortalize on its own, or cooperate with other viral and cellular oncogenes to transform cells. The E1A 12S gene product is much more efficient at this function(s). E1A functions are mediated, at least in part, through its binding to families of growth inhibitory and/or gene regulatory proteins. The E1A proteins contain five regions that are highly conserved among the various serotypes (**Fig. 1**). Three of these

(CR1-CR3) are located in the first exon and two (CR4, CR5) are located in the second exon. One of these, CR3, is unique to the 13S gene product and is necessary for the transcriptional transactivation of the viral early promoters to effect a lytic infection. CR3 is dispensable for the transforming functions of E1A, but can contribute to them. Thus, the E1A12S protein is fully competent at immortalization and cotransformation. These conserved regions seem to correspond to functions or activities carried by the E1A proteins. These, in turn, seem to be due to the interactions of the peptides encoded by these regions with cell cycle and/or transcriptional regulatory proteins in the host cell. In addition to the conserved regions, a non-conserved, N-terminal region (NTR) turns out to have several roles in regulating gene expression and to be important in immortalization and transformation with some, but not all, cytoplasmic oncogenes. The regions and interactions of the first exon are better characterized than those of the second exon, at this time. However, several functions have been assigned to the C-terminus of the protein and are under investigation. E1A may function at various control points during the cell cycle, which may be regulated by its phosphorylation status. The E1A proteins have many phosphorylation sites (serines and threonines) that can be phosphorylated by cyclin-dependent kinases, such as cdc2, cdk2 and cdk4, at true phosphorylation sites.

The 12S oncoprotein positively and negatively regulates the expression of cellular genes. It can thus redirect the metabolism of the host cell to bring about the transformed state. There are no data demonstrating that E1A proteins can directly interact with regulatory sequences in promoters of E1A-sensitive genes. The overall mechanism by which E1A affects both viral and cellular gene expression is by various regions or peptides in the E1A protein making contact with general transcription factors and/or gene-specific factors. In fact, in some cases, an E1A peptide serves as a bridge between these two classes of transcriptional regulatory proteins. E1A-sensitive sites include activating transcription factor (ATF) and TATAA sites in promoters and E2F and AP-1 enhancer sites.

Protein Complexes and Gene Expression

Several polypeptides have been shown to complex with E1A proteins, directly or indirectly (**Fig. 1**, **Tables 2** and **3**). Those most consistently and readily found include proteins with molecular masses of 400, 300, 130, 107, 105, 60 and 33 kDa (referred to as p400 etc.) and associate with peptides encoded by exon 1, which encodes the N-terminal 'half' of E1A. The identity of many of them has been made. More sensitive methods have led to the identification of additional protein interactions, including the first identification of a protein partner for the C-terminus, p48 CtBP, which interacts with CR5. CtBP has homology to NAD-dependent dehydrogenases. E1A immortalizing and co-transforming abilities are determined primarily by binding to cell cycle or transcriptional regulatory proteins and interfering with their functions. Mutants that are defective for binding to these cellular proteins are also defective for immortalization and cotransformation with E1B. The cell cycle regulatory proteins bind CR1 and CR2 and include the tumor suppressor pRB (p105; retinoblastoma gene product) and its family (nuclear pocket protein family) members p107 and p130, cyclins (p60 cyclin A and E; cdk regulatory proteins) and a cyclin-dependent kinase (cdk2, p33). The cyclins and cdks form complexes with E1A and p107 or p130.

Table 2 Cell cycle regulators affected by E1A

Positively	*Negatively*
E2F-DP1	p53
cyclinA	pRB family
cyclinE	cyclinD1
cdk2	cdk inhibitors
cdc25A phosphatase	p16 INK
p70S6kinase	p21 WAF/CIP/SDI
UBC9	p27 KIP
fos	TGFβ

The importance of pRB in immortalization/co-transformation is further underscored by the fact that it has not been possible to produce an E1A mutant that is defective for pRB binding while maintaining this phenotype intact. The importance of pRB and the other proteins in E1A-mediated immortalization and cotransformation is also supported by the observation that proteins of other DNA tumor viruses, namely the large T-antigens of SV40 and polyomavirus, and the E7 of human papilloma virus (HPV), also bind to pRB, p107 and p130 and all of these viral gene products are able to bring about immortalization and transformation of primary cells. Kinase activity has been detected in E1A complexes and probably derives from p60 cyclinA/E linked p33 cdk2, associated with p107 and p130, but not pRB. Phosphorylation of E1A and the other members of the complex may contribute to their functions in cell cycle regulation. In fact, phosphorylation of pRB also inactivates its tumor suppressor function. Thus, E1A has two means by

Table 3 Gene regulatory proteins affected by E1A

Regulatory protein	*Activator/repressor*	*E1A effect*	*E1A region*
Direct interaction with E1A			
p300/CBP	Activator	Repress	NTR
Dr1	Repressor	Repress	NTR
RB family	Repressor	Repress	CR1, CR2
RARβ	Activator	Activate	CR3-C
TBP (TFIID)	Activator	Activate	CR3-N
AP-1/ATF	Activator	Activate	CR3-C
c-jun	Activator	Activate	CR3-C
Sp1	Activator	Activate	CR3-C
USF	Activator	Activate	CR3
BS69	Activator	Activate	CR3
YY1	Repressor	Repress	CR3
Indirect interaction with E1A			
TBP (TFIID)	Activator	Repress	NTR
p53	Activator	Repress	NTR
TBP	Activator	Repress	NTR
ATF/CREB	Activator	Repress, Activate	NTR, CR1
c-jun	Activator	Repress	NTR, CR1
c-fos	Activator	Repress	NTR, CR1
AP-1, AP-2	Activator	Repress	NTR, CR1
YY1	Repressor	Repress	NTR, CR3
c-jun/ATF-2	Activator	Activate	CR1
E2F-DP	Activator	Activate	CR1, CR2
MYOD	Activator	Repress	CR1, CR2
Myogenin	Activator	Repress	CR1, CR2
BRG	Repressor	Repress	CR1, CR2
UBF	Activator	Activate	CR1, CR2
NFkB	Activator	Repress	spacer Ad12
COUP-TF	Repressor	Activate	spacer Ad12

which to inactivate pRB function. pRB regulates transcription during the cell cycle, by its underphosphorylated form binding to transcription factors, especially E2F/DP1 factors (**Fig. 2**). E2F1/DP1 are cell cycle regulating transcription factors. They activate genes whose products are required for S phase progression. During most of G_1, E2F/DP1 is repressed by pRB, which complexes with the activation domain and prevents E2F transactivation. Phosphorylation of pRB and/or its sequestration by E1A, releases E2F and enables it to bind to its cognate sequence in the promoters of the E2F-sensitive genes. E2F is also affected by other cell cycle regulators, such as p53, cdk2, cdk4 and some cyclins. Overexpression of E2F, itself, can be transforming, presumably by constitutively activating S-phase genes. E1A also competes for binding with pRB with RIZ, a 250 kDa nuclear, Zn finger, pRB binding protein with structural and antigenic homology to the E1A region concerned with RB binding and the C-terminus, which are juxtaposed in RIZ. UBC9, which has homology to ubiquitin-conjugating enzymes interacts with CR2 in both AD5 and 12 E1A proteins. It complements a cell cycle defect in the UBC9 gene in yeast and therefore may play a role in cell cycle regulation that can be usurped by E1A.

The extreme N-terminal 80 amino acids, the nonconserved NTR, also binds several proteins that are involved in regulating transcription, positively and negatively. The N-terminus is also involved in immortalizing/transforming functions. p400, p300 and CBP (CREB binding protein) directly bind the N-terminus and part of CR1 of E1A from both Ad5 and Ad12. p300 and CBP are homologous transcriptional adapters or co-activators and histone acetyltransferases, which are thought to enable passage of

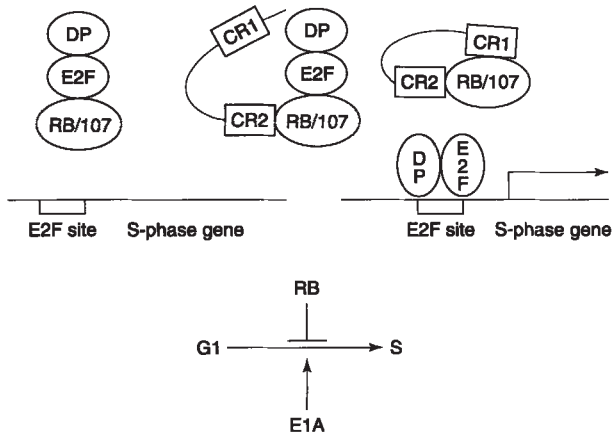


Figure 2 Model of the pRB pathway of gene activation by E1A 12S. CR1 and CR2, indicate the regions of E1A involved in making contact with the pocket protein family members. For details see text.

the BTC through chromatin. They participate in cell cycle arrest, mesenchymal cell differentiation, transactivation and co-activation of CREB [cAMP response element (CRE) binding protein]. p300 is a common mediator protein by which E1A can access multiple transcriptional regulatory pathways, including sequence specific transcription factors, such as YY1 (a transcriptional repressor), CREB/ATF (activating transcription factor) and the jun proteins of the AP-1 transcriptional complexes. p300 also bridges E1A with components of the basal transcriptional machinery (BTC), such as TFIIB. p300 and CBP form *in vivo* complexes with TBP (TATA binding protein) (**Fig. 3**). They interfere with cellular processes controlling cell cycle progression, which is needed for viral DNA replication. The E2F1 activation domain has sequence similarity with the N-terminal domains of 12S, which contains the CBP binding site and pRB binding site. CBP also interacts with E2F activation domain. The E1A NTR eliminates CBP-induced activation of E2F/DP1, thus CBP is a coactivator for E2F. The convergence of RB and CBP on E2F regulation helps explain the cooperativity of these proteins in carrying out E1A functions.

The TBP, which is part of TFIID of the BTC is also targeted by N-terminus of E1A, directly, resulting in transcriptional repression by blocking the interaction between TBP and the TATA box. This can be overcome by excess TFIIB. Thus, TBP interacts with E1A in two regions: CR3 transactivator and the N-terminal 35 amino acids. CR3 has two subdomains that interact with several transcription factors involved in activation of gene expression required for the lytic cycle of the virus and may contribute to the immortalizing/transforming functions of E1A (**Table 3**). The N-terminal region of CR3 interacts with TBP and the C-terminal domain interacts with ATF/CREB transcription factors. E1A complexes with the retinoic acid receptor (RAR), but not RXR, resulting in activation of the RARβ promoter in the presence of RA. This is mediated by CR3, which acts as a cofactor stabilizing the preinitiation complex with RAR and TBP. BS69 is a novel protein that binds to CR3 and inhibits its transactivation ability.

CtBP is a cellular phosphoprotein that complexes with six amino acids of CR 5 in the C-terminus of 12S, just upstream from the C-terminal NLS. This region is required for immortalization, cotransformation with E1B and negatively regulates ras transformation. Deletion of CR5 (and CR4) results in the metastasis of E1A+ras transformed cells. CR5 seems to control CR1-dependent activation. Thus, the C-terminus may down-modulate CR1 transcriptional regulation, via p48 binding. Perhaps CtBP regulates E1A immortalization/cotransformation by affecting CR1-regulated transcription. However, no differences in cell cycle activation or complex formation with E1A N-terminus binding partners are observed with CR4 or CR5 deletions. There is, however, a loss of maintenance of the proliferative response and consequently, the loss of immortalization in CR4 and CR5 deletions.

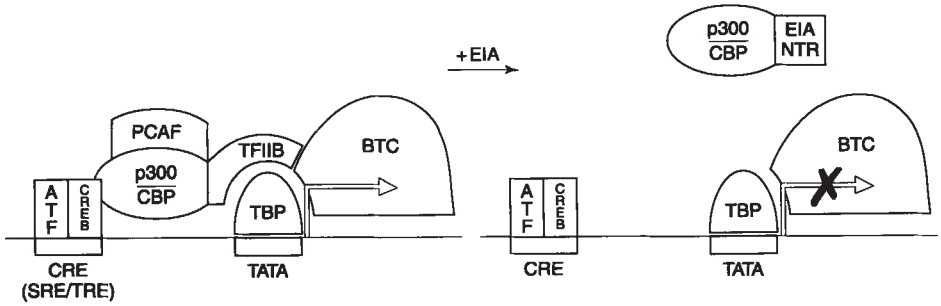


Figure 3 Model of the p300 pathway of repression of gene expression by E1A. E1ANTR indicates the region of E1A involved in contacting p300/CBP. For details, see text.

The bZIP proteins are transcription factors that contain a basic domain that interacts with DNA and a leucine zipper that dimerizes with other ZIP proteins. They regulate many different target genes with a broad range of consequences for the cell. Two of the subfamilies, AP-1 (activating protein)/ATF (activating transcription factor) and ATF/CREB are affected by E1A. The AP-1 family includes AP-1, jun and fos. The ATF/CREB family contains ATF1-4, CREB and CREM. These two subfamilies bind similar consensus DNA response elements [CRE (cAMP), S(serum)RE, T(TPA)RE)] and they can heterodimerize among themselves. There are altered repertoires of the transcription factors in E1A-expressing cells, which can generate novel transcription potentials. E1A affects c-jun, junB, c-fos, ATF-2, ATFa and ATF3. The expression of c-jun, junB and ATF3 is upregulated. A novel dimer between ATF3 and c-jun has been observed. E1A can activate or inhibit c-jun containing dimers depending on the dimeric partner. CR1 activates ATF-2 promoters that have jun1 and jun2 sites. c-jun can also associate with CBP, which can then be repressed by the E1A NTR and CR1. p300 interferes with c-jun/c-jun and c-jun/c-fos dimers. E1A 12S binds to AP-2 via a p300-independent region of the N-terminus. The relationship of all these transcriptional effects and immortalization and cotransformation has not been elucidated.

Cell Cycle Activation and Proliferation

E1A is able to overcome many growth inhibitory conditions. Ad normally infects quiescent cells and E1A, being an immediate early viral gene product, encodes functions to activate cells into the cell cycle, even in the absence of serum. These are accomplished by CR1, CR2 and the NTR in the first exon. These same regions are required for immortalization and cotransformation. E1A inhibits the breakdown of the E2F transcription factors that regulate cell cycle as well as releasing pocket protein (pRb)-E2F complexes (see above). The binding of CR2 to p107, but not to p300 or pRB, counteracts the p107-mediated repression required to activate transcription of cyclin A. This contributes to E1A being able to override negative growth controls. E1A CR1 enhances the expression and activity of the mitogen-inducible p70 S6 kinase (p70s6k), which is essential for G_1 progression.

Proliferating nuclear antigen (PCNA) is an E1A12S-inducible factor linked to DNA replication and cell cycle progression. Its promoter contains a 12S-sensitive, *cis*-acting element (PERE) that confers 12S inducibility. PERE binding proteins include ATF-1 (activating transcription factor) and CREB, which form heterodimers. CBP binds to CREB. Thus, 12S transactivates the PCNA promoter via the CBP-CREB-PERE pathway.

TGFβ is a regulator of proliferation and differentiation. It is a growth inhibitor for epithelial, endothelial, lymphoid and myeloid cells. E1A, especially the 12S protein, can overcome the growth inhibitory effects of TGFβ. This is due to the sequestration of pRB that blocks induction of cdk inhibitors such as p15/INK4, p21/WAF/CIP/SDI and p27KIP1 by TGFβ and provides another mechanism by which to override negative regulation of cell cycle progression. E1A also blocks transcriptional activation by TGFβ and this is dependent on the p300 interaction. E1A also binds to KIP and blocks its inhibitory effect, thereby restoring the activity of cyclin-cdk2 kinase and -cdk4 in TGFβ-treated cells. junB induction by TGFβ is reduced. E1A induces transcriptional downregulation of the TGFβ type II receptor due to decreased expression of transcription factor complexes that interact in the promoter at PRE1 and PRE2 sites.

The p53 tumor suppressor induces cell cycle arrest or apoptosis, depending on other conditions. Overcoming p53 and apoptosis are essential to the transformation pathway. E1A represses p53 transcriptional activation and abolishes p53 effects, including p53-mediated transactivation of the cdk inhibitor, p21/WAF/CIP/SDI, MDM2 or bax, a pro-apoptotic member of the bcl-2 family (*see also* **Apoptosis and virus infection**). This probably contributes to the ability of E1A to overcome apoptosis and effect immortalization/transformation. Repression of p53 effects requires the p300/CBP binding domain in the NTR.

Different cell types (neurons, skeletal and heart myocytes, adipocytes and keratinocytes) undergo terminal differentiation, which requires definitive withdrawal from the cell cycle. This is probably due to the induction of p21 WAF/CIP/SDI. Terminally differentiated cells are postmitotic and cannot be growth factor-stimulated. In addition to inducing the proliferation of reversibly growth arrested (quiescent) established or primary cells, 12S can induce full reentry into the cell cycle, including DNA synthesis, mitosis, cytokinesis and extended proliferation in otherwise permanently non-mitotic cells. p300 activates p21 expression and E1A binding to p300 represses it co-activator function, which enables E1A to overcome the block to cell cycle progression.

Immortalization

Normal primary cells exhibit a limited life span in culture and eventually undergo senescence. Immortal-

ization of cells, whereby the cells escape from senescence and apoptosis and acquire unlimited proliferative potential, has been considered an important and an early event in multistep tumorigenesis. Several studies indicate that immortalization itself is a multistep process. E1A 12S has been shown to be able to immortalize a variety of cell types (epithelial, endothelial and neuronal). These cells maintain many of their structural and ultrastructural and differentiated characteristics. Continuous expression of E1A is required for these cells to survive. E1A immortalized cells do not grow in soft agar or induce tumors in even nude mice or syngeneic animals. The various functions required for immortalization have been mapped to five regions, located in both exons of the 12S gene. Regions CR1 and CR2 in the first exon are necessary to activate quiescent cells into the cell cycle and increase the number of population doublings, however, they are insufficient to maintain the cells in a proliferative mode. The extreme N-terminus is also required, and its absence results in an earlier loss of proliferation, but the mechanism remains unclear. It most probably involves p300/CBP interactions and inhibition of p53 suppression of proliferation. Coexistence of strong E1A stimulation of the cell cycle and a conflicting growth suppressive signal can lead to apoptosis. Expression of the first exon (NTR + CR1 + CR2) enables primary cells to escape the M_1 phase of mortality, but not the M_2 phase and thus cannot extend their lifespan. The NTR, CR1 and CR2 are also required for cotransformation with E1B and *ras*. Expression of CR4 and CR5, in the second exon of 12S, is required to enable the cells to also overcome the M_2 mortality block and thus escape from senescence and become immortalized. The second exon seems to be required for performing a late crucial, function in immortalization, involving the reactivation of the cell cycle. Immortalization-competent 12S gene products induce the expression of a novel 110 kDa protein, p110, that forms a stable complex with rasGAP. Failure to induce the p110-rasGAP complex results in the concomitant loss of ability of 12S to immortalize primary epithelial cells. p110 induction is independent of the ability of 12S to activate the cell cycle and of the presence of adenovirus E1B and is not observed in the presence of the large T antigen of SV40. Thus, it is not a general response to proliferation or tumorigenic transformation, but rather seems to be specific to the immortalization function(s) of E1A 12S. The only identified protein to interact with the C-terminus is CtBP, which interacts with six amino acids in CR5. Efficient nuclear localization is required for immortalization and cotransformation with E1B, but not cotransformation with Ha-ras or v-src. A five amino acid nuclear localization signal (NLS) is found at the very C-terminus, and is part of CR5. Regions upstream of the NLS are also required for efficient nuclear targeting. Cooperation with E1B requires all the same E1A regions as immortalization by E1A, which is not surprising since the two genes have co-evolved.

Cotransformation

Expression of E1A on its own cannot bring about tumorigenic transformation, but rather enables the unlimited growth potential of cells, or immortalization. This is consistent with its function as a stimulator of the cell cycle in the virus lytic cycle. Coexpression of E1A with some viral or cellular oncogenes that cannot bring about transformation of primary cells on their own can result in transformed cells that can now grow in soft agar and form tumors in nude mice or syngeneic animals. These include AdE1B, Polyoma middle T, v-src and Ha-ras, but not v-raf, v-mos, V12rac, or V12cdc42. The eukaryotic translation initiation factor EIF-4E, which regulates the efficiency of translation, can transform in the presence of E1A or v-myc. Expression of antisense E1A in these transformed cells results in their apoptosis, thus they are continuously dependent on E1A for their viability. Individual oncogenes require different functions of E1A for cotransformation. E1B requires all the same regions and functions as immortalization does. Ha-ras only requires CR1, CR2 and the NTR and v-src only requires CR1 and CR2.

Oncogenicity

The highly oncogenic Ad serotypes of subgroups A (Ad12) and B (Ad3) are tumorigenic in immunocompetent animals, whereas the other serotypes are not. Ad12E1A suppresses multiple genes important for antigen processing and presentation. There is suppression in the transport and surface expression of all MHC class I antigens. There is a reduction in the TAP1 and TAP2 peptide transporters and proteasome associated gene expression. In addition, β_2-microglobulin is retained in the cell free of class 1 antigen, further subverting the major histocompatability complex. This enables the immunoescape of the Ad12-infected cells from CTL recognition and their development into tumors. Ad12-infected cells are also resistant to NK lysis. Ad12 E1A has a unique peptide segment that is contiguous with and separates CR2 and CR3. This unique peptide is also in the highly oncogenic simian adenovirus 7 E1A, but is not present in E1A from the non-oncogenic Ad5. This spacer region is an oncogenic determinant because it targets the MHC class I transcriptional enhancer and down-

regulates it. This enables immunoescape and, consequently, *in vivo* tumor formation. There is repression of MHC due to the loss of binding of the activator NFkB (p50/p65) to the region I enhancer (R1 element, H2TF1) and increased binding of COUP-TF (an orphan hormone receptor protein and a repressor) to region II (R2 element, CREII, class I-regulatory element II). There are not decreased levels of NFkB. Ad5 E1A has no such effects. In addition, CTL epitopes are encoded by Ad5, but not Ad12 E1A, further assisting immunoescape by Ad12 E1A expressing cells.

Differentiation

E1A has been shown to have two very different effects on cellular differentiation and these involve different regions of the gene. E1A12S can immortalize a variety of cell types (epithelial, endothelial and neuronal) that maintain their structural and differentiated phenotypes. E1A promotes epithelial characteristics in some tumor cell types, thereby partially reversing/suppressing the transformed phenotype, or at least diminishing the more aggressive growth characteristics and this has been attributed to CR4 and CR5. Mutations in the C-terminus result in the aberrant localization and loss of function of several cell–cell junctional complexes, including adherens junctions, which are cell–cell adhesion complexes that are hallmarks of epithelial differentiation. Loss of adherens junction function contributes to the progression of tumors to a more malignant phenotype. Introduction of an intact 12S gene suppresses this phenotype, without altering the proliferative potential of the cells. Similar results have been obtained with retinoic acid, a known inducer of differentiation, but this is accompanied by a slowing of cellular proliferation. E1A has been shown to induce differentiation of F9 teratocarcinoma and P19 embryonal carcinoma (EC) cells. An E1A-like activity in EC cells is required for differentiation by retinoids. c-jun is upregulated in response to RA or E1A during differentiation of F9 cells.

E1A inhibits terminal differentiation of skeletal and cardiac myoblasts and can reactivate the cell cycle in terminally differentiated skeletal muscle cells and suppress myocyte-specific expression. Expression of the myogenic, basic helix-loop-helix transcription factors, myogenin and MyoD also maintain cell cycle arrest. These transcription factors are repressed by E1A, but Myf5 is not. B-lymphocyte differentiation can also be inhibited by E1A. These cell types, whose differentiation can be inhibited by E1A, are of mesenchymal and not epithelial origin. This E1A phenotype has, in general, been attributed to the p300/CBP binding domain of the N-terminus. A role for CR1 has also been reported. It is also possible that the powerful stimulation of proliferation by the E1A N-terminus (NTR + CR1 + CR2) is antagonistic to such terminal differentiation and that this contributes to the loss of terminal differentiation in these cell types. Epithelial cells are more likely to be quiescent and able to be stimulated to proliferate without losing their differentiated properties. It is also possible that the presence of an intact C-terminus, which seems to be involved in maintaining epithelial characteristics, could participate in the 'epithelialization' or the prevention of a non-epithelial phenotype. The effect of C-terminal mutations on the inhibition of differentiation of mesenchymal cells has not been investigated.

Modulation of Transformation: Tumor and Metastasis Suppression

E1A is an immortalizing oncogene. Paradoxically, E1A has been shown to suppress transformation, *in vivo* tumorigenicity and metastasis, and consequently has also been considered an anti-oncogene or tumor suppressor. Cells transformed with a wild-type E1A give rise to cells that are transformed, but are equivalent to benignly transformed cells. They do not metastasize in immunocompromised rodents. The highly metastatic human melanoma cell line, BLM, is suppressed by E1A, along with the coordinate repression of a series of progression markers. Reduced oncogenicity in nude mice is also observed. Matrix metalloproteinases are important in the invasion and metastasis of tumor cells. E1A inhibits expression of matrix-degrading proteases, which are needed for cells to degrade the ECM and metastasize. This occurs through interfering with the DNA-binding capacity of cjun/cjun and cjun/cfos dimers. This requires CR1 and CR3. WT E1A suppresses ras and neu transformation. Neu (c-erbB, HER2) commonly occurs in human cancer and enhances tumor metastasis and chemoresistance. This can be suppressed by E1A and requires the NTR and CR1, but does not require CR2. This region of E1A represses neu overexpression by repressing promoter activity. E1A 12S reduced the anchorage-independent and tumorigenic growth of a variety of tumor cell types, but not their normal culture growth properties. E1A represses expression of the CD44 adhesion protein, which has been shown to confer metastatic potential. E1A can induce NM23, a nucleoside diphosphate kinase and a marker of low metastatic ability. E1A also promotes epithelial characteristics in some tumor cell types, thereby partially reversing/suppressing the transformed phenotype or at least diminishing the more

aggressive growth characteristics and this has been attributed to the C-terminus. Mutants that fail to bind to CtBP also give rise to metastases with an activated ras, whereas WT12S does not. It would appear that the region of E1A required for induction/maintenance of epithelialization and suppression of transformation progression, at least with ras, are the same. It is possible that the C-terminus could also contribute to the inhibition of non-epithelial differentiation pathways. The C-terminus of E1A 12S could be regulating epithelial–mesenchymal transitions (EMT) and deletions in this region result in EMT, contributing to tumor progression. EMT has been attributed to tumor progression.

Conclusions

E1A is a complex, multifunctional protein. It can affect a broad range of processes in cells. Analysis of E1A and its mechanisms of controling the cell has provided a vast amount of insight into the workings of the cell, from cell cycle to transcriptional regulation. It has led to the identification of many cellular proteins involved in these processes and to an understanding of how they function. E1A has provided and continues to provide a very fruitful model system to dissect proliferation, immortalization, tumorigenic transformation, metastasis, differentiation and transcriptional regulation. At times it seems as if E1A elicits contradictory behaviors. Some of these paradoxes may be a reflection of the multitude of different experimental systems in which it is used. But in all these cases, insights into the workings of cells, normal and otherwise, have been obtained. Underlying all the observed functions must be a unifying theme: to facilitate viral reproduction, since that is the function of any and all viral genes. It is important to keep this in mind when we interpret experimental results.

See also: **Adenoviruses (*Adenoviridae*): Animal viruses, General features, Molecular biology; Apoptosis and virus infection; Immune escape mechanisms; Retroviral Oncogenes; Papillomaviruses – human (*Papovaviridae*): General features, Molecular biology; Simian virus 40 (*Papovaviridae*); Transformation: Animal viruses; Tumor viruses – human.**

Further Reading

Bayley ST and Mymryk JS (1994) Adenovirus E1A proteins and transformation. *Int. J. of Oncol.* 5: 425.

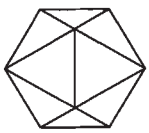
Doerfler W and Bohm P (eds.) (1995) *The Molecular Repertoire of Adenoviruses III: Biology and Pathogenesis*. Berlin: Springer-Verlag.

Hagmeyer BM, Angel P and van Dam H (1995) Modulation of AP-1/ATF transcription factor activity by the adenovirus E1A oncogene products. *BioEssays* 17: 621.

Jansen-Durr P (1996) How viral oncogenes make the cell cycle. *Trends Genet* 12: 270.

Mymryk JS (1996) Tumor suppressive properties of the adenovirus 5 E1A gene. *Oncogene* 13: 1581.

AFRICAN SWINE FEVER VIRUS (*ASFARVIRIDAE*)



María L Salas, Centro de Biología Molecular 'Severo Ochoa' (CSIC-UAM), Universidad Autónoma de Madrid, Madrid, Spain

History and Geographic Distribution

In 1921 Montgomery described African swine fever (ASF) as a peracute disease of domestic pigs caused by a virus. The disease was first recognized after the introduction of domestic pigs to Kenya in 1910. Montgomery established the likely transmission of ASF from wild swine and reported the lack of protection by passive immunization.

In 1957 the disease appeared for the first time outside Africa, in Portugal, and in 1960 spread to Spain. In the 1960s and 1970s the virus was found in France, Italy, Sardinia and Malta and, in America, in Cuba, Brazil, Dominican Republic and Haiti. In 1985 there was an ASF outbreak in Belgium and in 1986 in The Netherlands. Today, ASF is enzootic in sub-Saharan Africa and Sardinia.

Taxonomy and Classification

ASF virus (ASFV) is a deoxyvirus with an icosahedral morphology that multiplies in the cytoplasm of the infected cell. Because of these characteristics, the virus

urges an evaluation of the possibility to develop protective vaccines that are effective also in young children. The extended application of bone marrow, liver and kidney transplantation and improved care of immune-compromised patients demands introduction of rapid methods for the detection and typing of adenovirus infections. Subsequently development of immunoprofylaxis in the form of safer vaccines to be used before transplantation may become justified. Epidemiological surveillance is required to detect re-emerging and previously unrecognized genome types as has been observed with Ad7, Ad19, Ad21 and Ad41.

See also: **Adenoviruses (*Adenoviridae*): Molecular biology; Immune response: General features; Transplantation and virus infections; Vaccines and immune response.**

Further Reading

Doerfler W (ed.) (1983 and 1984) The molecular biology of adenoviruses 1, 2 and 3. *Curr. Top. Microbiol. Immunol.* vols 109, 110, 111.

Ginsberg HS (ed.) (1984) *The Adenoviruses*. New York: Plenum Press.

Horwitz MS (1990) Adenoviruses. In: Fields BN *et al.* (eds) *Virology*, 2nd edn. p. 1723. New York: Raven Press.

Mautner V (1989) Adenoviridae. In: Porterfield JS (ed.) *Andrewes' Viruses of Vertebrates*, p. 249. London: Baillière Tindall.

Smith GM (1998) Adenovirus-mediated gene transfer to treat neurologic disease. *Archives of Neurology* 55: 1061–4.

Wadell G (1990) Adenoviruses. In: Zuckerman AE, Banatvala JE and Pattison JR (eds) *Principles and Practices of Clinical Virology*, 3rd edn. Academic Press.

[This article is reproduced from the 1st edn (1994).]

Molecular Biology

Jörg Schröer, Institute of Genetics, University of Cologne, Koeln, Germany

Stefan Kochanek, Center for Molecular Medicine, University of Cologne, Koeln, Germany

Marianne Hösel, Institute of Genetics, University of Cologne, Koeln, Germany

Walter Doerfler, Institute of Genetics and Center for Molecular Medicine, University of Cologne, Koeln, Germany

Introduction

For a long time, adenoviruses have served as excellent tools for detailed studies on basic molecular mechanisms in mammalian cells, on the mechanism of viral oncogenesis and on gene transfer in mammalian organisms. In this brief article, it will not be possible to cover all of the recent progress accomplished in adenovirus research in a sensible way. We have therefore chosen to summarize current research on the following topics:

- The fate of viral DNA in mammalian cells.
- The abortive infection of hamster cells with human adenovirus type 12 (Ad12).
- Viral DNA integration and oncogenesis.
- A third generation adenovirus gene transfer vector.

References to more comprehensive surveys on ongoing adenovirus research have been listed under Further Reading.

Fate of Viral DNA in Mammalian Cells

The fate of foreign DNA in mammalian cells is of general biological interest. Foreign DNA can integrate into the genomes of mammalian cells, and this process plays major roles in viral oncogenesis and in the generation of transgenic organisms and will be important in evolving regimens for human somatic gene therapy. At least in cell culture, foreign DNA can be taken up by mammalian cells under appropriate conditions or in a stabilized conformation, e.g. in a complex with basic proteins.

Human adenovirus type 12 (Ad12) infects human cells productively and leads to viral DNA replication, whereas infection of Syrian hamster cells remains abortive with total blocks in viral DNA replication and late viral gene transcription. In abortively Ad12-infected BHK21 cells, the viral DNA can be integrated into the cellular genome early after infection. Viral oncogenesis by Ad12 has been demonstrated in

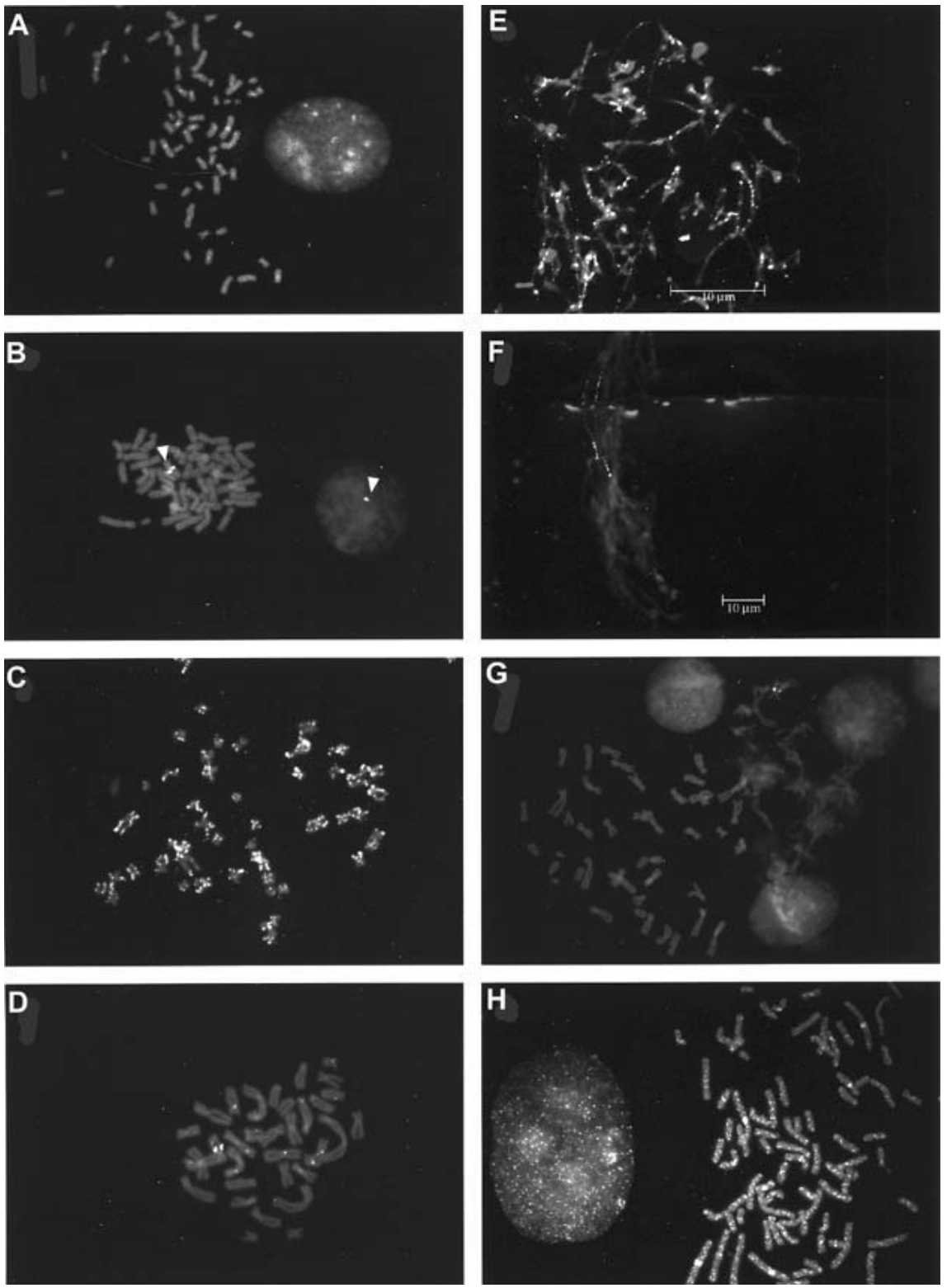


Figure 1 Association of Ad12 DNA with host cell chromosomes. (**A**) Human HeLa cells, uninfected control. (**B**) T637 cells, uninfected: arrowhead points to chromosomally integrated Ad12 genomes. (**C**) Productively Ad12-infected HeLa cells, 6 h p.i. (**D**) Abortively Ad12-superinfected T637 cells, 6 h p.i. (**E**) Productively Ad12-infected HeLa cells, 6 h p.i. (stretched chromosome preparation). (**F**) Ad12-transformed T637 cells (stretched chromosome preparation). (**G**) BHK21 cells, Ad12 DNA added to the medium. (**H**) BHK21, Ad12-DNA-TP added to the medium. Original magnification ×1250 (**A–D, G, H**); (**E, F**) see bar. (Reproduced with permission from Schröer *et al* (1997).) **(For color references see Color Plate 1.)**

newborn hamsters. In Ad12-transformed or Ad12-induced hamster tumor cells, the viral genome is found exclusively in a chromosomally integrated form.

The abortive infection of Syrian hamster cells with Ad12 facilitates monitoring the fate of viral DNA without interference by large amounts of newly synthesized Ad12 genomes. It is possible to introduce the viral DNA via different pathways. Cells can be naturally inoculated with the virus, the viral DNA can be transfected into the cell or, very gently, DNA can be directly added to the maintenance medium, either in protein complexes or as isolated DNA. The fate of Ad12 DNA at the single cell level can be followed by fluorescent *in situ* hybridization. This technique is sensitive enough to detect single copies or even fragments of an Ad12 genome per cell and to localize the foreign DNA in the nucleus and on cellular chromosomes.

Productive infection of human cells and abortive infection of hamster cells with Ad12

The inoculation of human or hamster cell lines with Ad12 leads to the transport of viral DNA into the nuclei of the cells and to the extensive association of viral DNA with the host cell chromosomes as early as 2 h postinfection (p.i.). Chromosomal association appears to be more efficient in productively infected permissive human cells (**Fig. 1C, E**) than in abortively infected nonpermissive hamster cells. Upon Ad12 superinfection, the Ad12-transformed Syrian hamster cell line T637, which carries multiple copies of integrated Ad12 DNA in a single chromosomal location (arrowhead in **Fig. 1B**), also exhibits extensive, apparently random chromosomal association of the superinfecting Ad12 genomes (**Fig. 1D**). The chromosomal association of adenoviral DNA remains stable even under conditions of high ionic strength or detergents and resists chromosomal stretching followed by mild fixation.

Transfection of human or Syrian hamster cells with Ad12 DNA

The application of the standard Ca^{2+}-phosphate precipitation method also leads to chromosomal association of the foreign DNA but seems to be less advantageous for cell survival and continuous growth.

Direct addition of Ad12 DNA to the maintenance medium

When Ad12 DNA is directly added to the growth medium of BHK21 hamster cells, an association of adenoviral DNA with the host cell chromosomes can also be documented (**Fig. 1H**), without any visible alteration of the thus treated cells. When plasmid DNA or bacteriophage M13 DNA are added, no significant chromosomal association can be observed. Chromosomal association of Ad12 DNA is markedly increased when Ad12 DNA still carrying the covalently linked terminal protein (Ad12-DNA-TP) is added to the medium. This finding raises the question of which function the terminal Ad12 protein might serve in the chromosomal association and integration of viral DNA.

Long-term studies on the chromosomal association of Ad12 DNA in abortively infected hamster cells reveal that the majority of the viral DNA will be eliminated from the cells after 90 h p.i. The loss of viral DNA may be due to a selective growth disadvantage of hamster cells carrying the foreign genome due to damages caused by the massive integration/association of foreign DNA and/or to an inherent instability of the chromosome-associated or integrated Ad12 DNA molecules. Stable integration of foreign DNA and concomitant survival of cells are probably rare events. The extensive association of Ad12 DNA with chromosomes of permissive human or nonpermissive hamster cells suggests a pathway for the interaction of foreign DNA with the chromatin of the target cells. In selected instances and at appropriately structured chromosomal sites, e.g., transcriptionally active regions, the association of foreign DNA may lead to its covalent linkage to the target cell genome.

Abortive Infection of Hamster Cells with Ad12

Upon the abortive infection of hamster cells with Ad12, defects in cell surface adsorption, entry and transport of Ad12 to the nucleus do not appear to account for the abortive infection in BHK21 cells. Ad12 virions enter the cells and viral DNA reaches the nucleus, where it can be integrated into the cellular genome. Early Ad12 mRNAs are synthesized in abortively infected hamster cells. *In vitro* translation studies have shown that most of the early Ad12 mRNAs isolated from BHK21 cells can be translated *in vitro* into viral proteins that are similar to the proteins translated *in vitro* from Ad12 mRNAs which have been isolated from productively Ad12-infected human cells. However, *in vitro* translation of Ad12 mRNAs isolated from Ad12-infected hamster cells reveals the lack of a 34 kDa protein, which is produced when mRNAs from productively infected human KB cells are translated. Moreover, in immunoprecipitation analyses of cellular extracts, Ad12 E1A-

specific proteins are detectable at 6 h p.i. in KB cell extracts, but not before 12 h p.i. in BHK21 cell extracts. The concentrations of Ad12 E1A and E1B proteins in abortively infected BHK21 cells are 6- to 20-fold lower than in productively infected KB cells. Although early genes of Ad12 are expressed in Ad12-infected BHK21 cells, the replication of Ad12 DNA is completely blocked.

Upon productive adenovirus infection, viral DNA replication is initiated within the inverted terminal repeats (ITRs) of the linear genome and proceeds by a strand displacement mechanism. Three viral gene products encoded in the E2 region of adenoviral DNA, the DNA-binding protein (DBP), the preterminal protein (pTP) and the adenoviral DNA polymerase (Ad pol), participate in adenoviral DNA replication. The initiation of adenoviral DNA replication is dependent on a protein-priming mechanism and requires pTP to be covalently bound to dCMP at the 5′-terminus. pTP must then interact with Ad pol. After this initiation reaction at either terminus, elongation proceeds from one or both termini to the opposite DNA end by the addition of nucleotides to the free 3′-OH group of the covalently bound dCMP. Elongation requires Ad pol and DBP. On the cellular side, nuclear factors I, II and III (NFI–NFIII) are essential for viral DNA replication. NFI and NFIII bind specifically to the origin of replication (ori) and form a stable preinitiation complex at the ori with the three viral proteins. NFII, which is related to topoisomerase I, is important for the elongation reaction. The activities of Ad pol and pTP for the initiation of DNA replication *in vitro* are equivalent in extracts from Ad12-infected BHK21 and KB cells. In contrast, the amount of Ad12 DBP is >100-fold lower in infected BHK21 cells than in infected KB cells. Thus, the reduced expression of the viral DBP may play a role in the mechanism of abortive infection of hamster cells by Ad12.

In BHK21 cells doubly infected by Ad2/5 and Ad12 or in Ad12-infected BHK297-C131 hamster cells, a cell line carrying and expressing the E1 region of Ad5, Ad12 DNA can replicate. On the other hand, the Ad12 early functions, although by themselves incapable of supporting the replication of Ad12 DNA in hamster cells, can substitute for the missing E1 functions in deletion mutants of Ad5 DNA, which are then capable of DNA replication and the completion of transcriptional and translational programs in permissive human cells. During complementation, the Ad2/5 E1 gene products may act directly on the Ad12 genome by substituting for the insufficient Ad12 E1 proteins in hamster cells. Ad12 recombinants in which the E1A and/or E1B regions are replaced with E1A and/or E1B of Ad5 are viable in human cells.

The levels of NFIII are markedly reduced in nuclear extracts prepared from mock-infected BHK21 cells as compared to complementing hamster BHK297-C131, or permissive human KB or HeLa cells. Upon the abortive infection of BHK21 cells with Ad12, nuclear extracts fail to show any change in the low NFIII levels. In contrast, Ad2 infection renders the levels of NFIII in BHK21 cells comparable to those in uninfected or Ad2-infected HeLa or BHK297-C131 cells. Another cellular factor which is required for efficient initiation of adenoviral DNA replication, NFI, does not appear to be limiting in the Ad12-BHK21 system. In productively Ad12-infected human cells, the structure of the promyelocytic leukemia (PML) protein arrays in the nuclei of infected human cells is markedly altered. Similar alterations have not been observed upon the abortive infection of hamster cells with Ad12. Since the redistribution of the PML protein is thought to be associated with adenoviral DNA replication in the nucleus, the absence of structural changes in the PML arrays may be related to the replication defect of Ad12 DNA in hamster cells.

The transcription of the late Ad12 genes and of the virus-associated (VA) RNA is also blocked in hamster cells. The major late promoter (MLP) of Ad12 does not function in Ad12-infected BHK21 hamster cells. The transcriptional defect of Ad12 DNA in hamster cells has thus been, at least partly, localized to the viral MLP. A 33 nt mitigator sequence, which is located in the downstream region of the Ad12 MLP, is responsible for the inactivity of the Ad12 MLP in hamster cells. Removal of this mitigator activates the Ad12 MLP in hamster cells and increases its activity in human cells. Because a similar negative regulator has not been found in the MLP of Ad2 DNA, the mitigator seems to be specific for the Ad12 MLP and might contribute to the abortive infection of Ad12 in hamster cells. The shutdown of major viral replicative functions may be a prerequisite for the malignant transformation of hamster cells by Ad12.

The late mRNAs of Ad12, which can be transcribed in the complemented system of Ad5-transformed BHK297-C131 cells, are polyadenylated, but are synthesized in reduced amounts as compared with the levels of Ad12 mRNAs in Ad12-infected human cells. The late Ad12 fiber gene-derived mRNA exhibits the authentic nucleotide sequence and carries the complete tripartite leader. Nevertheless, this RNA is not translated into fiber protein, even in the Ad5-complemented hamster cell system. It is therefore likely that cellular functions, either structural or enzymatic, which are required for the efficient translation of late Ad12 mRNAs, may also be missing in hamster cells. Thus, the abortive infection of

BHK21 cells with Ad12 is complex and related to the deficiency or altered activity of both viral and cellular functions.

Viral DNA Integration and Oncogenesis

The persistence of adenovirus DNA in an integrated state in hamster or other rodent cells is frequently associated with the transformed or oncogenic phenotype. In virus-transformed and virus-induced tumor cells, the integrated adenovirus genomes continue to be expressed. In general, early viral genes are transcribed and translated. Some of the early adenovirus proteins interact specifically with cellular proteins. The 289 amino acid protein encoded in the E1A region can bind to the gene product of the retinoblastoma (RB) gene, a tumor suppressor gene. Similarly, the 55 kDa protein encoded in the E1B region of the adenovirus genome has been shown to complex the p53 and similar cellular proteins. A strong case has been made for the notion that these specific interactions of certain adenovirus proteins with the products of cellular antioncogenes or tumor suppressor genes might interfere with the regulation of cell growth, in that the cells then override the apoptotic harnesses and are shifted on to a path of unlimited replication, possibly leading to tumor development. It remains to be proven that the sequestration of one or several tumor suppressor gene products in the cell would in fact suffice to transform a cell to the oncogenic phenotype. Cellular growth regulation seems to be subject to and safeguarded by several alternative pathways.

Viral DNA integration in Ad12-transformed or Ad12-induced tumor cells can have one or several of the following consequences for the cell whose genome has been targeted for foreign DNA insertion.

1. Insertion of foreign (viral) DNA leads to the stable and permanent genomic fixation of genes or DNA segments from sources outside the targeted cell or organism. Depending on the type of foreign genes inserted and its sequence environment at the site of insertion, the foreign DNA can be expressed, silenced, subjected to the cellular regulation or influence the regulation of neighboring cellular genes. Integrated foreign DNA in an established mammalian genome is in general *de novo* methylated extensively.
2. The cellular DNA sequences in the targeted cell that are directly affected by the insertion event could be silent or without an essential function. On the other hand, insertion might have a direct topical effect or, when a functional genetic center of the cellular genome is hit, the cell might not survive or alter its growth properties (insertional mutagenesis).
3. As a novel thought, we pursue the possibility that the insertion of foreign DNA into an established mammalian genome alters patterns of cellular DNA methylation, both at sequences close to and remote from the integrate. Such changes have been demonstrated in Ad12-transformed cells and in Ad12-induced tumor cells. Alterations of patterns in cellular DNA methylation have been shown to be associated with altered transcription patterns of the cellular genes affected. This model of the consequences of foreign DNA integration predicts *trans*-effects on regions of the cellular genome which might be located remote from the site of insertion. Alterations of the transcriptional program of the cell would amount to a novel type of functional insertional mutagenesis which would not be limited to genes at the site of integrated foreign DNA but might involve more extensive parts of the cellular genome.
4. The consequences of foreign DNA insertion on the overall chromatin structure of the targeted cell have not yet been investigated. Most frequently, foreign DNA molecules are inserted as multiple copies in pseudo-tandem arrays. With Ad12 as an example, up to 20, 30 or more copies of the viral DNA can thus be genomically fixed. With a genome length of 34 125 nucleotide pairs, 30 integrated copies of Ad12 DNA amount to the addition of >1 megabase of foreign DNA to the cell's highly organized genome. It is doubtful that perturbations of this magnitude can remain without functional sequelae for the cell.

Integrated Ad12 DNA can be lost from Ad12-transformed or Ad12-induced hamster tumor cells. At least in a few examples, the total or near-total loss of integrated foreign (Ad12) genomes has been shown to be still compatible with the maintenance of the oncogenic phenotype of the revertant cells. Detailed investigations on the presence of tiny segments of Ad12 DNA in some of the originally Ad12-induced hamster tumor cells by using the polymerase chain reaction method have revealed that different cells from this initially clonal population are losing the integrated Ad12 DNA at different rates. While some of the cells in the total cell population may have lost most or actually all traces of viral DNA integrates, others may still retain small Ad12 DNA segments. These results, which are based on the analyses of cell populations derived from clonal cells, argue in favor of a mechanism to eliminate the foreign DNA integrates that proceeds in a gradual, stepwise and clone-specific way. At present, it is unknown whether

these excisions are due to random events or the results of an active recognition and defense mechanism against foreign DNA in a mammalian genome. In this context, it is interesting to recall that in revertants of cell line T637, which have lost all but one or a few copies of the integrated Ad12 DNA, the remaining Ad12 DNA sequences are more heavily methylated than in the parent T637 cell line. This finding might imply that the levels of methylation in the integrated foreign DNA are somehow related to the ability of cellular defense mechanisms to recognize recently inserted DNA as foreign and lead to its elimination from the cellular genome.

In at least some of the Ad12-induced tumor cells, the continued persistence of integrated Ad12 DNA sequences is not a precondition for the maintenance of the oncogenic phenotype. A total population of between 10^2 and 10^7 revertant cells, which have lost almost all Ad12 DNA and are injected into hamsters to test for oncogenicity, is still sufficient to lead to tumor formation in the animals.

We have also demonstrated that the alterations in cellular DNA methylation patterns in the Ad12-transformed hamster cell line T637 persist in the revertant TR3, in which Ad12 DNA cannot be detected any longer by Southern transfer-hybridization experiments. It is thus likely that alterations in cellular methylation patterns and/or in cellular genome organization, which have been initially elicited by Ad12 DNA integration and viral genome products, are permanently imprinted onto the cellular genome.

Since cells originally transformed by Ad12 to tumor cells (like Ad12-induced hamster tumor cells) can maintain their oncogenic properties in spite of the loss of almost all of the integrated viral DNA from the cellular genome, the long-standing assumption that the human adenoviruses have no role in human oncogenesis has to be reconsidered. Obviously, failure to find adenoviral DNA sequences in human malignancies – even if it were confirmed with up-to-date technology – will not rule out a potential role of the virus or its genome, as it could have been lost from the human malignant cells.

A Third Generation Adenovirus Gene Transfer Vector

Somatic gene transfer has a considerable potential for the treatment of inherited or acquired diseases. Genetic diseases that are caused by loss-of-function mutations could be causally treated by introducing a functional copy of the defective gene into the appropriate tissue. Examples of genetic diseases that are good candidates for gene therapy are disorders like cystic fibrosis (CF), hemophilia A or B or hypercholesterolemia, disorders which are all caused by the absence or a low amount of a single functional gene product. In these diseases the pathogenesis has been characterized at the DNA level and the disease-causing mutations can be easily identified in each individual patient. Acquired diseases, like different types of cancer, are less well defined at the molecular level. Because of their frequency and their impact on public health, the interest in the development of gene therapy for these disorders is very high; hence, numerous clinical gene therapy trials have been conducted or have been approved. Nevertheless, so far there have been no unambiguous examples of the successful treatment of any human disease by gene transfer. Currently, one of the most significant reasons that prevent the broad application of gene transfer in the treatment of human diseases is the lack of safe and effective gene transfer technologies. Gene transfer encompasses the independent binding of the vector to the cell surface, its uptake through the cellular membrane, transport of the genetic material to the nucleus of the cell, and its expression. Several nonviral and viral gene transfer vectors have been tested *in vitro* and in different animal models of human diseases. What are the minimal requirements for the use of a viral or nonviral vector for gene transfer? The vector should be safe, nonpathogenic and stable and should be easily produced in large amounts. In addition, a vector should be able to transport and efficiently deliver foreign genes to different cell types. After gene transfer, the genes should be expressed at high levels, and these genes should preferably be regulated under tissue-specific control.

Adenoviruses have physical and genetic properties that fulfill most of these requirements. First-generation adenoviral vectors do not replicate in human cells under normal conditions because their early E1A and E1B functions have been deleted. These vectors are produced in a complementing cell line, usually in the 293 cell line, that provides the necessary E1 functions in *trans*. The results of clinical studies with first-generation adenoviral vectors have been instructive but disappointing and have led to the appreciation of several of their disadvantages.

1. Because in the 293 cell line only the E1A and E1B functions are expressed, all the other viral functions that are required for virus production need be expressed from the vector itself. Therefore, first-generation vectors still contain most of the adenoviral genes, which, if expressed in a tissue even at low levels, are believed to contribute to an antiviral immune response that

causes the clearance of transduced cells, resulting in only transient gene expression. Recent experimental data have shown that immune responses also against the transgene product are responsible for only short-term gene expression after gene transfer. For the permanent correction of a metabolic deficiency by gene transfer, however, the presence of numerous viral genes in the gene transfer vector is problematic.

2. Since the upper DNA packaging limit for adenoviruses is about 38 kb, the maximal size of foreign DNA that can be incorporated into these vector genomes is about 8 kb. Thus, the size constraints in the first-generation adenoviral vectors prevent or severely limit the use of this vector type in clinical applications. In order to decrease further the expression of late viral proteins, adenoviral vectors without the E2 and/or E4 functions, in addition to the deletion of the E1 region, have been generated. Presently, it is controversial whether these second-generation vectors have any significant advantages over the first-generation adenoviral vectors.

Recently, in an attempt to address several of the problems observed with first-generation adenoviral vectors, a new adenoviral vector has been developed that is predicted to be very useful for the functional analyses of genes *in vivo* and likely also for clinical applications. This vector, which has variably been called 'high-capacity adenoviral vector', 'gutless adenoviral vector' or 'helper-dependent (HD) adenoviral vector', has a capacity of more than 30 kb for the uptake of foreign DNA and at the same time does not contain adenoviral coding sequences. About two decades ago, several unusual types of adenoviruses were described that either had taken up large fragments of genomic cellular DNA after repeated passage of adenoviruses at high multiplicity of infection, that contained several copies of the SV40 genome, or expressed the large T-antigen of SV40. These recombinant viral genomes required for their replication the presence of functional wild-type adenovirus during productive infection of cultured cells. Interestingly, the recombinant adenoviral genomes always contained the adenoviral termini. Later it was demonstrated that the ITRs of the adenoviral genome are the adenoviral origins of replication. The only *cis*-element required for the packaging of DNA into adenoviral capsids, the packaging signal, resides between the left ITR and the E1 region. Thus, it was reasoned that it should be possible to rescue recombinant adenoviral vectors that contained large fragments of nonviral DNA and as the only adenoviral elements the left and right ITRs plus the packaging signal. The best-documented examples that demonstrate the validity of this concept concern the gene transfer of the murine dystrophin cDNA and of the human α_1-antitrypsin gene. The generation of vectors containing the 14 kb full-length dystrophin cDNA would not have been possible with first-generation adenoviral vectors. However, applying the helper virus concept, several groups could demonstrate that it was possible to rescue recombinant adenoviral vectors that did not contain any viral coding sequences. Initially, wild-type adenovirus was used as helper virus for production; later, replication-deficient adenoviruses served as helper virus. In one case the packaging signal of the helper virus was modified to impair packaging of the helper virus in order to package preferentially the recombinant vector DNA in viral capsids. It was possible to partially separate helper virus and vector by CsCl equilibrium centrifugation and thus purify the helper virus to approximately 99%. Gene transfer by intramuscular injection of the dystrophin vector into muscle of mdx mice, a genetic and biochemical model for Duchenne muscular dystrophy (DMD), resulted in the stable expression of the 400 kDa full-length dystrophin protein, correct localization of dystrophin to the sarcolemma membrane, redistribution of the dystrophin associated proteins (DAPs) to the muscle membrane and improvement of the histological phenotype. These were the first studies to demonstrate the usefulness of the new vector type for gene transfer *in vivo*. In a further development, the production system for the new vector was improved by taking advantage of the cre-lox recombination system of bacteriophage P1. In this production scheme, a helper virus is used that contains two lox-recognition sequences flanking the packaging signal. The vector is produced in 293 cells that express the cre-recombinase of bacteriophage P1. After infection of these cells by both helper virus and vector, the packaging signal of the helper virus is excised with relatively high efficiency. Thus, preferentially the vector is packaged. With this system, it is possible to facilitate production, reduce the contamination of vector with helper virus to less than 0.1% after CsCl equilibrium centrifugation, and also to increase vector yield. Using this improved production system a vector has been generated carrying the complete human α_1-antitrypsin gene locus as a 19 kb DNA fragment.

In vitro and *in vivo* gene transfer experiments with this vector were very instructive: gene transfer in mice with this genomic DNA fragment that contained the endogenous hepatocyte- and macrophage-specific promoters, all exons and introns and the endogenous polyadenylation signal of the α_1-antitrypsin gene resulted in tissue-specific and persistent gene expres-

sion over more than 40 weeks. It was demonstrated that, following liver gene transfer, transcriptional initiation was only from the liver-specific promoter, and that splicing of the primary transcript and processing of RNA and protein were normal. Gene transfer with increasing doses of this vector resulted in very high and stable α_1-antitrypsin serum levels that so far are unparalleled by gene transfer with any other tested combination of strong viral promoters and the α_1-antitrypsin cDNA. Significantly, the high serum α_1-antitrypsin levels that would be considered supraphysiological in humans were not accompanied by any significant hepatotoxicity. Gene transfer with the same dose of a first-generation vector carrying an α_1-antitrypsin expression cassette led to hepatic damage that was morphologically characterized by liver cell necroses and infiltration by granulocytes and was accompanied by significantly elevated liver enzymes in the serum. Improved expression and decreased toxicity with high-capacity adenoviral vectors were confirmed with another vector that carried the murine leptin cDNA under the control of a viral promoter. Taken together, these results indicate that high-capacity adenoviral vectors will be useful for functional studies on gene transfer and likely also for somatic gene therapy because of their improved safety and expression profiles. Their increased capacity for foreign DNA is a significant advantage over first and second generation adenoviral vectors.

See also: **Adenoviruses (*Adenoviridae*): General features; Latency; Persistent viral infection; Vectors: Animal viruses.**

Further Reading

Doerfler W (1991) Abortive infection and malignant transformation by adenoviruses: integration of viral DNA and control of viral gene expression by specific patterns of DNA methylation. *Adv. Virus Res.* 39: 89.

Doerfler W (1996) A new concept in (adenoviral) oncogenesis: integration of foreign DNA and its consequences. *BBA Rev. Cancer* 1288: F79.

Doerfler W and Böhm P (eds) (1995) The molecular repertoire of adenoviruses. *Curr. Top. Microbiol. Immunol.* 199/I–III.

Lucher LA (1995) Abortive adenovirus infection and host range determinants. *Curr. Top. Microbiol. Immunol.* 199/I: 119.

Schiedner G, Morral N, Parks RJ *et al* (1998) Genomic DNA transfer with a high-capacity adenovirus vector results in improved *in vivo* gene expression and decreased toxicity. *Nature Genet.* 18: 180.

Schröer J, Hölker I and Doerfler W (1997) Adenovirus type 12 DNA firmly associates with mammalian chromosomes early after virus infection or after DNA transfer by the addition of DNA to the cell culture medium. *J. Virol.* 71: 7923.

van der Vliet PC (1995) Adenovirus DNA replication. *Curr. Top. Microbiol. Immunol.* 199/II: 1.

Animal Viruses

William C Russell, School of Biomedical Sciences, University of St Andrews, St Andrews, Fife, Scotland

Mária Benkö, Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary

History

Members of the adenovirus family (*Adenoviridae*) can be recognized in virtually every class of vertebrate animals (**Table 1**). However, not all of the family members contain the same common antigen and initially two genera have been established each of them being characterized by a common (or group) antigen. The genus *Mastadenovirus* comprises viruses isolated from mammals (humans, monkey, cattle, pig, sheep, horse, dog, goat, tree shrew, deer, whale, mouse, etc.) whereas the *Aviadenovirus* genus contains viruses isolated from avian species (chicken, turkey, goose, pheasant, duck, etc.). The organization of the genome, though normally rather conserved within a genus, differs considerably between the two genera. Recently, strong evidence (based on genomic organization and phylogenetic analysis; **Fig. 1**) supported the establishment of a third genus incorporating several bovine adenoviruses (BAVs), the ovine adenovirus isolate 287 (OAV287) and the egg drop syndrome (EDS) virus which (lacking the genus-specific common antigens) had been recorded as 'atypical' members of the *Mastadenovirus* or *Aviadenovirus* genera. The proposed name of this possible third genus is *Atadenovirus* (referring to the characteristic AT-rich genome rather than the host animal taxon). The candidate BAV serotypes (4 to 8) of this proposed genus do not fit clearly into the *Mastadenovirus* genus and are presently separated as subgroup 2 from the subgroup 1 BAVs which obviously belong to the mastadenoviruses. A number of adenoviruses isolated from (or only detected in) lower vertebrate hosts (fish, frog, snake, chameleon, crocodile, etc.)

aggressive growth characteristics and this has been attributed to the C-terminus. Mutants that fail to bind to CtBP also give rise to metastases with an activated ras, whereas WT12S does not. It would appear that the region of E1A required for induction/maintenance of epithelialization and suppression of transformation progression, at least with ras, are the same. It is possible that the C-terminus could also contribute to the inhibition of non-epithelial differentiation pathways. The C-terminus of E1A 12S could be regulating epithelial–mesenchymal transitions (EMT) and deletions in this region result in EMT, contributing to tumor progression. EMT has been attributed to tumor progression.

Conclusions

E1A is a complex, multifunctional protein. It can affect a broad range of processes in cells. Analysis of E1A and its mechanisms of controling the cell has provided a vast amount of insight into the workings of the cell, from cell cycle to transcriptional regulation. It has led to the identification of many cellular proteins involved in these processes and to an understanding of how they function. E1A has provided and continues to provide a very fruitful model system to dissect proliferation, immortalization, tumorigenic transformation, metastasis, differentiation and transcriptional regulation. At times it seems as if E1A elicits contradictory behaviors. Some of these paradoxes may be a reflection of the multitude of different experimental systems in which it is used. But in all these cases, insights into the workings of cells, normal and otherwise, have been obtained. Underlying all the observed functions must be a unifying theme: to facilitate viral reproduction, since that is the function of any and all viral genes. It is important to keep this in mind when we interpret experimental results.

See also: **Adenoviruses (*Adenoviridae*): Animal viruses, General features, Molecular biology; Apoptosis and virus infection; Immune escape mechanisms; Retroviral Oncogenes; Papillomaviruses – human (*Papovaviridae*): General features, Molecular biology; Simian virus 40 (*Papovaviridae*); Transformation: Animal viruses; Tumor viruses – human.**

Further Reading

Bayley ST and Mymryk JS (1994) Adenovirus E1A proteins and transformation. *Int. J. of Oncol.* 5: 425.

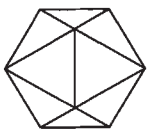
Doerfler W and Bohm P (eds.) (1995) *The Molecular Repertoire of Adenoviruses III: Biology and Pathogenesis*. Berlin: Springer-Verlag.

Hagmeyer BM, Angel P and van Dam H (1995) Modulation of AP-1/ATF transcription factor activity by the adenovirus E1A oncogene products. *BioEssays* 17: 621.

Jansen-Durr P (1996) How viral oncogenes make the cell cycle. *Trends Genet* 12: 270.

Mymryk JS (1996) Tumor suppressive properties of the adenovirus 5 E1A gene. *Oncogene* 13: 1581.

AFRICAN SWINE FEVER VIRUS (*ASFARVIRIDAE*)



María L Salas, Centro de Biología Molecular 'Severo Ochoa' (CSIC-UAM), Universidad Autónoma de Madrid, Madrid, Spain

History and Geographic Distribution

In 1921 Montgomery described African swine fever (ASF) as a peracute disease of domestic pigs caused by a virus. The disease was first recognized after the introduction of domestic pigs to Kenya in 1910. Montgomery established the likely transmission of ASF from wild swine and reported the lack of protection by passive immunization.

In 1957 the disease appeared for the first time outside Africa, in Portugal, and in 1960 spread to Spain. In the 1960s and 1970s the virus was found in France, Italy, Sardinia and Malta and, in America, in Cuba, Brazil, Dominican Republic and Haiti. In 1985 there was an ASF outbreak in Belgium and in 1986 in The Netherlands. Today, ASF is enzootic in sub-Saharan Africa and Sardinia.

Taxonomy and Classification

ASF virus (ASFV) is a deoxyvirus with an icosahedral morphology that multiplies in the cytoplasm of the infected cell. Because of these characteristics, the virus

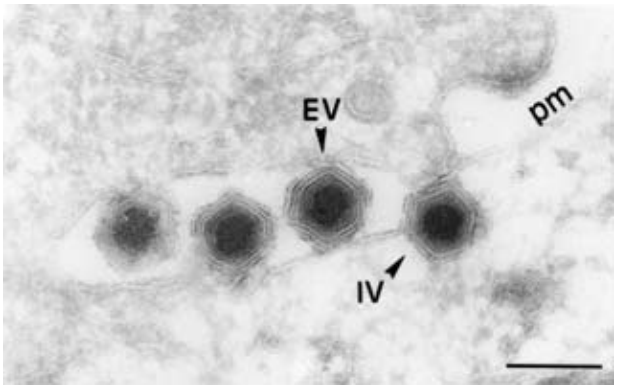


Figure 1 Electron micrograph of a cryosection of ASFV-infected Vero cells. The electron micrograph shows an intracellular virus particle (IV) and three extracellular virus particles (EV). pm, plasma membrane. Bar = 200 nm. (Courtesy of G. Andrés.)

has been classified for many years as an *Iridovirus*. However, several properties of ASFV are similar to those of poxvirus. The genome of ASFV resembles that of poxviruses in the presence of covalently closed ends and terminal inverted repetitions (see later). In contrast, the genomes of frog virus 3 and two other iridoviruses are both circularly permuted and terminally redundant. In addition, ASFV particles contain, like those of poxvirus, all the enzymatic machinery necessary for the synthesis and modification of early mRNA. Frog virus 3 particles do not contain RNA polymerase, and RNA synthesis in cells infected with frog virus 3 is sensitive to the host RNA polymerase II inhibitor α-amanitin, whereas transcription of the ASFV genome is resistant to this inhibitor.

ASFV has recently been assigned to a new family, *Asfarviridae*, as a member in the genus *Asfivirus*.

Virion Structure and Proteins

The ASFV particles have an icosahedral morphology and consist of a DNA-containing nucleoid of about 80 nm, a thick protein layer of about 30 nm, designated the core shell, a lipid envelope surrounding the core shell, the capsid, and, in the case of the extracellular virus, an outer membrane derived by budding through the plasma membrane (**Fig. 1**). The extracellular virions have a diameter of 200 nm. The capsid consists of a hexagonal arrangement of capsomers that appear as 13 nm long hexagonal prisms, each with a central hole. The intercapsomer distance is about 8 nm and the triangulation number is estimated to be between 189 and 217, corresponding to a number of capsomers between 1892 and 2172.

Two-dimensional gel electrophoresis analysis of purified virions has resolved 54 structural proteins with molecular weights ranging from 10000 to 150000. Some of these proteins have been localized in the virion by immunoelectron microscopy. The virus attachment protein p12 and protein p24 are present in the external region of extracellular virions, while proteins p150, p37, p34 and p14, products of polyprotein pp220, are found in the core shell. Protein p72, located in the surface of unenveloped intracellular virions, is the capsid protein.

ASFV particles contain a DNA-dependent RNA polymerase and other activities involved in the polyadenylation, methylation and capping of RNA. Other enzymes present in the virions are a protein kinase, two nucleoside triphosphate phosphohydrolases, an acid phosphatase, and a deoxyribonuclease active on single-stranded DNA.

The genes coding for 15 virion proteins have been identified and sequenced (**Fig. 3**). Two of these genes code for polyprotein precursors. Thus, gene *CP2475L* encodes a polyprotein of 220 kDa, which, after proteolytic cleavage, gives rise to four major structural proteins: p150, p37, p34 and p14; gene *CP530R* encodes polyprotein pp62, the precursor of proteins p35 and p15.

Properties of Genome

The genome of ASFV is a linear double-stranded DNA molecule with a size that oscillates between 170 and 190 kbp, depending on the virus isolate. The two DNA strands are covalently closed, at both ends, by a 37 nucleotide-long hairpin loop, composed, almost entirely, of incompletely paired A and T residues. The loops at each DNA end are present in two equimolar forms (fast and slow components) that, when compared in opposite polarities, are inverted and complementary (flip-flop), as in the case of poxvirus DNA (**Fig. 2A, B**). There is no homology between the hairpin loops of ASFV and vaccinia virus but, close to the hairpin loops, both viruses have a sequence 16 nucleotides long with a homology of about 80%. Following the hairpin loops there is a perfect terminal inverted repeat (TIR) with a length of 2134 nucleotides, which consists of unique sequences interspersed with five different sets of repeated sequences (**Fig. 2C**). The central region of the TIR contains 33 direct repeats in tandem of 34 nucleotides.

In addition to the TIRs, two types of internal repetitions have been described: short (10–50 bp) direct repeats in tandem at both intergenic and intragenic positions, and long (200 bp) tandemly repeated units found at the left end of the genome. The latter repeated sequences share similarities with eucaryotic scaffold-associated region (SAR)-like sequences.

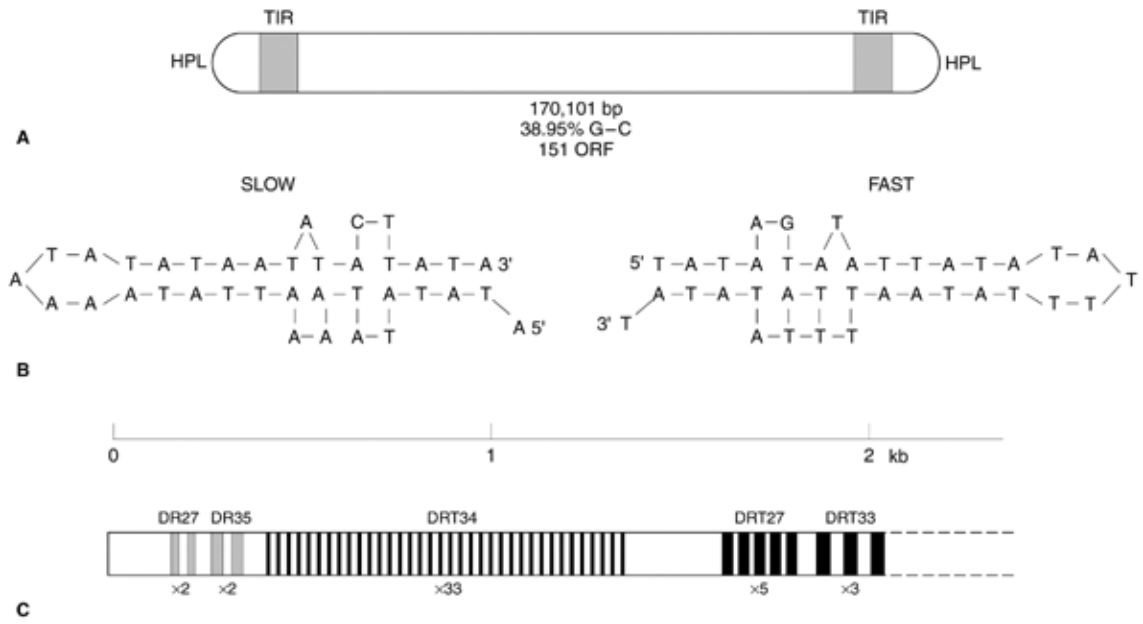


Figure 2 Structure of the ASFV genome. (**A**) ASFV DNA (BA71V strain). HPL, hairpin loop; TIR, terminal inverted repetition. (**B**) Predicted secondary structure of the 37 nucleotide-long fast and slow components in the HPLs. (**C**) Structure of the TIR. The TIR is 2134 nucleotides long and consists of unique sequences (in white) interspersed with two types of direct repeats (DR) and three sets of direct repeats in tandem (DRT).

The genome of ASFV strain BA71V has been completely sequenced and shown to contain 170101 bp, with a G-C content of 38.95%. Analysis of the DNA sequence has identified 151 major open reading frames (ORFs). A list of the ASFV genes encoding proteins for which there is functional information or significant homology is presented in Fig. 3.

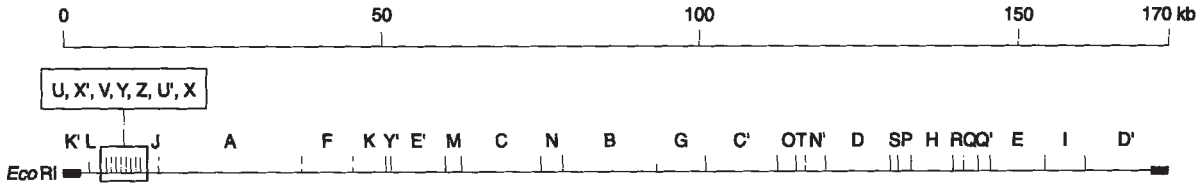
Penetration

ASFV infection starts with the interaction of the virus with a cell receptor. Titration experiments have shown the existence in susceptible Vero cells and swine macrophages of a specific receptor for the Vero cell-adapted virus. Virus penetration takes place by a mechanism of adsorptive endocytosis that is sensitive to lysosomotropic drugs. Nonproducing L cells and rabbit macrophages lack specific binding sites, indicating that the presence of receptors is necessary for a productive infection.

Protein p12, encoded by ORF *O61R*, has been identified as the virus attachment protein. ORF *O61R* codes for a polypeptide of 61 amino acids with a putative transmembrane hydrophobic domain in the central region that may anchor the protein in the virus envelope. Three forms of the protein with molecular masses of 17, 12 and 10 kDa have been observed in SDS-polyacrylamide gel electrophoresis, depending on the presence of 2-mercaptoethanol and the alkylation with 4-vinyl-pyridine, indicating that disulfide bonds are responsible for the multimerization of the protein. This result is in agreement with the existence of a cysteine-rich domain in the C-terminal region of the predicted amino acid sequence. In ASFV-infected cells, protein p12 is synthesized at late times of infection. The comparison of the amino acid sequence of protein p12 in different field virus isolates, deduced from the nucleotide sequence of the gene, reveals a high degree of conservation. No mutations were found after adaptation to Vero cells. Protein p12 is synthesized in swine macrophages infected with all the isolates tested. These findings suggest an essential role of the protein in the virus replication cycle, and indicate that the variability of protein p12 is not a mechanism used by ASFV to evade the host immune response.

DNA Replication and Repair

The replication of ASFV DNA does not occur in enucleated cells. The results obtained by autoradiography of ASFV-infected macrophages in pulse-label and pulse-chase experiments, and by *in situ* hybridization of infected macrophages and Vero cells, suggest a mechanism for viral DNA replication with an initial stage in the nucleus followed by a cytoplasmic phase. The viral DNA in the nucleus is



KP177R	Structural protein p22
A224L	Inhibitor of apoptosis IAP
*A104R**	Structural protein, histone-like
A240L	Thymidylate kinase
A238L	IκB-like (experimental)
A859L	Helicase superfamily II
A179L	Inhibitor of apoptosis *Bcl-2* (experimental)
*A137R**	Structural protein p11.5
F334L	Ribonucleotide reductase small subunit
F778R	Ribonucleotide reductase large subunit
F1055L	Helicase superfamily II
*K78R**	Structural protein p10, DNA-binding protein
K196R	Thymidine kinase (experimental)
EP1242L	RNA polymerase subunit 2
EP153R	C-type animal lectin-like region
EP402R	CD2 homologue
C147L	RNA polymerase subunit 6
B962L	Helicase superfamily II
B119L	*S. cerevisiae* ERV1 and vaccinia E10R
B318L	*trans*-prenyltransferase (experimental)
*B646L**	Structural protein p72, capsid protein
G1207R	DNA polymerase α-like
*CP2475L**	220 kD polyprotein precursor of structural proteins p150, p37, p14 and p34
*CP530R**	62 kD polyprotein precursor of structural proteins p35 and p15
CP80R	RNA polymerase subunit 10
O174L	DNA polymerase, pol X family (experimental)
*O61R**	Structural protein p12, virus attachment protein
NP1450L	RNA polymerase subunit 1
NP419L	DNA ligase (experimental)
NP868R	Guanylyltransferase (experimental)
D250R	8-hydroxy-dGTPase, MutT region
D1133L	Helicase superfamily II
*D117L**	Structural protein p17
D205R	RNA polymerase subunit 5
P1192R	Topoisomerase II
H359L	RNA polymerase subunit 3
*R298L**	Structural protein, serine protein kinase (experimental)
Q706L	Helicase superfamily II
QP383R	NifS-like protein
QP509L	Helicase superfamily II
*E183L**	Structural protein p54
E301R	Similar to proliferating cell nuclear antigen (PCNA)
E165R	dUTPase
*E120R**	Structural protein p14.5, DNA-binding protein
E296R	AP endonuclease class II
I243L	Transcription factor SII (TFIIS)
I215L	Ubiquitin-conjugating enzyme (experimental)
DP71L	Similar to HSV ICP34.5 neurovirulence factor

Figure 3 ASFV genes with known functions or significant homology. An *Eco*RI restriction endonuclease map of the ASFV genome (BA71V isolate) is shown. Below are listed ORFs encoding proteins with known functions or significant homology to other proteins in the databases. Asterisks indicate ORFs encoding structural proteins. The ORFs are designated by the name of the *Eco*RI fragment where translation is started (the letter 'P' substituted for the prime apostrophe), followed by the number of amino acids encoded and a letter, L or R, indicating the direction of transcription (leftwards or rightwards).

localized as a single focus in proximity to the nuclear membrane. The replication of ASFV DNA is thus similar to that of the iridovirus frog virus 3 in the two-stage mechanism, even though the structure of the ASFV genome is different from that of frog virus DNA, and therefore the molecular mechanisms of DNA synthesis are probably also different. An analysis of ASFV replicating DNA molecules has shown the existence of head-to-head and tail-to-tail linked molecules that may be replicative intermediates formed by some of the mechanisms proposed for poxvirus DNA replication.

The synthesis of ASFV DNA requires a virus-induced DNA polymerase which is sensitive to phosphonoacetic acid, and is probably encoded by ORF *G1207R*, homologous to α-like DNA polymerases. Three other enzymes involved in DNA and nucleotide metabolism are induced in cells infected with ASFV: a thymidine kinase, a ribonucleotide reductase and a DNA ligase. The genes coding for these enzymes, as well as the genes encoding proteins homologous to thymidylate kinase and the proliferating cell nuclear antigen (PCNA), which is a processivity factor for DNA polymerase δ, have been identified and sequenced (**Fig. 3**).

ASFV also encodes several enzymes of a DNA repair system of the base excision repair type, including an apurinic/apirimidinic (AP) endonuclease and the product of gene *O174L*, which has been characterized as a pol β-type DNA polymerase.

Transcription of the ASFV Genome

ASFV particles contain all the enzymatic machinery necessary for the synthesis and modification of virus-specific RNA. *In vitro* transcription by ASF virions requires high concentrations of ATP and is inhibited by the topoisomerase II inhibitor coumermycin A1, suggesting that transcription of ASFV DNA requires a topoisomerase II. A gene (*P1192R*) coding for a topoisomerase II has been identified and sequenced. Six subunits of the virus RNA polymerase have been identified by sequence similarity, suggesting that the enzyme is a multisubunit protein (**Fig. 3**). A gene homologous to the eucaryotic transcription factor SII (ORF *I243L*) has also been identified, and the protein

product of gene *NP868R* has been shown to be a guanylyltransferase.

The RNAs synthesized *in vitro* by the virion-associated RNA polymerase have methylated CAP structures and poly(A) tails. These RNAs are complementary to the same DNA regions as those synthesized in ASFV-infected cells in the presence of cycloheximide or cytosine arabinoside, and the two RNAs give rise to the same polypeptides after hybridization-selection with DNA restriction fragments and translation *in vitro*, suggesting that, before DNA replication, the early viral RNAs are synthesized by the RNA polymerase present in the virus particles. A subclass of early genes, designated immediate early, whose transcription is silenced before the onset of DNA replication, and an intermediate class of mRNAs, with the characteristics of the poxvirus intermediate transcripts, have also been identified. Late genes are expressed after the initiation of DNA replication. The existence of early, intermediate and late mRNAs suggests a cascade model for the regulation of ASFV gene expression.

The mRNAs have short leader sequences before the translational start site and the 3′ ends of several early and late genes map within a conserved sequence motif formed by at least seven thymidylate residues (7T).

The host RNA polymerase II is not involved in the transcription of ASFV genes, as cells infected in the presence of α-amanitin or 5,6-dichloro-β-D-ribofuranosyl benzimidazole synthesize RNAs and proteins that are indistinguishable from those made in the absence of the drugs.

Proteins Induced in ASFV-infected Cells

Two-dimensional gel electrophoresis analysis of proteins synthesized in ASFV-infected Vero cells has identified 81 acid and 14 basic virus-induced polypeptides, with molecular masses ranging from 220 to 10 kDa. Three classes of ASFV-induced proteins can be defined: early proteins whose synthesis is switched-off after virus DNA replication; early proteins synthesized along the whole replication cycle; and late proteins which are not synthesized in the presence of cytosine arabinoside.

Post-translational Processing

Proteolytic processing

Polyprotein processing is an essential mechanism for the generation of major components of the virus particle. As mentioned above (Virion Structure and Proteins), six ASFV structural proteins are derived from polyprotein precursors by proteolytic cleavage at the consensus sequence G-G-X. This sequence, together with other physical properties of the protein, seems to be a recognition sequence for the processing of a variety of viral and cellular proteins.

Glycosylation

Partially purified intracellular ASFV particles, adapted to grow in monkey kidney (MS) cells, have three minor structural glycoproteins of molecular masses 89, 56, and 51 kDa. However, highly purified, extracellular ASFV particles lack major glycoproteins but contain several species of glycolipids, and two sugar-labeled components of apparent molecular masses 230 and 95 kDa, which could account for the virus agglutination with lectins. The nonprotein glycosylated components present in extracellular ASF virions have a cellular origin.

ASFV induces in Vero cells the synthesis of 19 glycosylated components of molecular masses ranging from 9 to 220 kDa. At least five of the induced glycosylated components are probably virus-coded glycoproteins. Most of these glycocomponents are synthesized at early times after infection.

Myristoylation

Polyprotein pp220 and several other proteins induced during infection of Vero cells with ASFV are covalently bound to myristic acid. Two myristoylated proteins of 28 and 13 kDa are incorporated into the virus particle.

Phosphorylation

The most highly phosphorylated protein in extracellular virus grown in Vero cells in the presence of [^{32}P]phosphate is the structural protein p17, with a molecular mass of 17 kDa. A second phosphoprotein of 35 kDa is found in ASF virions. The most abundant and immunogenic protein induced by ASFV at early times of infection, protein p32 encoded by ORF *CP204L*, is a phosphoprotein. Two-dimensional gel electrophoresis has revealed the existence of 15 ASFV-induced phosphoproteins in infected monkey cells.

Two virion proteins are preferentially phosphorylated *in vitro* by the protein kinase associated with ASF virions, the structural proteins p10 and p9, with molecular masses of 10 and 9 kDa, respectively.

Ubiquitination

ASFV gene *I215L* codes for a ubiquitin-conjugating protein, an enzyme involved in the conjugation of ubiquitin to proteins, a modification that generally targets the protein for proteolytic degradation. Three

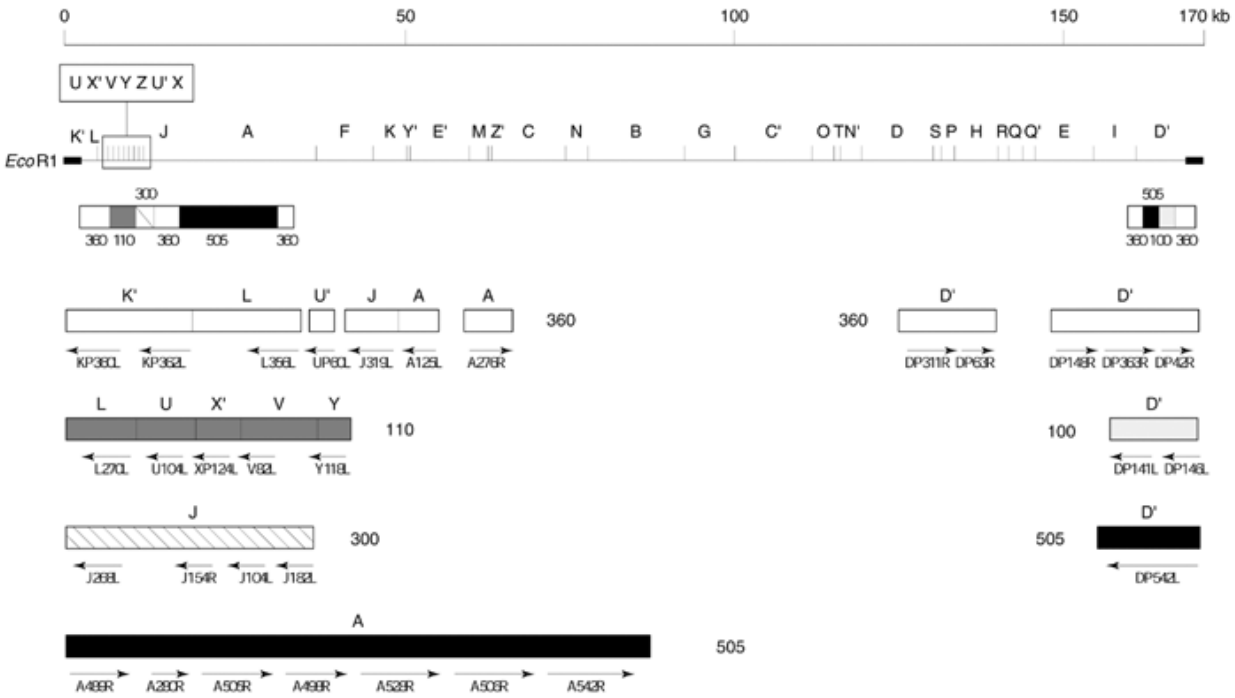


Figure 4 Multigene families in ASFV. Localization of multigene families 100, 110, 300, 360 and 505 in the *Eco*RI map of ASFV DNA (BA71V strain). The genes composing the different multigene families in the BA71V isolate are shown below. The arrows indicate the direction of transcription.

ubiquitinated proteins, with molecular masses of 5, 18 and 58 kDa, have been found in purified extracellular virions, the 18 kDa protein being externally located.

Morphogenesis

ASFV particles assemble from membranous structures present in discrete cytoplasmic areas termed viral factories. The viral membranes become polyhedral immature virions by the progressive formation of the capsid on their convex surface. Underneath the lipid envelope, two different domains are consecutively formed: first the core shell and then an electron-dense nucleoid, which is the DNA-containing domain (see above, Virion Structure and Proteins). The encapsidation of the viral genome is probably a late event in the ASFV assembly.

Host Range and Virus Propagation

ASFV infects domestic pigs and other members of the family Suidae. ASFV also propagates in ticks of the genus *Ornithodoros*, and is the only known arbovirus which contains DNA. In Spain the virus was isolated from the tick *O. erraticus* and in Africa from *O. moubata* collected from warthog burrows. ASFV may be primarily a virus of ticks. All efforts to infect other animal species with ASFV in an attempt to find a more adequate experimental model have been unsuccessful.

In pig tissues, viral antigens have been associated with macrophages and reticular cells, whereas in blood and bone marrow the main cells involved in the infection are monocytes, polymorphs and megakaryocytes.

ASFV replicates *in vitro* in pig monocytes and macrophages, a small fraction of polymorphs and in endothelial cells, but not in resting or mitogen-stimulated T or B lymphocytes. After adaptation, some ASFV isolates multiply in primary cell cultures or in established cell lines, such as pig kidney cells, Vero cells or other monkey kidney cells.

Genetic Variability

A comparison of restriction maps of different ASFV isolates has shown that the virus genome consists of a central, highly conserved region, with a constant length of about 125 kbp, and two variable regions close to the DNA ends, which show deletions or additions up to 8.6 kbp. Nucleotide sequence analysis of the variable regions has shown the existence of five multigene families, designated families 100, 110, 300, 360 and 505, of unknown function (**Fig. 4**). The deletions that occur in the variable regions result in

changes in the number of genes in the multigene families and may generate new members by recombination between homologous genes.

Twenty-three ASFV isolates (8 African, 11 European and 4 American) have been classified into five groups, according to the arrangement of *Sal*I sites in the central region. Four groups contain only African isolates, whereas all the European and American isolates belong to the same group. This distribution of isolates suggests that all non-African virus field isolates have a common origin.

A comparison of DNA sequences of ASFV strains BA71V, Malawi Lil 20/1 and LIS57 has shown that the proteins encoded are over 85% identical in the three virus isolates. The most variable proteins belong to the multigene families. In addition, the BA71V proteins EP402R (homologous to cellular CD2) and I177L are only 67.3 and 78.9% identical, respectively, to their counterparts in the Malawi strain. ASFV protein heterogeneity can also be generated by changes in the number of amino acid tandem repeats. These repeats have been found in 14 ASFV proteins, including the CD2 homologue, the α-like DNA polymerase, and the virion protein p54.

Evolution

The hairpin loop termini of ASFV DNA (see earlier) are very similar in structure, size and sequence to those found in linear plasmids of bacteria of the genus *Borrelia* (host-associated spirochetes that shuttle between arthropods and vertebrates). ASFV and *B. duttoni*, a relapsing fever species, share the same tick vector, *O. moubata*, in Africa. The similarities between these two DNA termini might indicate either a common ancestry or a horizontal genetic transfer.

The evolutionary relationships among the topoisomerase II sequences of ASFV, eucaryotes and procaryotes have been analyzed and a phylogenetic tree has been established. The tree indicates that the ASFV gene diverged from an ancestral cellular gene before the divergence of protozoa, yeasts and metazoa. The data support the hypothesis that an ancestor of ASFV was infecting a unicellular organism, possibly a lower eucaryote, and that the virus acquired the topoisomerase II gene at that time.

Serologic Relationships and Variability

A method of classifying different ASFV isolates is to compare different viruses and sera in a hemadsorption inhibition reaction. Animals surviving infection with ASFV or those infected with an attenuated virus develop hemadsorption-inhibiting antibodies, which seem to be virus-specific. Using this method, different virus isolates have been classified into three groups: A (Lisbon 57, Funchal 65 and Katanga 67), B (Lisbon 60, Madrid 60 and Angola 72) and C (Mozambique 64).

From stocks of hemadsorbing ASFV, nonhemadsorbing clones have been isolated by limiting dilution. Nonhemadsorbing viruses have also been isolated from nature. Hemadsorption of swine erythrocytes to ASFV-infected cells is a late function due to the virus protein EP402R, homologous to the cellular CD2.

A collection of monoclonal antibodies that recognize ten ASFV structural proteins have been used to determine the antigenic variability of ASFV passaged *in vitro* in either porcine macrophages or Vero cells, and to classify 23 field virus isolates from Africa, Europe and America into six antigenic homology groups, which indicates the existence of a complex variety of ASFV serotypes. No clear separation of virus isolates from different continents could be established, as virus isolates from Africa, Europe and America are present in the same antigenic homology group.

The African isolates differ among themselves more than the European or American isolates. This is probably because in Africa the virus has been circulating for a long time and has diverged extensively. The uniformity of the non-African isolates relative to the African ones might be due to the possibility that only one or few viruses entered Europe during 1957 to 1960.

Transmission and Tissue Tropism

Ticks can be infected with ASFV by feeding on viremic blood. The virus replicates in the insect's gut after ingestion, and then spreads to the sexual organs and salivary glands. ASFV can be transmitted between ticks transovarially and sexually. Natural transmission in wild African warthogs seems to occur by tick feeding. Once ASFV has been established in domestic pig populations, the disease can be readily transmitted by contact, as the virus is present in sufficient amounts in excretions and secretions. The virus is very stable in the blood and feces of infected animals. Airborne transmission over short distances can also occur.

Pathogenicity and Clinical Features of Infection

ASFV strains differ in pathogenicity. Highly virulent strains cause a fulminating disease in domestic pigs, with mortality rates of almost 100%. In these cases, pigs die within 1–3 days, before gross lesions can be detected. Since the introduction of ASFV in Europe in 1960, acute, subacute and chronic forms of the disease

Table 1 ASFV genes potentially involved in the regulation of immune response

Gene	*Homologous to*	*Proposed role*
EP402R	CD2-adhesion molecule of T lymphocytes and natural killer cells	Hemadsorption factor Prevents the interaction between infected cells and lymphocytes
A238L	IκB-inhibitor of NKκB transcription factors	Regulation of the host immune response
EP153R	C-type animal lectins	Control of the adhesion ability of infected cells
DP71L (*NL-S* gene of Malawi strain)	Neurovirulence gene *ICP34.5* of HSV	Virulence factor
A179L	Proto-oncogene *bcl-2*	Inhibition of apoptosis
A224L	Baculovirus *iap* gene	Inhibition of apoptosis

have evolved. Clinically, there is a longer incubation period, the course of the disease is slower and the lesions less severe. Chronic forms of the disease may develop in pigs that survive the subacute disease. Adult warthogs do not develop signs of disease, although ASFV has been found in their tissues. The clinical features of the infection are high fever, diarrhea accompanied by bleeding, generalized reddening of the skin and prostration. In some cases, nasal bleeding and swollen and hemorrhagic conjunctival membranes are observed.

The ASFV gene *NL-S* of the Malawi Lil 20/1 isolate, homologous to the herpes simplex virus neurovirulence gene *ICP34.5*, appears to be a viral virulence factor, as deletion of the gene converts the virulent Malawi strain into an attenuated form.

Pathology and Histopathology

After an initial replication of the virus in lymph organs, the spleen, liver and lungs are the main organs for secondary virus replication. Splenic enlargement has been observed and edema of the lungs has been frequently reported in pigs dying of acute ASF, while in chronic processes pneumonia is a common feature. Lesions in the liver consist of degeneration and necrosis of Kupffer cells and hepatocytes. Hemorrhage is the most severe symptom in ASF, and is often the cause of death in infected pigs. Hemorrhages are most consistently seen in lymph nodes, kidneys and heart. The development of disseminated intravascular coagulation seems to be responsible for the hemorrhagic syndrome.

Immune Response

The host immune response induced by ASFV is characterized by the lack of fully neutralizing antibodies, which has hampered the development of a vaccine to control the disease. Nevertheless, it has been reported that pigs infected with ASFV may recover and resist challenge exposure with virulent homologous, but not heterologous, virus. Partial protection by sera from ASF-resistant pigs or by several ASFV proteins has also been found. Although some antisera against whole ASFV or against several viral proteins exhibit high levels of virus inhibition *in vitro*, complete neutralization has not been achieved so far. Identification of the virus component(s) carrying the critical antigenic determinant(s) is one of the most important problems in ASF research.

The cellular immune system is not impaired by ASFV infection in pigs. Several studies have shown activity specific against ASFV of cytolytic T lymphocytes (CTLs) obtained from swine leukocyte antigen (SLA)-inbred minipigs surviving an experimental infection with ASFV. The CTL assays were performed using blood or alveolar macrophages as target cells. The specific lysis of these cells was mediated by purified CD8+ lymphocytes, and could be blocked by incubation with monoclonal antibodies against SLA class I proteins. The CD8+ population produced high levels of interferon γ (IFN-γ) this production being specific for the attenuated isolates of ASFV, suggesting that virulence could be related, at least in part, to isolate-specific IFN-γ production. CTL activity has been detected against cells infected with recombinant vaccinia virus expressing the ASFV p32 protein, an immediate early and one of the most immunogenic virus proteins.

The ASFV genome contains several genes coding for proteins potentially involved in mechanisms used by the virus to evade the host immune response (**Table 1**). One of these proteins, EP402R, homologous to the T cell adhesion molecule CD2, could prevent the interaction of CTLs with the infected

cells. Another ASFV protein (EP153R) that could be important in the control of the adhesion ability of infected cells is a homologue of C-type animal lectins. ASFV also codes for a protein homologous to the cellular IκB, an inhibitor of the transcription factors NFκB, which control the expression of genes involved in the inflammatory and immunological responses to viral infections. On the other hand, the proteins encoded by ASFV genes *A179L* and *A224L*, homologous to the cellular proto-oncogene *bcl*-2 and to the baculovirus gene *iap*, respectively, could prevent the death by apoptosis of the infected cell, thus allowing the development of a productive infection or, under certain conditions, the establishment of persistent infections.

Prevention and Control of ASF

ASFV is a menace to the pig population of the world because there is no vaccine. The control and eradication of ASF requires rapid diagnosis, drastic slaughter and quarantine. ASF diagnosis can be based on the demonstration of infectious virus, viral antigens and DNA or specific antibodies.

See also: **Frog virus 3 (*Iridoviridae*); Replication of viruses; Vaccinia virus (*Poxviridae*).**

Further Reading

Becker Y (ed.) (1987) *African Swine Fever*. Boston: Martinus Nijhoff.

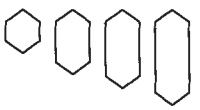
Costa JV (1990) African swine fever virus. In: Darai G (ed.) *Molecular Biology of Iridoviruses*, p. 247. Boston: Kluwer.

Viñuela E (1985) African swine fever virus. *Curr. Top. Microbiol. Immunol.* 116: 151.

Yáñez, RJ, Rodríguez JM, Nogal ML *et al* (1995) Analysis of the complete sequence of African swine fever virus. *Virology* 208: 249.

Aleutian Mink Disease Virus *see* **Parvoviruses**

ALFAMOVIRUS AND ILARVIRUSES (*BROMOVIRIDAE*)



JF Bol, Leiden University, Leiden, The Netherlands

History

Alfalfa mosaic virus was identified in 1931 as the causal agent of an economically important disease in alfalfa. In the literature the name of the virus has been abbreviated as AMV and AlMV. In their sixth report of 1995 the ICTV assigned AMV to alfalfa mosaic virus as the type member of the genus *Alfamovirus*, and AIMV to the potyvirus alstroemeria mosaic virus. Consequently, alfalfa mosaic virus is denoted as AMV in this chapter. Many strains of AMV are known which are closely related by serology and nucleotide sequence similarity and which are all characterized by having virions with a unique bacilliform shape. In 1971 it became clear that coat protein has a critical role in the initiation of infection by AMV. A few years later it was found that the coat proteins of ilarviruses play a similar role in the initiation of infection. The observation that the coat proteins of AMV and ilarviruses were interchangeable in this early function suggested a close relationship between the *Alfamovirus* and *Ilarvirus* genera.

Taxonomy and Classification

The term *Ilarvirus* was originally coined from the words *i*sometric, *la*bile, *r*ingspotting. The viruses in this group have quasi-isometric particles varying in size around 30 nm. The following eight subgroups have been suggested: (1) tobacco streak virus (TSV, and its isolates black raspberry latent virus and asparagus stunt virus), *Hydrangea* mosaic virus (HdMV); (2) tulare apple mosaic virus (TAMV), citrus leaf rugose virus (CiLRV), citrus variegation virus (CVV), elm mottle virus (EMoV), asparagus virus 2 (AV-2), spinach latent virus (SPLV); (3) prunus necrotic ringspot virus (PNRSV) (cherry rugose mosaic virus, some rose mosaic virus isolates and hop virus C are synonyms to PNRSV), apple mosaic

cells. Another ASFV protein (EP153R) that could be important in the control of the adhesion ability of infected cells is a homologue of C-type animal lectins. ASFV also codes for a protein homologous to the cellular IκB, an inhibitor of the transcription factors NFκB, which control the expression of genes involved in the inflammatory and immunological responses to viral infections. On the other hand, the proteins encoded by ASFV genes *A179L* and *A224L*, homologous to the cellular proto-oncogene *bcl*-2 and to the baculovirus gene *iap*, respectively, could prevent the death by apoptosis of the infected cell, thus allowing the development of a productive infection or, under certain conditions, the establishment of persistent infections.

Prevention and Control of ASF

ASFV is a menace to the pig population of the world because there is no vaccine. The control and eradication of ASF requires rapid diagnosis, drastic slaughter and quarantine. ASF diagnosis can be based on the demonstration of infectious virus, viral antigens and DNA or specific antibodies.

See also: **Frog virus 3 (*Iridoviridae*); Replication of viruses; Vaccinia virus (*Poxviridae*).**

Further Reading

Becker Y (ed.) (1987) *African Swine Fever*. Boston: Martinus Nijhoff.

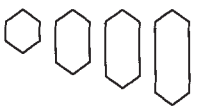
Costa JV (1990) African swine fever virus. In: Darai G (ed.) *Molecular Biology of Iridoviruses*, p. 247. Boston: Kluwer.

Viñuela E (1985) African swine fever virus. *Curr. Top. Microbiol. Immunol.* 116: 151.

Yáñez, RJ, Rodríguez JM, Nogal ML *et al* (1995) Analysis of the complete sequence of African swine fever virus. *Virology* 208: 249.

Aleutian Mink Disease Virus *see* **Parvoviruses**

ALFAMOVIRUS AND ILARVIRUSES (*BROMOVIRIDAE*)



JF Bol, Leiden University, Leiden, The Netherlands

History

Alfalfa mosaic virus was identified in 1931 as the causal agent of an economically important disease in alfalfa. In the literature the name of the virus has been abbreviated as AMV and AlMV. In their sixth report of 1995 the ICTV assigned AMV to alfalfa mosaic virus as the type member of the genus *Alfamovirus*, and AIMV to the potyvirus alstroemeria mosaic virus. Consequently, alfalfa mosaic virus is denoted as AMV in this chapter. Many strains of AMV are known which are closely related by serology and nucleotide sequence similarity and which are all characterized by having virions with a unique bacilliform shape. In 1971 it became clear that coat protein has a critical role in the initiation of infection by AMV. A few years later it was found that the coat proteins of ilarviruses play a similar role in the initiation of infection. The observation that the coat proteins of AMV and ilarviruses were interchangeable in this early function suggested a close relationship between the *Alfamovirus* and *Ilarvirus* genera.

Taxonomy and Classification

The term *Ilarvirus* was originally coined from the words *i*sometric, *la*bile, *r*ingspotting. The viruses in this group have quasi-isometric particles varying in size around 30 nm. The following eight subgroups have been suggested: (1) tobacco streak virus (TSV, and its isolates black raspberry latent virus and asparagus stunt virus), *Hydrangea* mosaic virus (HdMV); (2) tulare apple mosaic virus (TAMV), citrus leaf rugose virus (CiLRV), citrus variegation virus (CVV), elm mottle virus (EMoV), asparagus virus 2 (AV-2), spinach latent virus (SPLV); (3) prunus necrotic ringspot virus (PNRSV) (cherry rugose mosaic virus, some rose mosaic virus isolates and hop virus C are synonyms to PNRSV), apple mosaic

virus (ApMV), (Danish plum line pattern virus, some rose mosaic virus isolates and hop virus A are synonyms to ApMV), blueberry shock virus (BlShV), *Humulus japonicus* virus (HJV); (4) prune dwarf virus (PDV); (5) American plum line pattern virus (APLPV); (6) lilac ring mottle virus (LRMV); (7) *Parietaria* mottle virus (PMoV); (8) *Fragaria chiloensis* virus (FClV). For viruses within one subgroup serological relationships have been reported. AMV strains that have been studied in most detail are the isolates AMV-L and AMV-M of strain 425 and strain AMV-S. The nucleotide sequence similarity between AMV strains is over 95%.

Together with the genera *Bromovirus, Cucumovirus* and *Oleavirus,* AMV and ilarviruses constitute the family of *Bromoviridae.* The tripartite RNA genomes of bromo- and cucumoviruses are infectious as such whereas the tripartite genomes of AMV and ilarviruses are not infectious unless a few molecules of coat protein or RNA4, the subgenomic messenger RNA for coat protein, are added to the inoculum. This phenomenon is called 'genome activation'. Moreover, bromovirus and cucumovirus RNAs contain a 3′-terminal tRNA-like structure that is absent in AMV and ilarvirus RNAs. The particles of AMV and ilarviruses have bacilliform and spheroidal morphology, respectively. However, in addition to spheroidal particles, the preparations of several ilarviruses contain AMV-like bacilliform particles, notably those of TAMV, PNRSV and PDV. On the other hand, spheroidal particles are found in AMV preparations.

To date, the complete nucleotide sequence is known of the three genomic RNAs of AMV isolate AMV-L and the ilarviruses CiLRV, SPLV, EMoV, TSV and PDV. In addition, the sequence has been determined of RNA3 of LRMV, AV-2, HdMV, PNRSV, ApMV and CVV. **Figure 1** shows a phylogenetic tree of AMV and ilarviruses based on sequence similarities between the RNA3-encoded movement protein. Two major clusters can be distinguished, one containing CiLRV, CVV, LRMV, AV-2 and TSV, and the other containing AMV, PNRSV, ApMV and PDV. A tree based on the RNA3-encoded coat protein yields a similar clustering. A comparison of the replicase proteins encoded by RNAs 1 and 2 yields one cluster containing CiLRV, TSV and SPLV, and another cluster containing AMV, ApMV and PDV. Within one cluster the sequence similarity of these proteins is 45% or more whereas the similarity between clusters is 35–40%. Based on these data it has been suggested that AMV should be incorporated into the genus *Ilarvirus.* Another feature separating the two clusters is the presence of an open reading frame (ORF) 2b, similar

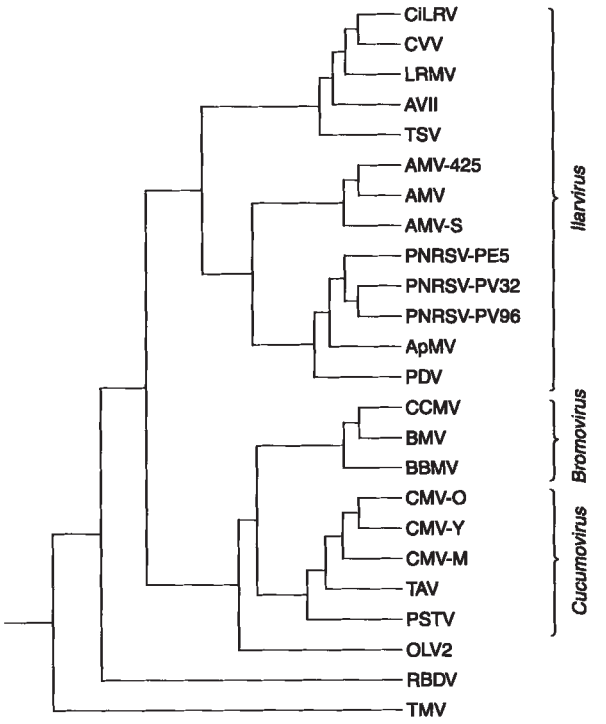


Figure 1 Phylogenetic tree obtained from the alignment of amino acid sequences of movement proteins encoded by RNA3 of viruses from the family *Bromoviridae.* In addition to the genera *Ilarvirus, Bromovirus* and *Cucumovirus,* the oleavirus OLV2, the idaeovirus RBDV and the tobamovirus TMV are included for comparison. (Reproduced with permission from Sánchez-Navarro and Pallás (1997) Evolutionary relationships in the ilarviruses: nucleotide sequence of prunus necrotic ringspot virus RNA3. *Arch. Virol.* 142: 749.)

to that found in RNA2 of several cucumoviruses. Such an ORF 2b is found in RNA2 of CiLRV, CVV, AV-2, TSV, SPLV and the related EMoV, but appears to be absent from RNA2 of AMV, ApMV and PDV. For comparison, **Fig. 1** shows the phylogenetic relationship of ilarviruses to bromo- and cucumoviruses, the idaeovirus RBDV, the oleavirus OLV2 and the tobamovirus TMV.

Host Range and Geographic Distribution

AMV occurs worldwide. Strains of this virus have been found in natural infections of about 150 plant species representing 22 families. The experimental and natural host ranges include over 600 species in 70 families. Although AMV infects mostly herbaceous plants, several woody hosts are included in its natural host range. Also, the host range of TSV is very wide, both experimentally and naturally, infecting many plant species in over 30 families. Both monocotyledonous and dicotyledonous hosts have been reported and both woody and herbaceous hosts. The original

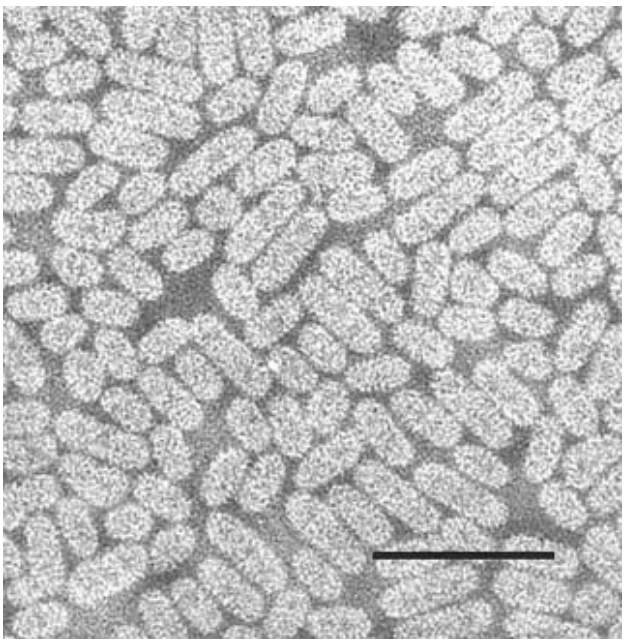


Figure 2 Electron micrograph of alfalfa mosaic virus. Scale bar = 100 nm.

description notes its occurrence near the edges of tobacco fields, suggesting that it spreads from perennial weeds. In contrast, TAMV was found in a single apple tree in California in 1955 and was discovered 20 years later in a hazelnut tree in France. Similarly, the *Citrus* species infected by CiLRV are restricted to one location in Florida. EMoV has been found in elm and lilac in Scotland and Germany. The distribution of PNRSV and PDV corresponds to those of their hosts as a result of their dissemination in vegetative propagating material as well as in seeds. Although some ilarviruses have a rather narrow natural host range they can all be transmitted experimentally to a wide range of herbaceous plants.

Structure and Composition

Preparations of AMV contain four major classes of particles called bottom component (B), middle component (M), top component b (Tb) and top component a (Ta) (**Fig. 2**). B, M and Tb are bacilliform and contain the genomic RNAs 1, 2 and 3, respectively. Ta contains two molecules of the subgenomic RNA4 and can be subdivided in bacilliform Ta-b and spheroidal Ta-t particles. The bacilliform particles are all 19 nm wide and have lengths of 56 nm (B), 43 nm (M), 35 nm (Tb) and 30 nm (Ta-b). The RNAs are encapsidated by a single type of coat protein which in the case of strain AMV -L has a length of 220 amino acids (mol. wt 24 280 Da). In solution, AMV coat protein occurs as dimers which under appropriate conditions of pH and ionic strength form a $T = 1$ icosahedral structure built from 30 dimers. This structure can be crystallized and has been studied by x-ray diffraction, cryoelectron microscopy and image reconstruction methods. The coat protein was found to have the canonical eight-stranded β-barrel fold with the N- and C-terminal arms as extended chains. Dimer formation in the $T = 1$ particle is based on the clamping of the C-terminal arms of the subunits. The dimer is further stabilized by interactions between the C-terminal arms and the icosahedral twofold axis.

From particle weight measurements and analysis of electron images it has been concluded that the number of coat protein monomers in the major virions is $60 + (n \times 18)$, n being 10 (B, 240 subunits), 7 (M, 186 subunits), 5 (Tb, 150 subunits) or 4 (Ta, 132 subunits). By gel electrophoresis at least 13 minor components have been resolved which probably represent other n values and are believed to contain monomers of genomic RNAs, multimers of genomic RNAs or RNA4 or specific degradation products of RNA3. Although details of the arrangement of the protein monomers have not been established, electron microscopical studies indicate that the cylindrical parts of the bacilliform particles have a hexagonal surface lattice with dimers of the coat protein associated with the twofold symmetry axes. The cylindrical part is believed to be capped by two halves of an icosahedron by changing the axes from sixfold symmetry in the cylinder into axes of fivefold symmetry. Neutron-scattering data suggested that the capsid structure of spheroidal Ta-t particles is represented by a deltahedron with 52-point group symmetry built from 120 subunits. The percentage of RNA in the virions decreases from 16.3 in B to 15.2 in Ta-b. The buoyant density in CsCl of the major components fixed with formaldehyde varies from 1.366 (Ta) to 1.372 (B) $g\,cm^{-3}$. The protein shell has an inner radius of 6.5 nm and an outside radius of 9.4 nm. The RNA is uniformly packed within the 6.5 nm radial limit, occupies about 20% of the interior volume available and slightly penetrates the protein shell. The particles are mainly stabilized by protein RNA interactions. The RNA is easily accessible to ribonucleases A and T1 through holes in the protein shell. At slightly alkaline pH the particles unfold reversibly. The structure of ilarviruses has been studied in little detail. By using sucrose gradient centrifugation virus preparations can be separated into four classes of spheroidal particles with diameters of 23 nm, 25–27 nm, 30–31 nm and 32–33 nm. The RNA content of all particles is about 16%. The capsids are built from one species of capsid protein with molecular weights between 23 635 Da (SPLV) and 26 346 Da (TSV). The

three largest particles of TSV contain the genomic RNAs and their capsids have been estimated to be built from 142, 179 and 225 subunits per particle. Like AMV RNAs, ilarvirus RNAs are degraded *in situ* by nucleases and the particles are readily disassembled into protein and RNA by high salt or low concentrations of sodium dodecyl sulfate (SDS).

Genome Structure

All four RNAs of AMV-L have been sequenced. RNA1 (3644 ribonucleotides) and RNA2 (2593 ribonucleotides) encode proteins of 126 and 90 kDa which are called P1 and P2, respectively. P1 and P2 are subunits of the purified AMV replicase. RNA3 (2142 ribonucleotides) contains a 5′-proximal reading frame for a 32 kDa protein called P3, and a 3′-proximal reading frame encoding the coat protein. P3 is required for cell-to-cell transport of the virus. Coat protein is translated from RNA4 which is identical in sequence to the 3′-terminal 881 ribonucleotides of RNA3. The length of the intercistronic region in RNA3 is 52 ribonucleotides including the leader sequence of RNA4 of 36 ribonucleotides. At the 5′-end, all four AMV RNAs are capped. The organization of the leader sequence of RNA3 varies between strains. For strains M, S, L and YSMV the length of this leader sequence is 240, 313, 345 and 391 ribonucleotides, respectively. The increased length of the last three strains is due to the presence of direct repeats of 56 (S), 75 (L) and 149 ribonucleotides (YSMV). At their 3′-termini the AMV RNAs do not contain a tRNA-like structure or poly(A) tail. There is about 80% sequence similarity between the 3′-terminal 145 ribonucleotides of the AMV genomic RNAs. The 3′-untranslated region (3′-UTR) of RNA3 is 182 ribonucleotides long and contains seven hairpins flanked by the single-stranded sequence AUGC. The 3′-UTR of AMV RNA3 contains at least two independent binding sites for coat protein, one being located in the region homologous to RNAs 1 and 2, and one in the region unique to RNA3. A minimal coat protein binding site is represented by the 3′-terminal 39 ribonucleotides containing two hairpins flanked by three AUGC-motifs. To initiate infection, each genome segment has to be complexed with a few dimers of the coat protein.

The genome organization of ilarviruses is very similar to that of AMV. Generally, the 5′-UTRs of RNAs 1 and 2 are relatively short (37–75 ribonucleotides) whereas those of RNA3 are longer (210–354 ribonucleotides). The 5′-UTR of SpLV RNA1 (73 ribonucleotides) and RNA2 (75 ribonucleotides) share a common 5′ sequence of 45 ribonucleotides; otherwise sequence similarity between the leaders is limited. The 3′-UTRs of SpLV and CiLRV vary in length from 174 to 418 ribonucleotides but the three genomic RNAs of the two viruses contain a highly conserved sequence of 190 ribonucleotides. In the 3′-UTRs of TSV (139–288 ribonucleotides) sequence similarity is limited to the 3′-45 ribonucleotides. Yet, the predicted secondary structure of the 3′-UTRs of ilarviruses is very similar to that of AMV and can be folded into a number of hairpins interspersed by AUGC motifs. These 3′-termini are fully equivalent in binding the homologous coat protein or heterologous coat proteins from other alfamo- or ilarviruses. The size of the ORF 2b protein that is encoded by RNA2 of several ilarviruses (SpLV, 21.1 kDa; TSV, 22.4 kDa) is twice the size of the corresponding cucumovirus 2b protein. The function of the 2b protein in ilarvirus replication is not known.

Replication

Replication of AMV can be initiated by inoculation of plants or protoplasts with a mixture of the viral particles B, M and Tb, with RNAs 1, 2, 3 and 4, or with RNAs 1, 2, 3 and coat protein. An infectious mixture is also obtained when purified B particles are added to a mixture of RNAs 1, 2 and 3. In this case the coat protein molecules of the B particle become readily distributed over the RNA molecules present in the inoculum. In protoplasts, RNA synthesis becomes detectable from 6 h after inoculation and levels off after 42 h. RNA3 is synthesized most rapidly whereas RNA4 accumulates rather late in the infection cycle. The accumulation of positive-strand RNAs is accompanied by the synthesis of three negative-strand molecules corresponding to RNAs 1, 2 and 3. In productively infected plants or protoplasts, massive amounts of coat protein are produced but the three nonstructural proteins are detectable only with specific antisera. On Western blots the P3 protein gives multiple bands suggesting a post-translational modification. RNAs 1 and 2 are able to replicate in protoplasts provided that some coat protein is added to the inoculum. However, in the absence of RNA3 or in the presence of RNA3 with a defective coat protein gene there is a strong reduction in positive-strand RNA accumulation. Apparently, the coat protein is required for (+)RNA accumulation *in vivo*. Infection of protoplasts with the complete genome or with RNAs 1 and 2 results in the accumulation of membrane-bound replication complexes. Replicase preparations purified from productively infected tobacco plants were found to contain P1, P2 and CP, and are able to transcribe plus-strand AMV RNAs into complementary minus-strands. Replicase can also be isolated from healthy transgenic tobacco

plants transformed with the P1 and P2 genes (P12 plants). Synthesis of RNA4 by this replicase on a (−)RNA3 template is strongly stimulated by coat protein *in vitro*. Available evidence indicates that all AMV components are synthesized and assembled in the cytoplasm.

Although the replication cycle of ilarviruses has been investigated in less detail, studies with purified particles, RNAs and coat protein of TSV, CiLRV, CVV, PNRSV and PDV demonstrated that for ilarviruses and AMV the requirements to initiate an infection are the same. As with AMV, the genomes of these viruses require coat protein or RNA4 in the inoculum to initiate infection. The coat proteins of AMV and these ilarviruses can reciprocally activate each other's genomes.

Genetics and Molecular Biology

Infectious cDNA clones of AMV RNAs 1, 2 and 3 have been used to map *cis*- and *trans*-acting functions in the genome by site-directed mutagenesis. The 3′-terminal sequence of 145 ribonucleotides that are homologous in the three AMV RNAs were found to be sufficient for a low level of minus-strand promoter activity *in vitro* and *in vivo*. Non-homologous upstream sequences from the 3′-UTRs of RNAs 1, 2 and 3 were required to enhance this promoter activity to wild-type levels. Coat protein is not required for minus-strand RNA synthesis *in vitro* or *in vivo*. Functions of the coat protein in genome activation, synthesis of plus-strand genomic RNA, synthesis of subgenomic RNA4, encapsidation and cell-to-cell movement could be mutated separately, suggesting that different domains of the protein may be involved in at least some of these functions. The P3 gene has no role in RNA replication but mutations affecting the expression of coat protein result in a 100-fold reduction of plus-strand RNA synthesis in protoplasts. When protoplasts are inoculated with wild-type RNAs 1 and 2 and a mixture of RNA3 mutants defective in the P3 gene or coat protein gene, the coat protein expressed by the P3 mutant permits asymmetric accumulation of RNA1, RNA2 and the RNA3 with the P3 mutation but does not stimulate plus-strand accumulation of RNA3 with the coat protein mutation. This indicates that coat protein is required in *cis* for replication of RNA3 and used in *trans* for replication of RNAs 1 and 2. Similarly, evidence has been obtained that P1 is required in *cis* for the replication of RNA1 whereas P2 is required in *cis* for the replication of RNA2. When plants rather than protoplasts are infected with a mixture of P3 and coat protein mutants, the mutant RNA3 molecules readily recombine to wild-type RNA3.

When the coat protein gene in AMV RNA3 was replaced by the coat protein genes of TSV or PNRSV, the ilarvirus coat proteins stimulated a relatively low level of plus-strand RNA accumulation in protoplasts and the PNRSV coat protein was able to encapsidate the AMV/PNRSV chimeric RNA. When both the P3 gene and the coat protein gene in AMV RNA3 were replaced by the corresponding genes of PNRSV, the mutant could hardly move in tobacco plants. This indicates that the RNA3-encoded proteins of AMV and PNRSV are not interchangeable in cell-to-cell movement.

Virus–Host Relationships

The fact that the AMV group is a large conglomerate of strains infecting a high number of susceptible hosts accounts for the tremendous range of symptoms displayed by AMV-infected plants. Mutations in the coat protein gene and 5′-UTR of RNA3 have been shown to affect symptom formation in tobacco. The ilarviruses mainly invade woody plants but also have a wide herbaceous host range. The symptoms of some of these viruses on their natural hosts are briefly listed here. TSV gives severe local and systemic necrosis in tobacco. TAMV induces mosaic in apple and hazelnut which can be followed by systemic necrosis. CiLRV is known for its rugosity and chlorotic flecks in species of *Citrus*. The symptoms of CVV in *Citrus* species range from patterns of chlorotic areas to severe distortion and variegation. EMoV causes ringspot and line patterns in elm, and white mosaic and chlorotic line patterns in lilac. The symptoms of PNRSV in *Prunus* species range from mild mottles to severe leaf necrosis, whereas chlorotic line patterns and rings are induced in roses. ApMV gives mosaic disease in apple and chlorotic lines in birch, plum and rose. PDV is responsible for thickened 'shoe-string' leaves on prune, chlorotic ringspot in sweet cherry and yellows in sour cherry.

In AMV-infected plants cytological modifications occur only in cells of organs showing symptoms. In these cells fragmentation of the ground cytoplasm and an increased accumulation of membrane-bound vesicles has been observed. Sometimes the lamellar system of chloroplasts is affected and invaginations of the nuclear membrane have been reported. The P3 protein has been localized in the middle lamellae of walls of those cells that had just been reached by the infection front and in which viral multiplication had just begun.

Virus particles are mainly found in the cytoplasm with a few records of particles in the nucleus. Depending on the strain, four types of intracellular aggregates of virus particles may occur. Also, ilar-

viruses induce only minor cytological changes in most of the virus–host combinations studied. These include an enhanced development of cytoplasmic membranes, production of vesicles and a slight degeneration of mitochondria. The virions of TSV are found in the cytoplasm, scattered randomly or packed in disordered or paracrystalline aggregates. In addition, tubular structures with single rows of virus particles are observed. The association of these structures with cell walls and plasmodesmata may suggest a function in cell-to-cell transport. Protoplasts infected with AMV produce tubular structures consisting of the P3 protein on their surface. These tubules appear to be filled with virus particles and are probably involved in cell-to-cell transport of the virus.

Transmission

AMV is easily transmissible manually. Field spread occurs predominantly by aphid transmission. At least 15 aphid species are known to transmit the virus in the stylet-borne or nonpersistent manner. Acquisition of the virus occurs within 10–30 s and is followed by immediate transmission without a latent period. The ability to continue transmission is lost by the aphid within 1 h. The variability of individual aphid species in their capacity to transmit different AMV strains suggests a specific virus–vector relationship which is probably governed by the structural properties of the coat protein. Seed transmission of AMV has been reported for alfalfa and seven other plant species with rates of transmission varying from 0.1 to 50%. Transmission of the virus between plants by parasitic dodder has been observed with five *Cuscuta* species.

Mechanical transmission of ilarviruses, particularly those of wood plants, frequently requires the addition of stabilizing compounds to the inoculum. Back transmission of these viruses from herbaceous plants to their woody hosts is generally difficult. In the field, transmission of ilarviruses occurs through seed and pollen. Seed transmission has been reported for AV-2, EMoV, LRMV, PDV, PNRSV and TSV. For EMoV, PDV, PNRSV and TSV transmission by pollen has also been observed. The effectiveness of pollen transmission is correlated with the activity of pollinating insects. For example, thrips have been shown to play a role in the spread of TSV-contaminated pollen.

Epidemiology and Control

Although there have been reports on resistance and tolerance of alfalfa to AMV, control of the virus in this crop can be done mainly by using virus-free seed and avoiding reservoir hosts of the virus. Because the virus occurs naturally in many different plant species this is practically impossible.

Ilarviruses are less widespread in nature, infecting many different plant families. Vegetative propagation of horticultural crops such as fruit trees, roses and berries is the most important factor in the dissemination of these viruses. Virus-free plantings should be used to prevent the rapid distribution of ilarviruses. Because of the seed transmission of these viruses the use of rootstocks grown from infected seeds is a particular risk. Transmission of the virus by pollen depends on the behavior of pollen-bearing insects. Honeybees, which are the most common visitors of flowers of fruit trees, tend to favor one flower species during pollen collection. Because bees forage only locally during a foraging trip, virus transmission by pollen in an orchard is most likely to occur between adjacent trees.

Tobacco plants transformed with the coat protein genes of AMV and TSV were found to be highly resistant to the homologous virus but not to the heterologous virus when infection was done by mechanical inoculation. Resistance to transmission of virus by aphids or pollen has not yet been tested.

Economic Significance

AMV is the causal agent of economically important diseases in alfalfa, clover, pea, potato, tobacco, pepper, tomato and celery. Ilarviruses of greatest economic importance are PNRSV, PDV, TSV and ApMV.

See also: **Bromoviruses (*Bromoviridae*); Plant virus disease – economic aspects.**

Further Reading

Francki RIB (1985) Polyhedral virions with tripartite genomes. In: Francki RIB (ed.) *The Plant Viruses*, vol. 1, p. 1. New York: Plenum.

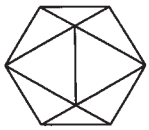
Fulton RW (1981) Ilarviruses. In: Kurstak E (ed.) *Handbook of Plant Virus Infections and Comparative Diagnosis*, p. 378. The Netherlands: Elsevier/North-Holland Biomedical Press.

Jaspars EMJ (1985) Interaction of alfalfa mosaic virus nucleic acid and protein. In: Davies JW (ed.) *Molecular Plant Virology*, vol. 1, p. 155. Boca Raton: CRC Press.

Reusken CBEM, Neeleman L, Brederode FT and Bol JF (1997) Mutations in coat protein binding sites of alfalfa mosaic virus RNA3 affect subgenomic RNA4 accumulation and encapsidation of viral RNAs. *J. Virol.* 71: 8385.

Sánchez-Navarro JA and Pallás V (1997) Evolutionary relationships in the ilarviruses: nucleotide sequence of prunus necrotic ringspot virus RNA3. *Arch. Virol.* 142: 749.

ALGAL VIRUSES (*PHYCODNAVIRIDAE*)



James L Van Etten, Department of Plant Pathology, University of Nebraska, Lincoln, Nebraska, USA

History

Since the early 1970s, viruses or virus-like particles (VLPs) have been reported in at least 44 taxa of eucaryotic algae, which include members in ten of the 14 classes of eucaryotic algae. However, most of these reports described isolated accounts of microscopic observations. The virus particles were not characterized because they were difficult to obtain in reasonable quantities. Several factors contributed to the low virus concentrations: (1) often only a few algal cells contained particles; (2) usually the cells only contained particles at one stage of the algal life cycle; (3) cells containing particles tended not to lyse; (4) in most cases the particles were not infectious; and (5) some hosts could not be easily cultured.

However, this situation began to change with the discovery of a family of large double-stranded (ds) DNA-containing viruses which infect and replicate in certain strains of unicellular, eucaryotic, chlorella-like green algae. The first of such 'chlorella viruses' was discovered in 1981 in chlorella symbiotic with the green coelenterate *Hydra viridis*, and subsequently in chlorella symbiotic with the protozoan *Paramecium bursaria*. The algae from *P. bursaria* can be grown free of the paramecium in culture, and these cultured, naturally endosymbiotic *Chlorella*, strains NC64A and Pbi or their equivalents, serve as hosts for many similar viruses (**Table 1**). These lytic viruses can be produced in large quantities and assayed by plaque formation using standard bacteriophage techniques. The prototype chlorella virus is PBCV-1, which stands for *Paramecium bursaria Chlorella* virus 1.

Large polyhedral, dsDNA-containing viruses which infect certain marine algae are also under active investigation. These include viruses that infect unicellular algae, *Micromonas pusilla* (MpV viruses) and *Chysochromulina* sp. (CbV viruses), as well as viruses that infect filamentous brown algae, including *Ecto-*

Table 1 Representative algal viruses

Virus	*Host*	*Genome size (kb)*	*DNA structure*	*%G+C*	*Level of methylation*	
					5mC[a]	*6mA*[b]
CA-4B	*Chlorella* NC64A	330	Linear	40.0	0.12	ND
PBCV-1	*Chlorella* NC64A	330	Linear	40.0	1.9	1.5
IL-3A	*Chlorella* NC64A	330	Linear	40.3	9.7	ND
NC-1A	*Chlorella* NC64A	340	Linear	40.5	7.1	7.3
SH-6A	*Chlorella* NC64A	330	Linear	41.1	12.6	10.3
XZ-6E	*Chlorella* NC64A	330	Linear	41.7	21.2	15.2
NYs-1	*Chlorella* NC64A	350	Linear	42.1	47.5	11.3
CA-4A	*Chlorella* NC64A	330	Linear	40.3	39.8	19.6
NY-2A	*Chlorella* NC64A	380	Linear	41.5	44.9	37.0
CVA-1	*Chlorella* Pbi	>300	Linear	46.1	43.1	ND
CVB-1	*Chlorella* Pbi	>300	Linear	45.4	42.7	17.7
CVM-1	*Chlorella* Pbi	>300	Linear	46.0	41.9	10.1
EsV	*Ectocarpus siliculosus*	320	Circular	50	1.0	3.0
EfasV	*Ectocarpus fasciculatus*	320	Circular	–	–	–
FsV-178	*Feldmania* sp.	178	Circular	–	–	–
FsV-158	*Feldmania* sp.	158	Circular	–	–	–
MpV-SP1	*Micromonas pusilla*	–	–	–	–	–
CpV-Pw1	*Chrysochromulina brevifilum*	–	–	–	–	–

[a]Percentage of 5mC per C.

[b]Percentage of 6mA per A.

ND, not detected.

carpus sp. (EsV viruses) and *Feldmannia* sp. (FsV viruses) (**Table 1**). EsV and FsV viruses have a lysogenic phase in their life cycle and are only expressed as virus particles in sporangial cells of their host.

Taxonomy and Classification

The algal viruses are polyhedra (100–190 nm in diameter) with large dsDNA (160–380 kb) genomes. The 320–380 kb chlorella virus genomes are linear, nonpermuted molecules with crosslinked hairpin ends. In contrast, brown algal viruses have circular dsDNA genomes. Both MpV- and CbV-virus genomes are probably linear dsDNA molecules.

Despite the differences in genome structure, phylogenetic trees indicate that all the algal viruses are more closely related to each other than to other dsDNA viruses and that they form a distinct phyletic group, suggesting a common, albeit ancient, ancestor. The viruses fall into several clades, which correlate with their algal hosts. Based on DNA polymerase gene sequences, herpesviruses are the most closely related outgroup.

The algal viruses are morphologically similar to the iridoviruses, such as frog virus 3 (FV3). Other shared properties between the chlorella viruses and FV3 include: aquatic habitats, a large dsDNA genome, the presence of methylated bases in their genomic DNA, and an internal lipid component comprising 5–10% of the virus weight. However, the chlorella viruses differ in several ways from FV3. (1) FV3 is uncoated inside the host cell, whereas the chlorella viruses specifically attach to the host cell surface and release DNA into the host cell like bacteriophages. (2) FV3 cannot replicate in UV-inactivated host cells, whereas the chlorella viruses initiate and complete viral replication (albeit slowly and with a small burst size) in UV-inactivated host cells. (3) FV3 virons do not contain glycoproteins, whereas some of the chlorella virion proteins are glycosylated. (4) FV3 mRNAs are not polyadenylated, whereas the chlorella virus early, but probably not all late, mRNAs are polyadenylated. (5) The FV3 genome is a linear and circularly permuted molecule, whereas the chlorella virus genomes are linear and nonpermuted with covalently closed hairpin ends.

Chlorella virus genomes share two characteristics with the vaccinia virus (poxvirus) genome even though the virions are morphologically distinct. Like PBCV-1, vaccinia virus DNA contains covalently closed hairpin termini and inverted terminal repetition. However, the terminal repetitive region of vaccinia virus DNA is larger (*c.* 10 kb) than that of PBCV-1 DNA (2.2 kb), even though the PBCV-1 genome is about twice the size of the vaccinia virus genome.

African swine fever virus (ASFV) (presently unclassified) is the only other large polyhedral virus containing dsDNA with hairpin termini and inverted terminal repetition. However, PBCV-1 and ASFV differ in several ways. Most significantly: (1) these two viruses infect different hosts; (2) the PBCV-1 genome is about twice the size of the ASFV genome; (3) the PBCV-1 genome contains methylated bases, whereas the ASFV does not; and (4) no glycoproteins are present in ASFV particles, whereas at least three PBCV-1 virion proteins are glycoproteins.

The 330 kb PBCV-1 genome has recently been sequenced. Comparison of the deduced amino acid sequences of the putative PBCV-1 encoded proteins with those from procaryotes, eucaryotes and their viruses did not reveal a common ancestor for the chlorella viruses. That is, some PBCV-1 genes are most closely related to bacteria and their viruses, whereas other viral genes are obviously more closely related to eucaryotic organisms and their viruses. Consequently, the chlorella virus genomes contain an interesting mosaic of procaryotic- and eucaryotic-like genes.

The chlorella viruses have family status, with the name *Phycodnaviridae*. It has been proposed that the brown algal viruses and the MpV- and CbV-viruses be included in the *Phycodnaviridae* family and that the four groups be assigned to separate genera.

Structure and Composition

The chlorella virus PBCV-1 is a 175–190 nm in diameter polyhedron with a multilaminate capsid. Some electron micrographs indicate that PBCV-1 virions contain flexible hair-like appendages that extend from at least some of the vertices. One unique virion vertex may contain a 20–25 nm spike-like structure. PBCV-1 sediments at about 2300 S and has an estimated size of 1×10^3 MDa. Virus composition is about 64% protein, 21–25% dsDNA and 5–10% lipid. The virus contains at least 50 structural proteins, ranging in size from 10–200 kDa. Four proteins, including the major capsid protein (Vp54), are located on the virion surface.

Vp54 comprises about 40% of the total PBCV-1 protein and is one of three viral glycoproteins. The glycan portion of Vp54 is on the external surface of the virus and probably contributes to the protease resistance of PBCV-1. Four PBCV-1 proteins, including Vp54, are myristoylated and at least six PBCV-1 proteins are phosphoproteins. The PBCV-1 lipid component, which is located beneath the outer glycoprotein shell, is required for infectivity, as organic solvents inactivate the virus.

Genome Structure

The 330 740 bp PBCV-1 genome is linear and non-permuted with covalently closed hairpin ends. The termini consist of 35 nucleotide-long, incompletely base-paired, covalently closed hairpin loops that exist in one of two forms (flip and flop). Each hairpin loop is followed by an identical 2221 bp inverted repeat sequence, after which the sequence diverges. The PBCV-1 genome encodes 702 open reading frames (ORFs) that have 65 or more codons, as well as a cluster of ten tRNAs located in the middle of the genome. Of the 702 ORFs, 377 are believed to encode proteins; about 40% of these 377 ORFs resemble proteins in the databases (some ORFs are listed in **Fig. 1**). Certain PBCV-1 genes are interrupted by one of two different types of introns: a transcription factor-like gene contains a self-splicing type I intron, whereas the DNA polymerase gene contains a spliceosomal processed type of intron. In addition, one of the viral tRNA genes is predicted to have an intron. One unusual feature of the chlorella virus genomes is that they contain methylated bases (**Table 1**).

The EsV and FsV viruses that infect filamentous brown algae have circular dsDNA genomes. EsV has a 320 kb genome, whereas FsV contains two genome size-classes, 158 and 179 kb, which may be packaged in separate particles. The EsV dsDNA genome contains several single-stranded gaps. The FsV genomes have many 173 bp direct repeats located in specific regions of the DNAs. Unlike the lytic chlorella viruses, the FsV genomes, and probably the EsV genome, are integrated into the host chromosome(s) in certain stages of the algal life cycle. In this integrated state they are transmitted in a mendelian fashion.

Virus Replication

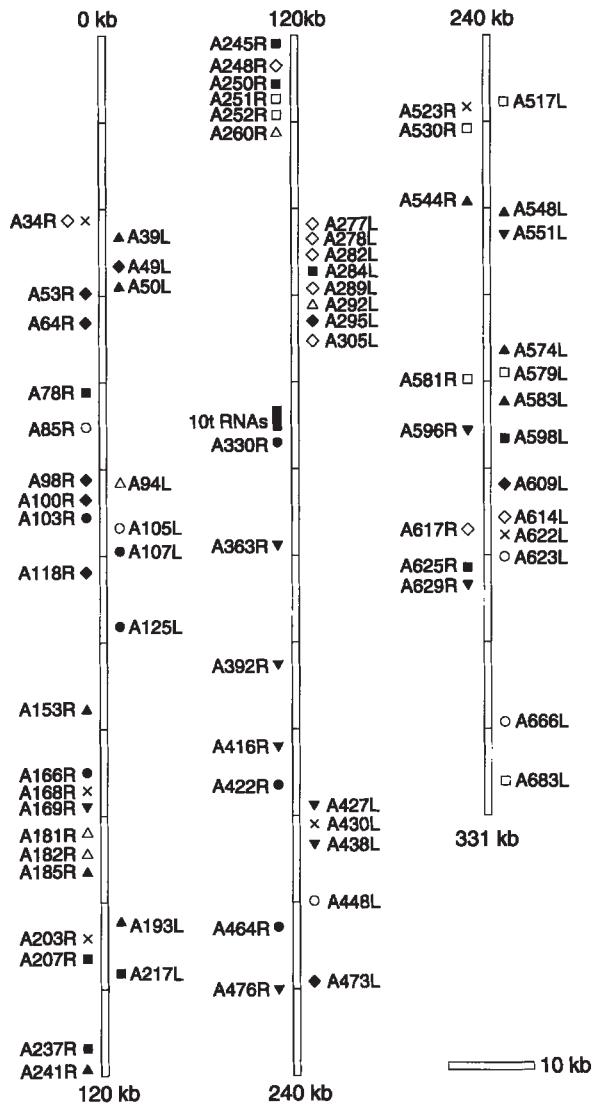
PBCV-1 attaches rapidly, specifically and irreversibly to cell walls, but not to protoplasts of host *Chlorella* NC64A. Following attachment, the host wall dissolves at the point of attachment and viral DNA enters the cell. An empty capsid remains on the cell surface. Since the virus also attaches to and digests wall fragments which have been boiled or extracted by harsh procedures, the virus carries the digestive enzyme(s). However, release of PBCV-1 DNA requires a host function, as attachment to wall fragments does not lead to empty capsids.

Presumably the infecting DNA, plus associated proteins, is rapidly transported to the host cell nucleus. Early PBCV-1 transcription is detected within 5–10 min postinfection. Three observations suggest that early virus transcription occurs in the nucleus. (1) PBCV-1 does not encode a recognizable RNA polymerase gene. (2) No RNA polymerase activity is detected in disrupted PBCV-1 virions. (3) The PBCV-1 DNA polymerase gene, which contains a spliceosomally processed intron, is an early gene product. Presumably the enzymatic machinery to process such an intron resides in the nucleus.

PBCV-1 translation occurs on cytoplasmic ribosomes and early PBCV-1 encoded proteins can be detected within 10–15 min postinfection. PBCV-1 DNA synthesis and late virus transcription begin about 1 h postinfection. Host nuclear and chloroplast DNAs are degraded beginning at about 1 h postinfection, even though the total DNA in the cell increases 4–10-fold by 4 h postinfection. Thus PBCV-1 DNA synthesis requires both energy and deoxyribonucleoside triphosphates from the host. While degraded host DNA may supply some intermediates, virus DNA replication requires 4–10-fold more deoxyribonucleoside triphosphates than recycling can provide.

At approximately 2 h postinfection host cells change cytologically. The nucleolus disintegrates and chromatin heterochromicity increases. The nucleus and cytoplasmic elements become appressed to the chloroplast, leaving one or more finely granulated electron-translucent areas in the cytoplasm (virus assembly centers). Membraneous material, which may function as templates for capsid assembly, appear in the virus assembly centers. This is followed by the accumulation of more dense material (presumably protein and/or glycoprotein) on the exterior membrane surface which gives rise to virus capsids at the periphery of the virus assembly centers. Apparent complete capsids are assembled prior to DNA packaging, which probably enters through an opening in the capsid. By 4–5 h postinfection, cells contain many filled virions which are distributed throughout the cytoplasm. PBCV-1 release, by cell lysis, begins at 4 h postinfection and is complete by 8–10 h postinfection. Mechanical disruption of cells releases infectious virus 30–50 min prior to spontaneous lysis. Consequently, PBCV-1 is completely assembled inside the host and does not acquire a membrane by budding through the host membrane.

The nuclear membrane and structurally intact mitochondria and chloroplasts are usually present at late stages of PBCV-1 infection. The role of organelles in virus replication is unknown. However, photosynthesis inhibitors do not prevent PBCV-1 replication; nor does PBCV-1 replication require host transcription, as PBCV-1 replicates in UV-irradiated cells. However, PBCV-1 replication is slow (latent period of 16–20 h versus 4–10 h) and the burst size is low (2–10 plaque-forming units (PFUs) per cell versus 200–350 PFUs per cell) in UV-treated cells; nor does



▲ DNA replication and repair

(E)	A185R	DNA polymerase
	A544R*	DNA ligase
	A583L*	DNA topoisomerase II
	A193L	PCNA
	A574L	PCNA
	A153R	Helicase
	A241R	Helicase
	A548L	Helicase
(E)	A50L*	T4 endonuclease V
	A39L	Cyclin A/cdk associated protein

▼ Nucleotide metabolism

(E)	A169R*	Aspartate transcarbamylase
	A476R	Ribo. reductase (small subunit)
	A629R	Ribo. reductase (large subunit)
	A427L	Thioredoxin
	A438L	Glutaredoxin
	A551L	dUTP pyrophosphatase
	A596R	dCMP deaminase
	A416R	dG/dA kinase
	A363R	Phosphohydrolase
	A392R	ATPase

● Transcription

	A107L	RNA transcription factor TFIIB
	A125L	RNA transcription factor TFIIS
	A166R	Exonuclease
	A330R	2-5A-dependent RNase
	A442R	Endonuclease
(E)	A103R*	RNA guanyltransferase
	A464R	RNase III

○ Translation

(E, L)	A666L	Translation elongation factor-3
	A85R	Prolyl 4-hydroxylase alpha-subunit
	A105L	Ubiquitin C-terminal hydrolase
	A448L	Protein disulphide isomerase
	A623L	Ubiquitin-like fusion protein
	▬	10 tRNAs

◇ Protein phosphorylation/dephosphorylation

	A34R	Protein kinase
(L)	A248R*	Phosphorylase B kinase
	A277L	Ser/Thr protein kinase
	A278L	Ser/Thr protein kinase
	A282L	Ser/Thr protein kinase
	A289L	Ser/Thr protein kinase
	A305L	Tyr phosphatase
	A614L	Protein kinase
	A617R	Tyr-protein kinase

◆ Sugar and lipid manipulating enzymes

	A64R	Galactosyl transferase
(E)	A98R*	Hyaluronan synthase
(E)	A100R*	Glucosamine synthetase
(E)	A118R	GDP-D-mannose dehydratase
	A295L	Fucose synthase
(E)	A473L	Cellulose synthase
(E)	A609L*	UDP-glucose dehydrogenase
	A49L	Glycerophosphoryl diesterase
	A53R	2-hydroxyacid dehydrogenase

△ Cell wall degrading enzymes

(E)	A181R*	Chitinase
(E)	A182R	Chitinase
(L)	A260R*	Endochitinase
(L)	A292L*	Chitosanase
	A94L	β-1,3 glucanase

□ Restriction/modification enzymes

	A251R*	Adenine DNA methylase (M. *Cvi*AII)
	A252R*	DNA restriction endonuclease (R. *Cvi*AII)
(E)	A517L*	Cytosine DNA methylase (M. *Cvi*AIII)
(L)	A530R*	Cytosine DNA methylase (M. *Cvi*AIV)
(E)	A581R*	Adenine DNA methylase (M. *Cvi*AI)
(E)	A579L*	DNA restriction endonuclease (R. *Cvi*AI)
	A683L	Cytosine DNA methylase (M. *Cvi*AV)

■ Extras

	A207R*	Ornithine decarboxylase
	A217L	Putrescine oxidase
(L)	A237R*	Homospermidine synthase
	A78R	β-alanine synthetase
	A245R	Cu/Zn-superoxide dismutase
	A284L*	Amidase
	A598L	Histidine decarboxylase
	A250R	K+ ion channel protein
	A625R	Transposase

× Virion proteins

A34R
A168R
A203R
A430L
A523R
A622L

Figure 1 Some putative genes encoded by the 330 740 bp chlorella virus PBCV-1. The genes listed to the left of the genome are transcribed in a left to right orientation; the ones listed to the right of the genome are transcribed in a right to left orientation. The genes labeled with an asterisk have been overexpressed and shown to encode a functional enzyme. Symbols (E) and (L) refer to early and late transcripts.

PBCV-1 replication require labile host factors, as the virus replicates in UV-inactivated cells incubated in the dark for up to 8 h before infection.

Virus replication has not been detailed to any extent for any of the other algal viruses. However, like PBCV-1, all of the algal viruses appear to be assembled and DNA is packaged in virus assembly centers located in the cytoplasm.

Virus Transcription

Detailed studies on transcription are lacking for all of the algal viruses and only a few general statements can be made about PBCV-1 transcription. (1) PBCV-1 infection rapidly inhibits host RNA synthesis. (2) Viral transcription is programmed and early transcripts appear within 5–10 min postinfection. Late viral transcription first begins about 1 h postinfection (3) Some early viral transcripts are synthesized in the absence of *de novo* protein synthesis. As expected, the synthesis of later transcripts requires translation of early virus genes. (4) Early and late genes are dispersed in the PBCV-1 genome. (5) Early PBCV-1 transcripts, but only a few late transcripts, are polyadenylated. (6) PBCV-1 ORFs are tightly packed on both DNA strands and the coding regions of some of the genes overlap. The largest distance between PBCV-1 ORFs is a 1788 nucleotide stretch in the middle of the genome. However, this region contains ten tRNA-like genes. (7) Consensus promoter regions for early and late genes have not been identified, although the 50 nucleotides preceding the ATG start codon of most functional PBCV-1 genes are AT-rich. (8) Transcription of some PBCV-1 genes is complex. For example, some gene transcripts exist as multiple bands and these patterns change between early and late times in the virus life cycle. Furthermore, a single chitosanase gene can produce two different-sized proteins.

Additional *Chlorella* NC64A Viruses

Hundreds of large polyhedral, plaque-forming viruses that infect Chlorella NC64A have been isolated from the USA, South America, China, Japan, Israel and Australia. Over 50 of these viruses, called NC64A viruses, have been partially characterized. Like PBCV-1, each of these viruses contains many structural proteins, a large (>300 kb) dsDNA genome with about a 40% GC content, and they are chloroform sensitive. The DNAs of some of these viruses hybridize strongly with PBCV-1 DNA, while others hybridize poorly.

The NC64A viruses can be grouped into at least 16 classes by plaque size, antiserum sensitivity, DNA restriction patterns, sensitivity of the DNAs to restriction endonucleases and the nature and abundance of methylated bases. DNA from each of the viruses contains 5-methylcytosine (5mC) in amounts varying from 0.12 to 47.5% of the total cytosine (**Table 1**). In addition, many viral DNAs contain N^6-methyladenine (6mA) in amounts varying from 1.5 to 37% of the total adenine.

Other Chlorella Viruses

Plaque-forming viruses of *Chlorella* Pbi (called Pbi viruses) have been isolated from fresh water collected in Europe, Australia, Canada and certain parts of the USA. *Chlorella* Pbi, like *Chlorella* NC64A, is a former symbiont of the protozoan *Paramecium bursaria*. *Chlorella* NC64A and *Chlorella* Pbi were originally isolated from American and European isolates of *P. bursaria*, respectively. Pbi viruses, which are serologically distinct from the NC64A viruses, neither infect nor attach to *Chlorella* NC64A and vice versa. Pbi viruses have large dsDNA genomes and are structurally similar to NC64A viruses. The Pbi virus genomes also contain 5mC and 6mA (**Table 1**). DNAs from the Pbi viruses hybridize poorly with the NC64A virus DNAs and have higher GC contents (*c.* 46%) than the NC64A virus DNAs (*c.* 40%).

Viruses morphologically similar to the NC64A and Pbi viruses have also been isolated from chlorella symbiotic in the coelenterate *Hydra viridis*. However, these algae have not been cultured and their viruses do not infect either *Chlorella* NC64A or *Chlorella* Pbi.

Other Algal Viruses

Field isolates representing at least six genera of filamentous brown algae contain virus particles that are morphologically similar to EsV and FsV. Virus expression is variable; virions are rarely observed in vegetative cells but are common in unilocular sporangia (FsV) or both unilocular and plurilocular sporangia (EsV). EsV viruses only infect the free-swimming, zoospore stage of *Ectocarpus* sp. All natural isolates of *Feldmania* sp. are infected with virus and so infection studies cannot be conducted.

Viruses that infect *Micromonas pusilla* and *Chrysochromulina brevifilum* have been isolated from many marine environments. These viruses can be distinguished by DNA restriction patterns.

Viruses Encode DNA Site-Specific Endonuclease and DNA Methyltransferase Enzymes

Since the chlorella virus DNAs contain 5mC and 6mA and the methylated sequences differ from those in

Table 2 Representative DNA methyltransferase and DNA site-specific endonuclease enzymes encoded by chlorella viruses

Virus	*DNA methyl transferase*	*Sequence*	*DNA site-specific endonuclease*	*Sequence*[a]
PBCV-1	M.*Cvi*AI	G^{m}ATC	R.*Cvi*AI	/GATC
	M.*Cvi*AII	C^{m}ATG	R.*Cvi*AII	C/ATG
	M.*Cvi*AIII	RC^{m}CG?		
	M.*Cvi*AIV	RC^{m}CG?		
	M.*Cvi* AV[b]	RG^{m}C(T/C/G)		
SC-1A	M.*Cvi*SI	TGC^{m}A		
	M.*Cvi*SII	C^{m}ATG		
	M.*Cvi*SIII	TCG^{m}A		
	M.*Cvi*SIV	G^{m}ATC		
	M.*Cvi*SV	RC^{m}CG?		
	M.*Cvi*SVI[b]	RG^{m}C(T/C/G)		
NY-2A	M.*Cvi*QI	GT^{m}AC	R.*Cvi*QI	G/TAC
	M.*Cvi*QII	R^{m}AR	NY2A-nickase[c]	R/AG
	M.*Cvi*QIII	TCG^{m}A		
	M.*Cvi*QIV	G^{m}ATC		
	M.*Cvi*QV	TGC^{m}A		
	M.*Cvi*QVI	G^{m}ANTC		
	M.*Cvi*QVII	C^{m}ATG		
	M.*Cvi*QVIII	RG^{m}C(T/C/G)		
	M.*Cvi*QIX	^{m}CC		
	M.*Cvi*QX	^{m}CGR		

[a]The slash indicates the cleavage site.

[b]Enzyme is not functional.

[c]Enzyme only cleaves one strand of the dsDNA.

host DNA, it is not surprising that the viruses encode multiple, sequence-specific, 5mC and 6mA DNA methyltransferases. However, unexpectedly, these viruses also code for DNA site-specific (restriction) endonucleases (**Table 2**). Thus algal viruses are a new source of DNA restriction endonuclease and DNA methyltransferase genes and the first source from a nonprocaryotic system. Some of the virus encoded site-specific endonucleases have the same recognition and cleavage specificities as bacterial restriction endonucleases (e.g. R.CviAI (/GATC) and R.CviBI (G/ANTC)); others are neoschizomers of bacterial endonucleases (e.g. R.CviAII(C/ATG) and R.CviQI (G/TAC)); and still others have novel recognition sites (e.g. R.CviJI (RG/CY) and R.CviRI (TG/CA)). Two of the site-specific endonucleases (NYs1-nickase (/CC) and NY2A-nickase (R/AG)) only cleave one strand of dsDNA, i.e. they are sequence-specific nicking enzymes.

The biological function(s) of the virus-encoded DNA site-specific endonucleases and DNA methyltransferases is unknown. However, the common occurrence of these genes in the viruses suggest that these enzymes have an important function in the natural history of the viruses. Bacterial restriction/methylation systems confer resistance to foreign DNAs and DNA viruses. In fact, the name 'restriction' refers to their role in excluding foreign DNA. Conversely, bacterial DNA methyltransferases serve to prevent self-digestion of bacterial DNA. We have considered two possible functions for the chlorella virus enzymes. (1) Chlorella virus endonucleases help degrade host DNA, providing deoxynucleotides that are recycled into virus DNA. Methylation of nascent DNA by the cognate methyltransferase would protect it from suicidal self-digestion. (2) Chlorella virus endonucleases prevent infection of a cell by a second virus. Experimental tests of these two hypotheses have yielded ambiguous results.

Although the genomes of most of the other algal viruses have not been characterized, preliminary studies indicate that methylated bases may be common in the genomes of these viruses.

Ecology

Eucaryotic algae are important components of both fresh water and marine environments; however, the

significance of viruses or VLPs in these systems is only beginning to be appreciated. The chlorella viruses are ubiquitous in fresh water collected throughout the world and titers as high as 40 000 infectious particles per ml have been reported in native waters. Typically the titer is 1–100 infectious particles per ml. The titers are seasonal with the highest titers in the spring. It is not known whether chlorella viruses replicate exclusively in algae symbiotic with paramecia or if the viruses have another host(s). In fact, it is not known if paramecium chlorellae exist free of their hosts in natural environments. It is known, however, that symbiosis protects paramecium chlorellae from infection and that the algae only become infected after they are released from the paramecium.

From an ecology standpoint, the MpV viruses that infect *Micromonas pusilla* have been the most extensively studied. Like the chlorella viruses, high titers of MpV viruses can be isolated from diverse marine environments. Detailed studies on the turnover of MpV viruses in nature indicate that these viruses probably play an important role in native populations of *M. pusilla*. Presumably similar viruses strongly influence populations of other marine algae.

Ectocarpus sp. and *Feldmania* sp. isolated from all over the world are infected with lysogenic EsV and FsV viruses, respectively. Lysogeny is consistent with the observation by many investigators that VLPs appear infrequently in eucaryotic algae and at certain stages of algal development. The apparent lack of infectivity by many of the previously observed VLPs in eucaryotic algae is also consistent with a lysogenic lifestyle. The VLPs might either infect the host and resume a lysogenic relationship or be excluded by preexisting lysogenic viruses.

Algae might also harbor viruses in a carrier-state relationship (also called pseudolysogeny), where at any one time a small population of algae are continually infected by virus. This type of relationship occurs with bacteriophages. A carrier-state-type relationship between PBCV-1 and *Chlorella* NC64A has been observed in the laboratory.

Future Perspectives

The algal viruses are similar in some respects to other large DNA viruses, in particular African swine fever virus, iridoviruses, poxviruses and herpesviruses. However, they also have some unique and interesting properties. (1) Algal virus genomes are the largest virus genomes characterized to date, more than one-half the size of the smallest free-living organism. (2) The chlorella viruses are a new source of DNA methyltransferase and site-specific endonuclease enzymes. (3) The chlorella viruses are the first viruses to encode many, if not all, of the enzymes involved in the glycosylation of their glycoproteins. (4) Chlorella virus PBCV-1 is the first virus to contain at least two different types of introns in its genome. (5) PBCV-1 is the first virus to code for enzymes such as aspartate transcarbamylase, translation elongation factor 3, hyaluronan synthase, UDP-glucose dehydrogenase, ornithine decarboxylase and histidine decarboxylase. (6) The brown algal viruses have a lysogenic phase as part of their normal life cycle, and convert to a lytic phase during the development of sporangia.

See also: **African swine fever virus (*Asfarviridae*); Frog virus 3 (*Iridoviridae*); Host-controlled modification and restriction; Vaccinia virus (*Poxviridae*).**

Further Reading

Fuhrman JA and Suttle CA (1993) Viruses in marine planktonic systems. Oceanography 6: 5.

Li Y, Lu Z, Sun L *et al* (1997) Analysis of 74 kb of DNA located at the right end of the 330-kb chlorella virus PBCV-1 genome. *Virology* 237: 360.

Muller DG, Kapp M and Knippers R (1998) Viruses in marine brown algae. *Adv. Virus Res* 50: 49.

Nelson M, Zhang Y and Van Etten JL (1993) DNA methyltransferases and DNA site-specific endonucleases encoded by chlorella viruses. In: Jost JP and Saluz HP (eds) *DNA Methylation: Molecular Biology and Biological Significance,* p. 186. Basel: Birkhauser.

Van Etten JL, Lane LC and Meints RH (1991) Viruses and virus-like particles of eucaryotic algae. *Microbiol. Rev.* 55: 586.

Alpha 3 Bacteriophage *see* **Coliphage ϕX174 and related phages (*Microviridae*)**

AMPHIBIAN HERPESVIRUSES (*HERPESVIRIDAE*)

Allan Granoff, St Jude Children's Research Hospital, Memphis, Tennessee, USA

History

In 1934, Baldwin Lucké, a pathologist at the University of Pennsylvania, discovered that kidney tumors (adenocarcinomas) of the common leopard frog, *Rana pipiens*, contained intranuclear acidophilic inclusion bodies typical of certain virus infections. On the basis of this observation and further study, Lucké concluded that the tumor was caused by a virus. More than 20 years later, electron microscopy revealed virus particles typical of herpesviruses in frog kidney tumor cells. Another decade passed before a causative relationship was established between the Lucké tumor herpesvirus (LTHV) and kidney adenocarcinoma in *R. pipiens*. Subsequent efforts to isolate and cultivate LTHV failed, but a second, distinct herpesvirus was isolated and grown in tissue culture. This virus (frog virus 4, FV4) was found not to be tumorigenic. To date, these remain the only two amphibian herpesviruses that have been identified. In contrast to the available information on the other animal herpesviruses, little is known of the properties of these viruses and their interactions with cells they infect.

Taxonomy and Classification

LTHV and FV4 are members of the family *Herpesviridae*. They have not been assigned to subfamily and genus.

Lucké Tumor Herpesvirus

Geographic distribution

The Lucké tumor and its associated LTHV has usually been limited to *R. pipiens* in north central and northeastern United States and adjacent southern Canada. For reasons that are unclear, the population of affected frogs in these regions has decreased drastically in recent years.

Properties of the virion

In size and structure, LTHV is similar to herpesviruses found in both higher vertebrates (humans) and lower vertebrates (fish). Virions in infected cell nuclei measure approximately 100 nm in diameter, with the size increasing after cellular membranes are acquired; extracellular virus increases to about 170 nm (**Fig. 1**).

The DNA of LTHV is a linear, double-stranded molecule with a base composition of 45–47% guanosine plus cytosine (G + C) and a molecular weight of 66×10^6, as determined by contour length measurement. If this value is confirmed by other methods of measurement, it is much lower than that of other herpesvirus DNAs.

Virus replication and gene expression

LTHV has not been cultured *in vitro*, a prerequisite for temporal studies of the kinetics of virus growth and molecular and biological events in the replication

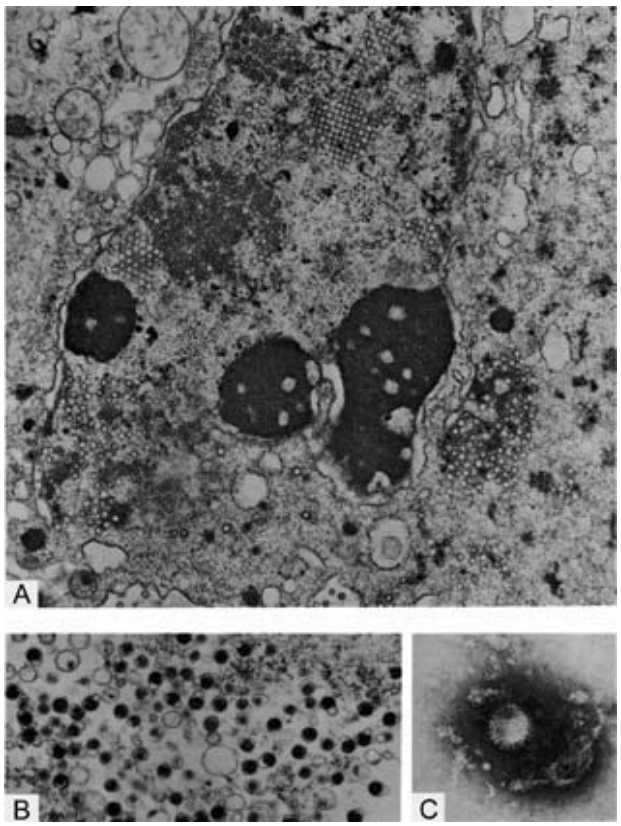


Figure 1 (**A**) Thin section of an inclusion-bearing Lucké tumor cell with typical herpesvirus particles in various stages of development in the nucleus. ×19 400. (**B**) Enveloped virions found extracellularly. ×24 000. (**C**) Negatively stained nonenveloped particle showing typical herpesvirus morphology. ×110 000. (Reproduced from *Oncogenesis and Herpesviruses*, 1972, p. 172. Hyon: International Agency for Research on Cancer.)

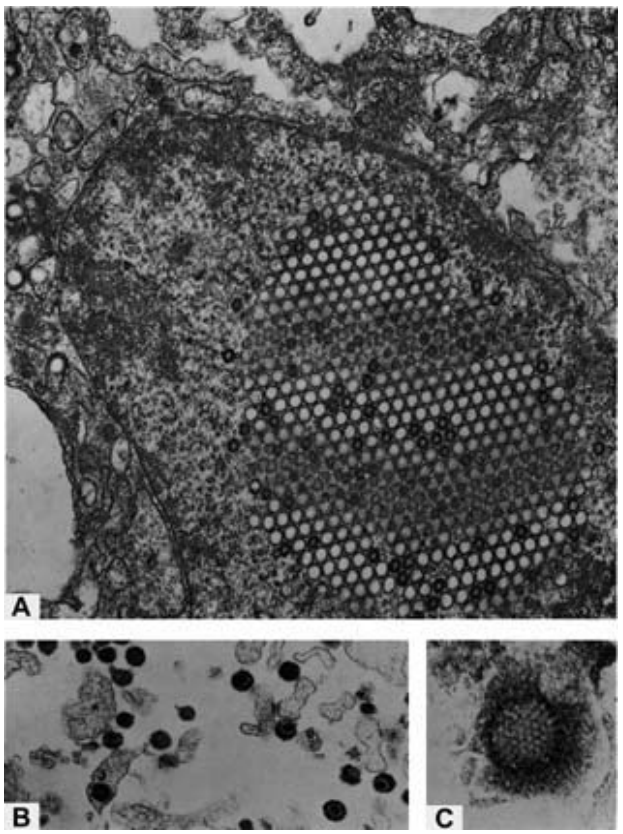


Figure 2 (**A**) Thin section of an FV4-infected *R. pipiens* embryo cell containing a nuclear crystalline array of typical herpesvirus in various stages of development. ×39 500. (**B**) Extracellular enveloped FV4. ×20 000. (**C**) Negatively stained unenveloped FV4 particle sedimented from infected fluid ×180 000. (Reproduced from *Oncogenesis and Herpesviruses*, 1972, p. 172. Lyon: International Agency for Research on Cancer.)

cycle. This has not been from lack of effort; cells from a number of amphibian, piscine and mammalian species have been tested under various conditions.

There is a direct relationship of temperature to virus replication and expression of virus genes. At temperatures below 11.5°C, tumor cells *in vivo* or *in vitro* have typical intranuclear inclusions and virus particles, whereas the cells are free of virus at higher temperatures. This is true for tumor cells in the intact animal, in fragment cultures *in vitro*, and in fragments explanted to the anterior eye chamber of *R. pipiens*. Also, tumor cells can become virus-free or can be induced to produce virus by shifting the temperature of either the intact animal or the *in vitro* tumor fragment culture system.

The immune system does not appear to play a role in the failure of LTHV to replicate at higher temperature in the intact animal because explants of virus-containing tumor tissue cultured *in vitro* at higher temperature become virus-free in the absence of antibody. Thus, temperature is the controlling factor in replication of LTHV. Although virus particles have not been demonstrated in tumor cells at the higher temperatures, virus genetic information is expressed in the form of virus-specific RNA and virus-associated membrane antigens. Therefore, the LTHV genome resident in virus-free tumor cells at elevated temperature transcribes RNA, the translational product of which is probably expressed as a membrane-associated antigen.

Oncogenicity

Tumors can be induced at a high frequency in *R. pipiens* embryos and larvae inoculated with virus-containing cell fractions from Lucké tumors. Although Lucké tumors have been found in nature only in *R. pipiens*, typical renal tumors have been induced in embryos of *R. clamitans* and *R. palustris* and in hybrids of *R. palustris* and *R. pipiens*. In addition, LTHV that has been purified by rate zonal centrifugation of cytoplasmic fractions of virus-containing tumors is oncogenic when injected into developing frog embryos. Tumor-bearing frogs frequently have ascites and the ascitic fluid contains virus that induces tumors when inoculated into developing frog embryos.

Transmission

Data on the natural transmission of LTHV and factors that influence tumor formation in nature are lacking. However, two possible modes of natural transmission have been suggested: (1) contact with urine, which has been found to contain virus particles; (2) infection of oocytes in tumor-bearing female frogs. In the past, Lucké tumors were found in *R. pipiens* with high frequency (1–9%) suggesting ready transmission of the virus.

Frog Virus 4

During attempts to isolate the Lucké tumor herpesvirus from homogenates of Lucké tumors and urine of tumor-bearing frogs, a herpesvirus was isolated that had a cytopathic effect on frog embryo cells derived from *R. silvatica*. Subsequently, two *R. pipiens* embryo cell lines were also found to be susceptible to this virus. In size, morphology and site of synthesis, the virus – designated frog virus 4 (FV4) – is indistinguishable from LTHV and from other members of the herpesvirus family (**Fig. 2**).

Properties of the virion

Several properties, however, distinguish FV4 from LTHV. The DNA of FV4, extracted from purified

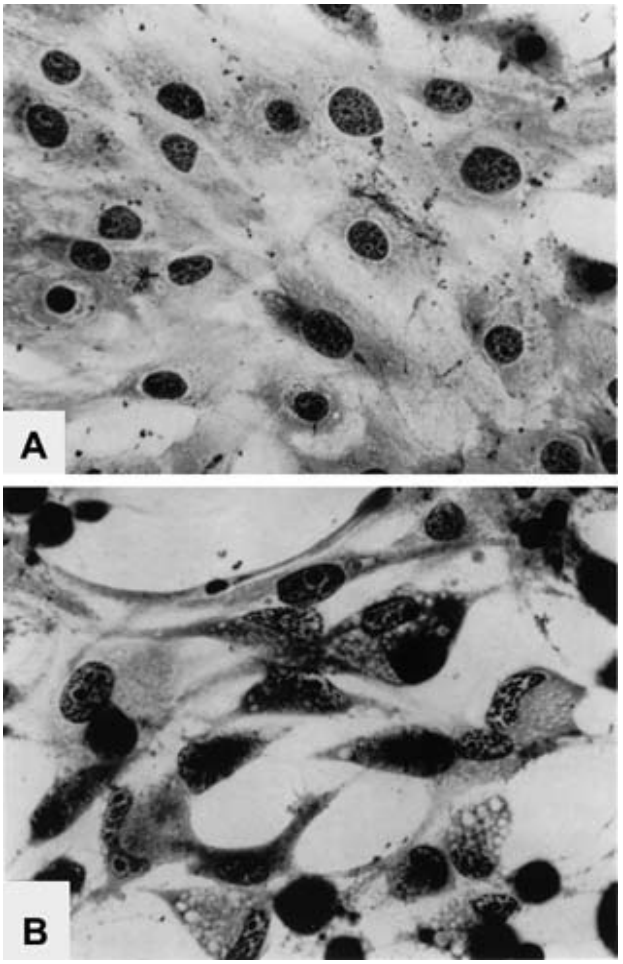


Figure 3 *R. pipiens* embryo cells infected with FV4 illustrating cytoplasmic vacuolization and intranuclear inclusions (**A**) uninfected, (**B**) FV4 infected (May–Gruenwald–Giemsa), ×356. (Reproduced from Merele Mizell (1969) *Recent Results in Cancer Research,* Special Supplement, *Biology of Amphibian Tumors,* p. 279. Springer-Verlag.)

virions, is a linear, double-stranded molecule. Its molecular weight, about 77×10^6, is comparable to the DNA of other herpesviruses that infect lower vertebrates, including channel catfish and trout, but significantly greater than that for LTHV. The base composition of FV4 also differs from that of LTHV, comprising 54–56% G+C. There is no sequence homology between FV4 and LTHV, as determined by DNA–DNA hybridization.

Virus replication

Unlike LTHV, FV4 can be readily cultured *in vitro*. The virus has a long growth cycle and produces a characteristic cytopathic effect in *R. pipiens* embryo and adult kidney cells at 25°C within 10–21 days after infection. This effect comprises rounding, vacuolization, enlargement of nuclei, polykaryon formation and intranuclear inclusions typical of the cytopathology of other herpesviruses (**Fig. 3**).

Transmission

Although FV4 infects *R. pipiens* embryos and larvae, animals that survive virus infection do not develop kidney tumors.

Future Perspectives

Further research on LTHV has been hampered by the lack of available virus-containing tumors and of a reproducible virus-susceptible cell culture system. The latter is an absolute requirement as a resource of substantive amounts of virus and for study of the various features of virus–cell interactions under controlled conditions. Initial information on FV4 suggests that this virus would be a useful model for furthering our understanding of comparative virology and the evolution of herpesviruses. However, research attention has focused on the more medically important herpesviruses and little additional work has been done on this potentially interesting one.

See also: **Fish herpesviruses (*Herpesviridae*); Herpes simplex viruses (*Herpesviridae*): General features, Molecular biology; Latency; Marek's disease virus (*Herpesviridae*).**

Further Reading

Granoff A (1983) Amphibian herpesviruses. In: Roizman B (ed.) *The Herpesviruses,* Vol. 2, p. 367, New York: Plenum.

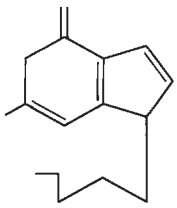
Gravell M. (1971) Viruses and renal carcinomas of *Rana pipiens*. X. Comparison of herpes-type viruses associated with Lucké tumor-bearing frogs. *Virology* 36: 467.

Naegele RF, Granoff A and Darlington RW (1974) The presence of Lucké herpesvirus genome in induced tadpole tumors and its oncogenicity: Koch–Henle postulates fulfilled. *Proc. Natl. Acad. Sci. USA* 71: 380.

Skinner MS and Mizell M (1972) The effect of different temperatures on herpesvirus induction and replication in Lucké tumor explants. *Lab. Invest.* 26: 671.

Tweedell KS (1967) Induced oncogenesis in developing frog kidney cells. *Cancer Res.* 27: 2042.

ANTIVIRALS



A Kirk Field, Department of Pharmacology and Toxicology, UMass Medical Center, Worcester, Massachusetts, USA

Catherine A Laughlin, NIAID, Bethesda, Maryland, USA

Introduction

Accomplishments in the discovery and development of antiviral drugs over the past few years have come at an ever increasing rate, so that the profile of available therapies today differs considerably from that described in the first edition of the *Encyclopedia of Virology*. In large part this has been driven by the increased emphasis on developing effective therapies for the treatment of human immunodeficiency virus (HIV) and diseases associated with immunosuppression, such as cytomegalovirus (CMV) retinitis and pneumonitis. But this progress has also been driven by the identification of novel targets (e.g. HIV and herpesvirus proteases) for antiviral therapy, and the considerable improvements in understanding the structure and functions of virus-encoded gene products. Just as new antiviral drugs emerge through improvements in understanding virus replication at the molecular level, so too is the improved understanding of virus gene function a result of new antiviral drug discovery and characterization of resistance mechanisms. Thus, the understanding of the mechanism of CMV-dependent phosphorylation of ganciclovir led to better definition of the CMV UL97 gene, and this occurred with the availability of ganciclovir-resistant mutants altered in the UL97 gene. Similarly, the discovery of the mechanisms of cytomegalovirus inhibition by the benzimidazole derivatives such as 1263W95 is helping to elucidate the mechanisms of DNA processing and virion maturation.

Another development that has driven advances in antiviral chemotherapy has been the realization that resistance to monodrug therapy accounts for considerable failure in the treatment of HIV. Thus the emphasis has shifted to drug combination therapy, with combinations of reverse transcriptase inhibitors and protease inhibitors getting most attention. An understanding of the dynamics of virus and host cell interaction and turnover and an appreciation of the importance of 'viral load' have led to these new strategies to inhibit the emergence of an array of virus mutants. As Dr Raymond Schinazi recently wrote 'Dead viruses don't mutate', recommending that 'we should learn rapidly from our experience with HIV and plan accordingly to hit the virus early with our best drugs' (Editorial (1997) *Int. Antiviral News* 5: 122). That philosophy is broadly applicable.

Here we have reviewed the newly approved antiviral drugs and drugs in clinical development, while trying to avoid redundancy with the first edition. Also, where applicable, we emphasize the strategies for overcoming treatment failure, including antiviral combination therapy. Only selected compounds will be mentioned in the text, so for ease as a quick and more complete reference, details of compounds and their development status are presented in **Table 1**.

Human Immunodeficiency Virus (HIV)

The emergence of HIV in the 1980s has had a devastating impact on the world's population. Although transmission in the USA is primarily through exchange of contaminated blood associated with male homosexual intercourse and illicit drug use, it is also increasingly transmitted by heterosexual intercourse, which is the primary mode of transmission in many parts of the world. Not only has the spread of HIV been a huge stimulus for new drug discovery and development, but also it has led to a revolution in the way clinical trials are conducted, including the use of surrogate markers, expedited Food and Drug Administration (FDA) review and expanded access to experimental agents outside of formal clinical trials. Numerous studies also now demonstrate that surrogate markers can be useful substitutes for the measurement of a drug's effect on disease progression and death. Changes in CD4 counts were initially, with some caveats, shown to be a reliable predictor for death. More recently, measurements of viral load appear to be more sensitive markers of therapeutic effect.

There are many stages of the viral replication cycle which can be targeted by anti-HIV therapeutics. These include: attachment and entry, uncoating, reverse transcription of genome RNA, DNA integration, transcription from the integrated genome, translation of viral proteins, and assembly and egress of progeny virions. To date, the approved drugs all act at the level of reverse transcription (reverse

Table 1 Approved and experimental antiviral drugs

Antiviral drug	*Chemical class*	*Probable mechanism of action*	*Potential virus/disease targets*	*Development status*
Human immunodeficiency virus				
Azidothymidine (AZT, Zidovudine, Retrovir)	Nucleoside analogue	RT inhibitor	First line Rx for adults and children 3–12 years; also to prevent transmission to newborns	Approved oral 1989; i.v. in 1990; transmission to newborns in 1994
Didanosine (ddI, Videx)	Same	Same	Adult and pediatric for advanced HIV refractory to AZT	Approved 1991
Hivid (ddC, Zalcitabine)	Same	Same	Combo with AZT	Approved 1992
			Monotherapy	Approved 1994
Stavudine (D4T, Zerit)	Same	Same	HIV unresponsive to other drugs	Approved 1994
			Full usage	Approved 1995
Lamivudine (3TC, Epivir)	Same	Same	Combo with AZT	Accelerated approval in 1995; full approval in 1997
Abacavir (GW 1592)	Same	Same	Combo therapy	Phase II/III
Lodenosine (F-ddA)	Same	Same	HIV	Phase I
Adefovir (PMEA, GS 393)	Nucleotide analogue	Same	HIV	Expanded access
Adefovir dipivoxil (GS 840, bisPOM-PMEA)	Adefovir prodrug	Same	HIV	Phase II/III
Delavirdine (Rescriptor, BHAP U-90152)	NNRTI		HIV combo therapy	Approved 1997
Nevirapine (BI-RG-587)	Dipyridodiazepinone	Same	HIV, in combo with nucleoside analogues	Approved 1996
Efavirenz (Sustiva, DMP-266)	Benzoxazin	Same	HIV, in combo with Indinavir	Phase III
HBY 097	Quinoxaline	Same	HIV	Phase II
Saquinavir (Inverase, Ro31-8959)	Peptide mimetic	Protease inhibitor	Combo with AZT for advanced HIV	Approved 1995
Fortovase	Gel formulation of saquinavir		Same	Approved 1997
Ritonavir (Norvir, ABT-538)	Same	Same	HIV, alone or in combo with nucleoside analogue RT inhibitor	Approved 1996 Pediatric approved 1997
Indinavir (Crixivan, MK 639, L-735,524)	Same	Same	Same	Approved 1996
Nelfinavir (Viracept, AG-1343)	Same	Same	HIV, adults and children	Approved 1997
Amprenavir (GW141W94, VX-478)	Carbamate derivative	Same	Combo with AZT & 3TC or NNRTI	Phase II/III
Zintevir (T30177)	Oligonucleotide	Virus binding and integrase inhibitor	HIV	Phase I/II
ISIS 5320	Same	Not well established	HIV	Studies on hold
GEM 92	Same	Same	HIV	Studies on hold
Herpesviruses				
Adenine arabinoside (Vira-A)	Nucleoside analogue	DNA Synthesis inhibitor	HSV-1, -2 VZV	Approved (i.v.) for herpes encephalitis, neonatal herpes
Acyclovir (Zovirax)	Same	Virus-activated DNA-synthesis inhibitor	HSV-1, -2 VZV	Approved, oral topical, i.v., ophthalmic

Table 1 Continued

Antiviral drug	*Chemical class*	*Probable mechanism of action*	*Potential virus/disease targets*	*Development status*
Valacyclovir (Valtrex)	Acyclovir prodrug	Same	HSV-1, -2 VZV	Approved(oral) for genital herpes, herpes zoster
Penciclovir	Nucleoside analogue	Same	HSV-1, -2 VZV	Topical for herpes labial
Famciclovir (Famvir)	Penciclovir prodrug	Same	HSV-1, -2 VZV, HBV	Approved (oral) for genital herpes, herpes zoster
Foscarnet (Foscovir)	Pyrophosphate analogue	DNA pol inhibitor	HSV-1, -2 VZV,HCMV	Approved (i.v.) CMV retinitis, resistant HSV
Docosanol (Lidakol)	Alkyl alcohol	Lipase inhibitor	Herpes labialis	Topical; phase III
348U87 +acyclovir	Combination	Ribonucleotide reductase inhibitor	HSV-1, -2	Phase II
Ro32-4397		Thymidine kinase inhibitor	HSV-1, -2	Experimental studies; orally active
HBPG	Nucleoside analogue	Thymidine kinase inhibitor	HSV-1	Experimental studies
BILS45BS	Thiazolyl phenyl-based	Helicase-primase inhibitor	HSV-1	Experimental studies; orally active
Ganciclovir (Cytovene)	Nucleoside analogue	Virus-activated DNA pol inhibitor	HCMV	Approved for HCMV retinitis; oral, injectable
Ganciclovir (Vitrosert)	Same	Same	HCMV	Approved vitreal implant for HCMV retinitis
Valgancyclovir	Valine ester prodrug of gancyclovir	Same	Same	Phase III
Cidofovir (Vistide)	Nucleotide analogue	DNA pol inhibitor	HCMV	Approved oral for HCMV retinitis
Lobucavir	Nucleoside analogue	DNA pol inhibitor	HCMV, HBV	Phase II oral for HCMV retinitis
1263W94	Benzimidazole analogue	DNA synthesis inhibitor	HCMV	Experimental studies
GW275175X	Same	DNA processing inhibitor	HCMV	Phase I
Unnamed	Same	DNA processing inhibitor	HCMV	Experimental studies
Bis-POMPMEA (Adepfovir dipivoxil, GS 840)	Nucleotide analogue	DNA pol inhibitor	HCMV	Phase III oral HCMV retinitis
Fomivirsen (Vitravene, ISIS 2922)	Oligonucleotide	Mechanism uncertain	HCMV	Approved 1998 for intravitreal HCMV retinitis
(ISIS 13312)		Same	Same	Phase I/II
GEM 132	Same	Antisense UL36/37 splicing inhibitor	HCMV	Phase I/II intravitreal HCMV retinitis Phase I/II i.v. for generalized HCMV
Myxoviruses				
Amantadine (Symmetrel)	Adamantane derivative	Inhibits viral M2 ion channel	Influenza A	Approved oral 1966
Rimantadine (Flumadine)	Same	Same	Same	Approved oral 1992
Zanamavir (GG167)	Sialic acid transition state analogue	Inhibits viral neuraminidase	Influenza A, B	Phase III
GS4104	Same	Same	Same	Phase III (oral)
2′-deoxy-2′-fluoroguanosine	Nucleoside analogue	Inhibits transcription	Same	Preclinical studies

Table 1 Continued

Antiviral drug	*Chemical class*	*Probable mechanism of action*	*Potential virus/disease targets*	*Development status*
Paramyxoviruses				
Ribavirin (Virazole)	Triazole nucleoside analogue	Inhibits RNA polymerases and transcription	RSV, PIV (also broad antiviral activities)	Aerosol approved for RSV in hospitalized infants Aerosol therapy of bone marrow transplant patients under evaluation
RespiGam	Immune globulin	Neutralizes viral infectivity	RSV	Prophylaxis in high-risk infants
Monoclonal antibodies	Same	Same	Same	Preclinical, early clinical, and phase III
Hepatitis viruses				
Interferons (INF) alpha/gamma (Intron A, Roferon)	Recombinant proteins	Immune modulator, direct antiviral	HBV, HCV	Approved i.m. injectable
Intron A/Rebetol, Rebetron (INF+ribavirin)			HCV	Approved 1998 for Intron A relapsed adults
INF+thymosin α_1		Immune modulators	HBV, HCV	Phase III
Famciclovir	See above	See above	HBV	Phase III
Lamivudine	See above	See above	HBV	Phase III
GS 840	See above	See above	HBV	Phase I
BMS-200475	Nucleoside analogue	DNA polymerase inhibitor	HBV	Phase I
Picornaviruses				
Pleconaril (VP-63843)	Isoxazole	Capsid binding, inhibits uncoating	Enteroviruses Common cold	Phase III, aseptic meningitis
Soluble ICAM (sICAM)	Peptide	Cell receptor decoy	Rhinoviruses	Phase II in prophylaxis in experimental rhinovirus infections
Papillomaviruses				
Interferon alpha (Intron, Aferon)	Protein	Immunomodulator direct antiviral	Intrawart injection	Approved 1996
Imiquimod (Aldera)	Quinoline	Immunomodulator	Topical for genital warts	Approved 1997
Condylox	Podophyllotoxin	Anti-mitotic	Same	Approved 1991
Cidofovir	Nucleotide analogue	Unknown	Topical/local genital warts, RRP	Phase III for genital warts
Ribavirin	See above	See above	Intravenous/oral laryngeal warts	Phase I/II clinical
5-fluorouracil (AccuSite)	Nucleoside analogue	Anti-mitotic	Intrawart injection	Approved in UK and Europe
Exotic Viruses (see text for discussion of antiviral agents)				

transcriptase inhibitors, or RTIs) or progeny assembly and egress (HIV protease inhibitors, or PIs); however, compounds addressing virtually every identified target are in stages of preclinical or clinical development.

For years it appeared that HIV replication smoldered between a burst at the time of primary infection and progressively higher chronic levels in the final stages of disease; however, it is now appreciated that replication is explosive throughout the disease course, with the early seeding of a large reservoir of latently-infected lymphocytes. Furthermore, host immune responses are continuously high in the desperate attempt to control viral replication and prevent the infection of new lymphocytes. If the assumption that pathogenesis is related to the rate of viral replication

is valid, then there is cause for optimism regarding the potential utility of antiviral therapeutics which can significantly reduce replication rates. This high level of virus replication also provides a rationale for the facility with which HIV develops resistance to monotherapies and underscores the urgency of the search for ever more potent aniviral drugs directed at novel gene targets for use in drug combinations.

The implementation of highly active antiretroviral therapy (HAART) has revolutionized the treatment of HIV infection. HAART has resulted in the restoration of immune function in patients with AIDS as well as a corresponding decrease in the incidence of opportunistic infections such as CMV retinitis. HAART generally involves the use of two RTIs in combination with a PI. It is important to change one or both RTIs at the time of addition of the PI, as resistance to the RTIs may have already developed. The stage of HIV disease also has an impact on the success of HAART, as patients with high viral loads ($>10^5$ RNA copies ml^{-1} blood) or low CD4 counts do not do as well as healthier patients. HAART increases the complexity of the dosing regimen as well as the number of pills, and strict patient compliance is also key to the success of HAART.

HAART has been so successful that it is the basis of the US Department of Health and Human Services (DHHS) treatment guidelines published in June 1997. These guidelines conclude that the damage to the immune system is the result of continued replication of HIV; and since replication of HIV is an error-prone process, the more replication, the likelier the selection of resistant mutants. Therefore the guidelines advise that the overriding goal of treatment is to decrease and maintain HIV replication below 10 000–20 000 copies of RNA ml^{-1}. Consequently, to reduce both the immune damage and mutant selection associated with viral replication, the guidelines recommend that initial treatment should consist of a combination of three anti-HIV agents with at least two others substituted at the same time if a patient's viral load increases. The current preferred form of HAART is two nucleoside RTIs combined with a PI. The guidelines also recommend that pregnant women receive azidothymidine (AZT) during pregnancy (at least from the 14th week) and intravenous AZT during labor and delivery, with the newborn receiving oral AZT for the first 6 weeks post partum.

Although HAART is dramatically effective in reducing or obliterating serum viral loads and preventing the emergence of drug-resistant virus, it has not been successful in eradicating virus from latently infected cells. The discovery that HIV remains dormant for years in a pool of latently infected lymphocytes led to the realization that HAART may not totally eliminate viral replication. Instead it may allow a replication level that is sufficient to establish new inducible latent infections, or the lifespan of the latently-infected cells may be sufficiently protracted to sustain infection. On an economic level, HAART is certainly financially beyond the reach of the vast majority of the world's HIV-infected population, emphasizing the need for prevention as well as treatment.

Approved agents

A. Nucleoside reverse transcriptase inhibitors (NRTIs)

Azidothymidine (AZT, retrovir), was the first drug to show clinical efficacy and receive FDA approval. Its licensure is for first-line therapy in early- and late-stage adults and in children over 3 months of age. The 1997 DHHS guidelines recommend its use with ddI, ddC or 3TC as the two nucleoside components of a three or more drug HAART regimen. AZT is a thymidine analogue in which the 3′OH is replaced by an azido group. AZT is phosphorylated in HIV-infected cells to AZT triphosphate and is preferentially incorporated instead of the natural thymidine triphosphate into replicating viral DNA by the HIV reverse transcriptase, resulting in chain termination. Thus, it inhibits reverse transcription at two levels, substrate specificity and elongation, with selectivity resulting from its low level of utilization by cellular DNA polymerase α. The first placebo-controlled study done with AZT in patients with advanced AIDS indicated that AZT therapy might prolong survival. Therapy has also been shown to reduce the number of opportunistic infections. The primary side effect is bone marrow toxicity, but it can also cause myopathy and gastrointestinal distress. Like the other nucleoside analogues, it is orally bioavailable and is excreted renally. AZT given during the last two trimesters of pregnancy and to the newborn after birth has been shown to prevent maternal to fetal transmission of HIV. The emergence of AZT-resistant virus is associated both with duration of therapy and stage of disease, and detailed characterization of HIV mutations is well documented. With the demonstration of the therapeutic potential of AZT, other dideoxynucleosides were also investigated for therapeutic potential and resulted in additional licensures.

ddC (Hivid, Zalcitabine): like AZT, ddC is converted to the active antiviral agent ddCTP by cellular kinases and incorporated by the HIV RT into proviral DNA as a chain terminator, in place of dCTP. Unfortunately, ddCTP is also a substrate for cellular polymerase β and mitochondrial polymerase γ. The

latter may be responsible for its primary toxicities, peripheral neuropathy occurring in about 25% of treated patients and pancreatitis in about 1% of treated patients. ddC is orally bioavailable and is excreted through the kidneys. Resistant mutants are often cross-resistant to ddI, d4T and 3TC. ddC is recommended in the 1997 DHHS Guidelines as a possible choice in combination with AZT for the two nucleosides in combination with a PI in HAART, but because of overlapping toxicities, ddC should not be used with ddI or d4T. The indication for which ddC was approved is as a combined therapy with AZT for patients with advanced AIDS.

ddI (Videx, didanosine): although extra steps are required for addition of the amino group to convert the inosine to adenosine, like the other nucleoside analogues ddI is processed by cellular kinases to produce the active antiviral agent, ddATP. The triphosphate competes with dATP for incorporation by the HIV RT into proviral DNA and results in chain termination. The intracellular half-life of ddATP is approximately 25 h, which is significantly longer than those of AZTTP and ddCTP. This suggests that it may be possible to decrease the number of daily doses needed from two to one. Didanosine is less orally bioavailable than the other nucleoside RTIs and must be taken on an empty stomach. Like ddC the principal toxicities are pancreatitis and peripheral neuropathy. The original approval was for adults who had already had long-term treatment with AZT and for treatment of adults or children who do not tolerate AZT or whose immune status deteriorated while on AZT. In the 1997 DHHS guidelines, ddI is recommended together with either AZT or d4T as a possible nucleoside combination to be used with a protease inhibitor as part of HAART.

d4T (Stavudine, Zerit) received approval for the treatment of patients who have failed to tolerate other approved therapies and for those who continue to decline on approved therapies. The 1997 guidelines list d4T, in combination with either ddI or ddC, as possible selection for the two nucleoside components of a triple drug regimen of HAART. Like the other nucleoside RTIs, d4T is phosphorylated to the active antiviral agent, stavudine triphosphate. Inhibition again is at two stages. First it competes with the correct RT substrate TTP and then further interferes by causing chain termination following incorporation. The three phosphorylation steps are all efficiently accomplished and there is not the accumulation of excess monophosphate with d4T that is seen with AZT. The primary toxicity is peripheral neuropathy.

3TC (Epivir, lamivudine, (−)-3′thiacytidine) received accelerated approval for use in combination with AZT on the basis of clinical trials using viral load and CD4 surrogate markers. Full approval was awarded in 1997 after improved survival was documented in an international study. Although 3TC is a potent inhibitor of both HIV and HBV RTs, resistant mutants are readily selected. Consequently it should always be used as part of a combination therapy. Its mechanism of action is similar to the other nucleoside analogues, e.g. phosphorylation to the triphosphate which is a competitive inhibitor of the HIV RT, with termination of growing proviral DNA chains. 3TC reacts less than the other nucleosides with the mitochondrial polymerase γ, a feature which is presumably responsible for its more attractive safety profile. The 1997 DHHS guidelines for HAART recommend its use as one of two nucleosides, preferably with AZT, combined with a PI.

B. Nonnucleoside reverse transcriptase inhibitors (NNRTIs)

The NNRTIs differ from the NRTIs in that they do not have a nucleoside structure and do not depend on phosphorylation for activity. They function as non-competitive substrate analogues and are selective inhibitors of HIV-1, with no activity against HIV-2 strains or even HIV-1 type O. Both approved NNRTIs have oral bioavailabilities greater than 60% and are highly protein bound. NNRTIs have been identified in multiple chemical classes, yet they all appear to bind the same region of the RT. This is separate from the binding region of the NRTIs, so the NNRTIs generally retain activity against AZT-resistant HIV variants. Although NNRTIs usually have potent antiretroviral activity, resistant mutants are readily selected and therefore these drugs are not recommended as monotherapy. The 1997 DHHS guidelines list the use of a combination of one NNRTI and two NRTIs as less preferred than a triple therapy of two NRTIs and a one PI.

Nevirapine (BI-RG-587, Viramune) in early clinical trials dramatically illustrated both the ease with which treatment with this class of compounds results in the selection of resistant mutants and the efficacy of combination therapy in preventing their appearance. One trial compared monotherapy with nevirapine to its use in combination with AZT. There was a quick drop in serum p24 levels in both groups, but the monotherapy arm returned to baseline levels within a few weeks. The combination group sustained the decreased p24 levels. Nevirapine is generally safe, but a pruritic rash, which can usually be managed by dose reduction, is potentially severe and may be life threatening.

Delavirdine (Rescriptor, U90152, a bis(heteroaryl)-piperazine) treated patients who received the drug as a monotherapy developed resistant virus within weeks of treatment. Accordingly, its approval is as part of a combination of antiretroviral agents and is based on surrogate markers rather than demonstrated clinical benefit. As with nevirapine, the primary toxicity is a rash which usually resolves, but can be serious.

C. Protease inhibitors (PIs)

The HIV protease is essential for the cleavage of the HIV polyprotein into the separate HIV proteins needed for virion assembly. The PIs are all highly potent enzyme inhibitors *in vitro*, and treatment with the first four PIs which were approved by the FDA have all resulted in improved CD4 cell counts and decreased viral loads. Improved clinical endpoints were also documented for the first three: *Indinavir, Ritonavir* and *Invirase*. Studies to determine the possibility of clinical benefit are underway for the fourth, *Nelfinavir*. These are the first drugs which attacked a viral target other than the RT and thus made highly potent combination therapies possible. In general, the pharmacokinetic properties of these drugs require strict observance of the dosing schedule to maintain efficacy and avoid the emergence of resistance. The PIs are principally metabolized by the liver cytochrome P_{450} oxidase system and consequently they can have serious interactions with other drugs in several classes. The PIs have been associated with a varied constellation of side effects which include the development of fatty masses, hyperglycemia and elevated triglycerides. New and exacerbated diabetes has also been reported during the postmarketing period, but it is not clear if this association is real or coincidental.

All four approved PIs are uncleaveable mimics of the HIV gag-pol polyproteins which occupy the active site of the protease. The inhibitors do not prevent the formation of progeny virions, but these virions are not mature and are noninfectious. Mutations which lead to resistance are usually (but not always) located in the drug-binding pocket and may require an accumulation of three or more for the development of significant resistance. Mutants resistant to one of these inhibitors often, but not always, show decreased sensitivity to other members of this class.

Inverase (saquinavir) has relatively poor oral bioavailability (about 5%) and has been formulated to maximize uptake and maintain plasma concentrations. It is metabolized by the liver P_{450} oxidase system, necessitating multiple dosing per day to maintain efficacy. Inverase's approval was based on both improvements in surrogate markers and a decline in disease progression and mortality in patients who received a combination of HIVID (ddC) and Inverase, as compared with patients who received either drug as a monotherapy. Safety for pediatric use has not been established. In 1997, *Fortovase*, a soft gel formulation of saquinavir which has significantly improved bioavailability, was approved.

Ritonavir (Norvir) is by contrast to Inverase much more readily orally bioavailable, requiring less frequent dosing. It is approved for use as a monotherapy or in combination with NRTIs on the basis of both improvements in surrogate markers of clinical progression as well as reductions in actual events of progression and mortality. Oral dosing is twice daily and a pediatric formulation is available for children aged 2 years or older. Interestingly, ritonavir enhances the plasma levels of saquinavir by up to 20-fold by blocking liver metabolism of the latter. This has the potential to provide a strategy to overcome the disadvantage of low bioavailability of saquinavir, but the predictability of the magnitude of this effect is not reliable and would necessitate extensive monitoring of patients receiving both drugs in combination.

Indinavir (Crixivan) obtained accelerated FDA approval with the demonstration of undetectable plasma virus load and increased CD4 cell levels when given in combination with two nucleoside analogue RTIs or as a monotherapy. It is not approved for pediatric use. To maintain adequate serum levels, Crixivan must be taken at 8 h intervals.

Nelfinavir (Viracept) is the most recently approved drug in this class and approval was based on changes in surrogate markers when it was administered either as a monotherapy or in combination with NRTIs. It is dosed orally three times a day and a pediatric formulation is available. A preliminary study suggested that some nelfinavir-resistant mutants may retain sensitivity to some other PIs, although nelfinavir was not always inhibitory to HIV mutants resistant to other PIs.

Experimental agents

New nucleoside (*Lodenosine*) and nucleotide (*Adefovir dipivoxil*) analogue RTIs and NNRTIs (*Efavirenz*) are in various stages of preclinical and clinical development, with the hope of achieving high plasma levels, overcoming resistance development, and also providing added benefit in combination with approved agents.

Also a new generation of protease inhibitors is in development, with the aim of providing greater efficacy in combination with PIs and RTIs (*Amprenavir, 141W94*), improved oral bioavailability (*DMP-*

450) and efficacy against virus resistant to other PIs through novel binding to the protease active site (*PNU140 690; BMS 232 632; PD 178 390*).

Numerous novel approaches against virtually every stage of the viral replication cycle are being pursued, and will just be mentioned in passing. These include the use of oligonucleotides (*GEM 92; Zintevir; ISIS 5320*) to inhibit by a combination of methods, potentially including antisense-mediated inhibition of RNA translation, blockade of virus adsorption and/or blockade of HIV provirus integration. A receptor-binding antagonist, RBC-CD4, uses the strategy of coating erythrocytes with surface CD4. These cells internalize free HIV, thereby preventing new infections, and also bind to gp120 on infected lymphocytes, resulting in phagocytosis of already infected cells. Peptide and/or nucleotide decoys which will interfere with the binding of the HIV TAT regulatory protein with the targeted TAR region of the HIV genome are also being investigated. Zinc fingers, which are highly conserved regions of HIV proteins that bind to the packaging region of the HIV genome RNA, are attractive targets as they are involved in multiple stages of the replication cycle including reverse transcription, genome integration, RNA packaging and protease function. Inhibition of multiple zinc finger functions make it theoretically likely that resistant viruses will not emerge. In addition, since they are part of the virus structure, drugs directed at zinc fingers may also be virucidal.

In addition to strictly antiviral approaches, several other strategies deserve mention. Multiple cellular functions have been targeted, including modulation of the immune system. The combination of HAART with *interleukin 2 (IL-2)*, to stimulate CD4 T cells and enhance resistance to secondary infections in AIDS, is in clinical evaluation. The therapeutic use of killed vaccines is also in clinical trials as a means of boosting residual immune function. Other cellularly targeted agents include *hydroxyurea (HU)*, which blocks cellular ribonucleotide reductase and depletes nucleotide pools for nucleic acid synthesis. The enhanced DNA incorporation of nucleotides derived from antiviral nucleosides is hypothesized to explain the potentiation of ddI's activity by HU when they are used in combination. Resistance is not a problem as the HU target is cellular.

Two approaches involving genetic alteration of the patient's stem cells are intriguing, although their practical utility remains to be demonstrated. The first, in phase I/II clinical trials, involves the transduction of HIV-targeted ribozymes into stem cells by means of retroviral vectors. Ribozymes are RNA enzymes which cleave at a specific HIV RNA nucleotide sequence following complementary base pairing. The second approach involves transfecting lymphocytes and stem cells *ex vivo* with genes encoding *intrakines* such as CDF, Rantes or MIP-1α. These serve to block the surface expression of both HIV coreceptors (CXCR4 and CCR5) and protect the intrakine-expressing cells from infection.

A possibly more feasible 'Trojan horse' strategy also is based on the use of cellular receptors for HIV. With this strategy, the envelope gene of vesicular stomatitis virus (VSV) is replaced with the genes encoding the T cell receptors (CD4 and CXCR4) for HIV. HIV-infected cells, displaying gp120 on their surface, now can fuse with the T cell decoy and are killed by the cytolytic virus. Similar decoy constructs are being developed to mimic macrophages. Although VSV is normally a mild pathogen for cows and pigs, and even milder for the accidentally infected humans, the decoy is noninfectious for these species owing to the loss of its envelope protein. The decoy can only infect and replicate in HIV-infected cells. The strategy has only been evaluated in tissue culture, but the clinical potential is exciting.

These myriad approaches underway inspire confidence that new therapeutic strategies will enable successful control of HIV, with the clear goal of providing drugs and strategies that will effect cures at costs that do not preclude their use.

Herpesviruses

Herpes simplex virus (HSV) and varicella virus (VZV) In spite of the awareness of the risks of sexually transmitted diseases during this era of the acquired immune deficiency syndrome (AIDS), the incidence of genital herpetic infection has continued to increase dramatically, so that today it is estimated that about 20% of the sexually active population of the USA may suffer from herpes genitalis. As with all herpesvirus infections, primary genital infection results in latency and periodic recurrences in the anal/genital area. The second major HSV-associated disease is generally the cosmetic nuisance resulting from HSV oral infection and resulting in herpes labialis (cold sores) in about 90 million people in the USA. Although experimental vaccines are being developed, none is approved, indicating the continuing need for effective antiviral therapy.

For VZV the situation is quite different. This ubiquitous virus is responsible for the systemic disease of chickenpox, usually in children, with possible recurrence from latency as herpes zoster (shingles) years later in a selected dermatome in the 'immune' adult. With the approval of an attenuated live VZV vaccine in 1995 in the USA, chickenpox is now a preventable disease. But the vaccine virus may

also establish latency, and the potential to recur as zoster or prevent wild-type infection over the long run is not yet resolved. Herpes zoster may be a very painful localized disease with postherpetic neuralgia. The recurrence incidence increases with advancing age, with an estimate that about one-third of the population may experience zoster by age 75. There is substantial incentive to develop effective antiviral drugs to halt reactivation and prevent postherpetic neuralgia.

Approved agents

The early success of *vidarabine* and the important discovery and success of *acyclovir (Zovirax)* in the clinical management of HSV genital and mucocutaneous infections has effectively pointed the way in antiviral drug researach. But acyclovir has limited oral bioavailability, requiring large multiple daily doses for the treatment of varicella zoster, since VZV is less sensitive to acyclovir than is HSU. Both its successes and its limitations have provided the incentive to develop new therapies. *Valacyclovir (Valtrex)* is the L-valyl ester prodrug of acyclovir and provides improved oral acyclovir availability. It is now approved in the USA for the oral therapy of varicella, and initial episodes or recurrences of genital herpes. *Penciclovir*, another acyclic nucleoside analogue, which is also selectively phosphorylated (like acyclovir) by HSV thymidine kinase (tk) and cellular kinases to the triphosphate inhibitor of viral DNA polymerase, has also been approved as a 1% cream for treatment of oral herpes. *Famciclovir (Famvir)*, a prodrug of penciclovir with enhanced oral bioavailability, has been approved for prophylactic suppression of recurring episodes of genital herpes.

In HSV-infected patients with normal immune responses, development of resistance to nucleoside analogues is not a significant problem. But in immunocompromised patients, infected neonates and in chronic skin infections acyclovir-resistant virus can be a clinical problem, presenting the need for alternative therapies. *Foscarnet (Foscovir)*, a DNA polymerase inhibitor, is considered a good alternative for treatment, although hospitalization is required for therapy and is associated with toxicity. Thus, there is a continuing need for new anti-HSV drugs with novel modes of action.

Experimental agents

The HSV ribonucleoside reductase inhibitor, *348U87* (from Glaxo Wellcome), has been used in combination with acyclovir in phase II clinical trials. Recently *lidakol* (10% *n*-docosanol cream), an inhibitor of enveloped viruses, has shown success in improving healing time as a topical treatment of herpes labialis and has moved into phase III clinical trials. HSV tk has long been recognized as a potential antiviral target because it is essential for virus reactivation and replication in neurons. A Roche Research Center team has identified an orally active tk inhibitor (*Ro 32-4397*) that is highly selective in inhibiting virus replication in dorsal root ganglia in mouse protection studies. Another tk inhibitor, HBPG, with *in vivo* efficacy is also under study at UMass Medical Center. Another exciting development is the identification of an inhibitor of HSV helicase/primase by a team at Boehringer Ingelheim and Bio-Mega. The lead thiazolyl phenyl-containing compound (*BIS45BS*) is orally active against both acyclovir-sensitive and -resistant viruses in animal protection studies, and further preclinical development seems warranted.

Human cytomegalovirus (HCMV) CMV is a common infection but an uncommon disease, associated with immunocompromised individuals such as late-stage AIDS patients, allograft recipients and the newborn. The rise in the incidence of CMV disease as an AIDS-opportunistic illness has been paralleled with the emergence of new products, primarily with the aim of halting the spread of CMV retinitis. Also the effect of HAART has resulted in a decrease in the incidence of CMV retinitis. But the needs go beyond AIDS, with life-threatening CMV still a problem for the bone marrow transplant patient. Furthermore, congenital CMV infection affects 1% of all American newborns and is the leading cause of hearing loss in children. Also emerging is an understanding that CMV infections may contribute to the development of atherosclerosis, and restenosis following angioplasty.

Approved agents

Ganciclovir (Cytovene, GCV) was initially approved for therapy in the treatment of CMV retinitis and later for the prevention of CMV disease in transplant recipients. Although GCV has poor oral bioavailability, an oral formulation has been approved as a prophylactic for the prevention of HIV- and solid organ transplant-related CMV disease, as well as an alternative to intravenous therapy for maintenance therapy for stable CMV retinitis in AIDS patients. However, the relatively poor oral bioavailability of GCV has been a significant deficiency, prompting the development of new drugs and new formulations. GCV has also been formulated, with approval in 1996, as an intravitreal implant (*Vitrasert*) for treatment of CMV retinitis. Furthermore, a valine prodrug of GCV (*Valganciclovir*) is in clinical evaluation for

the treatments of CMV. Unfortunately GCV lacks high anti-CMV potency for effective suppression of virus replication and the emergence of resistant mutants. Since CMV is a systemic infection, with localized sites of disease pathology such as retinitis in AIDS patients and pneumonitis in bone marrow transplant patients, it is important that effective therapy reduces the systemic virus load and reduces the chances of clinical resistance development. Prophylactic use of GCV results in emergence of resistant CMV strains in about 1% of patients; about 80% by mutation in the the *UL97* gene which is responsible for phosphorylation of GCV to GCV-monophosphate, and about 20% by mutations in the CMV DNA polymerase (DNA pol).

Unlike GCV, two drugs with antiviral potency not altered by the *UL97* mutation are *cidofovir (Vistide, HPMPC)*, which was approved in 1996 for intravenous treatment of AIDS-related CMV retinitis, and *foscarnet (Foscavir)*, which was approved for intravenous therapy of retinitis. Cidofovir is phosphorylated by cellular kinases and cidofovir diphosphate inhibits DNA pol. Foscarnet is a pyrophosphate analogue which inhibits CMV DNA synthesis. Both compounds are effective against GCV-resistant *UL97* mutants, but may not be effective against DNA polymerase mutants.

Experimental agents

Newer drugs now in different stages of clinical development have shown innovative approaches in the search for novel therapies. *Lobucavir*, a novel cyclobutyl nucleoside analogue, has been identified as active against the herpesviruses and hepatitis B virus, and is presently in phase I/II trials for oral therapy of CMV in AIDS patients, with the evaluation of CMV in semen. *Adefovir dipivoxil (GS 840, bis-pomPMEA)* a nucleoside phosphonate derivative, is in oral phase III clinical studies. Two benzimidazole derivatives which are in development have defined new targets for anti-CMV research. Glaxo Wellcome's *1263W94* inhibits CMV DNA synthesis but not by blocking DNA pol. Although mechanisms of inhibition are not fully elucidated, the compound is in phase I/II clinical trials. *GW275175X*, also from these labs, does not block CMV DNA synthesis but inhibits CMV *UL89* terminase, an apparently essential function for the processing and packaging of genomic CMV DNA into capsids. Lastly, three drugs have emerged from the antisense oligonucleotide approach to antiviral drug development. *Fomiversen (ISIS 2922)* (FDA approved 8/27/98), a phosphorothioate 21-mer oligonucleotide targeted to the *IE2* gene is presently in clinical phase III intravitreal injection studies for the treatment of CMV retinitis in AIDS patients. *ISIS 13312* is a chemically modified version of ISIS 2922 and has entered phase I/II trials. *GEM 132*, a 20-mer phosphorothioate RNA/DNA/RNA hybrid oligonucleotide targeted to the immediate early *UL36/37* splice site, is being evaluated in phase I/II studies by intravitreal injection in AIDS patients with CMV retinitis and also by intravenous injection in AIDS patients shedding CMV in the semen.

Perhaps the most exciting new potential targets for antiviral therapy of the herpesvirus group resulted from the identification of proteases of the HSV and CMV. This has stimulated the development of novel screens and structure/function studies in the anticipation that novel inhibitors will be developed to impact herpetic infections, just as the HIV protease inhibitors have proven useful in treating HIV. Although anti-herpesvirus proteases are in the preclinical development pipeline, the potential is exciting.

Myxoviruses

Influenza causes serious illness in most of the adults it infects and can result in death in vulnerable populations, such as the very young, the very old and those with underlying cardiac or pulmonary disease. On average in the USA alone, influenza epidemics kill approximately 30 000 people and cause 170 000 excess hospitalizations annually. Influenza has also been identified as a cause of fatal pneumonia in immunosuppressed bone marrow or stem cell transplant recipients. Typically, one or two influenza strains predominate in annual epidemics, requiring preparation of a customized killed vaccine as soon as these strains are identified. However, the vaccine successfully protects only 70–80% of recipients. Accordingly, both the magnitude of influenza morbidity and the vast numbers of its associated excessive mortality have made the identification of effective anti-influenza therapeutics a public health priority.

Approved agents

In 1966, the FDA for the first time approved a systemically administered anti-viral agent, the anti-influenza drug *amantadine (Symmetrel)*. A related drug, *rimantadine (Flumadine)*, was approved in 1993 and is equally effective but with a slightly better safety profile than amantadine. Both drugs are orally available and are excreted by the kidneys. The primary toxicities are gastrointestinal and central nervous system (CNS)-related, and can usually be controlled by dose reduction. Both drugs block the action of the influenza A M2 protein which normally establishes an ion channel in the viral membrane and

is essential both for the input virus uncoating and for progeny virus maturation.

The drugs are approved for prophylaxis in adults and children, and the treatment of adults. In addition, amantadine is approved for the treatment of influenza in children. The degree of protection afforded by prophylactic therapy is essentially complete, and the US Centers for Disease Control (CDC) have recommended rimantadine prophylaxis in residential settings with high-risk individuals, such as nursing homes, after an index case has been identified. A comprehensive nursing home study demonstrated that rimantadine prophylaxis provided significant benefits even when residents had been vaccinated.

Unfortunately, amantadine and rimantadine share three significant disadvantages: they are effective against only type A influenza; their therapeutic use yields only minimal clinical benefits; and therapeutic use results in the rapid emergence and shedding of resistant virus which is virulent and transmissable in approximately one-third of treated patients. To avoid the potential nightmare of epidemic-resistant virus, and because treatment benefits are only modest, investigators have recommended that infected close contacts of high-risk individuals forego the option of treatment in favor of the potentially more effective prophylaxis of the high-risk individuals.

Experimental agents

The influenza neuraminidase has been targeted by another class of antiviral compounds which are analogues of the neuraminidase's natural substrate, sialic acid. There are two candidate drugs in this class in clinical development (*Zanamavir* (or *GG167*) which is delivered by inhalation, and *GS4104* which is delivered orally), and at least one other in preclinical study. They share the significant advantage of activity against both types A and B influenza, and were designed using the known crystallographic structure of the influenza neuraminidase. Consequently, both are highly specific and selective, with no significant inhibitory activity against mammalian or bacterial neuraminidases. Inhibition of the influenza neuraminidase prevents the cleavage of terminal sialic acid residues from viral and cellular glycolipids and glycoproteins, preventing release of progeny virus from infected cells. In experimental phase II challenge studies, in which volunteers were inoculated with influenza virus, both drugs demonstrated good prophylactic and therapeutic activity, with drug resistance not an apparent serious problem.

Ribavirin has anti-influenza activity both *in vitro* and in animal models. Although it is approved as an aerosol for the treatment of respiratory syncytial virus (RSV) infections and has been used clinically either by aerosol or intravenous infusion for influenza, large controlled clinical efficacy studies have not been performed.

The influenza polymerase has also been identified as a possible target for the inhibition of influenza replication. The nucleoside *2'-deoxy-2-fluoroguanosine* interferes with transcriptional elongation, and inhibits types A and B *in vitro* and in mouse and ferret animal models by oral administration. The viral endonuclease which steals caps from cellular mRNAs provides another viral target for which potential antivirals (*2,4-dioxobutanoic acid derivatives*) are in the preclinical stages of development.

Paramyxoviruses

RSV is one of the common community-acquired respiratory infections that circulates in winter and early spring. It is the major cause of respiratory tract infections in infants and young children, infecting two-thirds in their first year of life. Children whose health is compromised by other conditions such as prematurity, bronchopulmonary dysplasia (BPD), cardiac problems or immune defects are at high risk for serious disease. RSV is estimated to result in 90 000 hospitalizations and 4500 deaths annually in the USA. Recently RSV, along with parainfluenza and influenza, was recognized as the cause of severe and often fatal pneumonia in immunosuppressed populations such as bone marrow transplant recipients and patients undergoing immunosuppressive treatments for leukemia and other cancers.

Approved agents

Aerosolized *ribavirin* (*Virazole*, a *triazole nucleoside*) was approved by the FDA in 1985 as a therapy for RSV infections in hospitalized infants. It has broad-spectrum antiviral activity and inhibits the replication of a wide variety of DNA and RNA viruses both in cell culture and in animal models. In the cotton rat model of RSV infection, the drug is effective both therapeutically and prophylactically when delivered orally or by aerosol. Ribavirin is believed to inhibit viral replication by several mechanisms, including ones which target host rather than viral activities. Consequently, it is not surprising that the emergence of ribavirin-resistant mutants has not been documented. The major mechanism probably involves inhibition of inosine-5'-monophosphate dehydrogenase activity by ribavirin monophosphate, leading to the depletion of intracellular pools of GTP. In addition inhibition of guanyl transferase by ribavirin triphosphate reduces mRNA capping. The triphosphate also appears to directly inhibit some viral polymerases.

Despite its well documented preclinical efficacy, the clinical use of ribavirin remains controversial. FDA approval of ribavirin and endorsement by the American Academy of Pediatrics (AAP) were based on several small placebo-controlled trials in nonventilated infants for whom treatment resulted in improved symptom scores and decreased RSV titers. However, some subsequent studies have failed to support benefit from ribavirin therapy in infants, and the AAP has now changed its recommendation from 'should be used' to 'should be considered'. The high cost of therapy as well as the need for careful environmental control due to the possibility of teratogenicity have also contributed to the lack of universal acceptance of ribavirin therapy for RSV in infants.

RespiGam, an intravenous immune globulin preparation enriched approximately sixfold for neutralizing antibodies to RSV (RSVIg) has recently been approved as a prophylactic use for infants in high-risk categories (BPD, prematurity), with the exception of underlying cardiac disease. Its prophylactic use every 30 days during the RSV season significantly reduced the incidence of hospitalization for RSV lower respiratory tract disease as well as the duration of the hospital stay. However, the use of RSVIg was not effective as a treatment for RSV in high-risk children hospitalized for RSV lower respiratory tract infection.

Because the mortality of RSV pneumonia in bone marrow transplant recipients is as high as 85%, a number of strategic approaches using drugs approved for other RSV indications have been tried. Although aerosolized ribavirin seems nonbeneficial once pneumonia is established, the results of an open label study suggested that the combination of aerosolized ribavirin with intravenous immunoglobulin reduced mortality below the expected rate, and may provide benefit as pre-emptive treatment. When symptoms of upper respiratory tract infection are observed and myxo- or paramyxovirus involvement is documented, then treatment with either ribavirin or rimantadine may be tried to prevent the progression of upper respiratory tract infection to lower respiratory tract disease.

Experimental agents

Monoclonal antibodies (MAbs) specific for the RSV F glycoprotein are under development, and in animal efficacy studies MAbs have been significantly more potent than RespiGam. Consequently, clinically effective levels of neutralizing antibody should be achievable from substantially smaller injection volumes than those required for the polyclonal product, and clinical evaluations of several potential MAb products are underway.

Parainfluenza virus (PIV) is also a common cause of respiratory infections, especially in children, in whom it is the classic cause of 'croup'. Ribavirin is effective against PIV in cell culture and in animal models, but no studies have been done to evaluate its clinical potential. In immunocompromised patients PIV, like RSV and influenza, can cause life-threatening lower respiratory tract disease. As with RSV infection, recent uncontrolled reports suggest that ribavirin might be useful for this indication.

Hepatitis Viruses

Hepatitis, or inflammation of the liver, can be caused by many different viruses and chemical substances. Viruses may cause acute infection (hepatitis A virus) or acute infection with the possibility for chronic liver infection leading to cirrhosis and hepatocellular carcinoma. Two major causative agents of acute and chronic disease are hepatitis B (HBV) and hepatitis C (HCV) viruses. The World Health Organization estimates the worldwide population of HBV carriers at about 350 million, with 75% of these living in the Far East. While less than 0.5% of the population of the USA and about 1% of that of Europe may carry HBV, 10 and 12% may be carriers in Southeast Asia and Africa, respectively. With the identification of HCV as the cause of most of the non-A, non-B hepatitis, and with the development of immunoassays for detection of antibody to HCV, it has become clear that HCV is a major worldwide problem. The CDC estimate that there are about 150 000 new cases in the USA each year, with 1.5% of the population antibody positive. In Japan there are about 2.5 million chronic HCV carriers, and infection is associated with high rates of hepatocellular carcinoma.

Approved agents

HBV is a preventable disease by immunization, with recombinant virus vaccines available worldwide and newer vaccines in development. Currently in the USA and in some countries of Europe, HBV immunization is recommended for all infants and adolescents. *Hepatitis B immune globulin (H-BIG)* preparations are also used as passive antibody prophylaxis in patients shortly after exposure to the virus to prevent transmission. Currently there are no approved HCV vaccines, but both vaccines and *HCV immune globulin (H-CIG)* are in preclinical development.

The only approved drugs for the treatment of hepatitis C virus (HCV) and hepatitis B virus infections in the USA are *interferons (IFNs)* α (*Intron* for HBV and HCV; *Roferon-A* for HCV), although

preparation of IFN-β and lymphoblastoid IFN are used elsewhere in the world. Interferons can be both direct inhibitors of virus replication and stimulators of specific and nonspecific immune responses. How they function to reduce hepatitis virus infection is uncertain, but they have been useful, as measured by virus DNA or RNA in the circulation. Unfortunately, only about 50% of chronic HCV or HBV patients treated with IFNs respond by virus suppression and half of these relapse back to elevated plasma virus levels. In addition, interferon therapy may also result in unwanted side effects, including depression, nausea, fever, fatigue, headaches and muscle aches. Recently a New Drug Application (NDA) for *Intron A/Rebetol (ribavarin) combination treatment* of chronic adult HCV patients who relapse following treatment with Intron was approved by the FDA.

Experimental agents

Since HCV and HBV are major causes of morbidity and mortality in the world, there are significant efforts to develop more effective therapies. IFNs are immunomodulators, and one approach in phase III studies is the combination of IFNs with thymosin α_1 (*Zadaxin*) for treatment of HCV and also for HBV. Although early in the drug discovery and development process, much research effort is now focused on the HCV protease/NPTase helicase as an antiviral target. The HCV serine protease is essential for virus replication and is associated with an NPTase domain which activates the protease, and a helicase domain that preferentialy unwinds double-stranded RNA. The three-dimensional structure has been determined and drug screening programs are underway in several laboratories to identify potent inhibitors.

A wider variety of therapies for HBV are now in development. Both *lamivudine (3TC)* and *famvir (famciclovir)* are in phase III studies, and are effectively demonstrating the capacity to reduce plasma HBV levels. However, resistance is also occurring in some patients after months of treatment. Newly emerging compounds with promise are *GS 840*, a phosphonate nucleotide analogue, and *BMS-200475*, a nucleoside analogue. Both are in early clinical studies.

It is important to note that antiviral drug development can be a risky venture. *Fialuridine (FIAU)* was a promising candidate for the treatment of HBV, and was in phase II clinical studies prior to the delayed emergence of severe toxicity resulting in deaths. This was not anticipated from the preclinical animal assessment studies. Thus, for the development of an antiviral agent that will be used for the repeated treatment of chronic disease, the potential for delayed or accumulative toxicity in the diseased patient must be appreciated and understood.

Picornaviruses

Although there is an abundance of human picornaviruses, including enteroviruses and the rhinoviruses, to date there are no approved antiviral agents. Yet there have been extensive studies on the structure of the virus capsids and also of the mechanisms of replication, providing attractive potential targets for antiviral intervention and resulting in the identification of novel experimental antiviral agents. The single-stranded picornavirus RNA genome is transcribed into a single polyprotein, which is cleaved by an essential protease into functional subunits, one of which is the RNA polymerase. *Enviroxime*, a benzimidazole derivative which inhibits rhinovirus polymerases and was promising in cell culture studies, was disappointing in the clinic against the common cold. However, studies are continuing with further modifications of enviroxime to increase oral bioavailability while retaining antirhinoviral potency and reduced toxicity.

The identification of promising isoxazole compounds that bind to a hydrophobic pocket within the capsid structure has led to extensive studies on the capsid structure and the inhibitors' capacity to prevent uncoating of the viral RNA. This similarity of structure/function of many enteroviruses and rhinoviruses has resulted in picornavirus inhibitors with a broad range of efficacy. *Pleconaril (ViroPharma VP-63843)* is an orally active low molecular weight pocket-binding compound that has been chosen for further development. Pleconaril reduces the duration and the severity of respiratory symptoms due to enterovirus infection, and is now in a multicenter phase II evaluations. Although laboratory studies have shown that resistance to pleconaril may occur due to mutations in the viral binding pocket, the significance of resistance in the clinic is not yet determined.

Ninety percent of rhinoviruses use ICAM-1 as their cell receptor, and *soluble ICAM-1 (sICAM)* acts as a decoy to inhibit effective virus–T cell binding and prevent infection. Recently sICAM has been successfully used as a prophylactic intranasal administration to prevent experimental rhinovirus infection in humans. The multiple treatments per day were well tolerated, indicating that this approach may be a promising novel preventative of the common cold.

Papillomaviruses (HPV)

Infections with HPV types are ubiquitous, with over 80 types that cause a variety of wart-associated

conditions. While most lesions are benign, some, especially those caused by HPV-16, -18 and other oncogenic HPV types, may progress to premalignant and malignant lesions. Papillomaviruses are causally associated with cervical cancer, which is diagnosed in 15 000 American women annually and causes approximately 4500 deaths. Types 6 and 11 are responsible for common genital infections and also for recurrent respiratory papillomatosis (RRP), a devastating but rare disease which may be acquired at birth as a result of vaginal delivery by an infected mother.

The HPV genome is small and encodes few enzymes. The virus consequently uses many host functions, such as DNA polymerase and other cellular replicative enzymes, for its own replication. This has made the goal of identifying safe and effective selective antiviral therapies especially challenging.

Approved agents

The currently approved therapies are not often satisfactory and generally involve physical ablation and/or antiproliferative or immunomodulatory agents as opposed to virus-specific strategies. These therapies include the crude ablative methods of surgery and electric cauterization, and the more precise laser vaporization. The primary disadvantage of these methods is high recurrence rates. Chemical ablative approaches include the use of trichloroacetic acid or podophyllotoxin and are no more successful than the physical methods.

Interferon α (Intron, Aferon) may provide a significant level of efficacy as intralesional therapy for condyloma acuminata (genital warts). Following a course of three times weekly intralesional injection, approximately 40% of IFN recipients achieved a complete response, as opposed to 20% of placebo recipients. As is the case with virtually all HPV therapies, approximately 25% of treated patients developed recurrences. The discomfort of intralesional injection has limited the wide use of this therapeutic strategy, and systemically delivered IFNs are not effective. Although IFN was discovered on the basis of its antiviral activity, it is also an immunomodulatory and antiproliferative agent. It is not known which of these properties is responsible for its anti-HPV effects.

Imiquimod (Aldara, R-837) is an immunomodulator which is approved as a topical therapy for genital warts. It is an interferon inducer and stimulates a natural immune response with the induction of several IFN-α subtypes and other cytokines associated with a Th1 immune response. These include tumor necrosis factor (TNF)α, IL-1α, IL-6 and IL-8. In clinical trials of a topically applied 5% cream, 40–55% of subjects experienced a complete clinical response following thrice-weekly use for 8–12 weeks. However, recurrences were observed in almost one-fifth of the complete responders.

Experimental agents

Both *ribavirin* and *cidofovir* were shown to be efficacious in a rabbit model of papillomavirus infection. On the basis of these studies, clinical trials for both agents have been undertaken. Ribavirin is being evaluated in phase I/II clinical studies as an oral therapy after laser ablation of laryngeal papillomas. In uncontrolled studies, cidofovir has been reported to have a dramatic degree of efficacy as an intralesionally administered agent for the treatment of RRP. Controlled studies are underway to determine whether these initial promising results can be confirmed. Cidofovir is also being evaluated as a topical therapy for genital warts in HIV-infected individuals. In a similar study of topical therapy for acyclovir-resistant genital herpes infections, the drug was not absorbed and no systemic toxicity was observed, suggesting the potential acceptability for this topical approach to therapy.

Another nonvirus-specific antimitotic agent, 5-fluorouracil, has been formulated for slow release in a collagen matrix gel and is designed to be injected intralesionally. Reponse rates with this product, *AccuSite*, were superior to those obtained with IFN. The product is approved in the UK and several European countries, although it has not been approved by the US FDA on the basis of toxicity concerns.

A number of virally targeted antipapillomavirus strategies are in very early stages of development. These include ribozymes directed at E6/7 mRNAs, peptides to interfere with the E7 transforming function, and agents designed to inhibit replicative activities of the viral E1 protein and transcriptional activities of the E2 protein. An antisense compound proceeded as far as early clinical evaluation but its development has since been terminated. Early-stage strategies targeting other than viral functions include the use of retinoids as inhibitors of angiogenesis and indole-3-carbinol, which downregulates estrogen activity and is being studied for possible prevention of cancer.

Exotic Viruses and Unmet Needs

At present there are no approved therapies for several types of 'exotic' viruses, those which are rare in the USA and Europe. However, several recent examples of emerging and life-threatening diseases due to Ebola

viruses and hantaviruses have made it clear that, with world travel requiring less time than many virus disease incubation periods, disease might spring up at any locale, and geographic containment alone is not realistic. There have been 15 outbreaks of Ebola virus infections reported since 1967, and the horrors of these infections have received considerable publicity. Hantaviruses have long been known to cause Korean hemorrhagic fever (KHF). More recently hantavirus pulmonary syndrome (HPS), with rapid disease course and mortality as high as 50% in less than a week, has occurred in regions of the USA. These examples have been part of the stimulus for investigations of immunotherapy, extended evaluation of available antiviral drugs, and new drug discovery to define effective strategies to treat and contain exotic virus outbreaks. Hyperimmune polyclonal equine antibody and MAb are being developed and tested to prevent acute Ebola disease and death in non-human primates. Furthermore, several inhibitors of *S*-adenosylhomocysteine hydrolase, including carocyclic 3′-deaza-adenosine, are also being evaluated in a mouse model of Ebola virus infection.

Ribavirin appears to effectively inhibit hantavirus infections, and is undergoing clinical evaluation in the intravenous treatment of HPS. Ribavirin also inhibits arenaviruses, which may cause hemorrhagic fever with CNS involvement, and the mosquito-borne bunyavirus, which causes La Crosse encephalitis. Controlled clinical evaluations of ribavirin for these indications are also in progress.

Cidofovir inhibits monkeypox virus, which can be spread from monkeys to and among humans. Monkeypox has been on the increase in Africa, perhaps as a consequence of the termination of smallpox vaccinations, which may have afforded a degree of crossprotection. Cidofovir has been effective in monkeypox animal protection studies and may be a candidate for clinical evaluation.

See also: **Refer to specific viruses in Table 1.**

Acknowledgment

The author wish to thank Mr Mark Alexander for assistance with literature searches, and recognize the generosity of Current Drugs Ltd (London) for providing access to their antiviral drug database.

Further Reading

Balzarini J, Schinazi RF and Kinchington D (1997) Current antiviral agents factfile. *Int. Antiviral News* 5: 74.

Galasso GJ, Whitley RJ and Merigan TC (eds) (1997) *Antiviral Agents and Human Viral Diseases*, 4th edn. Philadelphia: Lippincott-Raven.

Hirsch MS, Kaplan JC and D'Aquila, RT (1996) Antiviral agents. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 431, Philadelphia: Lippincott-Raven.

HIV Drug Database: http://www.niaid.nih.gov/daids/PDAT guide/overview.htm

Jeffries DJ and DeClercq E (eds) (1995) *Antiviral Chemotherapy*. London: Wiley.

Kinchington D, Balzarini J and Field HJ (1997) Current antiviral agents factfile. *Int. Antiviral News* 5: 161.

APOPTOSIS AND VIRUS INFECTION

Margaret P Quinlan, Department of Microbiology and Immunology, University of Tennessee, Memphis, Tennessee, USA

General Features

Maintenance of homeostasis between the cellular processes of proliferation and death is critical to a multicellular organism. Programmed cell death (PCD) is a genetically encoded and evolutionarily conserved, physiological process that induces a form of cellular suicide called apoptosis. Apoptosis is an important regulatory mechanism by which unwanted cells can be eliminated during normal development or because they are in excess or pose a threat to the organism, such as in the case of virus-infected cells. Thus, apoptosis protects the organism against cancer and viral infections and maintains homeostasis under a wide range of physiological and developmental stimuli. Defects in apoptosis can lead to diseases associated with increased cell types or numbers, such as autoimmunity or cancer, respectively. Increased apoptosis results in cell losses, such as found in immunocompromising or neurodegenerative diseases. The cell death program consists of the successive activation of unique apoptosis-specific genes and

viruses and hantaviruses have made it clear that, with world travel requiring less time than many virus disease incubation periods, disease might spring up at any locale, and geographic containment alone is not realistic. There have been 15 outbreaks of Ebola virus infections reported since 1967, and the horrors of these infections have received considerable publicity. Hantaviruses have long been known to cause Korean hemorrhagic fever (KHF). More recently hantavirus pulmonary syndrome (HPS), with rapid disease course and mortality as high as 50% in less than a week, has occurred in regions of the USA. These examples have been part of the stimulus for investigations of immunotherapy, extended evaluation of available antiviral drugs, and new drug discovery to define effective strategies to treat and contain exotic virus outbreaks. Hyperimmune polyclonal equine antibody and MAb are being developed and tested to prevent acute Ebola disease and death in non-human primates. Furthermore, several inhibitors of *S*-adenosylhomocysteine hydrolase, including carocyclic 3′-deaza-adenosine, are also being evaluated in a mouse model of Ebola virus infection.

Ribavirin appears to effectively inhibit hantavirus infections, and is undergoing clinical evaluation in the intravenous treatment of HPS. Ribavirin also inhibits arenaviruses, which may cause hemorrhagic fever with CNS involvement, and the mosquito-borne bunyavirus, which causes La Crosse encephalitis. Controlled clinical evaluations of ribavirin for these indications are also in progress.

Cidofovir inhibits monkeypox virus, which can be spread from monkeys to and among humans. Monkeypox has been on the increase in Africa, perhaps as a consequence of the termination of smallpox vaccinations, which may have afforded a degree of crossprotection. Cidofovir has been effective in monkeypox animal protection studies and may be a candidate for clinical evaluation.

See also: **Refer to specific viruses in Table 1.**

Acknowledgment

The author wish to thank Mr Mark Alexander for assistance with literature searches, and recognize the generosity of Current Drugs Ltd (London) for providing access to their antiviral drug database.

Further Reading

Balzarini J, Schinazi RF and Kinchington D (1997) Current antiviral agents factfile. *Int. Antiviral News* 5: 74.

Galasso GJ, Whitley RJ and Merigan TC (eds) (1997) *Antiviral Agents and Human Viral Diseases*, 4th edn. Philadelphia: Lippincott-Raven.

Hirsch MS, Kaplan JC and D'Aquila, RT (1996) Antiviral agents. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 431, Philadelphia: Lippincott-Raven.

HIV Drug Database: http://www.niaid.nih.gov/daids/PDAT guide/overview.htm

Jeffries DJ and DeClercq E (eds) (1995) *Antiviral Chemotherapy*. London: Wiley.

Kinchington D, Balzarini J and Field HJ (1997) Current antiviral agents factfile. *Int. Antiviral News* 5: 161.

APOPTOSIS AND VIRUS INFECTION

Margaret P Quinlan, Department of Microbiology and Immunology, University of Tennessee, Memphis, Tennessee, USA

General Features

Maintenance of homeostasis between the cellular processes of proliferation and death is critical to a multicellular organism. Programmed cell death (PCD) is a genetically encoded and evolutionarily conserved, physiological process that induces a form of cellular suicide called apoptosis. Apoptosis is an important regulatory mechanism by which unwanted cells can be eliminated during normal development or because they are in excess or pose a threat to the organism, such as in the case of virus-infected cells. Thus, apoptosis protects the organism against cancer and viral infections and maintains homeostasis under a wide range of physiological and developmental stimuli. Defects in apoptosis can lead to diseases associated with increased cell types or numbers, such as autoimmunity or cancer, respectively. Increased apoptosis results in cell losses, such as found in immunocompromising or neurodegenerative diseases. The cell death program consists of the successive activation of unique apoptosis-specific genes and

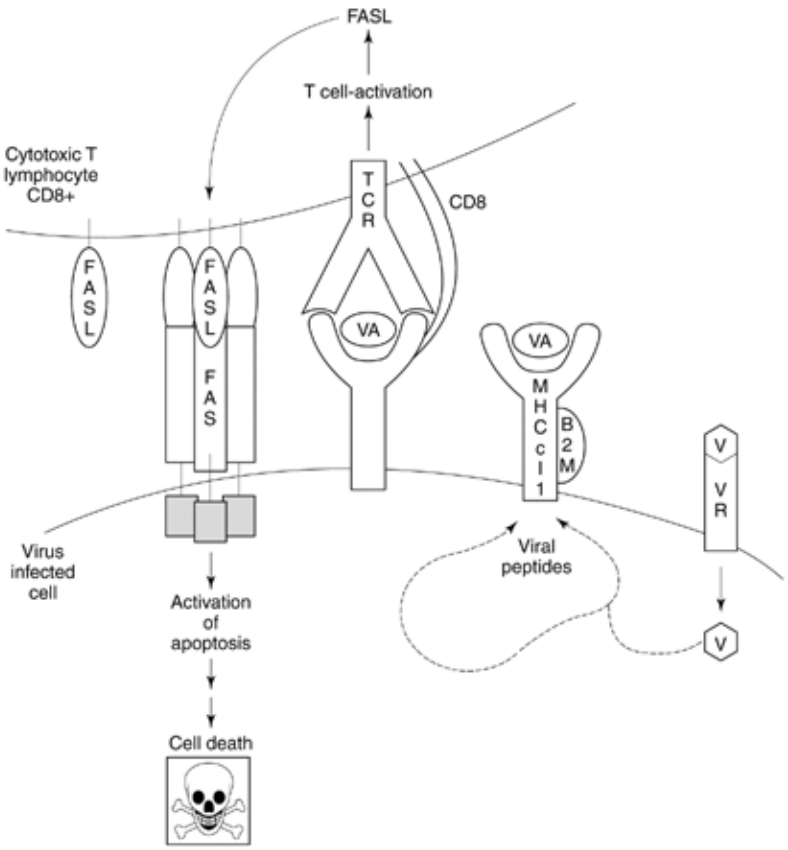


Figure 1 Immune mediated activation of apoptosis of virus infected cells. A virus (V) gains access to the inside of its target cell through a specific cell surface receptor (VR). Once inside, viral gene expression ensues. Viral peptides or antigens (VA) derived from the original virus (short dotted line) or newly synthesized by the cell (long dotted line) are presented by the class I MHC (MHC cl1)-β_2 microglobulin (B2M) complex on the surface of the infected cell. This is recognized as nonself by the T cell receptor (TCR) on the surface of a CD8+ cytotoxic T lymphocyte. The interaction between the viral peptide–MHC complex and the TCR activates the T cell to proliferate. This also results in the upregulation of FASL on the surface of the activated CTL. FASL interacts with FAS on the surface of the virus-infected cell and activates the apoptotic pathway in that cell leading to its death. ▤, death domain.

genes that are involved in the seemingly opposing functions of proliferation and development. Different cell types respond to different types of triggers, from inside and outside the cell, and possess different pathways to programmed cell death. The culmination in a death response is due to the extent, combination and timing of the collective set of genes activated. The changes observed in apoptotic cells include cell shrinkage, chromatin condensation, cytoplasmic blebbing, the generation of apoptotic bodies and internucleosomal fragmentation of the chromosomal DNA, an often used biochemical marker to identify apoptosis. Apoptotic cells or bodies are often phagocytosed by adjacent macrophages or parenchymal cells.

Rapid cell death of virus infected cells reduces virus spread. Apoptosis is used by the immune system to eliminate potentially dangerous cells, such as virus-infected ones. In fact, virus spread is limited by the immune response and apoptosis of infected cells. Killer lymphocytes are the primary immune effectors of virus and tumor immunity. Cytotoxic lymphocytes (CLs) play a pivotal role in the immune response to tumors, viruses etc. There are two effector populations with the ability to eliminate unwanted or harmful cells. These two cell types recognize the target cells in different ways. Cytotoxic T lymphocytes (CTLs) recognize target cell peptides, such as those derived from viral proteins, presented on the MHC class I glycoprotein at the cell surface (**Fig. 1**). This is accomplished by the recognition of the nonself antigen in the major histocompatability complex (MHC) by the T cell receptor (TCR) on the surface of the virus-immune T cell. The lymphocyte–target cell interaction activates the interacting T cell to proliferate and induce FASL expression. Natural killer (NK) cells mediate 'innate' immunity against virus-infected cells and do not require presensitiza-

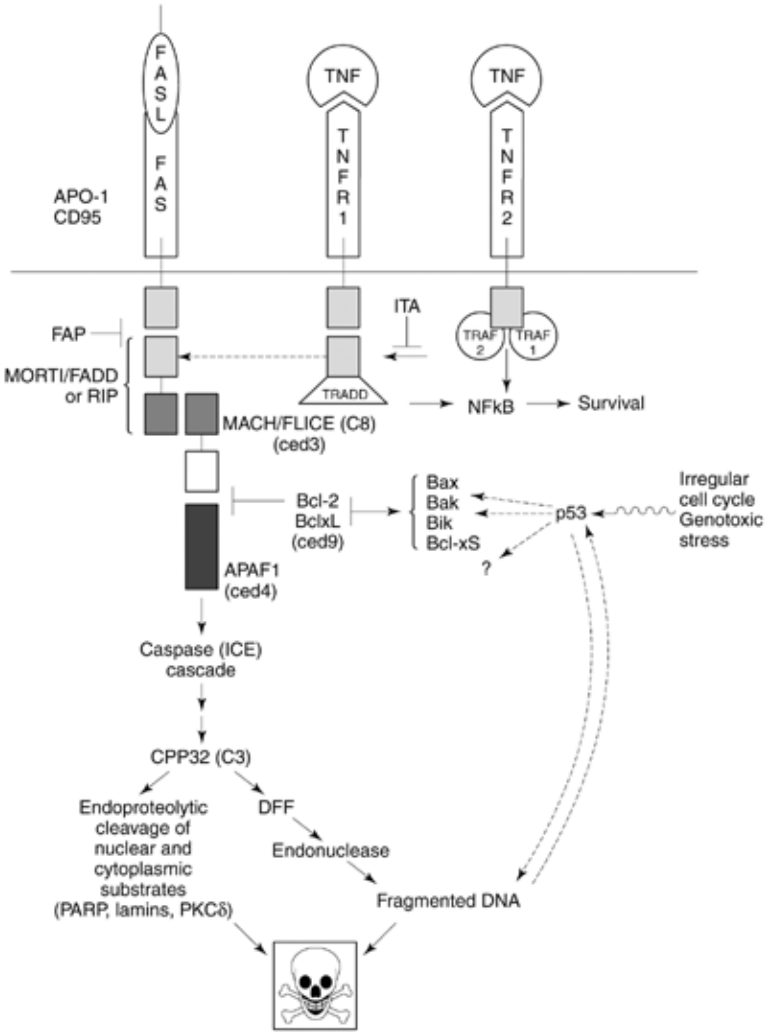


Figure 2 The apoptotic pathway. The apoptotic pathway can be initiated at the surface of a cell through the interactions of the tumor necrosis factor (TNF) with either of its receptors, TNFR1 or TNFR2 or FAS ligand (FASL) with its receptor FAS (APO-1, CD95). This initiates a series of intracellular, intermolecular events that are described in the text. ▒, death domains; ■ death effector domains. Solid lines indicate well-described events; dotted lines indicate less well described or hypothesized events.

tion. Target cells are killed through the induction of apoptosis by the secretory (perforin/granzyme) pathway or the nonsecretory pathway that involves triggering the cell surface death receptor, FAS (APO-1, CD95) by the membrane bound FASL (ligand) of the killer cell. These trigger an endogenous pathway of PCD resulting in the dissolution of the nuclear membrane, chromatin condensation and DNA fragmentation and ultimately, death of the virus-infected cell.

The expanded, activated CD8+ T cells arising in response to viral infection controls the virus, but can also be damaging to the host unless removed at the end of the immune response. This can be accomplished by the FAS-mediated apoptosis of the activated cells and is part of the regulation of the immune system.

The Apoptotic Pathway

The immune-mediated, apoptotic pathway (**Figs 1** and **2**) is initiated at the cell surface, with the interactions of the FAS (APO-1/CD95), which is ubiquitously expressed, or tumor necrosis factor (TNFR1, TNFR2) transmembrane receptors (death receptors) with their cognate, extracellular ligands, FAS ligand (FASL), which is predominantly expressed in activated T or NK cells, or TNF, respectively.

These death receptors are related. The death receptors contain 80 amino acid death domains, which are protein–protein interaction domains, in their intracellular, cytoplasmic, C-termini. The death domains are necessary for the receptor to trigger the cell death pathway. Once activated, the death domains of the death receptors interact with the death domains of cytoplasmic proteins. In the case of FAS, this is MORT1/FADD (FAS associated death domain protein) or RIP. The activation of FAS can be inhibited by FAP1 (FAS-associated phosphatase-1), which interacts with the C-terminal 15 amino acids of FAS. For the TNFR1, it is TRADD (TNFR associated-death protein). MORT1/FADD interacts with MACH (MORT1 associated CED-3/ICE homologue)/FLICE (FADD-like ICE), an ICE-(interleukin-1β converting enzyme) like enzyme. The ICE family members are now known as caspases. MACH/FLICE is also known as caspase 8. The ICEs or caspases are aspartate-specific, cysteine proteases that are the effectors or killer molecules (death proteases) of the apoptotic pathway. They are homologues to ced-3 in *Caenorhabditis elegans* and 10 have been identified in humans, thus far. This ced-3 homologue, MACH/FLICE binds to the ced-4 homologue, APAF1. This is the beginning of a caspase/ICE chain reaction, of which CPP32/caspase 3 is a part. CPP32 activates DFF (DNA fragmentation factor), which activates the endonuclease that results in DNA cleavage. CPP32 also brings about endoproteolytic cleavage of other cytoplasmic and nuclear proteins, such as PARP [poly(ADP-ribose) polymerase], lamins and protein kinase C (PKC)δ. In the TNFR1-TRADD path, TRADD is an adapter protein, which seems to link to the MORT1-MACH pathway, in an as yet unidentified manner. TNFR2 does not have a death domain, but interacts with two proteins, TRAF1 and TRAF2 (TNFR-associated factors). TRAF2 binds TRADD, which in turn connects to the FAS pathway, leading to the caspase/ICE cascade.

The ITA (inhibitor of T cell apoptosis) proteins, vertebrate homologues of the insect baculovirus IAP (inhibitor of apoptosis) form complexes with TRAFs, perhaps preventing TRADD from participating in the MORT1/FADD pathway. The bcl-2 family of ced-9 homologues are additional regulators of apoptosis. Presently, nine members are known and are related via their bcl-2 homology (BH) domains. Bcl-2 blocks a downstream step in the apoptotic pathway. It was originally discovered in B-cell lymphomas and contributes to neoplastic expansion by preventing programmed cell death or cellular turnover, disrupting homeostasis. Bcl-2 has subsequently been found in many other tumor types. The pro-death members of the bcl-2/ced-9 family include bad, bak, bax, bcl-xS and bik. The anti-death members are ced-9, bcl-2, bcl-w, bcl-xL, mcl-1, A1/Bfl-1 and Nr13. Their ability to form homo- and heterodimers with various members, and the ratio of inhibitors to activators regulates the final decision of life or death of the cell. Several viruses have evolved to encode BH domain-containing proteins that mimic bcl-2 to promote survival of infected cells (see **Table 1** and below).

Table 1 Viral gene products that inhibit apoptosis and their targets in the cell

Target	*Virus*	*Viral gene*
p53	Adenovirus	*E1B55K*
		E4ORF6
	Cytomegalovirus	*IE2*
	Epstein–Barr virus	*BZLF1*
		EBNA5
	Hepatitis B virus	*pX*
	Human papillomavirus	*E6*
	Simian virus 40	*LT*
bcl-2 family	Adenovirus	*E1B19K*
	CELO adenovirus	*GAM-1*
	African swine fever virus	*A179L*
	Epstein–Barr virus	*BHRE1*
		LMP1
	E26	v-*myb*
	Herpes saimiri	*ORF16*
	Rous sarcoma virus	v-*src*
Caspase/ICE	Baculovirus	*p35*
	Cowpox virus	*crmA*
	Orthopoxvirus	*SPI1–3*
	Vaccinia virus	*B13R*
DED proteins	African swine fever virus	*A22L*
	Baculovirus	*IAP*
	Kaposi sarcoma herpesvirus	*E8*
	Molluscipoxvirus	*MC159*
Death receptor	Adenovirus	*E3 10.4/14.7K*
	Myxoma virus	*M-T2*

The apoptotic pathway can also be activated by the p53 tumor suppressor, in response to genotoxic stress; DNA damage due to irradiation or unregulated cell cycles due to the expression of viral or cellular oncogenes. The mechanism remains unresolved. p53 is a sequence-specific, transcriptional transactivator, with an acidic domain. One cellular target for p53 seems to be bax, a pro-death member of the bcl-2 family and a downstream regulator of apoptosis. Inhibition or functional inactivation of p53 is a common feature of many DNA tumor viruses, although it is accomplished in different ways by each (see **Table 1** and below).

Viruses and Apoptosis

Viruses coevolve with their hosts to circumvent detection and destruction by the host. They have also evolved to usurp or take advantage of the host proteins and processes to enable or enhance their own replication and spread. Viruses encode immunomodulators to affect antigen presentation, function as cytokines or cytokine inhibitors, interrupt the complement cascade and affect apoptosis. Many viral immunomodulators have host counterparts (**Tables 1** and **2**).

Induction of early death of the infected cell could severely limit the yield of virus and result in the reduction or elimination of the virus. Some viruses have evolved distinct strategies to evade or delay apoptosis to facilitate replication, spread, latency or persistence. Different mechanisms have been employed: direct inhibition of the death proteases, stimulation of antideath pathways or regulation of transcription factors monitoring cell survival. Some common themes include inactivation of p53, encoding bcl-2 homologues, encoding protease inhibitors to inhibit the ICE/caspase cascade or homologues to members of the FAS or TNFR signaling systems (**Table 1**).

Induction of apoptosis later in the virus life cycle could assist the release of the newly produced virus particles, especially nonenveloped nonlytic viruses, from the cell. The presence of virus in apoptotic bodies, which are phagocytosed by other cells, helps the virus evade the host immune surveillance system and spread. Virus-induced apoptosis also contributes to the clinical manifestations and cytotoxicity associated with virus infections. The viral gene products involved in activating PCD and their targets in the cell are only starting to be identified (**Table 2**).

Table 2 Viral gene products that effect apoptosis and their targets in the cell

Target	*Virus*	*Viral gene*
p53	Human papillomavirus	*E2*
Bcl-2	Human immunodeficiency virus	*Tat*
		Protease
Death receptors	Hepatitis B virus	(*pX*)
	Hepatitis C virus	*core*
	Human immunodeficiency virus	*Tat*

Viruses Associated with Inhibition or Suppression of Apoptosis

DNA viruses

Adenoviridae Adenovirus (Ad) encodes several immunomodulatory proteins that function to inhibit or promote apoptosis. E1A inhibits the transcriptional transactivation of p53. E1A12S enables cells to overcome growth factor withdrawal and transforming growth factor β (TGFβ)-induced apoptosis. E1A13S upregulates p53, which can bring about apoptosis, but this depends on whether inhibitors of proliferation are present simultaneously, giving rise to a conflict for the cell, which will then result in apoptosis. E1B55K binds the p53 transactivation domain, blocking activation of p53-responsive genes in some, but not all, serotypes, such as subgroup C. E4ORF6 also binds to p53, but to a different region, although its role in cell death has not yet been shown. E1B19K has functional similarity to bcl-2. The actual homology is low and is only in the BH domain, but it interacts with other members of the bcl-2 family, such as bax and bak, preventing them from forming homodimers that promote cell death. E1B19K does not interact with bad, thus Ad-infected cells are resistant to bad-mediated death. Both E1B19K and bcl-2 inhibit p53-dependent apoptosis, but the mechanism is unknown. E1B19K also suppresses TNFα and FASL activated death pathways (**Fig. 2**). The E3 10.4K and 10.4K/14.7K heterodimer proteins inhibit TNF- and FAS-mediated cell death. Although not completely understood, it seems to be by preventing signaling from the TNFR and by the clearing of FAS from the surface, respectively. This enhances infection by enabling cells to escape immune destruction via CTL killing of Ad-infected cells. E4ORF6 also binds to p53, blocks its ability to transactivate and blocks p53-mediated apoptosis. The avian Ad, CELO AD, GAM-1 gene product is a novel protein that functions like bcl-2, but has no homology. E3 gp 19K sequesters MHC class I proteins in the endoplasmic reticulum, which prevents viral antigen recognition by CTLs.

African swine fever virus The African swine fever virus (ASFV) encodes the gene *A179L* (*5-HL* gene), which is a viral homologue of *bcl*-2. It has all the same domains as *bcl*-2, including the conserved BH1 domain, which is required to inhibit apoptosis, similarly to *bcl*-2. Thus, *A179L* probably works by a similar mechanism. The ASFV *A22L* gene is a homologue of the IAP proteins. It is expressed late in virus life cycle. A virulent strain of ASFV has been shown to induce apoptosis.

Hepadnavirus Hepatitis B virus (HBV) encodes pX that interacts with p53, interfering with DNA binding and transcriptional activation. pX is also an HBV viral oncogene involved in HBV-induced hepatocellular carcinomas, perhaps by blocking p53-activated

apoptosis. Conflicting with this, it has been shown that pX on its own can induce apoptosis in some cell types.

Herpesviridae This family of viruses contains members that bring about many different responses and diseases in the host. These viruses encode many different genes that affect PCD of the infected cell. Normally, herpes simplex virus type 1 (HSV-1) does not induce apoptosis, however, if ICP4 is deleted, apoptosis ensues. Thus, ICP4 blocks apoptosis, perhaps allowing cells to become latent. The HSV US3 gene, which encodes a serine/threonine protein kinase, also protects against apoptosis. The late γ34.5 gene inhibits apoptosis in infected neurons and promotes neurovirulence and could underlie HSV-mediated encephalitis. HSV-2-inhibits FAS ligand expression on the surface. PCD of cells infected with these herpesviruses results in decreased virus production.

A lytic infection with Epstein–Barr virus (EBV) causes apoptosis. Expression of *BHRF1*, a homologue of *bcl-2*, early in infection prevents TNFα- and FAS-induced apoptotic pathways. This is a means to maximize viral particles. BHRF1 also enables infected epithelial cells to overcome apoptosis induced by γ-irradiation and chemotherapeutics. It also provides an alternative mechanism of becoming multidrug resistant during chemotherapy. The latent membrane protein 1 (LMP1), an EBV oncogene, downregulates *myc* and upregulates *bcl-2* and *A20* expression, cellular anti-apoptotic genes. This prevents p53-mediated apoptosis and is part of the mechanism of B cell transformation by EBV. LMP1 may also contribute to the establishment of latency. EBV also encodes two polypeptides that interact with p53, EBNA-5 and BZLF1. EBNA-5 may also be able to stimulate cell cycle progression. BZLF1 binds to a region of p53 similar to that by Ad E4ORF6 and suppresses p53 transcriptional transactivation. The specific function of BZLF1 seems to be to regulate the dominance of the lytic versus latent paths of the virus.

CMV encodes two transcription factors, IE1 and IE2, that have been demonstrated to induce apoptosis. IE2 interacts with pRB (the retinoblastoma tumor suppressor) and p53, similar to other DNA virus anti-apoptotic proteins.

In herpes saimiri, ORF 16, is also a bcl-2 homologue. Several gamma-herpesviruses, including Kaposi's sarcoma herpesvirus (KSHV, HHV-8), E8 protein is a v-FLIP, which is a death effector domain (DED)-containing protein that interferes with apoptosis signaled through the death receptors. It interacts with FADD, preventing the recruitment of FLICE (caspase 8) and with the prodomain of FLICE. Thus, it uses DED-mediated interactions to interfere with apoptotic signaling pathways.

Iridiviridae The baculovirus p35 of *Autographica californica* nuclear polyhedrosis virus (AcMNPV) binds to and inhibits CPP32 protease, a caspase of the ced 3 family of ICE proteases. p35 is homologous to crmA of cowpox and inhibits TNFα and irradiation-activated apoptosis. Its expression enhances infectivity *in vivo* and *in vitro*. Another class of baculovirus genes that prevent apoptosis is IAP (inhibitor of apoptosis) (the *Drosophila* homologue is th). The function of IAP is currently not known, however, the presence of a RING zinc finger motif suggests the possibility that it could be a transcription factor. Other viral and cellular proteins associated with death prevention have a conserved double repeat sequence referred to as the baculovirus IAP repeat (BIR). Both the RING zinc finger and the BIR domains are required to overcome apoptosis. The human homologues, ITAs, interact with the TRADD/TRAF2 signaling complex. Both p35 and IAP prevent apoptosis and enable productive virus replication.

Papovaviridae The human papillomavirus (HPV) E6 binds to p53 and prevents its DNA binding and transactivation and/or results in the ubiquitin-mediated degradation of p53. The latter occurs in highly oncogenic, high-risk serotypes, such as HPV16 and 18. In these cases, E6 serves to deliver p53 to E6-AP (E6 associated protein), an ubiquitin–protein ligase. This, of course, prevents p53-mediated apoptosis due to unregulated cell cycle progression in response to the HPV E7 protein. Simian virus 40 (SV40) large T antigen (LT) binds and inhibits p53, thus preventing apoptosis due to constitutive activation of the cell cycle by LT.

Poxviridae Many poxviruses encode numerous immunomodulatory proteins, including homologues of cytokine receptors. These receptor mimics, viroceptors, bind and sequester cytokines preventing their signaling cascade prior to receptor engagement. The myxoma virus (virulent rabbit leporipoxvirus) M-T2 gene encodes a TNFR homologue. M-T2 is secreted, binds TNFα and inhibits the cytolytic activity. M-T2 is also an intracellular apoptosis inhibitor, via a different domain of the protein. It is the first viral immunomodulatory protein that exhibits two distinct anti-immune properties. The myxoma virus encodes another gene that inhibits apoptosis, the M11L gene, whose product is a novel transmembrane protein. The closely related, benign leporivirus cannot inhibit apoptosis.

Poxviruses also encode members of the serine proteinase inhibitor (serpin) superfamily. Cowpox encodes crmA (cowpox virus cytokine response modifier), which is an ICE/caspase protease inhibitor of the serpin family. Infected cells die by necrosis. CrmA is effective on CTL, FAS and TNFR, but not irradiation-induced apoptosis. CrmA does not inhibit CPP32. Orthopoxviruses encode 3 serpins, SPI-1–3, and these are also required for viral replication. SPI-2 is similar to crmA of cowpox. The rabbit poxvirus (RPV) SPI-1 serpin gene is required for its replication and inhibits apoptosis of RPV-infected cells. Vaccinia virus (VV) encodes the B13R gene, which is homologous to crmA and SPI-2, and inhibits ICE. B and T lymphoblast cells infected with VV are refractory to FAS and TNFα-activated apoptosis. B13R may contribute to the higher pathogenicity of some strains.

dsRNA seems to trigger apoptosis. The VV E3L gene encodes a dsRNA binding protein which prevents activation of the dsRNA-dependent, interferon-induced protein kinase R (PKR), which induces apoptosis. Suppression of apoptosis correlates with functional binding of proteins to dsRNA. The tumorigenic molluscipoxvirus (molluscum contagiosum virus) MC159 protein is a v-FLIP, similar to that seen in gamma herpesviruses (E8 gene). The MC159 protein interacts with FADD, via DED, and prevents recruitment of FLICE. In poxvirus-infected cells, apoptosis results in very few virus particles produced.

RNA viruses

Flavivirus The hepatitis c virus (HCV) viral core protein, which seems to be a transcriptional regulator, suppresses apoptosis through the CPP32 cysteine protease, but the mechanism is not known. The core protein seems to be involved in the pathogenesis of HCV.

Retroviridae Several of the oncoviruses inhibit apoptosis. The bovine leukemia virus (BLV), which is homologous to HTLV-1, induces a chronic expansion of the B cells (persistent lymphocytosis) and the development of B cell leukemia/lymphosarcoma. It is able to protect peripheral mononuclear cells and B lymphocytes from apoptosis. The viral G4 and/or R3 genes, which can attenuate the viruses, are not responsible for either the persistent lymphocytosis or B cell leukemia/lymphosarcoma. Human T-cell leukemia virus type 1 (HTLV-1) is an etiological agent for adult T cell leukemia. The *Tax* gene plays a causative role in transformation. The *Tax* gene of HTLV-1 inhibits APO-1-mediated apoptosis. *Tax* has also been reported to mediate apoptosis, when cells are serum deprived. The E26 avian leukemia virus oncogene, v-*Myb*, a transcription factor, upregulates *bcl*-2 transcription. The bcl-2 promoter has myb binding sites, which are required for its transcription. It suppresses apoptosis in myeloid cells and could, therefore, be part of its transformation mechanism. The Rous sarcoma virus (RSV) oncogene pp60 (v-*src*) activates NR-13, a *bcl*-2 related gene that is a potent anti-apoptotic factor. Activation of *NR*-13 may be involved in RSV transformation. The Abelson murine leukemia virus oncogene v-*abl* causes pre-B cell leukemia and inhibits apoptosis, functions which are probably related.

Viruses that Promote Apoptosis

DNA viruses

Adenoviridae Adenoviruses encode proteins that promote apoptosis at later stages in the productive cycle. These are encoded in the E3 and E4 regions. The E3 11.6K, Ad death protein (ADP) is expressed at high levels late in infection and may bring on apoptosis, but this has not been demonstrated. It is required at very late stages for efficient cell lysis and virus release.

African swine fever virus Monocytes, macrophages and mononuclear cells infected with AFSV undergo apoptosis. This gives rise to lymphoid organ impairment in acute infections.

Circoviridae The VP3 gene of chicken anemia virus (CAV) encodes apoptin, which is localized in the nucleus and has to be to induce apoptosis. It is p53 independent. The resulting apoptotic bodies are endocytosed by surrounding epithelial cells.

Hepadnaviridae Hepatitis B virus (HBV)-infected cells undergo apoptosis, contributing to fulminant hepatitis. Expression of FAS is upregulated and correlates with viral hepatitis, but not directly with HBsAg expression. Some data suggest that it is due to the viral pX gene product, a multifunctional regulatory protein. pX has also been shown to prevent apoptosis and contribute to hepatocarcinoma development.

Herpesviridae Varicella zoster virus (VZV) induces apoptosis as part of the lytic cycle. Protection of cells from apoptosis may lead to latency, but cannot be accomplished by bcl-2. HSV-1 has been shown to induce apoptosis in activated, peripheral blood lymphocytes, especially CD4+, but not CD8+, lymphocytes. This may underlie the immunosuppres-

sion and lymphocytopenia accompanying HSV-1 infections. A similar result was observed with Marek's disease virus (MDV) in latently infected chickens. MDV also causes thymic atrophy during the acute phase of infection of chickens. Immature CD4+ and CD8+ thymocytes undergo apoptosis, probably the cause of the immunosuppression seen with MDV infections.

Papovaviridae The HPV E2 gene product activates p53 transcription and inhibits E6 (which degrades p53 transcription leading to an overall increase in p53) leading to apoptotic death. E7 primes cells for apoptosis in response to γ-irradiation, and this is p53-dependent.

Parvoviridae Human parvovirus B19 strain induces apoptosis of fetal erythroid precursors. It may be due to a nonstructural protein, NSP, localized to the nucleus and able to bind DNA. It is lethal when expressed in several cell types. However, the mechanism is unknown.

RNA viruses

Arteriviridae Porcine reproductive and respiratory syndrome (PRRS) virus p25 (ORF 5) is a membrane-associated glycoprotein, also present in virus particles. p25 induces apoptosis that cannot be inhibited by bcl-2.

Bunyaviridae La Crosse virus causes apoptosis of neuronal cells by an unknown mechanism.

Coronaviridae Infections with mouse hepatitis virus result in transient atrophy of the thymus. CD4+ and CD8+ lymphocytes are depleted and apoptosis of lymphocytes increases. The viral A59 (MHV-A59) gene is responsible, but its function or mechanism is presently not known.

Flavivirus The hepatitis C virus (HCV) core protein activates the FAS-mediated cell death pathway, by upregulating FAS. It can be prevented by inhibiting the CPP32 cysteine protease, but not ICE. Thus, the CPP32 protease is involved in the apoptosis effector pathway of HCV and may contribute to HCV immune-mediated liver damage. It has also been reported that HCV core protein can overcome some types of apoptotic death and thus contribute to transformation. Dengue (DEN) virus induces apoptosis late in the virus life cycle. This may be a key element in the pathophysiology of hepatic failure associated with DEN hemorrhagic fever/shock syndrome. Another major target cell type of DEN type 1 are neurons, which apoptose after accumulation of viral proteins in the endoplasmic reticulum, resulting in stress that brings about apoptosis.

Orthomyxoviridae In influenza virus-infected cells, neuraminidase activates latent TGFβ, which activates apoptosis. TGFβ levels increase in response to influenza. FAS-associated apoptosis may also be due to double-stranded (ds) RNA, which activates a dsRNA-protein kinase (PKR), which augments FAS expression. FAS gene activation is regulated by the PKR/interferon system.

Paramyxoviridae Newcastle disease virus (NDV) brings about apoptotic death in virus-infected macrophages. Measles virus (MV) induces apoptosis of thymocytes and thus contributes to a long-term alteration in immune responses. Apoptosis induction reflects the virulence of the virus: the more virulent, rapidly replicated strains induce cell death. Parainfluenza virus type 3 (PIV3)-induced PCD inhibits T cell function.

Picornaviridae Theiler's murine encephalomyelitis virus (TMEV) results in the apoptosis of infected neurons or nonpermissive cells, which could be responsible for the fatal outcomes and chronic demyelination associated with different strains.

Reoviridae Reovirus sigma 1 (S1), but not sigma1s (which determines viral serotype), and M2 genes, which encode proteins of the outer capsid, may be involved in viral entry and may trigger apoptosis that can be overcome by *bcl*-2 expression. There is no effect on virus growth. Some reports indicate that it correlates with the neural pathology associated with reovirus. Rotaviruses also cause apoptosis, but the mechanism is unknown. It may also be responsible for the pathogenesis of rotavirus-induced diarrheal disease.

Retroviridae The lentiviruses seem to be very competent at inducing apoptosis. Both human (HIV) and simian (SIV) immunodeficiency viruses cause T cell apoptosis. For SIV, both CD4+ and CD8+ cells apoptose. HIV infection effects apoptosis of CD4+ T cells, leading to T cell depletion and ultimately immunocompromised individuals. This plays a major role in HIV pathogenesis. It is FAS-mediated. HIV-infected cells may induce FASL and increase surface expression of FAS in monocytes, this can be inhibited by a cysteine protease inhibitor (E64d). It has also been shown that bcl-2 is cleaved by the viral protease. High levels of bcl-2 protect against apoptosis, but result in reduced viral yields. The HIV transcription

factor, Tat protein, upregulates the FAS receptor and downregulates bcl-2, and increases bax, leading to apoptosis of T and B lymphoid cells and monocytoid cells. The HIV *vpr* gene arrests cells in G_2, which is followed by apoptosis. The extent of arrest and apoptosis are directly correlated. The viral envelope glycoprotein–CD4 interactions are required for apoptosis of cell–cell transmitted virus. This does not require new virus replication. HIV and SIV also bring about dementia, which seems to be due to the apoptosis of neurons as a consequence of infected lymphoid cells accessing the CNS. SIV infections of the CNS with neurovirulent, neuroendotheliotropic strains results in the apoptosis of neurons, glial cells and endothelial cells of the CNS. Caprine arthritis–encephalitis virus (CAEV) is an ungulate lentivirus that results in PCD, but no gene or mechanism has been identified.

Rhabdoviridae Infections of neurons of intact mice with rabies virus results in an increase in bax protein and apoptosis that can be inhibited by overexpression of bcl-2. This suggests that apoptosis is important in the pathogenesis of this virus.

Togaviridae For bovine viral diarrhea virus (BVDV), virulent strains induce apoptosis, whereas noncytopathic strains do not. For Sindbis virus (an alphavirus), apoptosis directly correlates with cytopathic effect and neurovirulence and is dependent on viral replication. Sindbis virus-infected neurons of the CNS die by apoptosis. Bcl-2 shifts the infectious cycle from lytic to persistent. For Semliki Forest virus (SFV), apoptosis is also seen with neurovirulent strains, but not avirulent strains. It can be inhibited by bcl-2, which acts early in the virus life cycle to inhibit viral replication and results in reduced numbers of infected cells. This leads to the establishment of chronically infected cells.

See also: **Cell structure and function in virus infections; Cytokines; Immune escape mechanisms; Immune response: Cell mediated immune response, General features; Retroviral Oncogenes; Pathogenesis: Animal viruses; Transformation: Animal viruses; Tumor viruses – human.**

Further Reading

Collins M (1995) Potential roles of apoptosis in viral pathogenesis. *Am. J. Respir. Crit. Care Med.* 152: S20.

Gillet G and Brun G (1996) Viral inhibition of apoptosis. *Trends Microbiol.* 4: 312.

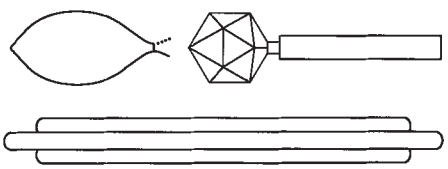
Shen Y and Shenk T (1995) Viruses and apoptosis. *Curr. Opin. Genet. Dev.* 5: 105.

Spriggs MK (1996) One step ahead of the game: viral immunomodulatory molecules. *Annu. Rev. Immunol.* 14: 101.

Teodoro JG and Branton PE (1997) Regulation of apoptosis by viral gene products. *J. Virol.* 71: 1739.

Yuan J (1997) Transducing signals of life and death. *Curr. Opin. Cell. Biol.* 9: 247.

ARCHAEAL PHAGES



Hans Peter Arnold, Kenneth M. Stedman and **Wolfram Zillig**, Max-Planck-Institute für Biochemie, Martinsried bei München, Germany

Phylogenetic Background

Complete genome sequencing has confirmed the previous notion that the *Archaea* represent a novel domain of life, distinct from *Bacteria* and *Eucarya* (formerly known as Eukaryotes). Despite this fact, very little is known about their viruses. The *Archaea* themselves are divided phylogenetically into two kingdoms, *Crenarchaeota* and *Euryarchaeota*. The latter contains the methanogens, the extreme halophiles, the extremely thermophilic, sulfur-dependent *Thermococcales* and the highly acidophilic *Thermoplasmales*. Most of the known crenarchaeota are extremely thermophilic and sulfur-dependent organ-

factor, Tat protein, upregulates the FAS receptor and downregulates bcl-2, and increases bax, leading to apoptosis of T and B lymphoid cells and monocytoid cells. The HIV *vpr* gene arrests cells in G_2, which is followed by apoptosis. The extent of arrest and apoptosis are directly correlated. The viral envelope glycoprotein–CD4 interactions are required for apoptosis of cell–cell transmitted virus. This does not require new virus replication. HIV and SIV also bring about dementia, which seems to be due to the apoptosis of neurons as a consequence of infected lymphoid cells accessing the CNS. SIV infections of the CNS with neurovirulent, neuroendotheliotropic strains results in the apoptosis of neurons, glial cells and endothelial cells of the CNS. Caprine arthritis–encephalitis virus (CAEV) is an ungulate lentivirus that results in PCD, but no gene or mechanism has been identified.

Rhabdoviridae Infections of neurons of intact mice with rabies virus results in an increase in bax protein and apoptosis that can be inhibited by overexpression of bcl-2. This suggests that apoptosis is important in the pathogenesis of this virus.

Togaviridae For bovine viral diarrhea virus (BVDV), virulent strains induce apoptosis, whereas noncytopathic strains do not. For Sindbis virus (an alphavirus), apoptosis directly correlates with cytopathic effect and neurovirulence and is dependent on viral replication. Sindbis virus-infected neurons of the CNS die by apoptosis. Bcl-2 shifts the infectious cycle from lytic to persistent. For Semliki Forest virus (SFV), apoptosis is also seen with neurovirulent strains, but not avirulent strains. It can be inhibited by bcl-2, which acts early in the virus life cycle to inhibit viral replication and results in reduced numbers of infected cells. This leads to the establishment of chronically infected cells.

See also: **Cell structure and function in virus infections; Cytokines; Immune escape mechanisms; Immune response: Cell mediated immune response, General features; Retroviral Oncogenes; Pathogenesis: Animal viruses; Transformation: Animal viruses; Tumor viruses – human.**

Further Reading

Collins M (1995) Potential roles of apoptosis in viral pathogenesis. *Am. J. Respir. Crit. Care Med.* 152: S20.

Gillet G and Brun G (1996) Viral inhibition of apoptosis. *Trends Microbiol.* 4: 312.

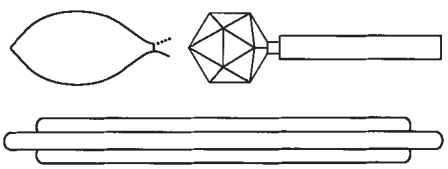
Shen Y and Shenk T (1995) Viruses and apoptosis. *Curr. Opin. Genet. Dev.* 5: 105.

Spriggs MK (1996) One step ahead of the game: viral immunomodulatory molecules. *Annu. Rev. Immunol.* 14: 101.

Teodoro JG and Branton PE (1997) Regulation of apoptosis by viral gene products. *J. Virol.* 71: 1739.

Yuan J (1997) Transducing signals of life and death. *Curr. Opin. Cell. Biol.* 9: 247.

ARCHAEAL PHAGES



Hans Peter Arnold, Kenneth M. Stedman and **Wolfram Zillig**, Max-Planck-Institute für Biochemie, Martinsried bei München, Germany

Phylogenetic Background

Complete genome sequencing has confirmed the previous notion that the *Archaea* represent a novel domain of life, distinct from *Bacteria* and *Eucarya* (formerly known as Eukaryotes). Despite this fact, very little is known about their viruses. The *Archaea* themselves are divided phylogenetically into two kingdoms, *Crenarchaeota* and *Euryarchaeota*. The latter contains the methanogens, the extreme halophiles, the extremely thermophilic, sulfur-dependent *Thermococcales* and the highly acidophilic *Thermoplasmales*. Most of the known crenarchaeota are extremely thermophilic and sulfur-dependent organ-

isms. Recent investigations have revealed that *Archaea* are not limited to extreme habitats like salterns or solfataras, but are ubiquitous.

This entry will focus on halophage ΦH and the virus SSV1. ΦH is the best characterized virus from euryarchaeota, SSV1 that of crenarchaeota. An overview of all other known viruses of the domain is also given and specific features of some will be discussed. Almost all known euryarchaeotal viruses resemble bacteriophages of the head and tail type, whereas the viruses of the *Crenarchaeota* exhibit novel morphologies (**Fig. 1**).

Viruses of Euryarchaeota

Halophages

All of the halophages isolated to date are head and tail phages similar to various coliphages (e.g. T phages and λ relatives). Halophages have isometric, icosahedral heads of widely differing sizes and tails, which in the case of ΦH, Hs1, HF1, HF2 and ΦCh1 are contractile. In the phages ΦH and ΦN tail fibers have been described, in the phages Hh3 and ΦCh1 collars. The DNA of halophages is double-stranded and varies between 30 and 60 kb in size, with the exception of Ja1 which carries an impressive 230 kb. Similar to their hosts, the guanine plus cytosine (G + C) content of the phage genomes is about 60–70%. Most of the halophages are inactivated in solutions of low ionic strength and are stable in solutions of high salt concentration, similar to their host's optimal growth conditions (typically 3–5 M). Their protein profiles in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) are relatively complex. Up to three major bands and a dozen or more minor bands are typical. Most halophages are stabilized by magnesium ions. These and other characteristics of halophages are listed in **Table 1.**

Halophage ΦH (*Myoviridae*) The best characterized of the halophages is ΦH. It is a temperate phage which causes lysis of the host cells when they enter the stationary growth phase. The phage persists in its host as prophage in the form of non-integrated, circular DNA, whereas the virion contains linear DNA. Several features of gene expression and genome organization have been elucidated. The structural and regulatory similarities to the coliphages are striking, despite the enormous evolutionary distance between these phage host systems.

Genome organization The phage particles contain linear double-stranded DNA with a length of 59 kb. This size differs from that of the viral episome, which is 57 kb. This difference is caused by the headful mode of packaging, leading to partial circular permutation and terminal redundancy of the phage genome. The terminal repetition comprises about 3% of the genome length. In comparison to bacterial phages the high rate of primary versus processive head filling is unusual; about 50% of all phage DNA molecules begin at the packaging start site of the genome (*pac* site). Because of the high genetic variability of *Halobacterium salinarum*, which is due to the high frequency of insertion elements, many variants of ΦH have been isolated (about 10% of any given phage population). This has facilitated the study of the genome organization and gene expression of the phage. All the changes are found in the so-called L region, a part of the genome which contains important regulatory elements and comprises the early genes and the insertion element ISH1.8 (in one or more copies). **Table 2** lists different host and phage variants.

The L region is flanked by the left and right arms of the viral genome. On the left arm are located the genes for the major structural proteins of ΦH: HP 20, HP 32 and HP 67 (9.3, 35.4 and 45.5 kDa respectively). The transcription of these late lytic genes proceeds across the terminal redundancy from the right to the left arm, which is possible because of the circularization of the viral genome upon its entry into the host cell. The most abundant phage variant, ΦH1, possesses the insertion element ISH1.8 at the left terminus of the L region (**Fig. 2**). In ΦH2 and ΦH5, which each carry two copies of ISH1.8 situated on both flanks, a genetic rearrangement can lead to inversion of the L region or to its looping out between the two copies of the IS element forming a plasmid, pΦHL, which harbors the L region. This plasmid can replicate autonomously and confers immunity to infection by ΦH, demonstrating the important role of the L region in the maintenance of the viral genome and in mediating immunity. The sequence of the L region has been determined. It contains two open reading frames (ORFs) which show similarity to bacterial and eucaryal genes encoding proteins that play a role in episome replication and maintenance.

Transcription of the L region and gene expression As mentioned above, ΦH can undergo a lytic cycle leading to host lysis or it can persist in a lysogenic state as a 'silent' episomal prophage. The decision between lytic cycle and lysogeny requires regulation of transcription in the L region (**Fig. 2**). The transcription of lytic phage genes is strictly time-dependent, with early gene products required for the expression of late genes. Three hours after infection

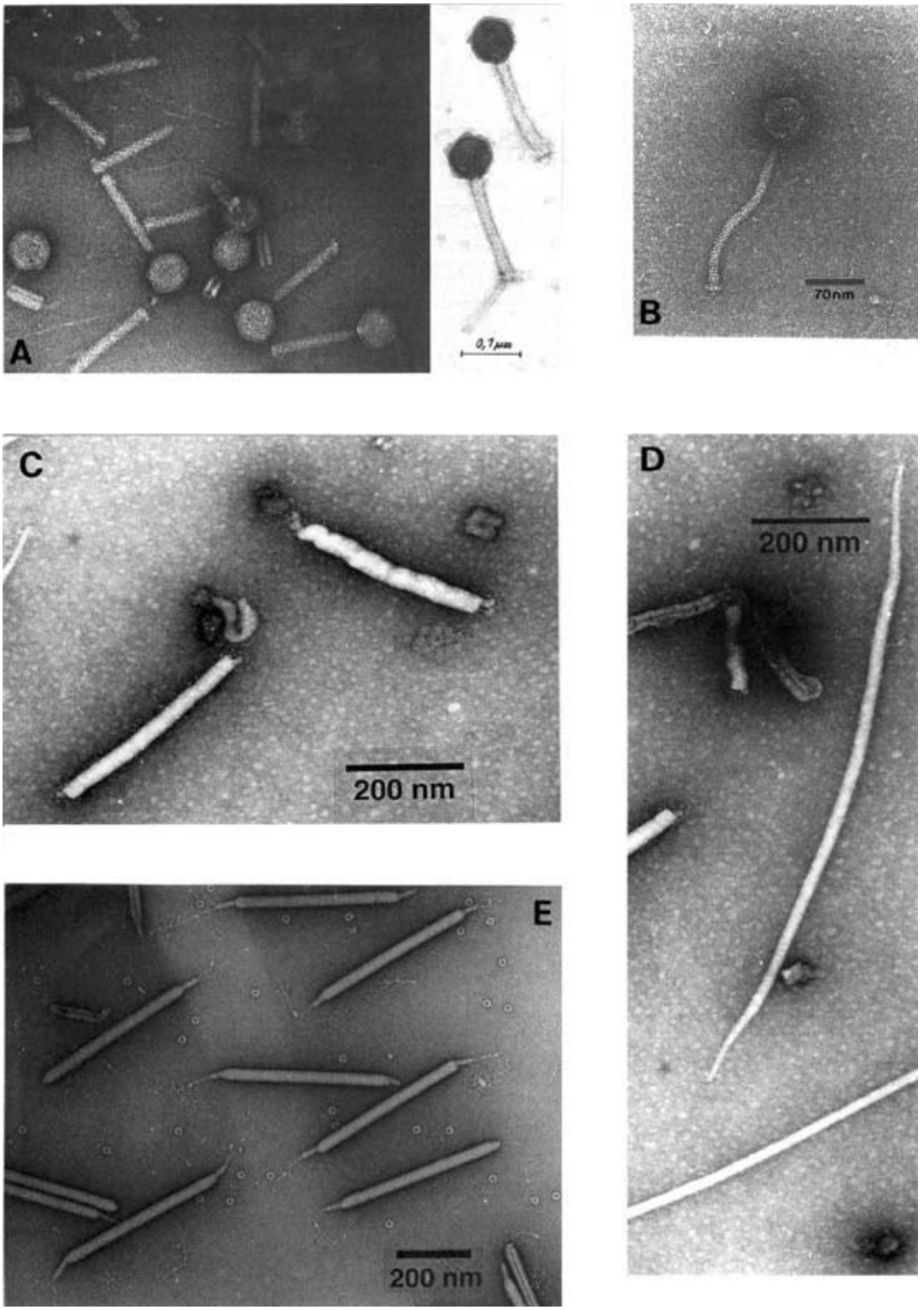


Figure 1 Electron micrographs of different archaeal viruses (negative stain). (**A**) Halophage ΦH of *Halobacterium salinarum*. On the left some with contracted tails. (Reproduced with permission from Zillig W *et al* (1986) Archaebacterial virus host systems. *Syst. Appl. Microbiol.* 7: 59.) (**B**) Methanophage ψM1 of *Methanobacterium thermoautotrophicum*. (Reproduced with permission from Meile L *et al* (1980) Characterization of ψM1, a virulent phage of *Methanobacterium thermoautotrophicum* Marburg. *Arch. Microbiol.* 152: 106. Copyright © Springer-Verlag.) (**C**) Viruses TTV1, (**D**) TTV2 and (**E**) TTV4 of *T. tenax*. (**F**) Virus SSV1 of *S. shibatae*. (**G**) Virus SIRV of *S. islandicus*. (**H**) Virus SNDV of *Sulfolobus* spp. from New Zealand. (**I**) Virus DAFV of *D. ambivalens*.

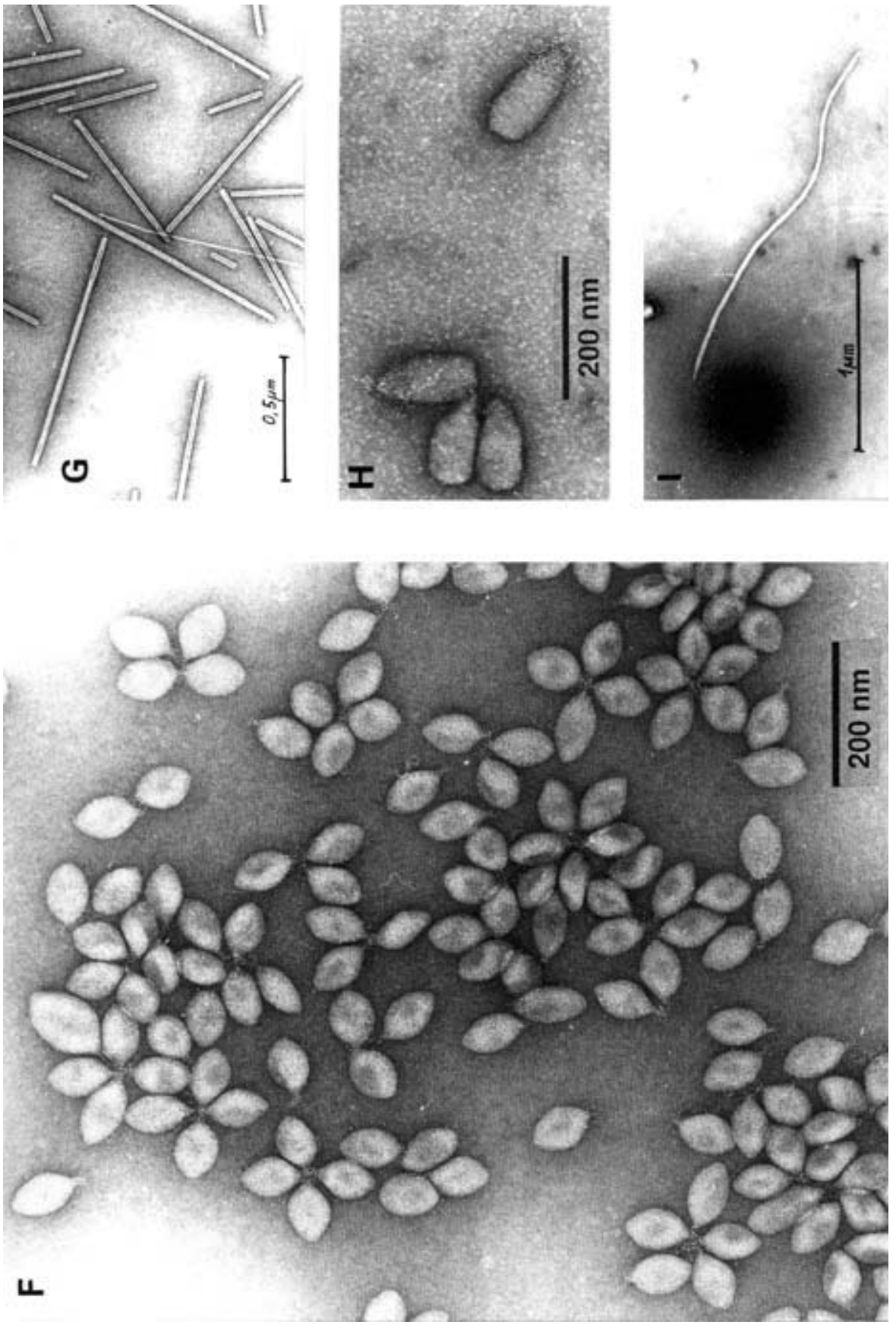

G
0,5µm
H
200 nm
I
1µm
F
200 nm

Table 1 All known halophages, their hosts and some characteristics

Phage	*Host*	*Morphology*	*Size (nm)*	*Genome type and size (kb), DNA*	*Proteins*	*Propagation*	*Eclipse (h)*	*Latent period (h)*	*Burst size per cell*
ΦH	*H. salinarum*	Head and contractile tail	64 170	Linear, ds (59), 3′ redundancy	3 major and several minor	Temperate, episomal prophage, lysis	5.5	7	170
HF1	*Haloferax*, *H. salinarum*	Head and contractile tail	55 94	Linear, ds (79)	1 major and several minor	Lytic	?	?	?
HF2	*H. saccharovorum*, Ch2	Head and contractile tail	55 94	Linear, ds (79.7), blunt ends	1 major and several minor	Lytic	?	?	?
ΦN	*H. salinarum*	Head and tail with fibers	60, 90	Linear, ds (56), terminal redundancy	1 major and several minor	?	10	14	400
Hs1	*H. salinarum*	Head and contractile tail	50 20	?	?	Stable carrier state, lytic at low salinity	12	17	200–300
Ja1	*H. cutirubrum*, *H. salinarum*	Head and tail	90, 150	Linear, ds (230)	?	?	2	6	140
Hh1	*H. cutirubrum*, *H. salinarum*	Head and tail	60, 100	Linear, ds (37.2)	1 major and several minor	Carrier state	6	12	1100 (2 cycles)
Hh3	*H. salinarum*, *H. cutirubrum*	Head and tail	74, 50	Linear, ds (29.4)	1 major and several minor	Carrier state	5	8	425
S45	*Halobacterium*, several spp.	Head and tail	40, 70	Linear, ds (?)	?	Strain-dependent	?	?	1300
ΦCh1	*Natronobacterium magadii*	Head and contractile tail with collar	70, 130	Linear, ds (55), partially modified	4 major and 5 minor	Temperate, lysis, integration into host chromosome	5	11	?

?, Unknown; ds, double-stranded; *H., Halobacterium.*

Table 2 Some features of *Halobacterium salinarium* strains and halophage ΦH variants

Strain/Variant	*Features*
H. salinarum	
R_1	*H. salinarum* strain which does not produce gas vacuoles
$R_1$24	R_1 lysogen harboring the complete circularized ΦH1 genome
R_1L	R_1 harboring the plasmid pΦHL which is derived from the L region of ΦH1, conferring immunity to ΦH1
Halophage	
ΦH1	Most abundant variant; >90% of the phage population, carrying a single copy of ISH1.8 at the right side of the L region
ΦH3, 4, 6	Like ΦH1, but differing by deletions or insertions outside the L region
ΦH 8	Like ΦH1, but carrying a single copy of ISH1.8 at the left side of L region
ΦH 7	Like ΦH 8, but with a deletion of 2.2 kb at the right border of the L region
ΦH2, 5	ΦH1 carrying one copy of ISH1.8 on both sides of the L region; capable of inverting the 12 kb L segment
pΦHL	Plasmid derived from ΦH1 consisting of the 12 kb L region, confers immunity to ΦH1 but not ΦHL1
ΦHL1	Variant in which an ISH23 element is inserted into the promotor of the transcript T4; this variant is able to overcome immunity of strain R_1L but not of strain $R_1$24
ΦHLA, B	Like ΦHL1, but carrying the L region in different orientation, variant A and B differ in carrying a flanking copy of ISH1.8 at one or the other end of the L region

the entire phage genome has been expressed, except for the left border of the L region and a small section in the right arm of the genome. Only these sections are expressed in lysogens and are thus most likely involved in lysogeny, including immunity to further phage infection. Another factor partially mediating immunity to superinfection is the *rep* gene product which can repress the formation of the major early lytic transcript T4. The promoters of the *rep* and T4 genes are situated back to back in a manner similar to that of *cI* and *cro* in bacteriophage λ. The region upstream of the T4 promoter contains two direct

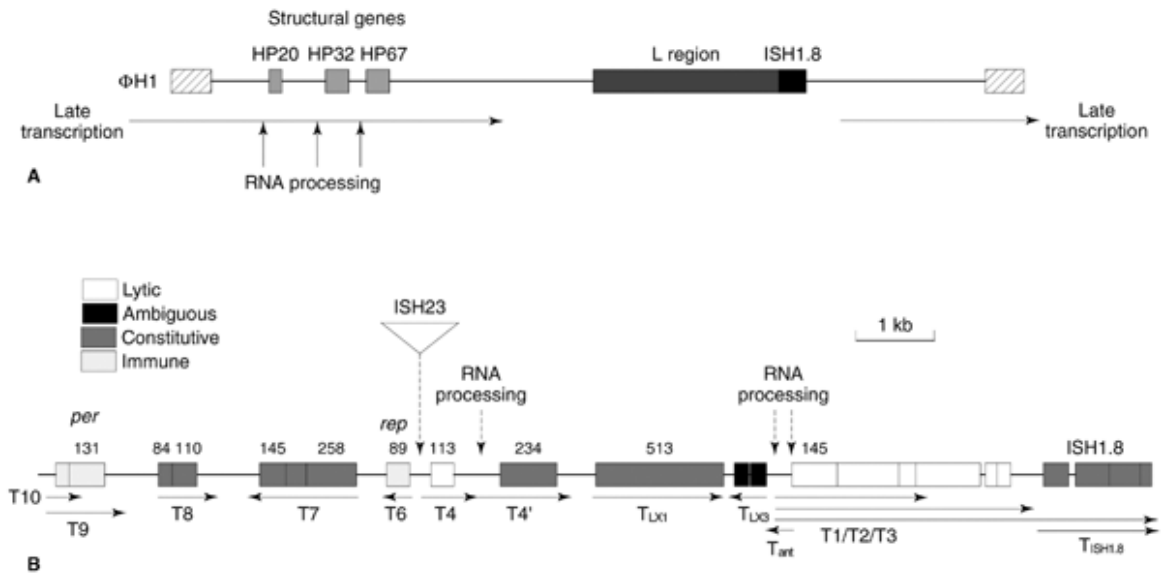


Figure 2 Map of the ΦH genome, showing genes and known transcription units. (**A**) Linear map of the phage variant ΦH1, which is the variant used in most studies on immunity and transcription. The hatched boxes at the ends of the DNA represent regions of terminal redundancy. The two arrows representing late transcription are parts of the same transcript, as the phage genome circularizes after entry into the cells. (**B**) Map of the L region. Boxes represent open reading frames. Transcripts denoted by a capital T followed by a designation are shown as arrows. The integration point of the IS element ISH23 in the phage variant ΦHL1 is indicated. The ISH1.8 insertion element shown is not part of the L region when present in plasmid pΦHL. (Reproduced with permission from Stolt P and Wolfram B (1994) Gene regulation in halophage ΦH; more than promoters. *Syst. Appl. Microbiol.* 16: 592.)

repeats with the sequence CTTCTCG separated by 15 bp. The repressor contains a helix-turn-helix motif and binds to this operator to repress transcription from the T4 promoter. The variant ΦHL1 can overcome the immunity of strain R_1L mediated by the plasmid pΦHL because of the insertion of element ISH23 into the promoter region of transcript T4 in ΦHL1. The insert functions as a spacer between repressor binding site (operator) and transcription start site, so that transcript T4 can be formed even in the presence of the repressor. Since ΦHL1 cannot overcome the immunity of *Halobacterium salinarum* carrying the wild-type prophage (strain $R_1$24), it is clear that immunity against ΦH is mediated by more than just the *rep* gene product. Transcription from the back to back promoters is mutually exclusive, with only the *rep* gene transcribed in the 'immune' (lysogenic) state and only transcript T4 produced during the lytic cycle. The product coded by transcript T4 has no effect on *rep* transcription but the transcription from the stronger promoter of the corresponding gene *per se* represses *rep* expression. These are the only two regulated promoters in the L region. All other L region promoters are transcribed constitutively.

Gene regulation also occurs at the level of RNA processing, particularly in the case of transcript T1. This lytic transcript is produced from a constitutive promoter 10 min after infection. In immune cells T1 is neutralized by the action of the antisense transcript T_{ant}. T_{ant} starts 151 bp downstream of the start site of T1 and is transcribed from a weak constitutive promoter in the opposite direction to T1. Consequently the first 151 nucleotides of T_{ant} and T1 form an RNA–RNA hybrid which is processed by an endonucleolytic RNase that removes the 3′ overhangs at the end of the RNA–RNA duplex but does not digest the double-stranded products. This leads to the removal of the 'Shine–Dalgarno' sequence (ribosome-binding site) of the lytic transcript T1 so that its RNA is not recognized by the translation apparatus. The RNase was shown to be host-encoded. Even though T_{ant} inactivates the early lytic transcript T1, its expression does not mediate immunity to phage infection but only a tenfold decreased production of phage particles. This is less than the immunity mediated by overexpression of the *rep* gene, which leads to a decrease in phage production by a factor 10^2, whereas pΦH1 mediates a 10^4-fold reduced phage production. Another example of the post-transcriptional modification of RNA in ΦH1 is 5′ processing of the large late lytic transcript on the left arm of the genome which encodes the three major structural proteins. Additionally, the transcripts T4 and T4′ are processed from a large common precursor RNA molecule by forming a loop structure and consecutive 3′ exonucleolytic removal of 40 nucleotides releasing both transcripts.

Halophages HF1 and HF2 (*Myoviridae*) *Morphology and hosts* Due to the genetic instability of *Halobacterium salinarum*, which is the only known host of the well-characterized halophage ΦH, investigators have also focused on viruses infecting members of the genus *Haloferax*, in particular *Haloferax volcanii*. Two novel viruses, HF1 and HF2, have been isolated from an Australian solar saltern. Both viruses are similar in their morphology, in their sensitivity to reduced ionic strength, their protein profiles, with one common major protein of 55 kDa and several minor proteins, and their linear, double-stranded DNA genomes of nearly the same size with similar restriction patterns (**Table 1**). DNA hybridization data also showed the two phages to be closely related. HF1 and HF2 are both lytic but differ in their host range. HF1 infects members of three halobacterial genera, including species of *Haloferax*, *Halobacterium salinarum* and *Haloferax volcanii*. In contrast to HF1 the host range of HF2 is confined to the closely related strains Ch2 and *Halobacterium saccharovorum*. HF2 also differs significantly from HF1 in plaque morphology.

Genome organization and replication strategy HF2 was chosen as a model for the study of the phage genome replication and the genome organization of the HF phages. The DNA of HF2 is not susceptible to cleavage by many type II restriction endonucleases, which is attributed to selection against palindromic restriction sites. The DNA termini of the linear HF2 genome exhibit homology to each other. Similar to bacteriophage T7, both ends are a 306 bp direct repeat, which contains five strings of C residues 3–6 bp in length separated by GT, TG or TGT motifs and two neighbouring sequences capable of forming stem–loop structures. The junction between the repeats and the rest of the HF2 genome contains two sequences which might be recognition sites for endonucleolytic cleavage. Similar sequences are flanking the cleavage sites of an endonuclease involved in replication in the genomes of the bacteriophages T3 and T7. This suggests a replication mode through concatameric intermediates also for HF2. As in the case of the bacteriophages T3 and T7, concatameric multimers of the genome have been observed in HF2-infected cells.

Other halophages *Phage ΦN of* Halobacterium salinarum (Siphoviridae) In contrast to all other known halobacterial phages, ΦN is not dependent on

high ionic strength or a high magnesium content in the medium to maintain infectivity. Curiously, in the genome of ΦN cytosine is completely replaced by 5-methylcytosine, a feature that has so far only been reported for the DNA of the bacteriophage XP12. Extensive C-methylation does not occur in the host and therefore a virus-encoded enzyme seems to be responsible for this modification.

Halobacterium salinarum *phage Hs1* (Myoviridae) The interaction of this phage with its host depends on the salt concentration of the medium. Lysis is retarded at higher salt concentrations, whereas the burst size remains unaffected. The rate of adsorption of the phage particles to their host cell is decreased at high salinity of the medium, whereas the growth rate of the host shows the opposite response. This is critical for the persistence of the phage in the host culture: at low salinity (17.5% NaCl) phage production exceeds archaeal growth, whereas at 25% NaCl the host can overgrow the phages via the segregation of cured, i.e. phage-free, cells. At concentrations between 25 and 30% NaCl a carrier state between Hs1 and its host is established. The frequency of lysis per cell and generation is 10^{-4} at 30% NaCl. Cells infected under low-salt conditions can be rescued by transfer to high-salt medium. This feature may be of selective advantage to the phage, as changes of the salt concentration in the natural habitat of the host often take place. The virus is lytic under conditions of low salinity, which are hazardous to its host. However, under conditions of optimal growth the phage propagates within its host in a stable carrier state.

Natronobacterium magadii *phage ΦCh1* (Myoviridae) The genera *Natronobacterium* and *Natronococcus* represent the alkalophilic group among the family of the *Halobacteriaceae*, with a pH optimum from 9 to 10. The phage ΦCh1 was detected via 'spontaneous' lysis of strains of *Natronobacterium magadii*. Morphologically ΦCh1 resembles other halophages. The tail has a collar at its end and an internal shaft which is visible after tail contraction. The host range of ΦCh1 is restricted to *Natronobacterium magadii*. The phage produces turbid plaques on lawns of *Natronobacterium magadii*, which is lysed by the phage in the stationary state. Incubation in medium of lower salinity than 2 M NaCl leads to the loss of the infectivity of the phage. But, as for most of the halophages, lower salt concentrations can be tolerated in the presence of magnesium. In addition to the partially methylated virus genome, host encoded RNA is found in the phage particles.

Viruses of methanogens

Although the methanogens represent the group of the *Archaea* with most known species and strains, knowledge of their viruses was, until recently, relatively sparse. In the past few years several new phages have been isolated and there is strong evidence that methanophages play an important role in lysis of methanogenic archaea in the rumen fluid. Almost all viruses of the methanogens are head and tail phages, except the virus-like particle (VLP) of *Methanococcus voltae*. Only two will be discussed here (see **Table 3** for more phages).

Phage ψM1 (*Siphoviridae*) ψM1 is the best characterized phage of the methanogenic archaea. It is an oxygen resistant phage of the thermophilic, strictly anaerobic, *Methanobacterium thermoautotrophicum* strain Marburg. The phage does not infect three other representatives of the genus *Methanobacterium* even though a defective prophage of ψM1 was found to be integrated into the chromosome of *Methanobacterium wolfei*. This methanogen is immune to infection by ψM1, but is not able to produce phage particles. In contrast to other species of *Methanobacterium*, *M. wolfei* lyses spontaneously upon energy starvation, a property possibly related to the possession of the integrated prophage. Only 85% of the DNA extracted from ψM1 phage particles is the phage genome, the rest are multimers of the cryptic plasmid pME2001 (4.5 kb) with the same size as the phage genome. The packaged pME2001 multimers are head to tail hexamers linked to an incomplete terminal copy of the plasmid. Despite lack of homology between the plasmid and phage DNA, the packaging appears to be the same for both and might be a headful mechanism. Similar plasmid encapsidation has been observed in mutant bacteriophage T4. Phage ψM1 also has the capacity to transduce a number of chromosomal markers at frequencies from 6×10^{-4} to 5×10^{-6} per PFU (plaque forming unit). Phage ψM1 thus appears to be a general transducing genetic element.

Virus-like particle from *Methanococcus voltae* A3 Electron-microscopic examination of the supernatant of *Methanococcus voltae* strain A3 revealed the presence of lemon-shaped particles, which morphologically resemble virus particles, particularly SSV1 (see below). Treatment of the particles with DNase showed that the DNA was protected from digestion until phenol extraction. However, neither infectivity nor inducibility of the virus-like particles could be demonstrated.

Table 3 Viruses and virus-like particles of methanogenic *Archaea*

Phage	*Host*	*Morphology*	*Size (nm)*	*Genome type and size (kb), DNA*	*Proteins*	*Propagation*	*Eclipse (h)*	*Latent period (h)*	*Burst size per cell*
ψM1	*Methanobacterium thermoautotrophicum* Marburg	Head and tail	55, 210	ds, linear (30.4)	?	Lytic	?	4	6
ΦF1	*Methanobacterium* sp.	Head and tail	70, 160	ds, linear (85)	?	Lytic	?	?	?
ΦF3	*Methanobacterium thermoautotrophicum* FF3	Head with flexible tail	55 230	?, ? (36)[a]	?	Lytic	?	?	?
PG	*Methanobrevibacter smithii* strain G	Head and tail	?	linear, ds (50)	?	Lytic	7–9	20	?
PSM1	*Methanobrevibacter smithii*	Head and tail	?	?, ds (35)	?	Lytic	?	?	?
VLP	*Methanococcus voltae* A3	Lemon-shaped	52 × 70	ds, ccc (23)	1 major and 3 minor	Temperate	?	?	?

[a] Terminally redundant linear or cccDNA.

Table 4 Viruses of *Thermoproteus tenax*

Phage	*Morphology*	*Size (nm)*	*Genome type and size (kb), DNA*	*Propagation*
TTV1	Flexible rod	40 × 400	linear, ds (15.9)	Lysogeny
TTV2	Filamentous	20 × 1250	linear, ds (16.0)	Lysogeny
TTV3	Filamentous	30 × 2500	linear, ds (27.0)	?
TTV4	Stiff rod	30 × 500	linear, ds (17.0)	Lytic

ds, Double-stranded.

Viruses of *Crenarchaeota*

In contrast to the phages of euryarchaeota, which to date are almost all of the head and tail type, the few known viruses of *Crenarchaeota* (*Thermoproteales* and *Sulfolobales*) all have unique morphologies. The only known organisms, which serve as hosts for filamentous or rod-shaped viruses containing linear, double-stranded DNA, are crenarchaeota.

Viruses of *Thermoproteus tenax*

Thermoproteus tenax is an extremely thermophilic, strictly anaerobic and facultatively chemolithoautotrophic archaeon with a growth optimum at 88°C. In the chemolithoautotrophic mode of growth, energy is supplied by the formation of H_2S from S^0 and H_2. *T. tenax* is the host for four different viruses, termed TTV1, 2, 3 and 4. TTV1 is a flexible rod, TTV4 a stiff rod. TTV2 and TTV3 are flexible filaments. They differ in their protein composition and their morphology. No homology between their genomes has been observed in Southern hybridization analysis (**Fig. 1** and **Table 4**).

TTV1 (*Lipothrixviridae*) TTV1 is the best characterized of the four viruses. The virus is temperate. Lysis is induced when the sulfur in the host culture is consumed. TTV1 shows genetic variability confined to a specific part of its genome indicating an, as yet undefined, recombination mechanism. Some lysates of *T. tenax* cultures yield only one variant, while others contain simple virus mixtures. The variance mostly results from the regrouping of sequences with high homology to each other located between two nonadjacent reading frames. These sequences have an unusually high G + C content of about 58%, compared with 37% for the entire virus genome. On their left ends they carry the consensus sequence ACXCCTAC. The lengths of the relevant DNA sequences vary between 66 and 84 bp but are always in multiples of three, conserving ORFs on either integration or excision. Insertion of a fragment always leads to the introduction of a stretch of alternating proline–threonine sequences into the protein, termed TPX, which is expressed by the infected host cells. Two variants of this protein differing in the alternating proline–threonine stretches have been described. The function of the protein TPX and of these DNA arrangements remains unclear.

TTV1 consist of a core of linear, double-stranded DNA, to which equimolar amounts of the highly basic proteins P1 and P2 are bound. This core is covered by an inner envelope which is a helical array of a protein P3 that is formed by self-interaction of this protein. The envelope is closed by caps of protein P4. P3 is able to form hollow filaments in the absence of core. These are shortened and partially closed when protein P4 is present during aggregation. The inner envelope is covered by an asymmetric unit membrane assembled from the pool of host lipid components. The termini of the genomic DNA are covalently masked by hydrophobic ligands, which could not be removed by proteases. Therefore only about 85% of the sequence of the TTV1 genome has been determined.

TTV2, TTV3 (*Lipothrixviridae*) TTV2 and TTV3 resemble each other in their morphology. Both are long and flexible filaments. Both viruses are composed of a filamentous inner core consisting of linear DNA covered with protein and an outer envelope of unknown composition. TTV2 contains two basic major proteins of equal molecular weight. TTV2 is associated with its host in a lysogenic state. Virus particles are normally produced in small quantities, but occasionally a titer of 10^9–10^{10} ml^{-1} results from lysis. The lysis-inducing agent is unknown. Nothing is known about the relationship between TTV3 and *T. tenax*.

TTV4 (unknown affiliation) TTV4 resembles TTV1 in its shape, though it is stiff and its projections at both ends are long and thin (**Fig. 1C, E**). The virus is highly virulent and causes lysis of the host cells independent of the mode of host growth. It is extremely thermostable. One hour of autoclaving at 120°C leaves it infective and does not destroy its structure. One of the major proteins of TTV4,

apparently from the envelope, is highly hydrophobic and has an unusually high cysteine content. Under aerobic conditions these cysteine residues are oxidized, leading to extensive crosslinking. This might explain the extreme thermostability of the virus. In addition to the coat protein, a putative DNA-binding protein has been identified. In contrast to TTV1 no membrane lipids have been found.

Because the envelope of TTV4 had not been characterized, this virus was erroneously assigned to the envelope-less *Rudiviridae*. Since its envelope, in contrast to that of TTV1, does not contain host lipids, it should rather be classified as belonging to its own family Lipothrixvirus.

Sulfolobus viruses

Sulfolobus is one of the best characterized genera of the *Archaea*. It grows aerobically and either facultatively or obligately heterotrophically and can be easily cultivated in liquid media and on solid supports. Some *Sulfolobus* species, like *S. solfataricus*, have become model organisms of the *Crenarchaeota*. Thus, most of the extrachromosomal elements of the *Crenarchaeota* have been described in this genus. Besides conjugative and nonconjugative plasmids, a few viruses have also been found in the *Sulfolobales*.

***Sulfolobus shibatae* virus SSV1 (*Fuselloviridae*)** SSV1 is the best-characterized virus of the *Crenarchaeota*. It has been a very useful tool for investigation of promoter elements and gene expression in *Archaea*. At the moment it is one of the best candidates for the development of a transformation system for *Sulfolobus*.

Morphology and hosts The members of the genus *Sulfolobus* are all extremely thermophilic and acidophilic and often sulfur-dependent archaea with a growth optimum around 80–85°C. SSV1 was isolated from the species *S. shibatae* strain B12 from Japan and is infectious for some other *Sulfolobus* species (e.g. *S. solfataricus*), but not *S. acidocaldarius*. SSV1 is a spindle-shaped virus 60 × 100 nm in size with a very short tail and tail fibers at one pole. Its characteristic spindle form has no counterpart in any known bacteriophage. The coat of the virus is resistant to the conditions of the harsh environment of its host (thermostable up to 97°C and acid-resistant at least until pH 2.0). The DNA packaged into virus particles, however, is sensitive to these acidic conditions. Longer exposure to medium of the 'normal' pH of 3.5 leads to progressive nicking. If the pH is raised to 5.5, however, the virus maintains infectivity for a longer time. Under these conditions it is quite stable up to 85°C. The virus coat consists of two very similar hydrophobic proteins termed VP1 (73 amino acid residues) and VP3 (92 amino acid residues). Both coat proteins have an identical hydrophobic polypeptide stretch of 20 amino acid residues in their sequence, which is due to an identical DNA sequence of 61 bp in their genes. The extremely basic VP2 (74 amino acid residues) is a DNA-binding protein which can be isolated out of the virus particle along with small amounts of a mixture of at least two polypeptides, of which at least one is a host-encoded DNA-binding protein of 6 kDa. The virus is temperate. After infection its genome is integrated into the host chromosome. Nevertheless, a few virus particles are produced constitutively. SSV1 proliferation can be induced, e.g. via ultraviolet irradiation, leading to temporary growth inhibition of the host cells, but not to their lysis. Virus propagation starts 3–4 h after induction, with a maximum titer of 4×10^{10} ml^{-1} after about 16 h, when virus production reaches a plateau. At this time, virus production stops and the host cells return to their normal growth rate. SSV1 attaches with its short tail fibers to the host membrane but not to its S-layer. This reaction is so effective that plating efficiencies are apparently reduced to 10–50% compared to total counts by electron microscopy. This is due to the affinity of SSV1 particles to cell-derived membrane vesicles and to putative small (invisible) membrane fragments, in the former case forming the so-called 'sunflowers', and in the latter what have been termed 'virus-rosettes', in which the virus particles appear to be attached to each others tails (**Fig. 1F**).

Genome organization The genome of SSV1 is a 15.5 kb cccDNA, and the entire sequence has been determined. DNA isolated from virus particles is positively supercoiled, a feature not shown to occur in any other known extrachromosomal element. Reverse gyrase, a host-encoded topoisomerase first described in *Sulfolobus*, is responsible for generating this topology. The pool of episomal SSV1-DNA being replicated in the host cell after induction is, however, a mixture of both negatively and positively supercoiled DNA. The genome of SSV1 integrates at a specific attachment site into a chromosomal $tRNA^{Arg}$ gene of its host. The integration leaves this host gene intact, but the viral gene encoding the integrase is interrupted. This integrase was shown to perform recombination also *in vitro*. In some other bacterial systems tRNA genes are the target site for the integration of the genomes of bacteriophages (e.g. P2, P22, HP1c1). A viral gene encoding a putative excisionase has also been found. However, in contrast to bacteriophage λ, the integrase of SSV1 does not

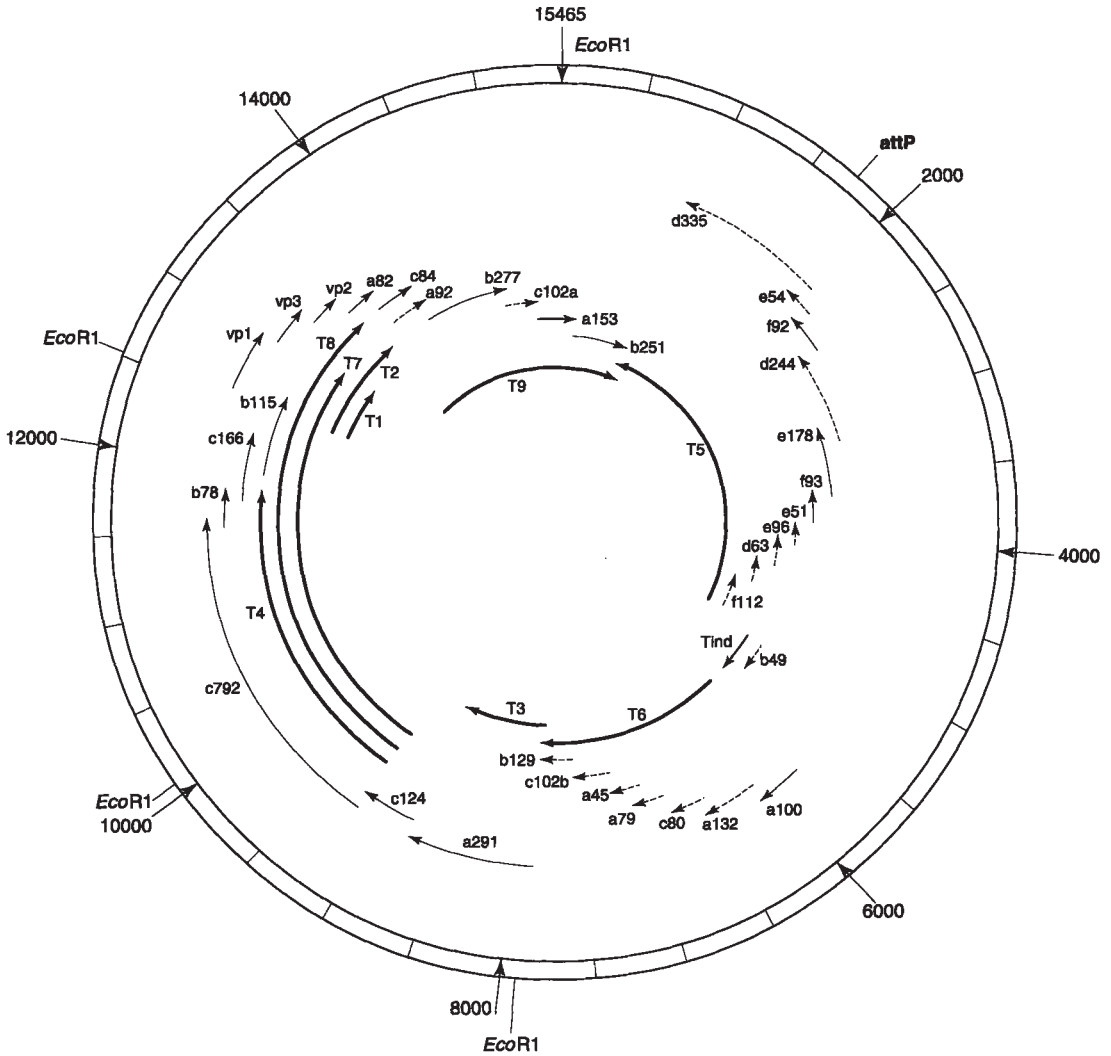


Figure 3 Map of the SSV1 genome, transcripts, proteins and open reading frames. Numbers refer to base pairs relative to the start of the largest *Eco*RI fragment. attP indicates the cleavage site for integration into the host genome. Bold arrows represent transcripts T1–T9 and T_{ind}; thin arrows are protein genes VP1–VP3 and all other open reading frames, without cysteine codons; dotted lines indicate open reading frames that contain cysteine codons. (From Palm *et al* (1991) *Virology* 185: 247.)

require accessory proteins like IHF and Xis for its *in vitro* activity.

Transcription All transcripts of the SSV1 genome have been identified (**Fig. 3**). In exponentially growing cells the major transcript is T1, although small but significant amounts of the transcripts T2, T3, T4, T7 and T8 are also detectable. Approaching stationary phase, the amount of the transcript T1 is amplified about twofold, whereas those of the transcripts T2, T3, T4, T7 and T8 are increased at least fivefold. The transcript T_{ind} appears as early as 2 h after ultraviolet irradiation, with a maximum between 3 and 4 h after induction, and has disappeared after 7 h. Transcripts T5 and T6 are also essentially absent in uninduced cells, but are transcribed 3 h after induction. They remain detectable longer than transcript T_{ind}. Although part of the amplification of the transcripts after ultraviolet induction could be due to an increase of gene dosage because of DNA replication, especially in the case of the transcripts T1 and T2, the transcripts T_{ind}, T5 and T6 are clearly induced.

Intriguingly, the promoter of the T_{ind} transcript lacks the typical, archaeal 'box A' motif about 26 bp upstream of the initiation site. The promoters of the transcripts T5 and T6, which are transcribed in opposite directions, are situated back to back. These transcripts start 5′ to the start and 3′ to the end of the T_{ind} gene, respectively, at the ends of long inverted repeat sequences. The entire length of the ultraviolet

inducible transcript T_{ind} maps between these two transcriptional start sites. The region between the promoters for the transcripts T5 and T6 contains stretches of DNA sequences exhibiting a complicated pattern of direct and inverted repeats. Regions of DNA containing such sequence repeats often constitute origins of replication, and it is likely that the region between the promoters of T5 and T6 is involved in the initiation of DNA replication. It has been claimed that this region allows a bacterial/archaeal shuttle vector to replicate autonomously in *Sulfolobus* and that the copy number of this vector is raised by ultraviolet irradiation (R. Cannio and M. Rossi, unpublished).

The constitutive transcripts T1 and T2 encode the three structural proteins of SSV1. Both transcripts share a common promoter. Transcript T2 is 1000 nucleotides long and encodes all of these proteins; T1 (500 nucleotides) terminates within the gene of VP3. Since the coat proteins VP1 and VP3 have been found to be integrated in the host membrane and since VP2, the DNA-binding protein, is also detectable in the cytoplasm during lysogeny, it appears that only DNA replication has to be induced for the maturation of virus particles. The newly synthesized DNA molecules then only need to be wrapped and packaged into their coat at 'virus membrane islands' in the host membrane. Virus particles have never been observed in the host cytoplasm. The transcript T_{ind} encodes only 49 amino acid residues and might serve as an RNA primer for DNA replication.

Other *Sulfolobales* viruses In contrast to SSV1 several other viruses from *Sulfolobus* isolates have not yet been extensively described (**Fig. 1G–I**). The rudivirus SIRV (*S. islandicus* rod shaped virus) was isolated from a *Sulfolobus* sp. strain from Iceland. It is a stiff rod and exhibits morphological similarity with the tobacco mosaic virus (TMV). A superhelix of the DNA with a DNA-binding protein forms a hollow tube which is terminally plugged and equipped with tail fibers at both ends. The virus appears to contain only one additional minor protein. There is no membrane or envelope coating the virus.

The lipothrixvirus DAFV (*D. ambivalens* filamentous virus) infects *Desulfurolobus ambivalens* (renamed *Acidianus ambivalens*), an obligately chemolithoautotrophic relative of *Sulfolobus*. It is filamentous, flexible and morphologically similar to TTV2 and TTV3 and contains linear double-stranded DNA of 56 kb.

Entirely different is SNDV (*S. newzealandicus* droplet-formed virus) which has not yet been classified. It looks like a droplet and is bearded with many fibres protruding around the pointed end. This virus is infectious for only a few strains from New Zealand and contains cccDNA of 20 kb.

Future Perspectives

Compared to the large number of characterized eucaryal viruses and bacteriophages, only a few viruses of archaea have been studied. Even so, the distribution of different morphological types within the two kingdoms of the *Archaea* is strikingly uneven. All known viruses of the *Crenarchaeota* are unique. Only in this group is found the combination of rod or filament shape and linear, double-stranded genomic DNA, whereas the phages of Euryarchaeota correspond to the head and tail morphology of many bacteriophages. However, the existence of head and tail phages in crenarchaeotes cannot be excluded. In electron micrographs of field samples, viruses with icosahedral heads have been observed attached via their tails to cells of *T. tenax* (length of the particles: 160 nm). The spindle-shaped SSV1 particle, which carries a short tail at one pole, could be regarded as a kind of head and tail virus, even though its head does not have the typical icosahedral form. Between SSV1 and the head and tail phages of Euryarchaeota and those of Bacteria exist additional similarities, particularly on the genetic level. This is an indication for the existence of head and tail viruses before the divergence of *Archaea* and Bacteria. A particularly striking argument is the similarity of the bacteriophage P1 and the halophage ϕH, which do not only resemble each other in their morphology but also both exist as episomal prophages in their lysogenic hosts. Moreover, both halophage ϕH and some λ related bacteriophages exert similar modes of control of transcriptional regulation of genes which are mutually transcribed from promoters situated back to back, with an operator for one or both of the genes in between. The genome of SSV1 is integrated into a tRNA gene in the host chromosome like that of some bacteriophages (e.g. P2, P22). Another similarity between SSV1 and lambdoid bacteriophages is the back to back arrangement of early genes and the switch from lysogeny to the lytic cycle after ultraviolet induction. These observations support the hypothesis that head and tail phages are derived of a common viral ancestor, already existing before the divergence of the three domains of life, even though the comparison of protein or DNA sequences between these viruses cannot yield direct evidence for this notion. After the divergence of the domains, the evolution of these phages could have been linked to that of their hosts, e.g. in the gene expression machinery and in coping with extreme environments. The extent of the differences between the modes of

gene expression in different domains and the divergent adaptation to the extreme living conditions make horizontal virus transfer between the domains after their divergence and stepwise adaptation of the phages to their novel hosts extremely unlikely.

Another incentive for research on archaeal viruses is the lack of a transformation system for *Sulfolobus*, which hinders progress of the molecular genetical investigation of the *Crenarchaeota*.

See also: **Enterobacteria phage P1 (*Myoviridae*); Mu-like phages (*Myoviridae*); P2, 186 and related phages (*Myoviridae*); T1-like phages (*Siphoviridae*).**

Further Reading

Nölling J, Groffen A and De Vos WM (1993) ΦF1 and ΦF2, two novel virulent, archaeal phages infecting different thermophilic strains of the genus *Methanobacterium*. *J. Gen. Microbiol.* 139: 2511.

Nuttall SD and Dyall-Smith ML (1995) Halophage HF2: genome organization and replication strategy. *J. Virol.* 69: 2322.

Reiter W-D, Zillig W and Palm P (1988) Archaebacterial viruses. *Adv. Virus Res.* 34: 134.

Schleper C, Kubo K and Zillig W (1992) The particle SSV1 from the extremely thermophilic archaeon *Sulfolobus* is a virus: demonstration of infectivity and transfection with viral DNA. *Proc. Natl Acad. Sci. USA* 89: 7645.

Stolt P and Zillig W (1994) Gene regulation in halophage ΦH; more than promoters. *Syst. Appl. Microbiol.* 16: 591.

Witte A, Baranyi U, Klein R *et al* (1997) Characterization of *Natronobacterium magadii* phage ΦCh1, a unique archaeal phage containing DNA and RNA. *Mol. Microbiol.* 23: 603.

Zillig W, Prangishvili D, Schleper C *et al* (1996) Viruses, plasmids and other genetic elements of thermophilic and hyperthermophilic *Archaea*. *FEMS Microbiol. Rev.* 18: 225.

ARTERIVIRUSES (*ARTERIVIRIDAE*)

Margo A. Brinton, Georgia State University, Atlanta, Georgia, USA

History

Lactate dehydrogenase-elevating virus (LDV) was discovered by accident in 1960 during a study on the development of methods for early detection of tumors in mice. Inoculation of mice with Ehrlich carcinoma cells resulted in a 5 to 10-fold increase in lactate dehydrogenase (LDH) levels in the serum in 4 days. Because this early rise in LDH levels was also observed in mice inoculated with cell-free extracts or sera from tumor-bearing mice, the presence of an infectious agent was suspected. LDV is now known to produce an asymptomatic, persistent infection in mice characterized by a lifelong viremia and elevation of the levels of at least seven serum enzymes in addition to LDH.

Equine arteritis virus (EAV) was first isolated in 1957 in Bucyrus, Ohio from the lung tissues of an aborted fetus during an epidemic of abortions and arteritis in pregnant mares. However, equine disease consistent with that caused by EAV, was first observed in the late 1800s. At the time of its discovery EAV was distinguished from equine (abortion) influenza virus. Serological evidence has indicated that, although EAV is widespread in the horse population, it rarely causes clinical disease. EAV can produce persistent infections in seropositive horses.

Simian hemorrhagic fever virus (SHFV) was first isolated in 1964 during outbreaks of a fatal hemorrhagic fever in macaque colonies in the United States, Russia and Europe. A number of additional SHFV outbreaks in macaque colonies have occurred since the 1960s. The most 'famous' of these was the one in Reston, Virginia, which occurred in conjunction with a Reston Ebola virus outbreak. SHFV causes asymptomatic acute or persistent infections in several genera of African monkeys that are thought to be the natural hosts for this virus.

Porcine respiratory and reproductive syndrome was first detected in North America in 1987 and in Europe in 1990. This disease has also been referred to as porcine epidemic abortion and respiratory syndrome (PEARS), swine infertility and respiratory syndrome (SIRS) and mystery swine disease (MSD). The causative agent of this disease is now referred to as porcine respiratory and reproductive syndrome virus (PRRSV). The extent of the sequence divergence observed between American and European strains of

gene expression in different domains and the divergent adaptation to the extreme living conditions make horizontal virus transfer between the domains after their divergence and stepwise adaptation of the phages to their novel hosts extremely unlikely.

Another incentive for research on archaeal viruses is the lack of a transformation system for *Sulfolobus*, which hinders progress of the molecular genetical investigation of the *Crenarchaeota*.

See also: **Enterobacteria phage P1 (*Myoviridae*); Mu-like phages (*Myoviridae*); P2, 186 and related phages (*Myoviridae*); T1-like phages (*Siphoviridae*).**

Further Reading

Nölling J, Groffen A and De Vos WM (1993) ΦF1 and ΦF2, two novel virulent, archaeal phages infecting different thermophilic strains of the genus *Methanobacterium*. *J. Gen. Microbiol.* 139: 2511.

Nuttall SD and Dyall-Smith ML (1995) Halophage HF2: genome organization and replication strategy. *J. Virol.* 69: 2322.

Reiter W-D, Zillig W and Palm P (1988) Archaebacterial viruses. *Adv. Virus Res.* 34: 134.

Schleper C, Kubo K and Zillig W (1992) The particle SSV1 from the extremely thermophilic archaeon *Sulfolobus* is a virus: demonstration of infectivity and transfection with viral DNA. *Proc. Natl Acad. Sci. USA* 89: 7645.

Stolt P and Zillig W (1994) Gene regulation in halophage ΦH; more than promoters. *Syst. Appl. Microbiol.* 16: 591.

Witte A, Baranyi U, Klein R *et al* (1997) Characterization of *Natronobacterium magadii* phage ΦCh1, a unique archaeal phage containing DNA and RNA. *Mol. Microbiol.* 23: 603.

Zillig W, Prangishvili D, Schleper C *et al* (1996) Viruses, plasmids and other genetic elements of thermophilic and hyperthermophilic *Archaea*. *FEMS Microbiol. Rev.* 18: 225.

ARTERIVIRUSES (*ARTERIVIRIDAE*)

Margo A. Brinton, Georgia State University, Atlanta, Georgia, USA

History

Lactate dehydrogenase-elevating virus (LDV) was discovered by accident in 1960 during a study on the development of methods for early detection of tumors in mice. Inoculation of mice with Ehrlich carcinoma cells resulted in a 5 to 10-fold increase in lactate dehydrogenase (LDH) levels in the serum in 4 days. Because this early rise in LDH levels was also observed in mice inoculated with cell-free extracts or sera from tumor-bearing mice, the presence of an infectious agent was suspected. LDV is now known to produce an asymptomatic, persistent infection in mice characterized by a lifelong viremia and elevation of the levels of at least seven serum enzymes in addition to LDH.

Equine arteritis virus (EAV) was first isolated in 1957 in Bucyrus, Ohio from the lung tissues of an aborted fetus during an epidemic of abortions and arteritis in pregnant mares. However, equine disease consistent with that caused by EAV, was first observed in the late 1800s. At the time of its discovery EAV was distinguished from equine (abortion) influenza virus. Serological evidence has indicated that, although EAV is widespread in the horse population, it rarely causes clinical disease. EAV can produce persistent infections in seropositive horses.

Simian hemorrhagic fever virus (SHFV) was first isolated in 1964 during outbreaks of a fatal hemorrhagic fever in macaque colonies in the United States, Russia and Europe. A number of additional SHFV outbreaks in macaque colonies have occurred since the 1960s. The most 'famous' of these was the one in Reston, Virginia, which occurred in conjunction with a Reston Ebola virus outbreak. SHFV causes asymptomatic acute or persistent infections in several genera of African monkeys that are thought to be the natural hosts for this virus.

Porcine respiratory and reproductive syndrome was first detected in North America in 1987 and in Europe in 1990. This disease has also been referred to as porcine epidemic abortion and respiratory syndrome (PEARS), swine infertility and respiratory syndrome (SIRS) and mystery swine disease (MSD). The causative agent of this disease is now referred to as porcine respiratory and reproductive syndrome virus (PRRSV). The extent of the sequence divergence observed between American and European strains of

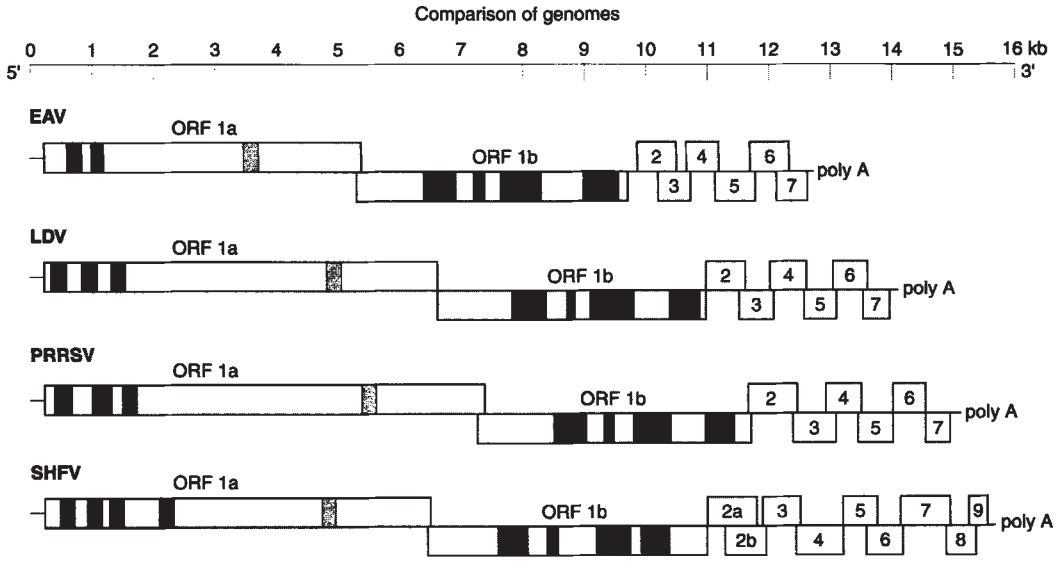


Figure 1 Organization of the genomes of LDV,EAV, PRRSV and SHFV. The nonstructural polyproteins are encoded by open reading frame (ORF) 1a/1b, which is located in the 5′ portion of the genome. ORF 1b is translated only when a −1 frameshift occurs. The conserved motifs in ORF 1a/1b are indicated by black boxes. From left to right these are: one to three papain-like cysteine proteases (stippled boxes), a cysteine protease, a serine protease, an RNA polymerase, a zinc finger, a helicase and a nidovirus-specific motif. The 3′ portion of the genome encodes the structural proteins. Adjacent 3′ ORFs are in different frames. (The genome sequences utilized are from: EAV, den Boon *et al* (1991) *J. Virol.* 65: 2910; PRRSV (Lelystad), Meulenberg *et al* (1993) *Virology* 192: 62; LDV, Godeny *et al* (1993) *Virology* 194: 585, Smith *et al* (1997) *Gene* 19: 205 and Brinton, unpublished data.)

PRRSV suggests that the ability to cause porcine disease emerged independently in these two virus populations.

Taxonomy and Classification

On the basis of the size and morphology of their virions as well as the infectious nature of their RNA genomes, LDV and EAV were originally classified within the *Togaviridae* family. After it was reported that EAV transcribed multiple subgenomic mRNAs, EAV was reclassified as the sole member of the *Togaviridae* genus *Arterivirus*. LDV was subsequently designated as a member of this genus. The genus *Arterivirus* was then designated as a 'floating genus'. SHFV was first classified as a togavirus and then as a flavivirus. In 1996, EAV, LDV, SHFV and PRRSV were designated as members of a new family, the *Arteriviridae*. At the same time, the family *Arteriviridae* was classified with the family *Coronaviridae* in the new Order *Nidovirales*.

The genomes of EAV, LDV, PRRSV and SHFV have been sequenced and their genome organization is similar to that of the coronaviruses (**Fig. 1**). Although the genomes of the four known arteriviruses contain the general features and conserved motifs characteristic of coronavirus genomes, they are only about half as long as coronavirus genomes. In addition, arterivirus particles are about half the size of coronavirus particles and differ from them morphologically; the surface of arterivirus envelopes is fairly smooth, and arteriviruses have icosahedral nucleocapsids.

Properties of the Virion

Virions are spherical, enveloped and 50–60 nm in diameter (**Fig. 2**). Unfixed virions undergo distortion and disintegration during standard negative staining procedures. The virion surface appears smooth and is thought to contain short, if any, spike-like projections. The virion capsid is icosahedral and about 30–35 nm in diameter. The general appearance of these viruses is similar to that of the alpha togaviruses and the flaviviruses and this morphological similarity resulted in some of the arteriviruses initially being classified as members of these virus families. Buoyant densities of 1.13–1.17 g cm^{-3} have been reported for arteriviruses in sucrose. The spherical capsid is composed of the nucleocapsid (N) protein. Virion envelopes contain the M protein, one major glycoprotein, and one to three minor glycoproteins. The major glycoprotein and the M protein are present as heterodimers in virions.

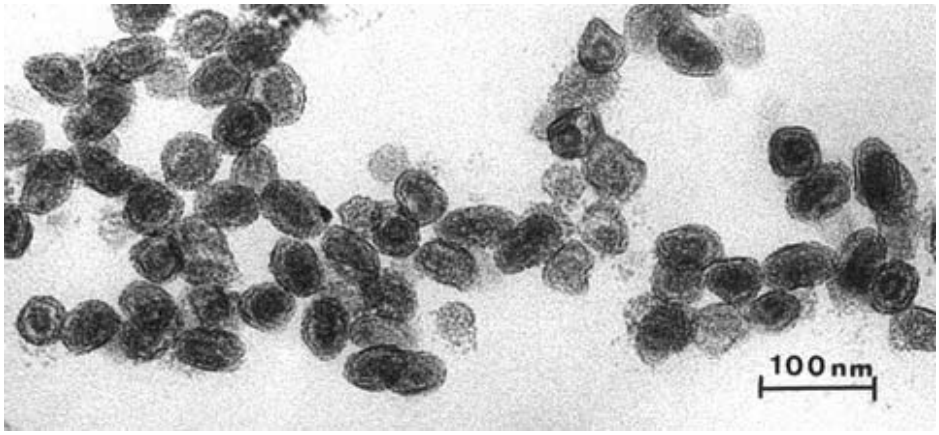


Figure 2 An electron micrograph of a positively stained thin section of gradient-purified LDV.

Properties of the Genome

The genomes of the arteriviruses are single-stranded RNAs of positive polarity. They contain a 3′ poly(A) tract of approximately 50 nucleotides in length and a 5′ type I cap. The length of the genome of EAV is 12.7 kb, that of LDV is 14.1 kb, that of PRRSV is 15.1 kb and that of SHFV is 15.7 kb (**Fig. 1**). The nonstructural polyproteins are encoded by ORFs 1a and 1b. The ORF 1b proteins are expressed only when a −1 frameshift occurs. The frameshift region is characterized by a slippery sequence upstream of a pseudoknot. Three different types of virion proteases are encoded in ORF 1a and these post-translationally cleave the ORF 1a and ORF 1a/1b polyproteins into 12 or more mature nonstructural proteins. There are six 3′ ORFs in the genomes of EAV, PRRSV and LDV, whereas SHFV encodes nine 3′ ORFs. Limited sequence homology suggests that the SHFV ORFs 2a, 2b and 3 may be duplications of the SHFV ORFs 4, 5, and 6, respectively (**Fig. 1**). Adjacent arterivirus 3′ ORFs are in different reading frames and in most cases overlap. Only the 5′ terminal ORF of most of the subgenomic mRNAs is translated. However, mRNA 2 of EAV, LDV, and PRRSV and mRNAs 2 and 4 of SHFV are bicistronic.

Properties of the Proteins

Two polyproteins are translated from the ORF 1a/1b region of the arterivirus genome. The ORF 1a polyprotein contains one or more papain-like cysteine proteases, a cysteine protease, and a serine protease. The serine protease cleaves at five sites within the ORF 1a polyprotein and eight sites within the ORF 1a/1b polyprotein. Three of the ORF 1a encoded proteins have hydrophobic domains which may be important for membrane association of the viral replication–transcription complexes. The ORF 1a/1b polyprotein is translated only after a −1 frameshift occurs. Motifs characteristic of a viral RNA polymerase, a zinc-finger, a helicase, and a conserved nidovirus-specific region are found in ORF 1b (**Fig. 1**). The capsid protein is encoded by ORF 7 (ORF 9 in SHFV), the nonglycosylated, triple-membrane spanning envelope M protein is encoded by ORF 6 (ORF 8 in SHFV), and the major virion glycoprotein is encoded by ORF 5 (ORF 7 in SHFV). The remaining 3′ ORFs each encode proteins of about 20 kDa in size that contain a number of putative N-linked glycosylation sites and may function as minor virion envelope glycoproteins. The ORF 2 (ORF 4 in SHFV) protein is a class I integral membrane protein. A soluble, non-virion-associated form of the ORF 3 protein is released from infected cells.

Physical Properties

Virions can be stored indefinitely at −70°C. At −20°C, samples of LDV lost half of their infectivity by 4 weeks. At 4°C, LDV in mouse plasma decreased in titer by about 3.5 logs in 32 days; at room temperature, plasma virus was stable for 24 h, whereas infectivity was completely inactivated by heating at 58°C for 1 h. LDV suspended in media supplemented with 10% serum was more heat labile than virus in plasma. Virions are fairly stable between pH 6 and pH 7.5, but are rapidly inactivated at high or low pH. Virus is efficiently inactivated by lipid solvents and is very sensitive to nonionic detergent treatment. A brief incubation with low concentrations (0.01%) of nonionic detergents, such as NP40 and Triton X-100, efficiently disrupts the virion envelope.

Replication

Viral replication occurs entirely in the cytoplasm. Infected cells contain full-length genome RNA,

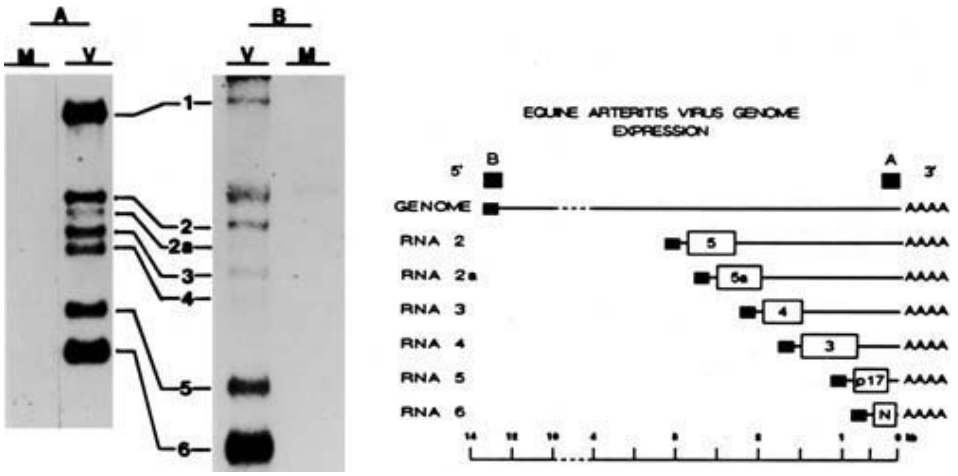


Figure 3 EAV proteins encoded at the 5′ end of the genome are translated from the genome RNA whereas proteins encoded at the 3′ end are translated from subgenomic mRNAs. The EAV genomic and subgenomic mRNAs are designated by numbers 1–6. The autoradiographs show Northern-blot hybridizations obtained with mock-infected (M) and EAV-infected (V) cell extracts reacted with either (**A**) a 3′ probe or (**B**) a 5′ probe. The open boxes represent ORFs, and the closed boxes represent the 5′ leader sequence. The bicistronic nature of mRNA2 is not indicated. (Reproduced with permission from Spaan *et al* (1989) In: Brinton MA and Heinz FX (eds) *New Aspects of Positive Strand Viruses*, p. 12. Washington: ASM Press.)

subgenomic viral mRNAs and full-length and subgenomic viral minus-strand RNAs. The subgenomic mRNAs form a 3′ coterminal nested set (**Fig. 3**) and are thought to be transcribed by an uncharacterized discontinuous mechanism which utilizes the conserved junction sequences that precede each 3′ ORF. Each subgenomic mRNA contains a common 5′ leader sequence which is identical to the 5′ terminal sequence of the genome RNA. Conserved RNA structures and cis-acting signals for plus-strand RNA replication have been identified in the 3′ noncoding region of the complementary full-length minus-strand RNA. The 3′(−)non-coding region has also been shown to bind specifically to four cellular proteins thought to be involved in viral RNA replication.

Assembly, Uptake and Cytopathology

Evidence for a specific saturable, but as yet unidentified receptor for LDV on a subpopulation of murine macrophages has been reported. LDV-immune complexes are infectious and apparently infect target cells via Fc receptors. Ventral motor neurons in susceptible mouse strains can be infected by 'free' LDV-C, but not by viral immune complexes. A heparin-like molecule on the surface of MARC-145 cells may serve as a receptor for PRRSV. PRRSV has been reported to enter cells via a low pH-dependent, endocytic pathway. Soon after infection, virus particles have been observed in small vesicles that appear to be clathrin-coated. Virus replication occurs in the perinuclear area of infected cells. Virions are observed to bud through endoplasmic reticular membranes into intracellular vesicles. Virions presumably move to the exterior of the cell via these vesicles. Assembled nucleocapsids have not been observed in the cytoplasm, except in association with budding virions. Infection of primary macrophages with EAV, SHFV, PRRSV and, probably, also LDV is cytocidal. The formation of double-membrane cytoplasmic vesicles is a characteristic of virus-infected cells. Laboratory strains of EAV, SHFV, and PRRSV cause obvious cytopathology in the continuous cell cultures which they infect. Cells become rounded and are released from the tissue culture flask. Apoptosis has been observed in PRRSV-infected porcine alveolar macrophages, MA-104 cells and testicular germ cells.

Geographic and Seasonal Distribution

Viruses with biological properties identical to those of LDV have been isolated from small groups of wild mice (*Mus musculus*) in Australia, Germany, the US and UK. EAV-infections and EAV-induced disease have been well documented in North America and Europe and antibodies to EAV have been detected in horse sera from Africa and South America indicating that EAV infection is geographically widespread. Natural PRRSV infections have been reported in North America and Europe. SHFV infection in captive patas monkeys has been documented and SHFV has been detected in the blood of wild patas monkeys, African green monkeys, and baboons

suggesting that these African primates are the natural hosts for SHFV.

Host Range and Virus Propagation

LDV replicates efficiently in all strains of laboratory mice and wild *Mus musculus* and somewhat less efficiently in the Asian mouse *Mus caroli*. Numerous attempts to infect other rodents such as rats, hamsters, guinea pigs, rabbits, deer mice (*Peromyscus maniculatus*) and dwarf hamsters (*Phodopus sungorus*) with LDV have not been successful. LDV grows very efficiently in mice reaching titers of 10^{11} ID_{50} ml^{-1} of plasma within the first day of infection. By a week after infection, viral titers have declined to a steady-state level of about 10^6 ID_{50} ml^{-1}. *In vitro*, LDV replicates only in an as yet uncharacterized subpopulation of primary murine macrophages. In peritoneal macrophage cultures obtained from 1–2-week-old mice, up to 70% of the cells are infectible. However, only 40% or less of the macrophages are virus susceptible when the cells are obtained from older mice. The highest number of infected cells was observed when peritoneal cell cultures were infected during the first 24 h plating. Thereafter, the number of infectible cells progressively declined, until by 7 days, the cultures were no longer infectible. However, incubation of fresh peritoneal macrophage cultures with colony-stimulating factor resulted in the continued differentiation of virus-susceptible cells from stem cells. Attempts to infect numerous continuous cell lines with LDV, including all attempts to infect murine macrophage cell lines, have been unsuccessful with one exception. A line of mouse macrophage–human hybrid cells was reported to be transiently permissive for LDV replication.

EAV infects horses and donkeys. Field isolates are rarely obtained and are difficult to propagate in cell culture. However, laboratory strains of EAV have been successfully grown in primary cultures of horse macrophages and kidney cells, rabbit kidney cells and hamster kidney cells and also in cell lines, such as BHK-21, RK-13, MA-104 and Vero.

Natural infections with PRRSV were thought to be restricted to pigs. However, a recent report suggested that chickens and mallard ducks may also be susceptible to infection with PRRSV. PRRSV replicates in primary cultures of porcine alveolar macrophages. Some, but not all, isolates of PRRSV can be adapted to replicate *in vitro* in MA-104 cells.

SHFV naturally infects several species of African primates, namely *Erythrocebus patas*, *Cercopithecus aethiops*, *Papio anuibus* and *Papio cynocephalus*. SHFV infection of members of the genus *Macaca* has occurred in a number of primate facilities. Some isolates of SHFV can replicate in primary cultures of rhesus aveolar lung macrophages and MA-104 cells.

Genetics

Evidence for the existence of virulence mutants of LDV, SHFV and EAV has been obtained. Virulent and avirulent mutants of EAV have been identified on the basis of the severity of the diseases they cause. A number of temperature-sensitive mutants of EAV have also been selected. One strain of LDV, designated LDV-C, was shown to induce neurologic disease in a few susceptible inbred mouse strains. SHFV isolates that produce acute infections and ones that cause persistent infections in patas monkeys have been reported. EAV and PRRSV infectious clones have recently been constructed.

Evolution

Comparison of the genomes of EAV, LDV, PRRSV and SHFV (**Fig. 1**) suggest that these genomes have evolved via both point mutations and copy choice RNA recombination mechanisms. Sequence comparisons of various field isolates of either PRRSV or EAV indicated that the sequences of the M and N proteins are more conserved than those of the glycoproteins. The extent of the divergence between the sequences of European and North American PRRSV isolates indicate that these two virus populations represent subspecies. Phylogenic analyses have shown that PRRSV is most closely related to LDV and that SHFV is more closely related to these two viruses than to EAV.

The organization of the genomes of LDV, EAV, SHFV and PRRSV is very similar to that of the coronaviruses (**Fig. 1**). Both types of viral genomes have the same set of conserved nonstructural protein motifs arranged in the same order within ORF 1a/b. Although there are significant differences in the structural protein regions of the arterivirus and the coronavirus genomes, both families of viruses encode their capsid protein as the terminal 3′ gene and a nonglycosylated envelope M protein as the penultimate 3′ gene. Coronaviruses have helical nucleocapsids, whereas LDV, SHFV, PRRSV and EAV have icosahedral nucleocapsids. The capsid protein of the coronaviruses is about twice as large as the one encoded by the arteriviruses. Coronavirus particles have large peplomer spikes on their surface, whereas LDV, SHFV, PRRSV and EAV particles have 'smooth' surfaces. The coronaviruses and the arteriviruses most likely evolved as two distinct lineages from a common progenitor. The prototype virus may originally have had an iscosahedral nucleocapsid, and

then via a copy choice recombinational event acquired a helical capsid gene from another virus. This change in the type of capsid utilized would have removed the packaging restrictions on the genome size. The additional genetic information found in the current coronavirus genomes could then have been gained during further evolution via recombination.

Serologic Relationships and Variability

All attempts to demonstrate antigenic crossreactivity between EAV, LDV, PRRSV and SHFV have been unsuccessful. Even though antiviral antibodies are detected in infected animals by a week after infection, all four viruses can cause persistent infections. The mechanism by which these viruses evade clearance by the immune system is not known. Neutralization escape virus variants have been reported to arise during persistent LDV infections. Antiserum obtained from animals chronically infected with LDV and PRRSV contains infectious viral immune complexes. Plasma from LDV-infected mice has a higher non-specific binding activity than plasma from uninfected mice; virus-specific binding measured by an ELISA usually cannot be detected until the plasma has been diluted at least 1:400.

Transmission

No insect vector has been associated with LDV or SHFV transmission in wild animal populations. In the laboratory, LDV infection is rarely transmitted between mice housed in the same cage, even though infected mice excrete virus in their feces, urine, milk and saliva, unless the cage mates are fighting males. Since LDV in mice and SHFV in patas monkeys is produced throughout the lifetime of an infected animal, the transfer of fluids or tissues from an infected animal to an uninfected one results in the inadvertent transfer of infection. Historically, the most frequent mode of transmission of LDV among laboratory mice and of SHFV from patas monkeys to macaques has been through experimental procedures such as the use of the same needle for sequential inoculation of several animals. Currently, the most frequent sources of LDV contamination are pools of other infectious agents or tumor cell lines that have been repeatedly passaged in mice, especially those first isolated in the 1950s. Such materials should be checked for the presence of LDV and, if found to be contaminated, can be 'cured' of LDV by repeated passage in culture. It has been suggested, but not proven, that SHFV can be transmitted between macaques via the respiratory route.

No evidence for transmission of EAV and PRRSV by insect vectors in domestic animal populations has been reported. Horizontal transmission of EAV and PRRSV can occur via the respiratory route as well as via the sexual route in semen. Vertical transmission of PRRSV *in utero* has been reported.

Tissue Tropism

The primary target cells of all four arteriviruses are macrophages. LDV replicates in an uncharacterized subpopulation of murine macrophages. Virus target cells are located in tissues as well as in the blood. Cells containing LDV-specific antigen have been identified in sections of liver and spleen by indirect immunofluorescence. In spleen, the virus-infected cells were nucleated and located in the red pulp. In liver, only Kupffer cells contained LDV-specific antigen. In C58 and AKR mice infected with a neurotropic strain of LDV, designated LDV-C, virus replication was demonstrated in ventral motor neurons. Measurement of the amount of virus in various tissues during natural EAV infections indicated that lung macrophages were the first host cells to be infected. Bronchial lymph nodes subsequently became infected and then the virus spread throughout the body via the circulatory system. In fatally infected horses, lesions are found in subcutaneous tissues, lymph nodes and viscera. PRRSV has been reported to replicate in testicular germ cells which could result in excretion of virus into the semen.

Pathogenicity and Clinical Features of Infection

Avirulent and virulent strains of EAV and PRRSV have been isolated. Attenuated vaccine strains of EAV and PRRSV have been selected and used as vaccines. Although these vaccines induce immunity against disease, immunized animals are not protected from reinfection. Horses infected with the virulent Bucyrus strain of EAV develop a high fever, lymphopenia and severe disease symptoms. LDV-C differs from other LDV isolates in its ability to infect ventral motor neurons in immunosuppressed C58 and AKR mice and induce poliomyelitis. Isolates of SHFV that induce persistent, asymptomatic infections and ones that cause acute, asymptomatic infections in patas monkeys have been reported. All SHFV isolates cause fatal hemorrhagic fever in macaque monkeys.

Mice infected with LDV usually display no overt symptoms of disease. A distinguishing feature of LDV infections is the chronically elevated levels of seven serum enzymes, LDH (8–10-fold), isocitrate dehydrogenase (5–8-fold), malate dehydrogenase (2–3-fold),

phosphoglucose isomerase (2–3-fold), glutathione reductase (2–3-fold), aspartate transaminase (2–3-fold) and glutamate–oxaloacetate transaminase (2–3-fold). The elevated LDH levels result primarily from a decreased rate of clearance of normal turnover enzyme from the blood. A permanent reduction in the population of Kupffer cells involved in receptor-mediated endocytosis of LDH has been shown to occur soon after infection of mice with LDV. A decrease in the humoral and cellular immune response to non-LDV antigens is observed during the first 2 weeks after LDV infection. Thereafter, the immune response to other antigens is normal. In immunosuppressed C58 and AKR mice, one isolate of LDV, LDV-C, can induce a sometimes fatal poliomyelitis. Immunosuppression is required to delay antibody production so that virus can reach the central neurons system (CNS) and infect the susceptible ventral motor neurons. LDV-infected neurons become the targets of an inflammatory response. In mice 6 months of age or older, paralysis of one or both hind limbs and sometimes a fore limb is observed. In younger C58 mice, poliomyelitis is usually subclinical.

Both EAV and PRRSV can cause either asymptomatic infections or induce various disease symptoms such as respiratory disease, fever, necrosis of small muscular arteries and abortion. The severity of disease caused by EAV and PRRSV depends on the strain of virus as well as the condition and age of the host animal. The most common symptoms of natural EAV infections in horses are anorexia, depression, fever, conjunctivitis, edema of the limbs and genitals, rhinitis, enteritis, colitis and necrosis of small arteries. If clinical symptoms occur, they are most severe in young animals and pregnant mares. Infections in pregnant mares are often inapparent, but can result in a high percentage (50%) of abortions. Young animals sometimes develop a fatal bronchopneumonia, but natural infections are usually not life-threatening. In contrast, about 40% of pregnant mares and foals experimentally inoculated with EAV die as a result of the infection.

SHFV causes asymptomatic acute or persistent infections in patas monkeys, but a fatal hemorrhagic fever in macaques. Infected macaques develop fever and mild edema followed by anorexia, dehydration, adipsia, proteinuria, cynosis, skin petechia, bloody diarrhea, nose bleeds and occasional hemorrhages in the skin. The pathological lesions consist of capillary-venous hemorrhages in the intestine, lung, nasal mucosa, dermis, spleen, perirenal and lumbar subperitoneum, adrenal glands, liver and periocular connective tissues. These signs and symptoms are not unique to SHFV-infected animals, since they are also observed after infection with other types of viruses that cause hemorrhagic fevers. Although the SHFV-induced lesions are widespread, the level of tissue damage is not severe. Even so, the mortality of SHFV infections in macaques approaches 100%.

Pathology and Histopathology

Although most LDV infections are inapparent in mice, some histopathogenic changes are observed in infected animals. As described above, the serum levels of seven enzymes are chronically elevated in LDV-infected mice. Normally, an increase in the serum levels of tissue enzymes is the result of tissue damage, but in LDV-infected animals little tissue damage is observed. Although there are five naturally occurring LDH isozymes in mouse plasma, only the level of isozyme LDH 5 is elevated. Studies have indicated that the increase in enzyme levels is primarily the result of a decreased rate of enzyme clearance. A recent report showed that a subpopulation of Kupffer cells involved in receptor-mediated endocytosis of LDH is severely diminished in mice by 24 h after LDV infection. It has been postulated that LDV replication in these cells causes their death and the depletion of these cells results in the slower turnover of LDH.

Splenomegaly, characterized by a greater than 30% increase in spleen weight, occurs in about 40% of the mice infected with LDV. The increase in spleen weight is observed by 24 h after infection and persists for up to a month. A marked necrosis of lymphocytes in thymic-dependent areas occurs during the first 4 days after LDV infection together with a transient decrease in the number of circulating T lymphocytes between 24 and 72 h after infection. A transient decrease in peritoneal macrophages is also observed between the first and 10th day of infection.

Despite the lifelong presence of circulating viral immune complexes and the demonstration of LDV antibody deposits in the kidneys of LDV-infected mice as early as 7 days after infection, infected animals do not develop kidney disease. It has been suggested that nephritis does not develop in chronically infected mice because of the inability of the majority of the LDV-antibody complexes to bind C1q. Low levels of C1q-binding activity can only be detected between days 10 and 18 after LDV infection.

The central nervous system lesions in LDV-C-infected C58 and AKR mice are located in the gray matter of the spinal cord and, occasionally, in the brainstem and consist of focal areas of inflammatory mononuclear cell infiltrate in the ventral horn. Virus-specific protein and nucleic acid have been detected and maturing virions have been observed in ventral motor neurons.

In horses experimentally or fatally infected with EAV, the most common gross lesions are edema, congestion and hemorrhage of subcutaneous tissues, lymph nodes and viscera. Microscopic investigation of tissues from chronically infected horses, which have mildly swollen lymph nodes and slightly increased volumes of pleural and peritoneal fluids, revealed extensive lesions consisting of generalized endothelial damage to blood vessels of all sizes and severe glomerulonephritis. Both types of lesions are thought to be caused by the deposition of viral immune complexes. Extensive capillary necrosis leads to a progressive increase in vascular permeability and volume, hemoconcentration and hypotension. During the terminal stages of the disease, lesions are also found in the adrenal cortex, and degenerative changes are observed in the bone marrow and liver. Virus infection causes focal myometritis which is thought to be the cause of deficiencies in the fetal and placental blood supply. The resulting anoxia is probably the cause of abortion.

Immune Response

The persistence of LDV infectivity in the plasma of infected mice and the failure of initial attempts to demonstrate the presence of neutralizing antibodies led investigators to postulate that LDV-specific antiviral antibodies were not produced. It was subsequently demonstrated that antiviral antibodies were efficiently produced in LDV-infected animals, but were complexed with virions. Whereas 99% of the infectivity in sera collected 24 h after infection could be neutralized with ether-extracted murine anti-LDV immunoglobulin, no neutralization of the LDV infectivity in sera from chronically infected mice could be demonstrated unless antibody to mouse immunoglobulin was also used. Anti-LDV antibody not complexed to virus can be detected by 15 days after infection, indicating that antibody is present in excess of virus in chronically infected mice. Studies with nude mice suggest that LDV is a T-independent antigen. LDV-infected mice display a polyclonal humoral response, but anti-LDV antibody apparently accounts for only a small portion of this polyclonal response. The mechanism by which LDV infection activates B cells polyclonally is currently not known, but mice immunized with inactivated virus do not develop a polyclonal response. Autoantibodies to a variety of cellular components (autoimmune antibodies) have been detected in mice chronically infected with LDV. LDV-infected animals develop cytotoxic T cells that can specifically lyse virus-infected macrophages after infection. However, the cytotoxic response does not clear the infection. A T cell response has also been detected in PRRSV-infected pigs.

Anti-EAV antibodies can be detected in horses 1 to 2 weeks after infection with virulent or avirulent strains of the virus by either serum-neutralization or complement-fixation assays. Virus neutralization is enhanced by the presence of fresh complement. Newborn foals with mothers that are immune receive protective antibodies in the colostrum. Complement-fixing, antiviral antibodies peak at 2–3 weeks after the initiation of infection and then decline. Neutralizing antibody levels peak between 2 and 4 months after infection. Often after 8 months, anti-EAV antibody can no longer be detected by complement-fixation or neutralization assays, but in some animals the virus persists and viral immune complexes may continue to circulate.

Neutralizing antibodies in sera obtained from EAV- and LDV-infected animals are ORF 5 protein-specific and the neutralizing epitope has been mapped to the ectodomain of this protein. The number of gylcosylation sites in the ectodomain of the ORF 5 protein varys in different LDV strains and it has been postulated that antibodies bind less efficiently to virions with extensive glycosylation in this region. The induction of neutralizing antibodies by the ORF 4 protein of PRRSV has also been reported. SHFV isolates that induce acute infections in patas monkeys induce high levels of neutralizing antibody, whereas SHFV isolates that induce persistent infections induce low titers of non-neutralizing antibody. Antibodies to virus that causes acute infection do not cross-neutralize virus that causes persistent infection.

Prevention and Control

The most effective means of prevention of LDV, SHFV, PRRSV and EAV infection is interruption of animal to animal transmission. Infected animals should be destroyed or isolated. However, the current lack of rapid diagnostic assays for the detection of LDV and SHFV in persistently infected animals means that it is still a time-consuming task to identify animals with inapparent infections. Materials obtained from animals that might be infected with an arterivirus should be checked for viral contamination before they are injected into a susceptible animal. Multiple animals should not be injected using the same needle.

Killed and attenuated live vaccines for EAV and PRRSV are commercially available. The live vaccines are more effective and induce a longer-lasting immunity than the killed vaccines. Although animals immunized with the live vaccines are protected from disease, they are not protected from reinfection and

can spread virus. Current serological assays can not distinguish field strains from vaccine strains.

Future Perspectives

Arteriviruses have so far been isolated from mice (LDV), horses (EAV), pigs (PRRSV) and monkeys (SHFV). It seems likely that other host species, including humans, may harbor additional members of this virus family. Such viruses will be especially difficult to find in natural hosts that develop asymptomatic infections. Because of the inapparent and persistent nature of infections caused by LDV, PRRSV, SHFV and EAV it is important to develop rapid and reliable diagnostic tests for these viruses to easily identify infected animals. The availability of complete genomic sequences for EAV, PRRSV, SHFV and LDV should facilitate the development of new diagnostic assays and vaccines and may also provide the means for detecting additional arteriviruses.

See also: **Immune response: Cell mediated immune response, General features; Vaccines and immune response.**

Further Reading

Brinton MA (1986) Lactate dehydrogenase-elevating virus. In: Bhatt PN, Jacoby RO, Morse HC and New AE (eds) *Viral and Mycoplasmal Infections of Laboratory Rodents*, p. 389. New York: Academic Press.

Cavanagh D (1997) *Nidovirales*: a new order comprising *Coronaviridae* and *Arteriviridae*. *Arch. Virol.* 142: 629.

De Vries AAF, Horzinek MC, Rottier PJM and de Groot RJ (1997) The genome organization of the *Nidovirales*: similarities and differences between arteri-, toro-, and coronaviruses. *Semin. Virol.* 8: 33.

Plagemann PGW (1996) Lactate dehydrogenase-elevating virus and related viruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Virology*, 3rd edn, p. 1105. Philadelphia: Lippincott-Raven.

Plagemann PGW and Moenning V (1992) Lactate dehydrogenase-elevating virus, equine arteritis virus, and simian hemorrhagic fever virus: a new group of positive-strand RNA viruses. *Adv. Virus Res.* 41: 99.

Rowson KEK and Mahy BWJ (1985) Lactate dehydrogenase-elevating virus. *J. Gen. Virol.* 66: 2297.

Snijder EJ and Meulenberg JM (1998) The molecular biology of arteriviruses. *J. Gen. Virol.* 79: 961.

ASCOVIRUSES (*ASCOVIRIDAE*)

Brian A Federici, Department of Entomology and Interdepartmental Graduate Program in Genetics, University of California, Riverside, California, USA

Introduction

Viruses are the causative agents of a wide variety of diseases, and occur in virtually all forms of life. Owing to their medical, veterinary and economic importance, the viruses that have been studied the most are those that cause important diseases of humans and their domesticated plants and animals. In contrast, viruses that attack insects have received relatively little study. The best-known insect viruses are those that attack honeybees or silkworms, or the viruses that attack crop-eating pests such as caterpillars. Studies of honeybee and silkworm viruses are aimed at controlling the viral diseases of these economically important insects, whereas the studies of caterpillar viral diseases emphasize development of the viruses that cause these diseases as microbial insecticides.

Though insect viruses have not been studied nearly as well as many of the vertebrate and plant viruses, they are no less interesting with respect to their general biology, pathology, biochemistry and molecular biology. Moreover, because insects are a remarkably diverse and evolutionarily successful group of organisms, with more species described – well over one million – than any other group of organisms, they represent a source of new and interesting viruses that can differ significantly in their biologies from known viruses. The ascoviruses are an example of just such a group. Initially discovered about two decades ago, these large, enveloped, double-stranded (ds) DNA viruses, known primarily from insects of the order Lepidoptera (butterflies and moths), are unique in their structure, pathology, and general biology. As a result, they take their place as a new family of enveloped, dsDNA viruses alongside

can spread virus. Current serological assays can not distinguish field strains from vaccine strains.

Future Perspectives

Arteriviruses have so far been isolated from mice (LDV), horses (EAV), pigs (PRRSV) and monkeys (SHFV). It seems likely that other host species, including humans, may harbor additional members of this virus family. Such viruses will be especially difficult to find in natural hosts that develop asymptomatic infections. Because of the inapparent and persistent nature of infections caused by LDV, PRRSV, SHFV and EAV it is important to develop rapid and reliable diagnostic tests for these viruses to easily identify infected animals. The availability of complete genomic sequences for EAV, PRRSV, SHFV and LDV should facilitate the development of new diagnostic assays and vaccines and may also provide the means for detecting additional arteriviruses.

See also: **Immune response: Cell mediated immune response, General features; Vaccines and immune response.**

Further Reading

Brinton MA (1986) Lactate dehydrogenase-elevating virus. In: Bhatt PN, Jacoby RO, Morse HC and New AE (eds) *Viral and Mycoplasmal Infections of Laboratory Rodents*, p. 389. New York: Academic Press.

Cavanagh D (1997) *Nidovirales*: a new order comprising *Coronaviridae* and *Arteriviridae*. *Arch. Virol.* 142: 629.

De Vries AAF, Horzinek MC, Rottier PJM and de Groot RJ (1997) The genome organization of the *Nidovirales*: similarities and differences between arteri-, toro-, and coronaviruses. *Semin. Virol.* 8: 33.

Plagemann PGW (1996) Lactate dehydrogenase-elevating virus and related viruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Virology*, 3rd edn, p. 1105. Philadelphia: Lippincott-Raven.

Plagemann PGW and Moenning V (1992) Lactate dehydrogenase-elevating virus, equine arteritis virus, and simian hemorrhagic fever virus: a new group of positive-strand RNA viruses. *Adv. Virus Res.* 41: 99.

Rowson KEK and Mahy BWJ (1985) Lactate dehydrogenase-elevating virus. *J. Gen. Virol.* 66: 2297.

Snijder EJ and Meulenberg JM (1998) The molecular biology of arteriviruses. *J. Gen. Virol.* 79: 961.

ASCOVIRUSES (*ASCOVIRIDAE*)

Brian A Federici, Department of Entomology and Interdepartmental Graduate Program in Genetics, University of California, Riverside, California, USA

Introduction

Viruses are the causative agents of a wide variety of diseases, and occur in virtually all forms of life. Owing to their medical, veterinary and economic importance, the viruses that have been studied the most are those that cause important diseases of humans and their domesticated plants and animals. In contrast, viruses that attack insects have received relatively little study. The best-known insect viruses are those that attack honeybees or silkworms, or the viruses that attack crop-eating pests such as caterpillars. Studies of honeybee and silkworm viruses are aimed at controlling the viral diseases of these economically important insects, whereas the studies of caterpillar viral diseases emphasize development of the viruses that cause these diseases as microbial insecticides.

Though insect viruses have not been studied nearly as well as many of the vertebrate and plant viruses, they are no less interesting with respect to their general biology, pathology, biochemistry and molecular biology. Moreover, because insects are a remarkably diverse and evolutionarily successful group of organisms, with more species described – well over one million – than any other group of organisms, they represent a source of new and interesting viruses that can differ significantly in their biologies from known viruses. The ascoviruses are an example of just such a group. Initially discovered about two decades ago, these large, enveloped, double-stranded (ds) DNA viruses, known primarily from insects of the order Lepidoptera (butterflies and moths), are unique in their structure, pathology, and general biology. As a result, they take their place as a new family of enveloped, dsDNA viruses alongside

other viruses with similar structural and biochemical properties, namely the herpesviruses of vertebrates, poxviruses of vertebrates and invertebrates, baculoviruses of invertebrates, and plasmaviruses of bacteria.

History

The first ascoviruses were discovered during the late 1970s in southern California where they were found causing disease in larvae of moths belonging to the lepidopteran family Noctuidae. The diseased larvae where recognized by the presence of blood that was very white and opaque, in marked contrast to the blood of healthy larvae which is translucent and slightly green. The color and opacity of the blood in diseased larvae was shown to be due to the presence of high concentrations of vesicles that contained virions. The white blood and virion-containing vesicles are diagnostic for the disease, and the name for this group, *Ascovirus,* derived from the Greek 'asco' meaning 'sac,' was chosen on the basis of the latter characteristic. Since the discovery of the first ascovirus, ascoviruses have been isolated as the cause of disease in many species of noctuid larvae, and recently in France from larvae of the family Yponomeutidae.

Taxonomy and Classification

Because ascoviruses have been discovered only recently, little is known about their distribution, diversity or taxonomy. They are all placed in the *Ascovirus* genus of the *Ascoviridae* family. So far, four distinct types or species have been identified. These are the viruses isolated from, respectively, the noctuid species *Trichoplusia ni* (the cabbage looper), *Spodoptera frugiperda* (the fall armyworm) and *Autographa precationis* (which lacks a common name), and from the yponomeutid species, *Acrolepiopsis assectella* (the leek moth). The latter virus is unusual in that it is known to be vectored by the endoparastic wasp, *Diadromus pulchellus* (family Ichneumonidae), in which the viral genome is carried in wasp nuclei as an episome. Its unusual biology, discussed later, suggests it represents a second type of ascovirus.

For convenience, these four ascoviruses are referred to as *Trichoplusia* ascovirus, *Spodoptera* ascovirus, *Autographa* ascovirus and *Diadromus* ascovirus, with the last virus being named after its wasp vector. The primary characteristic used to establish these as different viral species is the failure of their DNAs to hybridize to each other under conditions of low stringency. In addition, as will be noted below, these viruses exhibit differences in G + C content, host range, tissue tropism and cytopathology, which further validate their being recognized as distinct species. Variants also exist, with the most notable being the *Helicoverpa* ascovirus, a variant of the *Trichoplusia* ascovirus isolated from *Helicoverpa zea* (= *Heliothis zea,* the corn earworm). The genome of this variant has a restriction enzyme fragment pattern distinct from that of the *Trichoplusia* ascovirus, and the virus differs from the latter virus in its histopathology. However, *Helicoverpa* and *Trichoplusia* ascovirus DNAs hybridize strongly even under conditions of high stringency, and thus *Helicoverpa* isolate is considered a variant of the *Trichoplusia* ascovirus.

Ascoviruses probably occur worldwide, but at present they are only known from the USA and France. Based on their likely worldwide distribution, it is probable that there are numerous species.

Virion and Genome Structure and Composition

Depending on the species, the virions of ascoviruses are either bacilliform, allantoid or ovoidal in shape, with complex symmetry, and are very large, measuring about 130 nm in diameter by 200–400 nm in length (**Fig. 1**). The virion consists of an inner particle surrounded by an outer envelope. The inner particle itself is complex, containing a DNA/protein core as well as an apparent internal lipid bilayer surrounded by a distinctive layer of protein subunits. Thus, the virion appears to contain two lipid membranes, one associated with the inner particle, the other forming the lipid component of the envelope. In negatively stained preparations, virions have a distinctive reticulate appearance, which is thought to be due to superimposition of subunits on the surface of the internal particle with those in the envelope.

As indicated by the size and complexity of the virions, the genome of ascoviruses is large, and consists of a single molecule of dsDNA, which, depending on the viral species, is either in the range of 116 kb in size (*Diadromus* ascovirus), 140 kb (*Spodoptera* ascovirus) or 180 kb (*Trichoplusia* ascovirus). The G + C content of the *Spodoptera* ascovirus is 60%, whereas for the *Trichoplusia* ascovirus it is 42%. Supercoiled circular DNA has not been detected in cesium chloride gradients, nor have circular forms been observed by electron microscopy. The DNA genome is therefore referred to as linear, though each terminus may consist of a short single-stranded loop connecting the stands, making the molecule circular, as occurs in herpes-

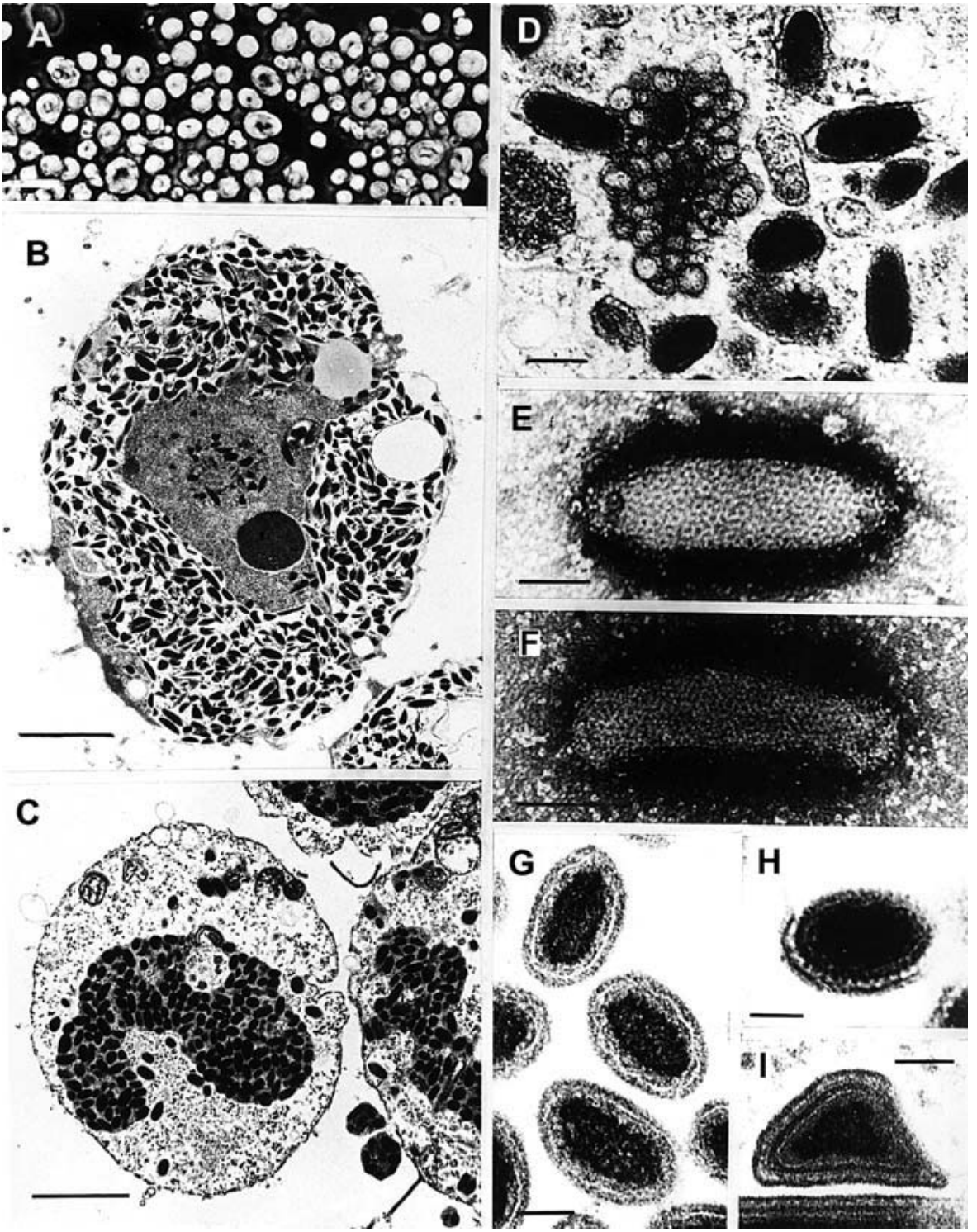


Figure 1 Structural and morphological characteristics of ascovirus virions and virion-containing vesicles. (**A**) Wet mount preparation viewed with phase microscopy of blood from a diseased *Spodoptera frugiperda* larva infected with the *Spodoptera* ascovirus. The spherical refractile bodies are virion-containing vesicles. (**B, C**) Ultrathin sections through viral vesicles produced by the *Trichoplusia* and *Spodoptera* ascoviruses. (**D**) The matrix of the occlusion body produced by *Spodoptera* ascovirus. The occlusion consists of virions, protein and small spherical vesicles. (**E, F**) Negatively stained virions, respectively, of the *Spodoptera* and *Trichoplusia* ascoviruses. (**G, H**) Ultrathin cross-sections through inner particles of the *Spodoptera* ascovirus after formation (**G**) and during envelopment (**H**). (**I**) Ultrathin cross-section through a fully developed virion of the *Trichoplusia* ascovirus. Bar markers: (**A**) and (**B**), 10 μm; (**C**), 2 μm; (**D**), 200 nm; (**E**) and (**F**), 100 nm; (**G**), (**H**), (**I**), 50 nm.

viruses. Recent studies of *Diadromus* ascovirus DNA indicate it is in the form of a relaxed circle.

Based on gel analyses, ascovirus virions contain at least 12 structural polypeptides, ranging in size from 12 to 200 kDa. In addition to proteins and the DNA genome, the presence of an envelope, as detected by electron microscopy as well as experiments with detergents and organic solvents, indicates that virions contain a substantial lipid component. And, as in other enveloped viruses of eucaryotes, it is likely that the virion also contains carbohydrate in the form of glycoproteins, though none have yet been identified.

Transmission and Ecology

One of the most interesting features of ascoviruses is that their transmission from host to host appears to be dependent on their virions being transmitted by female endoparasitic hymenoptera (wasps). Ascoviruses are extremely difficult to transmit *per os*, with typical infection rates averaging less than 15%, even when larvae are fed as many as 10^5 virion-containing vesicles in a single dose. In contrast to this, infection rates for caterpillars injected with as few as 10 virion-containing vesicles are typically greater than 90%. Moreover, experiments with parasitic wasps show that they can effectively transmit ascoviruses to their noctuid hosts. For example, when female wasps are allowed to lay eggs in ascovirus-infected noctuid caterpillars, thereby contaminating their ovipositor, and then allowed to lay eggs in healthy larvae, the majority of the latter contract ascovirus disease. Interestingly, though the parasite eggs hatch in their noctuid hosts, the parasite larvae die as the ascovirus disease develops in the caterpillar. Under field conditions, the prevalence of ascovirus disease in caterpillars is correlated with high rates of parasitization by endoparasitic hymenoptera. Thus, laboratory and field studies provide sound evidence that the primary mechanism for the transmission of ascoviruses attacking noctuid larvae is through being vectored mechanically by parasitic wasps. No evidence has been found in the lepidopteran hosts for transovum or transovarial transmission.

In the case of the *Diadromus* ascovirus, the association of the virus with its wasp and lepidopteran host is much more intimate. The ascovirus DNA is carried in wasp nuclei as an episome, and small numbers of virions are produced in the oviducts, but the virus does not cause noticeable pathology in the wasp host. The females lay eggs in the pupal stage of the lepidopteran host, introducing small numbers of ascovirus virions along with the wasp eggs. These virions invade lepidopteran host cells, replicate and initiate destruction of major host tissues. The wasp larva then emerges from the egg and feeds on the host tissues and ascovirus virions. The ascovirus genome is carried by both male and female wasps, where it is apparently transmitted from generation to generation transovarially.

These observations make ascoviruses the only known group of viruses pathogenic to insects primarily dependent on vectors for their transmission.

Now that the characteristics of the disease are known, field studies in southeastern USA and California are beginning to show that ascoviruses are probably the most common type of virus to occur during most of the year in populations of several important noctuid pests, including the cabbage looper, *T. ni*, fall armyworm, *S. frugiperda*, and the corn earworm, *H. zea*. Prevalence rates range from 10 to 25%, depending on the species and time of the year, with the highest rates of infection, as noted above, being correlated with high levels of parasitization.

Host Range and Virus Propagation

The experimental host range of ascoviruses varies with the viral species. The variants of the *Trichoplusia* ascovirus have a broad host range and are capable of replication in a variety of noctuid species, as well as in selected species belonging to other families of the order Lepidoptera. Alternatively, the experimental host range of the *Spodoptera* ascovirus is limited to other species of the genus *Spodoptera*. The *Diadromus* ascovirus can replicate in hymenopteran and lepidopteran hosts closely related to its natural host species. To propagate virus in the laboratory, all ascoviruses can be grown in their caterpillar hosts. To infect caterpillars, they are injected with virus in the fourth or early fifth instar, and virion-containing vesicles are harvested from the blood 5–7 days later.

Pathology and Pathogenesis

Gross pathology

The gross pathology of ascovirus disease is very indistinct, and probably accounts for the fact that ascoviruses were discovered only recently. The most obvious sign of disease is that, within 24 hours of infection, larvae cease to gain weight or progress in development. Healthy larvae, particularly in the early stages of development, will easily quadruple their weight and size in a period of 3–4 days, whereas ascovirus-infected larvae cease to grow and may actually lose weight. This is a characteristic that is virtually impossible to detect in the field, but which is easily noticed under laboratory conditions when infected and healthy larvae are reared side-by-side over a period of a few days. A second feature easily

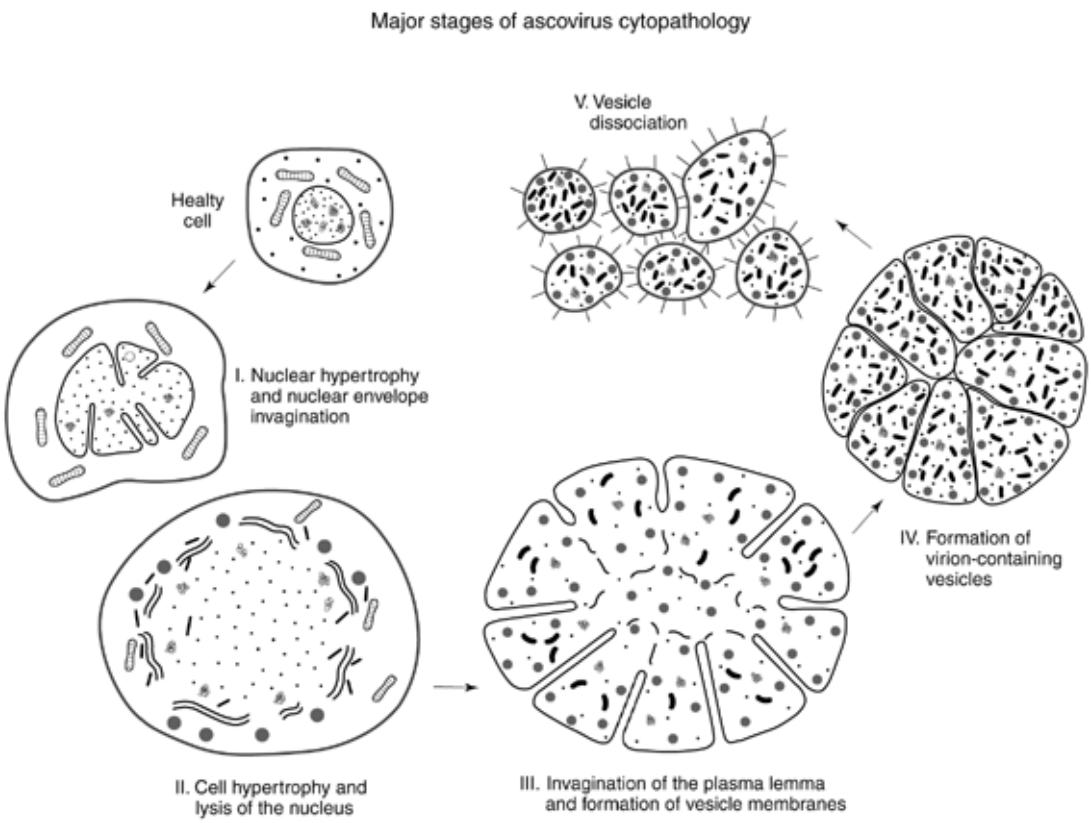


Figure 2 Major stages of cellular pathogenesis caused by a typical ascovirus. After infection, the nucleus enlarges and the nuclear membrane invaginates, and then lyses. Subsequently, the plasmalemma of the cell invaginates and coalesces with cytoplasmic membranes, apparently formed *de novo*, thereby dividing the cell into a cluster of virion-containing vesicles. These vesicles dissociate and are liberated into the blood as the basement membrane of infected tissues degenerates. Virion assembly becomes apparent as the nuclear membrane lyses, and continues throughout all subsequent stages of vesicle formation.

noted in the laboratory is that ascovirus diseases are chronic, though invariably fatal. When infected during early stages of development, ascovirus-diseased larvae often survive for 2–3 weeks beyond the time at which most healthy larvae have completed their development and pupated. Signs of disease other than these are minor, but include the inability to completely cast the molted cuticle, and an occasional white discoloration and hypertrophied appearance of the larval body at advanced stages of disease development.

Cytopathology

In comparison to all other known viruses, the most unique property of ascoviruses is the unusual cytopathology that leads to the formation of the virion-containing vesicles. At the cellular level, the disease begins with extraordinary hypertrophy of the nucleus followed by a corresponding enlargement of the cell (**Fig. 2**). Cells will typically grow from five- to tenfold the diameter of uninfected cells. As the nucleus enlarges, the nuclear envelope ruptures and disintegrates into fragments. At about this stage the cell plasmalemma begins to invaginate along 'planes' toward the now anucleate cell center. Concomitantly, sheets of membrane form closely adjacent to mitochondria that accumulate along the planes. As this process continues, the membrane sheets coalesce and join the invaginating plasmalemma, thereby cleaving the cell into a cluster of from 20 to more than 30 vesicles, ranging in size from 5 to 10 μm in diameter. The vesicles typically remain in the tissue until the basement membrane ruptures, though on occasion cell hypertrophy can be so great that the enlarging cell erupts out through the basement membrane of the infected tissue, releasing large fragments of the infected cell directly into the blood.

Despite the chronic nature of the disease caused by ascoviruses, virion-containing vesicles are present in

Table 1 Tissue tropism of four ascovirus species

	Major tissues infected[a]			
Viral species	*Epidermis*	*Tracheal matrix*	*Fat body*	*Midgut*
Trichoplusia AV	++	++	+	−
Heliothis AV[b]	+++	++	+	−
Spodoptera AV	−	−	+++	−
Autographa AV	−	−	+++	−
Diadromus AV	+++	++	+	+++

[a]The number of + signs indicates the degree of infection; +++ indicates the tissue is very heavily infected and almost completely destroyed by 5–7 days postinfection.

[b]Based on DNA hybridization data, the *Heliothis* ascovirus is a variant of the *Trichoplusia* ascovirus, i.e. a pathovar, not a distinct species.

the blood within 2–3 days of infection. Moreover, when the virus replicates in cells *in vitro*, the vesicles are formed within 12–16 h of infection.

Tissue tropism

Though the cytopathology of ascoviruses is consistent among different viral species, considerable variation occurs with respect to the tissues attacked, i.e. in which replication occurs (**Table 1**). The *Trichoplusia* ascovirus variants from *T. ni* and *H. zea* exhibit a relatively broad tissue tropism infecting the tracheal matrix, epidermis and connective tissue. Differences exist between these variants in that the variant from *H. zea* infects the epidermis much more extensively than the *T. ni* variant, whereas the *T. ni* variant can also replicate in fat body cells, but appears to only do this extensively when larvae are infected early in their development. Alternatively, the *Spodoptera* and *Autographa* ascoviruses have a very narrow tissue tropism, with the fat body being the primary site of infection. The *Diadromus* ascovirus occurs in the nuclei of all tissues of its wasp host, but appears to only produce progeny in ovarial tissues. In its pupal host, it attacks and replicates in a wide variety of tissues.

In all ascoviruses, the virion-containing vesicles are cleaved from cells as the disease progresses. Typically, the vesicles accumulate in the tissues where they are formed, but as these tissues degenerate during disease progression, the basement membrane of infected tissues deteriorates and ruptures, allowing the vesicles to spill out into the blood. There they accumulate, reaching concentrations as high as 10^8 vesicles ml^{-1} within 3–4 days of infection. There is some evidence that viral replication proceeds within the vesicles as they circulate in the blood, and thus this tissue must also be considered one of the tissues attacked by ascoviruses. If fact, because such high concentrations of viral vesicles are found in the blood, this tissue could be considered a major site of infection, particularly if it is eventually shown that these viruses continue to replicate in the vesicles as they circulate in the blood.

Replication and Morphogenesis

Though there have been no biochemical studies of viral DNA replication or protein synthesis, studies carried out with the *Trichoplusia* and *Spodoptera* ascoviruses *in vitro* in insect cell lines show that progeny virions first appear about 12 h after infection. Virion morphogenesis is initiated after the nucleus ruptures, and occurs before and during the cleavage of the cell into vesicles. The first recognizable structural component of the virion to form is the multilaminar layer of the inner particle. Based on its ultrastructure, this layer consists of a unit membrane and an exterior layer of protein subunits. As the multilaminar layer assembles, a dense nucleoprotein core aggregates on the interior surface. This process continues until the inner particle is complete. After formation, the inner particle is enveloped by membranes within the cell or vesicle. These membranes are apparently synthesized *de novo*. Thus, the assembly of the virions is reminiscent of that in other viruses with complex virions, such as the herpesviruses and poxviruses, where the virions differentiate after the association of the precursors of virion structural components.

After formation, the virions of the *Trichoplusia* ascovirus accumulate toward the periphery of the vesicle, where they often form inclusion bodies, i.e. aggregations of virions. In the *Spodoptera* ascovirus, occlusion bodies are formed in which the virions are actually occluded in a 'foamy' vesicular matrix that consists of a mixture of protein and minute spherical vesicles. When viewed with phase microscopy, these viral inclusion and occlusion bodies are phase bright,

and are largely responsible for the highly refractile appearance of the vesicles.

Evolution

The subject of viral evolution over millions of years has received little study due to the lack of a fossil record. Moreover, viruses are considered polyphyletic, with the more then 50 families of viruses thought to have originated independently. In this regard, the ascoviruses may provide a unique opportunity to obtain insight into virus evolution over long periods. The only ascovirus gene sequenced to date is gene encoding a DNA polymerase. Phylogenetic comparisons of this gene with other viral DNA polymerase genes confirm that the ascoviruses represent a unique and stand-alone virus family. However, ascovirus virions are structurally and morphologically similar to the particles formed by ichnoviruses of the family *Polydnaviridae*. As ichnovirus particles are produced in the reproductive tracts of endoparasitic wasps of the family Ichneumonidae, and the wasp host of the *Diadromus* ascovirus is a member of this family, there is a reasonable possibility that ascoviruses and ichnoviruses are related phylogenetically, and thus share a common ancestor. This possibility is currently under investigation, and should be resolved over the next few years through a comparative analysis of the molecular evolution of genes of ascoviruses and ichnoviruses after more of their genes have been cloned and sequenced. A major question to be addressed is whether the *Diadromus* ascovirus represents an intermediate evolutionary form of ascovirus between viruses such as those found in noctuids and the particles of ichnoviruses.

Economic Importance and Future Perspectives

At present, too little is known about ascoviruses to assess whether they are, or will turn out to be, of economic importance. Because of their poor infectivity *per os*, it is unlikely they hold promise for development as microbial insecticides. However, as more entomologists become familiar with the disease caused by ascoviruses, it may be shown that in habitats not treated with synthetic chemical pesticides, ascoviruses are responsible for significant levels of natural pest suppression, particularly where parasitic wasps are abundant. Such findings would encourage even greater emphasis on the development of biological control and other more environmentally sound methods of pest control. With respect to the cytopathology of the disease and viral molecular biology, ascoviruses provide interesting models for the study of novel replication strategies. The unusual process of cell cleavage by which the virion-containing vesicles form probably involves highly specific virus-directed mobilization of the cell cytoskeleton and mitochondria. In addition, it is possible that the virion-containing vesicles will provide a unique anucleate cellular system for studying the replication of a complex type of enveloped dsDNA virus *in vitro*.

See also: **Baculoviruses (*Baculoviridae*): Granuloviruses, Nucleopolyhedrovirus; Entomopoxviruses (*Poxviridae*); Herpes simplex viruses (*Herpesviridae*): General features, Molecular biology; Polydnaviruses (*Polydnaviridae*); Vaccinia virus (*Poxviridae*).**

Further Reading

Bigot Y, Rabouille A, Doury G *et al* (1997) Biological and molecular features of the relationships between Diadromus pulchellus ascovirus, a parasitoid hymenopterna wasp (*Diadromus pulchellus*) and its lepidopteran host, *Acrolepiopsis assectella*. *J. Gen. Virol.* 78: 1149.

Federici BA (1983) Enveloped double-stranded DNA insect virus with novel structure and cytopathology. *Proc. Natl Acad. Sci. USA* 80: 7664.

Federici BA and Govindarajan R (1990) Comparative histopathology of three ascovirus isolates in larval noctuids. *J. Invertebr. Pathol.* 56: 300.

Federici BA, Vlak JM and Hamm JJ (1990) Comparison of virion structure, protein composition, and genomic DNA of three *Ascovirus* isolates. *J. Gen. Virol.* 71: 1661.

Govindarajan R and Federici BA (1990) Ascovirus infectivity and the effects of infection on the growth and development of Noctuid larvae. *J. Invertebr. Pathol.* 56: 291.

Pellock BJ, Lu A, Meagher RB, Weise MJ and Miller LK (1996) Sequence, function, and phylogenetic analysis of an ascovirus DNA polymerase gene. *Virology* 216: 146.

ASTROVIRUSES (*ASTROVIRIDAE*)

Stephan S. Monroe, Centers for Disease Control and Prevention, Atlanta, Georgia, USA

History

Astroviruses were first described in 1975 by two independent research groups using electron microscopy (EM) to study viruses in stool samples from young children with gastroenteritis. The name astrovirus was coined by Madeley and Cosgrove and is derived from the characteristic five-or six-pointed star seen on the surface of some, but not all, particles (**Fig. 1**). Over the succeeding years, viruses with similar appearance were detected by EM in stool samples from a variety of young animals with gastroenteritis, including sheep, cows, deer, pigs, cats, mice, dogs, turkeys and ducks. A breakthrough in astrovirus research was the report by Lee and Kurtz in 1981 of the isolation of human astrovirus strains in primary human embryonic kidney (HEK) cells. Inclusion of trypsin in the growth medium was a strict requirement for both primary isolation and the subsequent adaptation of isolates to growth in LLCMK2 cells, a rhesus monkey kidney continuous cell line. The availability of a cell culture system allowed for the identification of virus serotypes, the development of immune reagents for virus detection and characterization, and ultimately, the molecular cloning and sequence analysis of the astrovirus genome.

Taxonomy and Classification

Astroviruses were recently classified by the International Committee on Taxonomy of Viruses in a distinct family of plus-sense, single-stranded (ss), RNA viruses, the *Astroviridae*. The family consists of a single genus, *Astrovirus,* with human astrovirus type 1 as the type strain. Virus species are identified by the host species of origin (e.g. human astrovirus [HAstV], bovine astrovirus [BAstV]), with serotypes identified by number (e.g. HAstV-1).

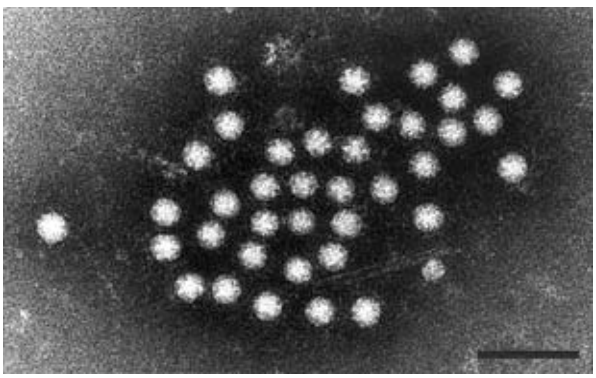


Figure 1 Negative contrast electron micrograph of human astrovirus in a stool sample from a child with gastroenteritis. Bar = 100 nm. (Photo courtesy of C. D. Humphrey, Centers for Disease Control and Prevention.)

Properties of the Virion

As viewed by EM, astrovirus virions are non-enveloped particles, with icosahedral symmetry, approximately 28 nm in diameter, with a smooth outer edge and the characteristic star apparent on the surface of some particles. Particles with a rough edge containing spike projections are often seen in preparations of virus propagated in cell culture. The particles sediment with $s_{20,w}$ of approximately 160, and their buoyant density in CsCl is 1.36–1.39 g cm^{-3}. Virions are composed of a single copy of the genome RNA and multiple (presumably 60) copies of two to four capsid proteins. Different numbers and sizes of the capsid proteins have been reported, depending on the host species of origin of the virus and the conditions of virus purification and analysis. For instance, two capsid proteins with M_r 33 000 were reported for ovine astrovirus (OAstV), whereas two to four capsid proteins have been reported for human astrovirus. The consensus composition of human astrovirus strains is three capsid proteins with M_r of 26 000–36 000, which have been referred to as VP32, VP29 and VP26 (for HAstV-2), or, more generally, as P1, P2 and P3. The apparent size of the smallest protein, P3, varies as a function of virus serotype.

Properties of the Genome

The astrovirus genome is a single molecule of infectious, positive-sense, ssRNA, 6.8–7.9 kb in size. There is a poly(A) tract at the 3′ end, but the structure of the 5′ end has not been determined. Analysis of the complete sequence of two human astrovirus strains, HAstV-1 and HAstV-2, has shown that the genome contains three major open reading frames, ORF 1a, ORF 1b, and ORF 2 (**Fig. 2**). ORFs 1a and 1b are presumed to encode the proteins involved in viral replication, including a chymotrypsin-like protease (pro) and an RNA-dependent RNA polymerase (pol) identified by comparative sequence analysis. Between these two ORFs is a ribosomal

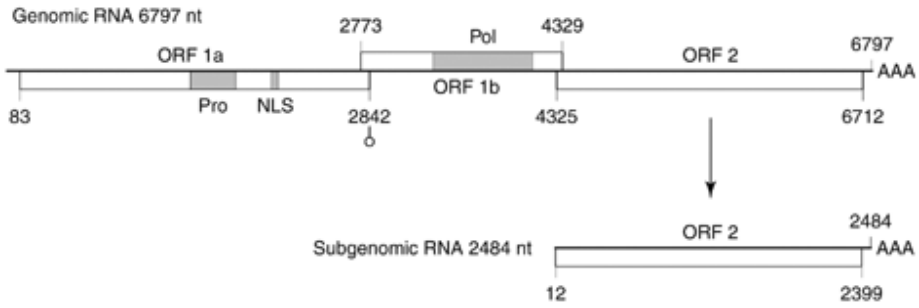


Figure 2 Genome organization of human astrovirus type 2. The arrangement of the three major open reading frames (ORFs) and locations of the motifs for the protease (Pro), polymerase (Pol) and ribosomal frameshifting site (δ) are indicated. The region corresponding to the subgenomic RNA detected in the cytoplasm of infected cells is also indicated. NLS, nuclear localization signal.

frameshifting signal that includes a heptanucleotide 'slippery sequence' and a predicted stem–loop structure. ORF 2, which overlaps ORF 1b by five nucleotides, encodes the capsid protein precursor. A conserved stem–loop structure is predicted at the 3′ end of the genomic RNA of human, ovine, porcine and turkey astroviruses.

Physical Properties

Astrovirus virions do not contain a lipid envelope and are resistant to treatment with organic solvents, such as chloroform, and to nonionic, anionic and zwitterionic detergents. The virus particles are relatively stable, being resistant to treatment at pH 3 and to incubation at 50°C for one hour.

Replication

The receptor by which astroviruses attach to and the mechanism by which they enter susceptible cells have not been determined. Purified astrovirus RNA is infectious; thus immediately following entry and uncoating of the virus particles, the first step in the virus life cycle is translation of the incoming genome RNA to generate the proteins involved in virus replication. For HAstV-2, ORF 1a encodes a 920-amino acid polyprotein with a predicted M_r of 103 000. The proteins encoded in ORF 1b are expressed as an ORF 1a–1b fusion protein (predicted M_r 161 000) generated via a −1 ribosomal frameshift, which occurs with an efficiency of approximately 25%. The heptanucleotide slippery sequence and the adjacent stem–loop structure are absolutely required for efficient frameshifting, but downstream sequences are dispensable, indicating that a pseudoknot structure is not involved. A chymotrypsin-like protease domain has been identified in ORF 1a, but the precise pattern and sequence of the proteolytic cleavages of the primary translation products of ORFs 1a and 1b have not yet been determined.

Approximately 12 h postinfection, two actinomycin D-resistant, virus-specific RNA species appear in the cytoplasm of infected cells. The larger RNA, ~7 kb, is progeny genome RNA, whereas the smaller species, ~2.7 kb, is a subgenomic mRNA encoding ORF 2, as indicated in **Fig. 2**. The subgenomic RNA is colinear with the 3′ end of the genome, and both species are polyadenylated. Concomitant with the appearance of the subgenomic RNA is the production of the viral structural protein precursor (M_r 88 000). This protein is processed to the mature capsid proteins through a cascade of proteolytic steps, for which the enzymes involved are not known. The amino termini of HAstV-2 proteins VP29 (P2) and VP26 (P3) have been mapped by microsequencing to residues 362 and 395, respectively, both adjacent to arginine residues, consistent with cleavage by trypsin.

Although most of the astrovirus replication takes place in the cytoplasm, at least one step in the replication cycle appears to involve the nucleus. There is a canonical nuclear localization signal in ORF 1a, and viral proteins have been detected in the nucleus of infected cells by using immunofluorescence. Assembly of viral particles occurs in the cytoplasm of the infected cell with the formation of paracrystalline arrays of particles seen late in infection by using thin-section EM. Release of progeny infectious virus is dependent on the presence of trypsin in the growth medium.

Geographic and Seasonal Distribution

Human astroviruses are distributed worldwide and have a distinct winter seasonality in temperate climates. Animal astroviruses are less well studied, but strains have been detected in all locations where serious efforts have been made to look for them.

Host Range and Virus Propagation

Astroviruses have been detected in a wide variety of mammals and some birds, with each host species supporting the replication of a unique virus species. The human astroviruses grow in a primate cell line, but direct infection of primates with human astrovirus strains has not been reported. In cases where it has been examined, serum from animals infected with a homologous virus does not react with heterologous virus from other species. The bovine and ovine astroviruses have been passaged in experimentally infected gnotobiotic animals, with symptomatic infection occurring only in the latter species. Human, bovine and porcine astroviruses have been adapted to growth in cell culture, in each case using a cell line derived from the host species and with the inclusion of trypsin in the growth medium. The human colon carcinoma cell line, Caco-2, is widely used for primary isolation and propagation of human astrovirus strains.

Genetics

The use of naturally occurring, or chemically induced, temperature-sensitive mutants, or other genetic approaches, has not been applied to the study of astrovirus gene expression. With the recent report of an infectious cDNA clone of HAstV-1, the potential exists for future studies to map gene functions by using site-directed mutagenesis.

Evolution

The genome organization of astroviruses and sequence comparisons of conserved protein motifs indicate that astroviruses are distinct from all other families of nonenveloped, plus-sense, ssRNA viruses. Astroviruses do, however, share several features with caliciviruses, including expression of the capsid protein(s) from a 3′-coterminal subgenomic RNA and sequence similarity in the putative chymotrypsin-like protease. In contrast, expression of the nonstructural proteins via ribosomal frameshifting, the absence of an RNA helicase motif, and similarity in the RNA-dependent RNA polymerase domain, are more typical of the plant luteoviruses.

One unusual feature of astroviruses is the marked difference among human serotypes in the degree of sequence similarity as a function of the coding region analyzed. Within a portion of ORF 1a, the pairwise nucleotide sequence distances between serotypes 1 through 5 range from 4.1% to 7.4%. In contrast, the corresponding range of values for a portion of ORF 2 is 19.3–22.7%. Moreover, when the recently identified serotypes, 6 and 7, are included in the analysis, they

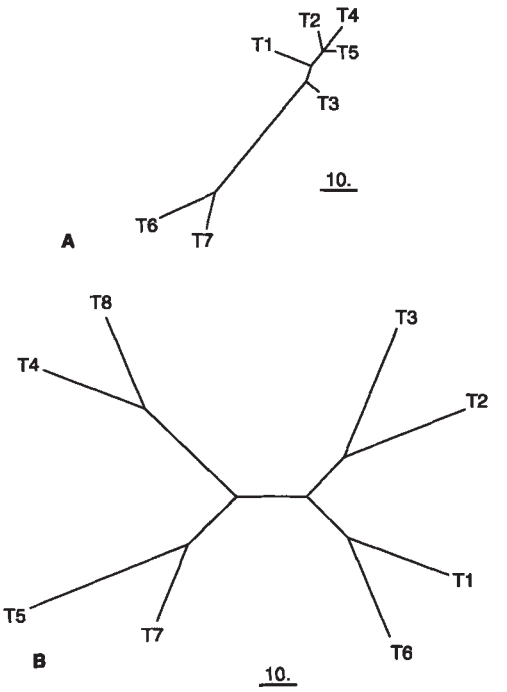


Figure 3 Phylogenetic analysis of human astrovirus nucleotide sequences. Unrooted trees were generated by maximum parsimony analysis with the PAUP program, using a 244-nucleotide region of ORF 1a (**A**) or a 348-nucleotide region of ORF 2 (**B**). Scale bars indicate number of nucleotide changes.

constitute a distinct clade in ORF 1a, as indicated in the phlyogram in **Fig. 3A**. This contrasts with the nearly equidistant arrangement of the sequences analyzed in ORF 2, as indicated in **Fig. 3B**. One possible explanation for this pattern is the occurrence of an RNA recombination event leading to discontinuous evolution in serotypes 6 and 7.

Serologic Relationships and Variability

Human astrovirus serotypes are defined on the basis of greater than 20-fold differences in two-way cross-reactivity as measured by immunofluorescence, immune EM, or neutralization using reference antisera raised in rabbits. Seven serotypes (HAstV-1 to HAstV-7) are currently recognized, with a possible eighth serotype not yet fully characterized. More recently, typing of human astroviruses has been done by using an enzyme immunoassay (EIA) or by phylogenetic analysis of nucleotide sequence information. Two serotypes of bovine astrovirus have been defined by crossneutralization, with a possible third serotype not yet fully characterized.

Several neutralizing monoclonal antibodies have been described, and the target epitopes have been mapped to proteins VP29 or VP26, or both. In

addition to type-specific epitopes, human astrovirus strains share one or more common epitopes recognized by group-reactive monoclonal antibodies.

Epidemiology

Our picture of the epidemiology of human astrovirus infection has changed as the methods to detect the virus have become increasingly more sensitive. On the basis of early studies, which relied on direct detection of virus particles in stool specimens by EM, astroviruses were thought to account for approximately 2% of the cases of gastroenteritis in young children. The development of specific EIAs for detection of astroviruses allowed for larger studies to be conducted using a simpler and more sensitive detection method. On the basis of several prospective studies, astroviruses are associated with 6–9% of the cases of gastroenteritis in children under 5 years of age, with most cases detected in children between 9 and 18 months of age. Most children will have an episode of astrovirus gastroenteritis by age 5 years.

The more recent use of reverse transcriptase–polymerase chain reaction (RT-PCR) assays for detection of astroviruses in stool samples from an outbreak in a daycare facility indicated that this assay is more sensitive than EIA for detecting prolonged shedding in symptomatic children and that asymptomatic infection was common in the older children. In addition to young children, the elderly are at increased risk for astrovirus infection, with outbreaks of illness occurring in nursing homes. Astrovirus infection was significantly associated with diarrhea in a cohort of persons infected with human immunodeficiency virus (HIV), indicating that immunocompromised persons are also at increased risk.

Serotype 1 is the predominant type of HAstV, accounting for approximately half of all infections. Serotypes 2–5 are associated with approximately 10% of the infections each, whereas types 6 and 7 are rarely identified. Additional insights into the frequency of astrovirus infection have been obtained by seroprevalence studies which have demonstrated that acquisition of antibodies commonly occurs in the first 5 years of life, and that 90–100% of adults have antibodies to one or more astrovirus serotypes.

Transmission and Tissue Tropism

Human astroviruses are most commonly spread by fecal–oral transmission, although increased levels of community sanitation appear not to have an impact, since the rates of infection in young children are similar for developing and developed countries. Person-to-person spread is the most likely mode of transmission in outbreaks in daycare centers and nursing homes. Infection may also occur via contaminated water or foods, particularly shellfish. Most human and animal astroviruses are associated with symptoms of gastroenteritis, and replication has been documented in the gastrointestinal tract. A noteworthy exception is duck hepatitis virus which has been detected by EM in the livers of animals with histologic evidence of hepatitis.

Pathogenicity

The pathogenic potential of astroviruses ranges from asymptomatic infection of calves with bovine astrovirus to severe hepatitis, with as high as 50% mortality in ducklings with duck hepatitis virus. The mortality rate of human astrovirus infection is extremely low, but deaths have been reported in young children and the elderly.

Clinical Features and Infection

Astrovirus infection of young children results in a mild, self-limiting gastroenteritis with an incubation period of 2–4 days and a duration of 2–3 days. Typical symptoms include watery diarrhea, vomiting, fever and abdominal pain. Experimental infection of adult volunteers with human astroviruses results in low levels of symptomatic infection, although seroconversions can be measured in a majority of the patients. This observation suggests that infection early in life may protect against symptomatic infection later in life.

Pathology and Histopathology

Little is know about the pathology of astrovirus infection in humans. A single report describes astrovirus particles in intestinal epithelial cells in two children who were shedding virus in their stools. Histologic changes have been well documented during experimental infection of gnotobiotic lambs with OAstV. Astrovirus particles were seen in columnar epithelial cells on the apical two-thirds of villi beginning 14 h postinfection (PI). By 23–38 h PI, there was sloughing of villus epithelial cells with release of virus particles, and by 120 h PI the villi had returned to normal. In contrast, experimental challenge of gnotobiotic calves with BAstV resulted in asymptomatic infection. Virus particles were detected in M cells overlying Peyer's patches in the small bowel.

Immune Response

Primary infection with human astroviruses results in the appearance of type-specific serum antibodies

detectable by immune EM, EIA or neutralization. The seroprevalence rates among 242 persons in the Netherlands as measured by neutralizing antibody to HAstV types 1 to 7 were 91, 31, 69, 56, 36, 16 and 10%, respectively. Antibody levels declined in the elderly, a finding that may account for the increased risk for symptomatic infection in this group.

Prevention and Control

Improvements in community sanitation are not likely to reduce the incidence of astrovirus infection since similar rates of disease have been reported in developing and developed countries. Strict adherence to standard enteric infection control practices may help to limit the spread of infection during outbreaks in daycare centers or nursing homes. Because primary infection with astrovirus appears to confer long-term immunity, as evidenced by the low rate of symptomatic infection in adults, there is good potential for the development of a protective vaccine.

Future

Much remains to be learned about the basic molecular virology of astroviruses. The number, size and genome location of the mature capsid proteins need to be precisely defined, as do the recognition sites and enzymes responsible for proteolytic processing. The putative replicative proteins encoded in ORFs 1a and 1b should be fully characterized. We have a relatively clear picture of the epidemiology of astrovirus infection, but the burden of disease in hospitalized children should be more carefully assessed as a step toward consideration of the development of a protective vaccine.

See also: **Rotaviruses (*Reoviridae*): General features, Molecular biology.**

Further Reading

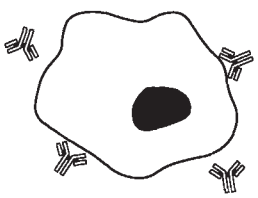
Carter MJ and Willcocks MM (1996) The molecular biology of astroviruses. *Arch. Virol.* 12(suppl): 277.

Glass RI, Noel JS, Mitchell D *et al* (1996) The changing epidemiology of astrovirus-associated gastroenteritis: a review. *Arch. Virol.* 12(suppl): 287.

Matsui SM and Greenberg HB (1996) Astroviruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 811. Philadelphia: Lippincott-Raven.

Monroe SS, Carter MJ, Herrmann JE, Kartz JB and Matsui SM (1995) *Astroviridae*. In: Murphy FA, Fauquet CM, Bishop DHL *et al* (eds) *Virus Taxonomy: Classification and Nomenclature of Viruses*. Sixth Report of the International Committee on Taxonomy of Viruses, p. 364. Vienna: Springer-Verlag.

AUTOIMMUNITY



Robert S Fujinami, University of Utah, Salt Lake City, Utah, USA

Introduction

Several hypotheses have been proposed to explain the development of autoimmunity and subsequent disease in humans and animals. These include a viral etiology for the initiation of autoimmune disease. Potential mechanisms include: polyclonal activation of autoreactive T and B cells by virus infection; expression of altered self or neoantigens presented during infection; alterations in anti-idiotypic responses to self peptides generated by infection; infection of lymphoid and/or antigen-presenting cells (APCs) causing modulation of the ensuing immune antiviral response; and cross-reacting immune responses generated by the occurrence of common determinants between virus and self proteins, known as molecular mimicry.

Potential Mechanisms

Viruses can cause polyclonal activation of autoreactive T and B cells. This results in the production of activated self-reactive T cells and the production of autoantibodies. An example of this is the activation of self-reactive T cells by superantigens encoded by certain retroviruses. T cell activation normally takes place when the T cell receptor encounters a peptide in association with either major histocompatibility complex (MHC) class I or class II molecules on APCs (**Fig. 1**). In addition, the APCs provide auxiliary costimulatory signals that help drive the T cells to respond. The T cell receptor (TCR) provides the specificity in this interaction. Superantigens have the ability to bind to families of MHC class II molecules

detectable by immune EM, EIA or neutralization. The seroprevalence rates among 242 persons in the Netherlands as measured by neutralizing antibody to HAstV types 1 to 7 were 91, 31, 69, 56, 36, 16 and 10%, respectively. Antibody levels declined in the elderly, a finding that may account for the increased risk for symptomatic infection in this group.

Prevention and Control

Improvements in community sanitation are not likely to reduce the incidence of astrovirus infection since similar rates of disease have been reported in developing and developed countries. Strict adherence to standard enteric infection control practices may help to limit the spread of infection during outbreaks in daycare centers or nursing homes. Because primary infection with astrovirus appears to confer long-term immunity, as evidenced by the low rate of symptomatic infection in adults, there is good potential for the development of a protective vaccine.

Future

Much remains to be learned about the basic molecular virology of astroviruses. The number, size and genome location of the mature capsid proteins need to be precisely defined, as do the recognition sites and enzymes responsible for proteolytic processing. The putative replicative proteins encoded in ORFs 1a and 1b should be fully characterized. We have a relatively clear picture of the epidemiology of astrovirus infection, but the burden of disease in hospitalized children should be more carefully assessed as a step toward consideration of the development of a protective vaccine.

See also: **Rotaviruses (*Reoviridae*): General features, Molecular biology.**

Further Reading

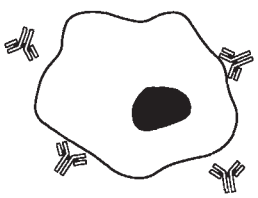
Carter MJ and Willcocks MM (1996) The molecular biology of astroviruses. *Arch. Virol.* 12(suppl): 277.

Glass RI, Noel JS, Mitchell D *et al* (1996) The changing epidemiology of astrovirus-associated gastroenteritis: a review. *Arch. Virol.* 12(suppl): 287.

Matsui SM and Greenberg HB (1996) Astroviruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 811. Philadelphia: Lippincott-Raven.

Monroe SS, Carter MJ, Herrmann JE, Kartz JB and Matsui SM (1995) *Astroviridae*. In: Murphy FA, Fauquet CM, Bishop DHL *et al* (eds) *Virus Taxonomy: Classification and Nomenclature of Viruses*. Sixth Report of the International Committee on Taxonomy of Viruses, p. 364. Vienna: Springer-Verlag.

AUTOIMMUNITY



Robert S Fujinami, University of Utah, Salt Lake City, Utah, USA

Introduction

Several hypotheses have been proposed to explain the development of autoimmunity and subsequent disease in humans and animals. These include a viral etiology for the initiation of autoimmune disease. Potential mechanisms include: polyclonal activation of autoreactive T and B cells by virus infection; expression of altered self or neoantigens presented during infection; alterations in anti-idiotypic responses to self peptides generated by infection; infection of lymphoid and/or antigen-presenting cells (APCs) causing modulation of the ensuing immune antiviral response; and cross-reacting immune responses generated by the occurrence of common determinants between virus and self proteins, known as molecular mimicry.

Potential Mechanisms

Viruses can cause polyclonal activation of autoreactive T and B cells. This results in the production of activated self-reactive T cells and the production of autoantibodies. An example of this is the activation of self-reactive T cells by superantigens encoded by certain retroviruses. T cell activation normally takes place when the T cell receptor encounters a peptide in association with either major histocompatibility complex (MHC) class I or class II molecules on APCs (**Fig. 1**). In addition, the APCs provide auxiliary costimulatory signals that help drive the T cells to respond. The T cell receptor (TCR) provides the specificity in this interaction. Superantigens have the ability to bind to families of MHC class II molecules

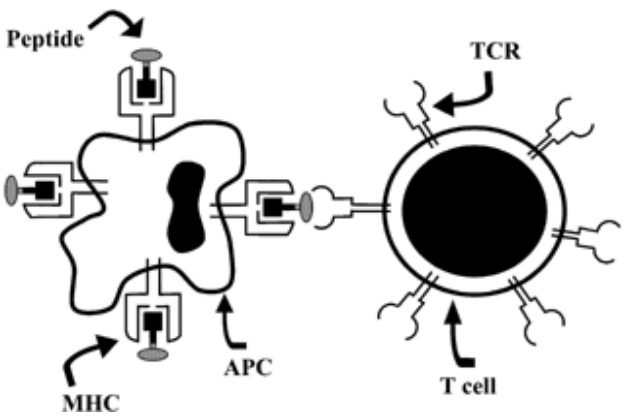


Figure 1 T cell–APC interaction. T cells recognize antigen, either foreign or self, on APCs. Recognition occurs by engagement of the TCR present on the surface of the T cells with the MHC molecule containing the self or foreign peptide on the surface APC. This interaction can lead to activation, anergy or unresponsiveness or deletion of the T cell, depending on the context in which the interaction takes place.

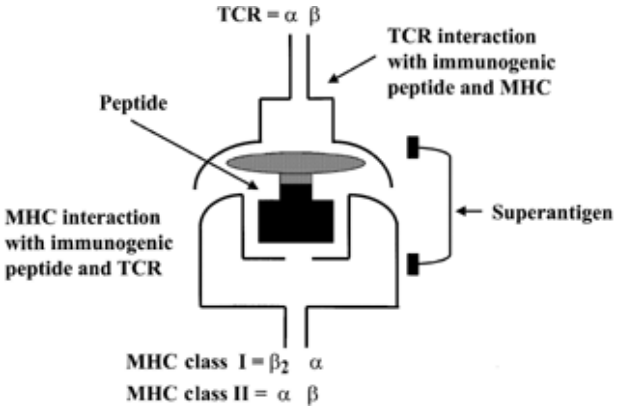


Figure 2 TCR–peptide–MHC interaction. TCRs are present on the surface of T cells and are heterodimers comprising an α and a β chain. The TCR interacts with the MHC class I or class II molecule on the surface of APCs or target cells. CD8+ T cells recognize peptide with class I molecules and CD4+ T cells recognize peptide with class II molecules. MHC class I molecules are heterodimers comprising β_2-microglobulin (β_2) and an α chain. Similarly, MHC class II molecules are heterodimers comprising an α and β chain. Superantigens can activate CD4+ T cells by linking the class II molecule and the TCR. This type of stimulation is thought to be involved in some forms of autoimmunity. The peptides can be either self or foreign peptides that are recognized.

and engage T cells recognizing the particular MHC class II families (**Fig. 2**). Thus, populations of T cells, some of which recognize self peptides, are activated and potentially could initiate autoimmunity. For B cells (production of autoantibodies), infection by Epstein–Barr virus (EBV) leads to the production of autoantibodies. B cells differentiate into plasma cells and secrete antibody of a single specificity. This can be antiviral or of antiself specificity. Normally, B cells require interleukin (IL)-2, IL-4, IL-5, IL-6 and interferon γ (IFN-γ) secreted by T cells for growth and differentiation into plasma cells. Infection of B cells with EBV drives these B cells to produce antibodies. Should the B cells (antibody) be specific for self protein, EBV-infected cells would produce antibodies reactive with self.

Neoantigens

Virus infection can cause the induction of neoantigens or self proteins not normally present in or on cells. For example, infection of cells with paramyxoviruses can lead to the expression of heat shock proteins not normally present in the uninfected cell. Self antigens expressed in cells can be presented in the context of MHC class I or class II pathways. Of potential concern is the scenario where peptides from heat shock proteins are presented to T cells in the context of class II molecules. If this were to occur in the environment of an inflammatory response where there are proinflammatory cytokines, the heat shock protein-reactive T cells could become activated and initiate a self-reactive immune response. In animal models, injection of heat shock protein in adjuvant can result in arthritis. The autoimmune disease can be transferred to naive animals with activated heat shock protein-reactive T cells taken from immunized animals. Therefore, a possible mechanism of autoimmunity is one where infection with the virus induces neoself antigens recognized by T cells which could cause disease. Even though the virus is cleared, an antiself response is generated owing to the continued presence of the self antigen. This is consistent with many chronic autoimmune diseases.

Anti-idiotypic response

Another mechanism to initiate autoimmunity by virus infection is the generation of anti-idiotypic responses. Viruses attach to cells due to specific regions or sites on the virus. This can be specific viral glycoproteins such as the influenza virus hemagglutinin or conformational sites made up of several viral capsid proteins such as the canyon for rhinoviruses. These virus proteins or sites bind to distinct receptor proteins on susceptible cells. Infection by a virus could generate antibodies, which react with those specific regions of the virus that bind to the host cell receptor. Hypothetically, these antibodies could induce the production of other idiotypic antibodies, which would bind to the combining site of the

original antibody and also to the receptor protein on the cell. Such anti-idiotypic antibodies that bind to the cells could initiate cell lysis or death due to the activation of the complement cascade. Similarly, lymphocytes are also thought to be regulated by idiotypic networks.

Infection of lymphoid cells

A third mechanism for virus-induced autoimmunity is infection of lymphoid cells and/or APCs. Different types of lymphoid cells can regulate the immune response. In addition, different viral proteins or epitopes comprising these proteins can help skew the immune response. Responses to self have to be more stringently regulated than antimicrobial responses, otherwise autoimmunity would be more pronounced. Negative selection eliminates many autoreactive cells (both T and B cells), but not all self-reactive cells are eliminated. Infection of lymphocytes can alter the cytokines produced by these cells. Different cytokines will favor a Th1 (delayed-type hypersensitivity, DTH) versus a Th2 response. CD4+ Th1 T cells (class II restricted) are the favored T cell type for autoimmune disease. Autoimmune disease can be transferred by Th1 autoreactive T cells in a variety of animal models of human autoimmune disease. Release of proinflammatory cytokines, for example IFN-γ, can push an immune response toward a Th1 type. Early clinical trials treating patients with multiple sclerosis (a proposed human autoimmune disease) with IFN-γ have led to exacerbation of clinical symptoms, suggesting enhancement of Th1 T cells increasing disease. Release of cytokines in areas of inflammation can also lead to upregulation of MHC class I and II antigens. These molecules can present additional self peptides to autoreactive T cells and could further diversify an autoimmune response through determinant spreading. This is the spreading of an immune response to other determinants or epitopes, not the first inciting epitope.

Molecular mimicry

Another potential mechanism by which viruses may incite autoimmune reactions is molecular mimicry. Viruses that have common determinants with self-antigens can induce crossreacting immune responses. These determinants (epitopes) can be recognized by B and T cells. Several predictions can be made for this type of mimicry/crossreaction. First, T and B cells reactive to the crossreacting determinant must not be eliminated by negative selection. Second, although crossreacting determinants are relatively common, the crossreacting epitope must be a disease-inducing determinant. For example, having an immune response to a nondisease-inducing epitope would result in the expansion of autoreactive T cells, but no disease or tissue injury would occur. Here, autoreactive T cells (Th2 cells) that downregulate the harmful autoaggressive T cells (Th1) could be generated. Third, it is probable that viruses, being facultative intracellular parasites, have acquired genes from the host cell encoding proteins necessary for replication and also to subvert the immune response, allowing the virus to replicate efficiently in the cell and persist in the host. Lastly, it is not unreasonable that viruses which look like self (mimicry) have a selective advantage over those that do not.

Models of Autoimmunity

Diabetes, particularly type I (insulin-dependent diabetes mellitus, IDDM) has been associated with viral infections. A potential mechanism described above is molecular mimicry. The principle here is that a virus encoding a crossreacting epitope is also present in the pancreas of the genetically susceptible individual. Infection with a virus having the crossreacting protein and/or epitope could initiate a crossreacting immune response. Here, the cell in the pancreas that contains the peptide in association with MHC molecules would be recognized by T cells and attacked. Two laboratories, those of Oldstone and Zinkernagle, have constructed transgenic mice to test this hypothesis. Lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP) or nucleocapsid protein (NP) genes controlled by the insulin promoter were inserted into the germ line. Such transgenic mice expressed the viral GP or NP protein in the islet cells of the pancreas. These mice therefore consider the viral protein as self. Infection of the transgenic mice with LCMV led to the generation of NP- or GP-specific cytotoxic T lymphocytes (CTLs). These T cells migrate to the pancreas and kill the islet cells, leading to hyperglycemia and hypoinsulinemia. Two types of IDDM were observed in the Oldstone model: a fast onset and slow onset of IDDM after infection with LCMV. In the first instance, LCMV-infected mice developed IDDM within 1–2 weeks after infection. The slow onset progressed 1–2 months after infection. In both instances diabetes was dependent on the effector cell being a CD8+ T cell. The difference between the fast and slow onset is the number and affinity of the CD8+ T cells specific for the viral/self protein. In the fast-onset diabetes, the viral protein is not expressed in the thymus (where negative selection occurs) but only in the pancreas. The LCMV-specific T cells were not selected against in the thymus and frequency of CD8+ T cells was higher and affinity higher to peptide–MHC. In the slow-onset diabetes model,

transgenic mice express the viral protein in the thymus as well as the pancreas. Therefore, the high-affinity anti-LCMV-specific T cells are selected against and eliminated in the thymus. The numbers and affinity of LCMV-specific CD8+ T cells that enter the periphery are reduced. For disease to occur, CD4+ T cells are required to provide help for sufficient CD8+ T cell expansion. Depletion studies show that without the CD4+ T cell population, no expansion occurs and animals remain disease free. The CD4+ T cells are not cytotoxic for virus-infected cells. Therefore, a critical threshold is needed for disease to occur. CD8+ T cells can transfer disease to uninfected transgenic mice. Thus, this model reflects systems where class I effector T cells are important for elaboration of disease, and CD4+ T cells can regulate the CTL effector response. Myositis is an example of MHC class I restricted T cells playing a role in autoimmune disease.

Another model of viruses modulating autoimmune disease is an animal model for multiple sclerosis. This model is called experimental autoimmune encephalomyelitis (EAE). Here animals (mice) are injected with myelin proteins or disease-inducing peptides from these proteins in adjuvant. Mice develop clinical signs of ataxia and incontinence and inflammatory central nervous system (CNS) demyelinating disease. The mice have many of the clinical and pathological features of multiple sclerosis. CD4+ T cells obtained from immunized mice can transfer disease to naive mice.

The following experiments were initiated to determine whether virus encoding self proteins could modulate the outcome of autoimmune disease. A recombinant vaccinia virus was constructed to encode a disease-inducing region of myelin basic protein, MBP. Infection of PL/J mice with the recombinant virus encoding a portion of MBP did not induce EAE by itself. Antibody to MBP could be detected in the vaccinated mice. In contrast, PL/J mice injected with the recombinant virus encoding a portion of MBP and subsequently challenged with MBP or an encephalitogenic peptide (disease-inducing epitope AC 1–11) were protected from the development of EAE. PL/J mice vaccinated with a control recombinant vaccinia virus encoding β-galactosidase developed EAE. The protection is specific for MBP, as it does not extend to mice challenged with whole spinal cord. The major self-CNS antigen in whole spinal cord is proteolipid protein. The region of MBP encoded by the recombinant vaccinia virus encompasses the PL/J epitope and not the SJL/J strain. The disease-inducing region is specific for different mouse strains. For example, the first 9–11 amino acids (AC 1–11) of MBP represent the encephalitogenic peptide/epitope for the PL/J strain of mice. A different region (89–108 of MBP) is encephalitogenic for the SJL/J strain of mouse.

To further determine the specificity of the protection, SJL/J mice were vaccinated with the recombinant virus encoding the PL/J epitope (AC 1–11) and 5 weeks later were injected with the SJL/J encephalitogenic peptide (89–108). The SJL/J mice were not protected and developed EAE. Therefore, the protection was strain specific. These cells have the Th1 phenotype and this cell type is responsible for DTH reactions. In mice vaccinated with the recombinant vaccinia virus encoding AC 1–11 of MBP and not a control vaccinia virus, a decrease in DTH to MBP was observed. Thus, vaccination decreased the numbers of or anergized the MBP-specific Th1 T cells. As with many of these models of autoimmunity, disease can be transferred with self-reactive T cells. In the case of EAE, the disease can be transferred by the Th1 CD4+ MBP-specific T cells. Vaccinated mice infected with the recombinant virus encoding a portion of MBP and challenged with the encephalitogenic peptide from MBP do not develop EAE. T cells from these mice are unable to transfer EAE to other mice, whereas T cells from control virus vaccinated mice. T cells from mice injected with AC 1–11 of MBP (the encephalitogenic peptide) can transfer EAE to naive mice. These T cells were also able to transfer EAE to vaccinated mice, indicating that the block is at the activation stage. Once the effector cells are generated, vaccination cannot inhibit disease. In this system, depletion of CD8+ T cells does not alter the protection.

In a modification of the virus model for CD4+-mediated T cell disease, a recombinant vaccinia virus was constructed to encode the entire proteolipid protein (PLP) sequence. This is the major myelin protein found in the CNS. PLP or peptides derived from PLP can induce EAE in mice when injected with adjuvant. Infection of mice with the recombinant virus does not lead to autoimmune disease. However, it does prime mice for autoimmune disease. Mice vaccinated with this virus produce self-reactive antibody to three encephalitogenic epitopes (disease inducing) contained in PLP. When vaccinated mice are later challenged with encephalitogenic peptides from PLP, mice develop an enhanced acute disease which occurs much sooner and is more severe than mice vaccinated with a control virus or no virus. The extent of demyelination and inflammation in the CNS was enhanced in the mice vaccinated with the virus encoding the self protein. Interestingly, while mice vaccinated with the control virus or no virus went on to develop a relapsing and remitting clinical course of disease, mice vaccinated with the recombinant PLP virus had a much reduced clinical course with very few relapses.

In summary, virus infections can have negative or positive influences on the development of autoimmune disease.

Future Perspectives

Some of the initial studies on molecular mimicry and virus-induced autoimmunity indicated that viruses share common sequences with host components. It has been demonstrated that some viruses have acquired host genes and modified these to subvert the immune system. Herpesviruses encode MHC-like molecules, Fc receptors, IL-10-like factor and complement regulatory proteins, and poxviruses have incorporated into their genome, IL-1 receptor, tumor necrosis factor (TNF) receptor, IFN-γ receptors and complement control proteins. In addition, viral proteins need to perform specific functions in the cell to replicate its genome and assemble new virions. These functions use the existing cellular machinery and therefore mimic many of the host cells, which also perform similar tasks. The key question is still: how are these infections which are associated with autoimmune disease able to initiate the pathway leading to self-reactive immune responses?

See also: **Immune response: General features, Cell mediated immune response; Immune escape mechanisms; Lymphocytic choriomeningitis virus (*Arenaviridae*): General features, Molecular biology; Epstein-Barr virus (*Herpesviridae*): General features, Molecular biology.**

Further Reading

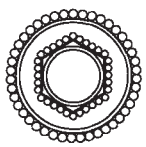
Barnett L and Fujinami RS (1992) Molecular mimicry: a mechanism for autoimmune injury. *FASEB J.* **6**: 840.

Dyrberg T, Mackay P, Michelsen B *et al* (1996) Viruses and diabetes mellitus. In: Friedman H, Rose NR and Bendinelli M (eds) *Microorganisms and Autoimmune Diseases*, p. 105. New York: Plenum Press.

Fujinami RS, Oldstone MBA, Wroblewska Z, Frankel ME and Koprowski H (1983) Molecular mimicry in virus infection: crossreaction of measles virus phosphoprotein or of herpes simplex virus protein with human intermediate filaments. *Proc. Natl. Acad. Sci. USA* **80**: 2346.

von Herrath MG and Oldstone MBA (1995) Role of viruses in the loss of tolerance to self-antigens and in autoimmune diseases. *Trends Microbiol.* 3: 424.

AVIAN TYPE C RETROVIRUSES (*RETROVIRIDAE*)



John M Coffin, Department of Molecular Biology and Microbiology, Tufts University, Boston, Massachusetts, USA

History

Avian leukosis virus (ALV) and related viruses are among the oldest retroviruses known. The first description (of a leukemia virus) was in 1907, but the continuing history of these viruses starts with the isolation of Rous sarcoma virus (RSV) by Peyton Rous in 1911. Numerous isolates of ALV, as well as transforming viruses which cause sarcomas as well as a variety of hematopoietic neoplasms, were reported in the decades that followed, but progress in understanding their nature was very slow until the development of cell culture assays in the late 1950s and the development of *in vitro* genetic and cell biological approaches to study replication and transformation. The discovery of reverse transcriptase in 1970 and of the origin and mechanism of action of viral oncogenes in the decade following led to an explosion of research activity, with results justifying the intensive study.

Taxonomy and Classification

The avian leukosis viruses comprise a single genus *Alpharetrovirus*, of the family *Retroviridae*. Although they share structural and biological characteristics with the mammalian C-type viruses (such as murine leukemia virus) these two groups are not closely related. All ALVs are closely related to one another sharing considerable sequence and antigenic identity. Isolates are differentiated by subgroup (i.e. receptor utilization) and the presence or absence of oncogenes.

In summary, virus infections can have negative or positive influences on the development of autoimmune disease.

Future Perspectives

Some of the initial studies on molecular mimicry and virus-induced autoimmunity indicated that viruses share common sequences with host components. It has been demonstrated that some viruses have acquired host genes and modified these to subvert the immune system. Herpesviruses encode MHC-like molecules, Fc receptors, IL-10-like factor and complement regulatory proteins, and poxviruses have incorporated into their genome, IL-1 receptor, tumor necrosis factor (TNF) receptor, IFN-γ receptors and complement control proteins. In addition, viral proteins need to perform specific functions in the cell to replicate its genome and assemble new virions. These functions use the existing cellular machinery and therefore mimic many of the host cells, which also perform similar tasks. The key question is still: how are these infections which are associated with autoimmune disease able to initiate the pathway leading to self-reactive immune responses?

See also: **Immune response: General features, Cell mediated immune response; Immune escape mechanisms; Lymphocytic choriomeningitis virus (*Arenaviridae*): General features, Molecular biology; Epstein-Barr virus (*Herpesviridae*): General features, Molecular biology.**

Further Reading

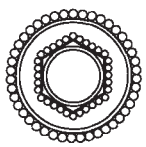
Barnett L and Fujinami RS (1992) Molecular mimicry: a mechanism for autoimmune injury. *FASEB J.* **6**: 840.

Dyrberg T, Mackay P, Michelsen B *et al* (1996) Viruses and diabetes mellitus. In: Friedman H, Rose NR and Bendinelli M (eds) *Microorganisms and Autoimmune Diseases*, p. 105. New York: Plenum Press.

Fujinami RS, Oldstone MBA, Wroblewska Z, Frankel ME and Koprowski H (1983) Molecular mimicry in virus infection: crossreaction of measles virus phosphoprotein or of herpes simplex virus protein with human intermediate filaments. *Proc. Natl. Acad. Sci. USA* **80**: 2346.

von Herrath MG and Oldstone MBA (1995) Role of viruses in the loss of tolerance to self-antigens and in autoimmune diseases. *Trends Microbiol.* 3: 424.

AVIAN TYPE C RETROVIRUSES (*RETROVIRIDAE*)



John M Coffin, Department of Molecular Biology and Microbiology, Tufts University, Boston, Massachusetts, USA

History

Avian leukosis virus (ALV) and related viruses are among the oldest retroviruses known. The first description (of a leukemia virus) was in 1907, but the continuing history of these viruses starts with the isolation of Rous sarcoma virus (RSV) by Peyton Rous in 1911. Numerous isolates of ALV, as well as transforming viruses which cause sarcomas as well as a variety of hematopoietic neoplasms, were reported in the decades that followed, but progress in understanding their nature was very slow until the development of cell culture assays in the late 1950s and the development of *in vitro* genetic and cell biological approaches to study replication and transformation. The discovery of reverse transcriptase in 1970 and of the origin and mechanism of action of viral oncogenes in the decade following led to an explosion of research activity, with results justifying the intensive study.

Taxonomy and Classification

The avian leukosis viruses comprise a single genus *Alpharetrovirus*, of the family *Retroviridae*. Although they share structural and biological characteristics with the mammalian C-type viruses (such as murine leukemia virus) these two groups are not closely related. All ALVs are closely related to one another sharing considerable sequence and antigenic identity. Isolates are differentiated by subgroup (i.e. receptor utilization) and the presence or absence of oncogenes.

Distribution, Host Range and Propagation

ALVs are endemic in flocks of domestic chickens (*Gallus gallus*) worldwide, and natural infections seem limited to this species, within which they are of some economic importance. Related endogenous viruses are found in ring-necked and golden pheasant (but not other related birds, such as turkeys), but exogenous viruses have not been isolated from other species. ALV will replicate efficiently in species closely related to the chicken such as quail, turkeys and pheasants, but less so in more distant species such as ducks. RSV of some subgroups can transform mammalian cells and induce tumors in mammals, but with greatly reduced efficiency, and virus replication in mammalian cells cannot be reproducibly observed. The restriction of the virus to avian species is due both to a lack of suitable receptors for most subgroups as well as a block to expression of virus-encoded proteins. The rare transformants that arise in RSV-infected mammalian cells often display rearrangements in proviral DNA that relieve this block.

A variety of cell types from gallinaceous birds (including chickens, turkeys and quail) can be used to propagate ALVs and their relatives. Primary and secondary fibroblast cultures are most commonly applied to this purpose, since permanent cell lines with the exception of those derived from some quail tumors (e.g. QT6) have only recently become available for most avian species. The cell lines can be productively infected and are useful for some purposes. To avoid problems associated with frequent recombination, it is advisable to use cells that do not contain related endogenous proviruses, such as cells from species other than chickens or from chickens bred to contain no such proviruses.

Properties of the Virion

Like all retroviruses, avian leukosis viruses are transmitted as enveloped virions of about 100 nm diameter derived by budding from the host cell membrane. Within the retrovirus family, they are defined as having a C-type morphology. Virions are modified by the inclusion of peplomers consisting of trimers of the two *env* encoded proteins, SU (surface) and TM (transmembrane). The internal core of the virion is of uncertain symmetry in mature virions but appears in electron micrographs as a centrally located, roughly spherical structure about 30 nm diameter. Immature virions seen during or shortly after budding have a more open, spherical core structure, substantially larger in diameter than the processed one. The core comprises about 2500 copies each of the four *gag*-encoded proteins (as well as protease) and about 100 copies each of reverse transcriptase and integrase.

Properties of the Genome

The avian leukosis virus genome consists of a homodimer of plus sense single-stranded (ss) RNA about 7500 bases in length. Transforming viruses, in which an oncogene has been inserted, have genomes varying in length from about 3.2 kb (for UR2 virus) to about 9.3 kb (for nondefective Rous sarcoma virus). In most of these viruses, the oncogene has replaced some of the normal genome, leading to genetically defective virus, which requires co-infection with helper ALV for replication. The genome is modified in ways resembling cellular mRNAs, reflecting its synthesis and processing by cell machinery. It contains a 5′ m^7GpppGm capping group and a 3′ poly(A) sequence, as well as some internal m^6A residues. In *in vitro* translation systems, it is capable of serving as mRNA for the *gag-pro* and *gag-pro-pol* gene products. As with all retroviruses, the order of genes is 5′-*gag-pro-pol-env*-3′.

Important noncoding regions found near the end of the genome are necessary to provide signals for virus replication. These include an 18–21 base sequence (R) repeated at each end as well as unique sequences U3 (ca 250 bases) near the 3′ end and U5 (ca 80 bases) near the 5′ end which are duplicated into the long terminal repeat (LTR) during reverse transcription. Adjacent to these are the sites for initiation of reverse transcription: the primer binding (PB) region consisting of 18 bases complementary to the 3′ end of $tRNA^{trp}$ next to U5, and a polypurine (PP) sequence next to U3. Between PB and the beginning of *gag* is an approximately 300-base leader region which contains signals important for the packaging of the genome into virions.

Properties of the Proteins

The virion contains nine proteins, the products of four coding regions. The Gag proteins constitute the major structural components and are sufficient to form recognizable virions if expressed alone. The Gag-Pro precursor is processed during release of virus into four proteins: MA (matrix, about 19 kDa) which interacts with the cell membrane; p10, a 10 kDa protein of unknown function and location; CA (capsid, about 27 kDa) which forms the core shell structure; and NC (nucleocapsid, about 12 kDa) an RNA-binding protein necessary for specific encapsidation of genome RNA. The Gag-Pro precursor also contains the 15 kDa PR (protease) peptide necessary for processing all internal virion proteins. The Pol read-

ing frame is expressed as a fusion protein with Gag and Pro and processed to yield reverse transcriptase (RT, usually present as a heterodimer of 98 and 66 kDa reflecting a partial processing by PR), and integrase (IN, about 32 kDa) the two enzymatic activators necessary for synthesis and integration of the DNA provirus. The env protein encodes a precursor (Pr^{env}) which is processed as a membrane protein and cleaved by host cell proteases to yield the SU glycoprotein which has an apparent molecular weight of about 85 kDa, about half of which is due to the provision of ca. 14 N-linked carbohydrate side chains and the TM glycoprotein, which has an apparent molecular weight of about 37 kDa. The SU and TM products remain as a disulfide-bonded heterodimer with SU containing the activity necessary for receptor binding and TM anchoring the structure into the virus envelope.

Physical Properties

Virions of ALV have an equilibrium density in sucrose solutions of about 1.16 g ml^{-1} and a sedimentation coefficient of about 600S. They are quite labile and are readily inactivated by extremes of pH, as well as by heat or mild detergent treatments. They are somewhat radiation resistant, perhaps reflecting the recombinational repair capability provided by the dimeric genome.

Replication

Replication of ALV is like that of other retroviruses, and this group of viruses provided some of the important early models for studying the process. Entry of the virion follows interaction with a specific receptor on the cell surface. Genetically, at least eight subgroups (A–I) have been identified on the basis of distinct receptor recognition. The presence of receptors for specific subgroups is polymorphic among birds. Three unlinked genetic loci for ALV receptors have been genetically identified in chickens. These are called *Tv-a, Tv-b* and *Tv-c*. The dominance of susceptibility over resistance alleles at each of these loci implies that they encode the receptor directly. The *tv-b* locus has several alleles, controlling susceptibility to subgroups B, D and E. The Tv-a and Tv-b receptors have been cloned. They encode quite different membrane proteins with Tv-a resembling a portion of the receptor for low-density lipoprotein and Tv-b, the Fas receptor. Entry of the virion core into the cell is by fusion of viral and cellular members, perhaps following endocytosis.

Once within the cytoplasm of the infected cell, the process of reverse transcription within the poorly defined core structure copies the ssRNA genome into a molecule of double-stranded (ds) DNA. This process – which varies little from that of other retroviruses – includes a series of 'jumps' from one end of the template to the other. The product is a dsDNA molecule which differs from the genomic RNA by the presence at either end of the LTR. The LTR contains sequences necessary for DNA integration, and for synthesis and processing of viral RNA.

Integration of viral DNA into more or less random sites in the cell genome is accomplished by the IN protein which has entered the cell with the virion and remains with the DNA in an ill-defined structure. The process of integration leads to the insertion of the viral DNA into cell DNA in the same general organization as both genome and unintegrated DNA. Integrated ALV DNA is characterized by the loss of two bases from each end of the viral sequence and the duplication of six bases of cell DNA at the integration site.

Transcription of the provirus into genome and mRNA is mediated by cellular RNA polymerase II directed to the correct initiation site by promoter and enhancer sequences in the LTR. The strength of the enhancer elements is a major factor distinguishing pathogenic from nonpathogenic ALV isolates. Unlike some other retroviruses, there is no apparent role of virus-encoded proteins in regulating the transcription process. Processing of the viral transcripts includes addition of poly(A) following a canonical signal (AAUAAA) in the RNA derived from the 3′ LTR and splicing of the fraction of the transcripts destined to become mRNA for the *env* gene. The splicing removes most of the *gag*, *pro* and *pol* sequences, leaving the beginning of *gag* fused to *env*.

Translation of the full-length RNA leads to two products: the Gag-Pro precursor of about 76 kDa and the Gag-Pro-Pol precursor of about 180 kDa. Synthesis of the latter molecule is made possible by a −1 translational frameshift about 5% of the time, bypassing the termination codon at the end of Pro. Assembly of these precursors is at the cell surface, and is coincident with budding, implying a simultaneous association of the precursors with the genome, with the cell membrane, and with one another. The use of readthrough synthesis apparently ensures the correct ratio of RT to Gag proteins in the virion. Release of the immature particle (characterized by a hollow, symmetrical core which almost fills the virion) is rapidly followed by cleavage of the Gag-Pro and Gag-Pro-Pol precursors to yield the finished proteins. This cleavage is accompanied by condensation of the core into its mature form. Since the PR protein embedded in the Gag-Pro precursor contains only one-half of the active site, dimerization of this domain is necessary

for cleavage to occur. This requirement probably helps to delay cleavage until the appropriate time.

Once infected, the host cell is usually not killed by the virus, but can continue to divide and release virions indefinitely. A strong superinfection resistance due to blockade or loss of viral receptors develops soon after infection and prevents accumulation of proviruses by reinfection. In some cases, weak or slow development of superinfection resistance is associated with a cytopathic interaction of the virus with its host cell.

Transformation

A unique characteristic of ALV and a few other retroviruses is their ability to incorporate certain host sequences into their genome and alter the function of these proto-oncogenes into oncogenes. The presence of an oncogene renders the virus capable of inducing malignant transformation of cells in culture and one of a variety of malignant and rapidly fatal diseases in birds. At least 20 distinct cell sequences have been incorporated by ALV into a very large number of distinct isolates. RSV which contains *src* is the prototype oncogene-containing virus. Other notable oncogene-containing ALV variants include avian myeloblastosis virus (AMV; containing *myb*); avian myelocytomatosis virus-29 (MC-29; *myc*); avian erythroblastosis virus (AEV; *erb*-A and *erb*-B); Fujinami sarcoma virus (FSV; *fps*) and University of Rochester sarcoma virus-2 (RU-2; *ros*). Study of the genetic alterations that distinguish these oncogenes from proto-oncogenes, and the enzymatic and physiological function of the proteins they encode has been a keystone of modern cancer research. Incorporation of oncogenes into the virus genome is usually at the expense of some viral genes and co-infection of a cell with a wild-type (helper) ALV is thus necessary to provide viral proteins for replication of oncogene-containing viruses.

Endogenous Viruses

Another unique feature shared by ALV and a few other retrovirus groups is their ability to become established in the germline and inherited stably as endogenous proviruses. Naturally occurring endogenous proviruses form a distinct lineage of ALVs, showing a specific host range (subgroup E) for which many domestic chickens lack receptors (a phenomenon known as xenotropism), and a reduced replication capacity and pathogenicity relative to exogenous viruses. Endogenous viruses are usually expressed at a very low rate, due largely to methylation of CpG residues in the proviral DNA, and are often (but not always) defective in sequence.

Genetics

Strain differences among ALV isolates are primarily in host range and are encoded by differences within the central portion of SU; other parts of the genome, with the exception of the U3 end of the LTR, are quite highly conserved. Like other retroviruses, ALVs exhibit very high rates of homologous recombination – a consequence of the diploid genome and the 'jumping' mechanism of reverse transcription. The latter also permits relatively high rates of non-homologous recombination, leading to frequent (but usually lethal) rearrangements of the genome as well as the occasional acquisition of foreign sequences such as oncogenes.

Evolution

Amino acid sequence relationships reveal a common origin of all retroviruses, but the ALV group forms a divergent branch, with its closest relative being the mouse mammary tumor virus. Whereas the recent spread of viruses among chickens is probably due largely to human intervention, the virus group is of considerable antiquity, since distantly related endogenous viruses are widespread in the genomes of avian and even mammalian species. The closely related endogenous viruses seem to be recent introductions derived by germline infection with exogenous virus since they are found only in *Gallus gallus*, and not in other species of *Gallus*, although they do appear in more distantly related pheasants.

Transmission and Tissue Tropism

Transmission of virus is principally vertical by infection of the offspring through virus secreted into the egg. Indeed, high titers of ALV are often detectable in commercial hen's eggs. Horizontal spread of virus is naturally much more rare, requiring close contact, but virus can be readily spread from infected birds via contaminated needles during vaccination or through vaccines prepared from infected eggs or cell cultures.

All isolates of ALV replicate efficiently in fibroblast cultures and in the bursa. Tropism for other tissues varies among isolates and is determined by both *env* and LTR sequences.

Pathogenicity

ALVs induce a wide spectrum of disease in naturally or experimentally infected animals. The prototypic

disease induced by ALV is a B cell lymphoma arising in the bursa of Fabricius starting a few months after infection and spreading to the liver and other organs during its course (hence the synonym visceral lymphomatosis). Other malignancies, including erythroleukemia, sarcoma and others, are not uncommon depending on the strain of virus and bird and the time and route of inoculation. The malignant diseases induced by viruses which do not contain oncogenes are the consequence of insertional activation of cellular proto-oncogenes (such as c-*myc* in the case of lymphoma, c-*erb*-B in erythroleukemia, and others). In addition to malignancies, these viruses also induce hemangiomas, osteopetrosis and wasting diseases the mechanisms of which are not as clear. In some cases, an immune response against infected cells may be important; in others, cytopathic effects of the sort noted above may play a significant role.

Acquisition of oncogenes by ALVs greatly alters the nature and course of the disease. Infection of newly hatched chicks, with AMV, for example, can lead to their death from myeloblastic leukemia in as few as 5 days. Moribund animals display enormously elevated myeloblast counts and a level of viremia sufficient to render the plasmid noticeably turbid. Similarly, birds inoculated with RSV develop rapidly growing, usually fatal, sarcomas at the site of injection. It should be noted that the oncogene-containing viruses are not efficiently transmitted from one animal to another due to their rapid pathogenicity. In most cases, they have probably arisen in the animal from which they were isolated and would have died out if not brought into the laboratory.

Not all members of this group are highly pathogenic. RAV-0 (an endogenous virus) can infect susceptible chickens and induce viremia, but disease is rare and occurs only after a long latent period. The reduced virulence is probably an important feature of viruses inherited in the germline.

Immune Response

In infected birds, the only significant immune response is the appearance of type-specific neutralizing antibodies which apparently recognize the regions of Env involved in receptor recognition. Group specific responses against Env or other proteins are not usually observed in infected chickens, although inoculation of virus into mammals induces antibodies capable of recognizing all virion proteins in the absence of subgroup-specific reactivity. The limited immune response observed in infected chickens has been attributed to the presence of endogenous proviruses whose expression (even at a low level) can induce tolerance to antigens in common with infecting virus. Indeed, it has been suggested that induction of tolerance might be a desirable feature for the animal, since it could prevent or limit immunopathological sequelae of infection. Postinfection immune response seems to be of little consequence in preventing subsequent malignant disease, since the cells which will eventually form the tumor are probably infected quite soon after infection, and the long latency reflects the necessity for subsequent rare events (such as mutations in other genes) rather than a continuing period of virus replication.

Prevention and Control

ALV-induced disease is a cause of some economic loss to the poultry industry in the United States, and occasional more serious epizootics (such as a recent outbreak of hemangioma in Israel) due to ALV have occurred. Control of infection is generally by detection and culling of infected individuals. No useful vaccination strategy has been developed. In principal, it should be possible to virtually eliminate the disease by breeding the appropriate *Tv-a* and *Tv-b* alleles into commercial strains; in practice this has not been done very often. A more recent strategy is to introduce defective proviruses encoding envelope protein into the germline of birds; these can block infection by inducing superinfection resistance.

Future

Although of economic importance to the poultry industry, the value of ALV and the related oncogene-containing viruses to science has been far greater. Although the use of ALV as a model for retrovirus replication has been largely supplanted by the more directly relevant human retroviruses and advances in technology have led to the development of ways to study oncogenes without complications introduced by viruses. Nevertheless, the study of these viruses as models will continue to illuminate fundamental aspects of retrovirus biology. Also, continued searches for new transforming viruses are likely to yield novel and important oncogenes. Indeed, two of the most interesting viral oncogenes – jun (a transcription factor) and crk (a hybrid tyrosine kinase) – were identified only within the past decade. The goal of eradication of ALV disease from commercial chickens is attainable with present technology; its realization is largely a matter of logistic and economic considerations.

See also: **Bovine leukemia virus (*Retroviridae*); Feline leukemia and sarcoma viruses (*Retroviridae*); Immune response: Cell mediated immune**

response, General features; Murine leukemia viruses (*Retroviridae*).

Further Reading

Coffin JM, Hughes SH and Varmus HE (1997) *Retroviruses*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

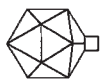
Coffin JM (1996) Retroviridae and their replication. In: Fields B, Knipe D, Howley P (eds) *Fields Virology*, 3rd edn, p. 763. Philadelphia: Lippincott-Raven.

Levy J (ed.) (1992) *The Retroviridae*, vol 1. New York: Plenum.

B

Baboon Herpesvirus *see* **Herpesvirus – Baboon and Chimpanzee**

BACILLUS PHAGE ϕ29 (*PODOVIRIDAE*)



Margarita Salas, Centro de Biología Molecular 'Severo Ochoa' (CSIC-UAM), Universidad Autónoma, Cantoblanco, Madrid, Spain

ϕ29 and Related Phages: General Features

Phage ϕ29, first isolated from soil by B.E. Reilly, and the related phages PZE, PZA, ϕ15, Nf, M2Y, B103, SF5 and GA-1, are the smallest *Bacillus subtilis* phages isolated so far. The phages fall into three serological groups: phages ϕ29, PZE, PZA and ϕ15 form one group, phages Nf, M2Y and B103 another, and phage GA-1, and probably SF5, form a third group.

Phage ϕ29 forms very small plaques on lawns of *B. subtilis* 168, although it productively infects this bacterium in liquid medium. Large plaques are formed on asporogenous variants of strain 168 carrying the A12 mutation. Glucosylated teichoic acid has been shown to be essential for ϕ29 adsorption. Phage ϕ29 infects very efficiently *B. amyloliquefaciens* H. The burst size is about 200, although it varies with the growth conditions. ϕ29 also infects varieties of *B. liqueniformis* and *B. pumilus*. The host ranges of phages GA-1 and SF5 differ from those of the other phages.

ϕ29 is a lytic phage, although it has very little effect on the host cell functions, and macromolecular synthesis remains essentially unaltered until very late in phage development.

When ϕ29 infects sporulating cells phage multiplication is inhibited at a very early stage of cell development and the phage DNA is incorporated into spores in a heat-stable form. After germination and outgrowth of the spore, expression of the incorporated phage genome takes place.

Classification

Phage ϕ29 is an unassigned species in the *Podoviridae* family.

Genetics

Temperature-sensitive (*ts*) and suppressor sensitive (*sus*) mutants of phage ϕ29 are available. Quantitative complementation allowed to locate the mutants in 17 cistrons. Recombination frequencies determined by two-factor crosses were used to construct a linear genetic map of 24.4 recombination units. The genes are numbered sequentially from left to right (1 to 17) according to their relative map position. In addition, gene 8.5 was located between genes 8 and 9 and was shown to code for the head fibers, that are dispensable. Genes 1 to 6 and gene 17 are early genes, whereas genes 7 to 16 are late ones. *Sus* mutants are available in all genes, except gene 5; *ts* mutants are available in most of the genes, except in genes 1, 4, 8.5, 14, 15 and 17.

Comparison of the nucleotide sequence of phages ϕ29, ϕ15 and PZA has shown that deletions in the central and carboxy-terminal parts of gene 17 are tolerated, whereas the region corresponding to 83 amino acids from the amino-end of the protein has to be conserved to encode a functional protein.

Cells infected with a spontaneous deletion mutant of phage ϕ29 with a 1117 bp deletion, that covers almost the complete sequence of genes 14 and 15, including the early promoter B2, support normal phage development, but lysis is very delayed. Gene 14

mutants exhibit a delayed lysis phenotype. Gene 14 encodes a holin, and sequence and biochemical analysis of gene 15 suggest that it encodes a lysozyme, although a possible role in phage morphogenesis has also been described. Therefore, the products of genes 14 and 15, as well as the early B2 promoter, seem to be dispensable for ϕ29 development.

The EcoRI cleavage map of ϕ29 DNA has been ordered relative to the genetic map by marker rescue experiments.

DNA Structure

The ϕ29 genome consists of a double-stranded DNA 19 285 bp long with a protein covalently linked at the two 5′ DNA ends. This protein is the product of the viral gene 3 and it is named p3 or terminal protein (TP). The TP is linked to the DNA as a result of the initiation reaction that it primes in the replication of the viral DNA. The linkage is a phosphoester bond between the OH group of residue serine 232 in the TP and 5′ dAMP, the terminal nucleotide at both 5′ DNA ends. The genome of the ϕ29-related phage PZA is 19 366 bp long and that of phage B103 is 18 630 bp long. The DNA of the other related phages has a similar size, except that of phage GA-1, which is a little longer. The sequence of phage PZA DNA is very similar to that of ϕ29 DNA. Phage M2Y seems to have originated from phage Nf, by deletion of a particular region of the genome. In most of the ϕ29-related phages a TP covalently linked at the 5′ DNA ends has been characterized.

Phage ϕ29 and all the related phages have a short, inverted terminal repeat six nucleotides long (5′ AAAGTA) for ϕ29, PZA, ϕ15 and B103 DNAs, eight nucleotides long (AAAGTAAG) for Nf and M2Y DNAs, and seven nucleotides long (AAATAGA) for GA-1 DNA.

In addition, the sequence of the first 18 nucleotides at the left DNA end and that of the first 13 nucleotides at the right end is identical for phages ϕ29, PZA, ϕ15, Nf, M2Y and B103. There are homologies from nucleotides 19 to 50 at the left DNA end of the latter phages, and an identical sequence from nucleotide 27 to 38 at the right end. The remainder of the known sequence of the DNA of phages ϕ29, PZA and ϕ15 is very similar and different from that of phages Nf, M2Y and B103, which share a similar sequence. The sequence of GA-1 DNA is unrelated to that of the other phages, except for the three terminal nucleotides and the sequence from nucleotides 29 to 41 at the right end, which is almost identical to that present from nucleotides 27 to 38 in the other phage DNAs.

Virion Structure and Proteins

The phage ϕ29 particle, of molecular mass about 18×10^6 Da, consists of a prolate head, a neck and a short, non-contractile tail. The head is 415 Å long and 315 Å wide and contains a high amount of fibers. The neck consists of two collars, an upper one or connector, required for head assembly, and a lower one, from which twelve spindle-shaped appendages are attached. The connector is assembled in a circular structure with a hole in its center and twelve morphological units in the periphery. The appendages are required for adsorption of the phage to the bacterial cell wall. The tail is about 300 Å long and is enlarged at the distal part. Some of the ϕ29-related phages, like M2Y, GA-1 and SF5, lack the fibers, which in ϕ29 have been shown to be dispensable.

By sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis, six structural proteins have been characterized. Protein p8 is the major head protein, p8.5 forms the fibers, p10 forms the connector, p11 is the lower collar protein, p12 forms the neck appendages and p9 forms the tail. Protein p12 is synthesized as a precursor, p12*, that is processed before its assembly in the phage particle.

The open reading frames (ORFs) corresponding to the different genes have been characterized in the ϕ29 DNA sequence. They all start with the ATG triplet and end with either TAA, TAG or TGA termination triplets. In general, the initiation triplet of the different genes is very close to the termination triplet of the preceding one. Overlapping sequences exist between the gene coding for the TP p3 and the transcriptional regulator p4. In the case of genes 8 and 8.5, the termination triplet of gene 8 overlaps with the initiation codon of gene 8.5; for genes 10 and 11, the termination triplet of gene 10 overlaps 8 bp with gene 11. In general, the Shine–Dalgarno sequences are strong and with the correct spacing. The predicted size of the different gene products and their function are shown in **Table 1.** By SDS–polyacrylamide gel electrophoresis all proteins, except the gene 13 and 14 products, have been characterized. All PZA genes are identical in size to the corresponding ones of ϕ29, except genes 1 and 17 that are 8 and 7 amino acids longer, respectively.

In addition to the ORFs corresponding to the genes characterized, three small ORFs that could code for proteins of 47 to 58 amino acids are present downstream of gene 1, at the left end of the ϕ29 and PZA genomes, where early genes are located, and one ORF, that could code for a protein of 66 amino acids, is found between genes 5 and 6. At the left of gene 17, five ORFs are present in ϕ29, PZA, ϕ15 and B103 that could code for proteins of 37 to 130 amino acids.

Table 1 Genes of phage ϕ29

Gene	Protein or phenotype	nt[a]	aa[b]	kDa[b]
1	DNA replication	1126–857	89	10.3
2	DNA polymerase[c]	2863–1145	572	65.2
3	Terminal protein	3686–2886	266	31.1
4	Transcriptional activator	3952–3575	125	15.1
5	ssDNA binding protein	4345–3971	124	13.4
6	dsDNA binding protein	4921–4609	104	12.0
7	Scaffolding protein	5222–5518	98	11.2
8	Major head protein	5549–6895	448	49.7
8.5	Head fiber protein	6895–7737	280	29.6
9	Tail protein	7751–9550	599	67.7
10	Upper collar protein (connector)	9555–10484	309	35.9
11	Lower collar protein	10477–11358	293	33.6
12	Preneck appendage protein	11371–13935	854	92.4
13	Morphogenesis (tail assembly)	13947–15044	365	41.0
14	Lysis	15063–15458	131	14.9
15	Lysozyme, morphogenesis	15460–16236	258	26.9
16	DNA encapsidation	16247–17245	332	38.9
17	DNA replication	19083–18583	167	19.4

[a] Both the initiation and termination codons have been considered.
[b] The initiation methionine was considered for the calculations.
[c] The position of two possible initiation codons is given.

These ORFs have been named 16.9, 16.8, 16.7, 16.6 and 16.5. ORFs 16.7 and 16.5, encoding for 130 and 137 amino acids, respectively, are the most conserved ones among these phages. Computer-assisted analysis of the deduced amino acid sequence of ORF16.7 revealed it to contain a membrane-spanning domain located at its N-terminus which is followed by three positively charged lysine residues. The gene product of ORF16.7 was detected in ϕ29-infected *B. subtilis* cells early after infection. The native protein p16.7 was shown to be an integral membrane protein.

Replication of the Phage DNA

Replicative intermediates

Two types of replicative intermediates are observed by electron microscopy in ϕ29-infected *B. subtilis:* type I molecules which consist of double-stranded DNA with single-stranded tails coming from one or from the two DNA ends, and type II molecules which are partially double-stranded and partially single-stranded. Analysis of these molecules showed that replication starts at either DNA end, non-simultaneously, and proceeds towards the other end by strand-displacement. The same type of replicative intermediates produced *in vivo* are formed in the *in vitro* system described below.

Proteins involved in replication

Genes 1, 2, 3, 5, 6 and 17 are required for the synthesis of ϕ29 DNA *in vivo*. Genes 2 and 3, coding for the DNA polymerase and the TP, respectively, are essential for the initiation of ϕ29 DNA replication, both *in vivo* and *in vitro*. Gene 5 codes for a single-stranded DNA binding protein (SSB) that is essential for elongation in *in vivo* replication; it stimulates *in vitro* replication. Gene 6 codes for a double-stranded DNA binding protein that is also required for *in vivo* replication; it stimulates the initiation of replication *in vitro*. The gene 1 product is a membrane-associated protein, essential for *in vivo* replication, that is likely to recruit TP and DNA polymerase to form a membrane-associated multiprotein complex that would enable nascent viral DNA molecules to become membrane-associated when its synthesis begins. The product of gene 17 is dispensable for *in vivo* replication when infection takes place at high multiplicity, although it is required when low multiplicity of infection is used. Protein p17 stimulates *in vitro* replication under conditions of low input DNA and low amounts of initiation proteins. Development of ϕ29 occurs in most of the *B. subtilis* replication mutants available. Three genes, G, E and T, are involved in the replication of phage M2Y DNA. Genes G and E correspond to the ϕ29 genes 2 and 3, respectively.

ϕ29 genes 2, 3, 5, 6 and 17 have been cloned in *Escherichia coli* under the control of the P_L promoter of phage lambda or under the control of the ϕ10 promoter of phage T7, and the proteins have been overproduced and highly purified in a functional form. Proteins p5 and p6 are very abundant in ϕ29-infected *B. subtilis* and they have also been purified from phage-infected bacteria. Gene 1 has also been cloned under the control of the phage lambda P_L promoter. The overproduced protein has been purified from an insoluble fraction.

Role of TP and DNA polymerase in replication

Replication of ϕ29 DNA occurs by a protein-priming mechanism in which, in the presence of the ϕ29 TP–DNA complex (TP–DNA) as template, dATP and Mg^{2+}, the viral DNA polymerase catalyzes the formation of a covalent linkage between dAMP and the OH group of residue Ser232 of a free TP molecule (priming protein). Mn^{2+} ions are better activators of the initiation reaction than Mg^{2+}, due to a 25-fold decrease in the K_m value for dATP. The initiation reaction is greatly stimulated by NH_4^+ ions because they stabilize the formation of a heterodimer between ϕ29 DNA polymerase and TP. The TP–dAMP complex formed is elongated by the viral DNA polymerase to produce full-length ϕ29 DNA in a very processive way. When primed M13-DNA is used as template, the ϕ29 DNA polymerase synthesizes DNA chains greater than 70 kb, also in a very processive way. Therefore, the ϕ29 DNA polymerase is able to produce strand-displacement very processively without need of accessory proteins. The ϕ29 DNA polymerase, in addition to initiation and polymerization activities, has $3' \rightarrow 5'$ exonuclease activity that is about 10-fold higher on single- than on double-stranded DNA, and shows a marked preference for excision of a mismatched relative to a correctly paired 3′ terminus. These characteristics enable the ϕ29 DNA polymerase to act as a proofreading enzyme. A pyrophosphorolytic activity has also been characterized in the ϕ29 DNA polymerase. This activity requires a high concentration of pyrophosphate and a duplex DNA with a protruding 5′ single-strand, and it is reversed by polymerization.

Replication origins

Proteinase K-treated ϕ29 DNA is not a template for the initiation reaction. However, piperidine-treated ϕ29 DNA is active, although the activity is reduced 5–10-fold from that obtained with TP–DNA. Terminal fragments from the left and right ϕ29 DNA ends have been cloned; fragments released by restriction nuclease digestion, containing the ϕ29 DNA terminal sequences at the DNA ends, are active templates. However, no template activity is obtained with the circular plasmid or when the terminal sequences are not placed at the DNA end. The minimal origins of replication are located within the terminal 12 bp at each ϕ29 DNA end. A change of the second or third A in the ϕ29 DNA sequence into a C completely abolishes the template activity, whereas changes at positions 4 to 12 are tolerated to a great extent.

Although the parental TP seems to be partially dispensable *in vitro*, no replication is obtained *in vivo* after transfection of *B. subtilis* protoplasts with ϕ29 DNA molecules lacking the parental TP.

Single-stranded oligonucleotides with the sequence corresponding to the 3′ terminus of ϕ29 DNA give rise to the formation of the TP–dAMP initiation complex, the preferred nucleotide being dAMP. Initiation activity is also obtained with unspecific single-stranded DNA such as poly(dC), with the formation of TP–dGMP complex. Deoxynucleotidylation of the TP with any of the four dNMPs, catalyzed by the ϕ29 DNA polymerase, is also detected in the absence of any template, although in this case the affinity for the dNTPs is greatly reduced relative to the template-dependent reaction.

Replication of the displaced strand

When recombinant ϕ29 DNA molecules containing parental TP at only one DNA end are used to transfect *B. subtilis* protoplasts, no replication is obtained, suggesting that the fully displaced DNA strand is not an active template for replication *in vivo*. The same type of ϕ29 DNA molecules used as templates in the *in vitro* replication system leads to both an accumulation of full-length single-stranded DNA molecules and a lack of type II replicative intermediates. Moreover, when the TP–DNA complex is used as template, type II replicative intermediates are found at incubation times at which no synthesis of full-length ϕ29 DNA is detected, indicating that the appearance of type II molecules does not require synthesis of full-length DNA and displacement of the strand. Altogether, the results support a model in which initiation of replication can occur from both DNA ends, and type II molecules are produced by separation of the two displacement forks when they meet.

Functional domains in the ϕ29 TP

By deletion analysis, two DNA polymerase-binding regions in the TP have been found, at positions 72–80 and 241–261. On the other hand, the amino acid regions of the TP at positions 13–18, 30–51 and 56–71 are important for DNA binding.

By site-directed mutagenesis, Ser232 in the TP has been changed into Thr. The purified mutant protein is completely inactive in the initiation reaction. A change of Ser232 into Cys results in a TP with about 0.7% of the priming activity of the wild-type TP. Changes of Leu220, Ser223 and Ser226 into Pro give rise to mutant proteins with 3%, 140% and 1% of the priming activity of the wild-type protein, respectively. All the mutant TPs can interact with DNA polymerase and DNA, suggesting that Leu220 and Ser226 are involved in the initiation reaction, in addition to Ser232. These three amino acid residues are conserved in the TP of phages PZA, Nf and B103.

The ϕ29, PZA, Nf, M2Y and B103 TPs have, at positions 256–258, the sequence Arg-Gly-Asp (RGD), found in cell-adhesive proteins. The synthetic peptide RGD, but not RGE, inhibits transfection of ϕ29 and M2Y DNAs, as well as the *in vitro* initiation reaction. A role of the RGD motif in the interaction of the ϕ29 TP with DNA polymerase has been shown.

Residues Asp80 and Tyr82, conserved in the TPs of phages ϕ29, PZA, Nf, B103 and GA-1, are important for the interaction between the primer TP and the parental TP.

By using an *in vivo trans*-complementation assay, it has been shown that the ϕ29 TP functionally substitutes the TP of PZA but not that of Nf.

Functional domains in the ϕ29 DNA polymerase

The ϕ29 DNA polymerase is inhibited by aphidicolin, phosphonoacetic acid and the nucleotide analogues butylanilino dATP (BuAdATP) and butylphenyl dGTP (BuPdGTP), known inhibitors of eukaryotic DNA polymerase α. These functional, as well as structural criteria, have allowed classification of the ϕ29 DNA polymerase as belonging to the family of α-like or eukaryotic-like DNA polymerases. Less related to this group are the *E. coli* DNA polymerase I (pol I)-like DNA polymerases, although significant amino acid sequence similarity has been found among the two groups of DNA polymerases, both at the amino-terminal part and at the carboxy-terminal region.

Three regions of amino acid sequence homology have been found at the amino-terminal part of eukaryotic- and pol I-like DNA polymerases, containing the 3′ → 5′ exonuclease domain in Pol I, that have been named Exo I, Exo II and Exo III. Site-directed mutants in the ϕ29 DNA polymerase at residues Asp12 and Glu14 in region Exo I, Asp66 in region Exo II, and Tyr165 and Asp169 in region Exo III demonstrated a role of these residues in catalysis of the 3′–5′ exonuclease, supporting the idea that the geometry of the Pol I 3′–5′ exonuclease active site and the two-metal ion mechanism proposed for this enzyme can be extrapolated to ϕ29 DNA polymerase. None of those mutants were affected in the initiation and polymerization activities. However, the strand-displacement activity was impaired, indicating that this activity of the ϕ29 DNA polymerase is located in the amino-terminal domain, somehow overlapping with the 3′–5′ exonuclease active site.

Other amino acid residues are structurally and functionally conserved at the exonuclease domain of most prokaryotic and eukaryotic DNA polymerases. Among them, ϕ29 DNA polymerase residues Thr15 and Asn62, located at the Exo I and Exo II motifs, respectively, act as single-stranded DNA ligands, having a critical role in the stabilization of the frayed primer terminus at the 3′–5′ exonuclease active site. These residues do not have a role in the strand-displacement capacity of the ϕ29 DNA polymerase.

An invariant lysine residue, found in the eukaryotic-type DNA polymerases between the Exo II and Exo III motifs, has been shown by site-directed mutagenesis in the ϕ29 DNA polymerase (residue Lys143), to be involved in catalysis at the 3′–5′ exonuclease active site. Mutants at Lys143 also showed a reduced polymerization capacity, but only when DNA synthesis was coupled to strand-displacement.

At the carboxyl part of the DNA polymerases, containing the polymerization domain in Pol I, five main regions of amino acid sequence homology have been found and named 1, 2a, 2b, 3 and 4. Site-directed mutants in the five regions of the ϕ29 DNA polymerase, characterized by the consensus motifs Dx_2SLYP, Kx_3NSxYG, Tx_2GR, YxDTDS and KxY, respectively, have been obtained. The mutational analysis demonstrated that the C-terminal two-thirds of the ϕ29 DNA polymerase polypeptide constitutes the polymerization and protein-primed initiation domains, containing sites for interaction with the metal activator, dNTPs and DNA. Three Asp residues, invariant in all members of the eukaryotic-type DNA polymerases, are involved in metal binding and catalysis at the polymerization active site. These ϕ29 DNA polymerase residues, Asp249 (motif Dx_2SLYP), and Asp456 and Asp458 (motif YxDTDS), are predicted to form a metal binding tripod, analogous to that found in other DNA polymerases. In addition, Arg458 (motif Tx_2GR) was also proposed to play a role in catalysis of the polymerization reaction. Three Tyr residues, and one Lys, invariant or highly conserved in the eukaryotic-type DNA polymerases, are directly or indirectly involved in interaction with dNTPs. These residues are Tyr254 (motif Dx_2SLYP), Lys383 and Tyr390 (motif Kx_3NSxYG), and Tyr454 (motif YxDTDS). Tyr254 and Tyr390 are also involved in nucleotide binding

selection, playing a critical role in the fidelity of DNA replication. Eight residues, invariant or highly conserved in eukaryotic-type DNA polymerases, are involved in binding template-primer structures. These residues are Ser252 (motif Dx_2SLYP), Asn387, Gly391 and Phe393 (motif Kx_3NSxYG), Thr434 and Arg438 (motif Tx_2GR) and Lys498 and Tyr500 (motif KxY). In addition, residues Thr434 and Arg438 (motif Tx_2GR) are involved in TP binding. The 3′–5′ exonuclease activity is not affected in any of the mutants mentioned above.

A conserved motif (YxGG/A), located between the 3′–5′ exonuclease and polymerization domains of eukaryotic-type DNA polymerases, is a DNA binding motif that plays a role in the coordination between DNA synthesis and proofreading.

A C-terminal deletion derivative of ϕ29 DNA polymerase, containing the first 188 N-terminal amino acid residues (including the three Exo motifs), was independently expressed in *E. coli* cells and purified. This N-terminal domain was devoid of any synthetic activity (TP-primed initiation and DNA polymerization), but retained some 3′–5′ exonuclease activity. In addition, a N-terminal deletion derivative of ϕ29 DNA polymerase, lacking the first 188 N-terminal amino acid residues has been expressed in *E. coli* cells and purified. As expected, the 44 kDa C-terminal fragment lacks 3′–5′ exonuclease activity and strand-displacement capacity. On the other hand, it retained polymerase activity when Mn^{2+} was used as metal activator, although the catalytic efficiency was greatly reduced with respect to that of the complete enzyme, and polymerization was distributive, in contrast to the high processivity of the complete ϕ29 DNA polymerase. These polymerization defects could be related to a strong impairment in DNA binding, suggesting that contacts present in the N-terminal domain are important for an optimal stabilization and translocation of the DNA during polymerization. The C-terminal domain also showed a very reduced capacity to initiate TP-primed DNA replication, as a consequence of a weakened interaction with the TP primer and a lack of activation by protein p6. Thus, the C-terminal portion of ϕ29 DNA polymerase (residues 188–575), although having a structural entity as the domain responsible for the synthetic activities, requires the N-terminal domain to provide important contacts for the two different substrates, DNA and TP, that prime DNA synthesis.

Temperature-sensitive mutants available in the ϕ29 DNA polymerase gene, *ts*2 (24) and *ts*2 (98), consist in the change Ala355 into Val (A355V) and Ala492 into Val (A492V), respectively. The A355V mutation is located between regions 1 and 2a, in a conserved region present only in protein-primed DNA polymerases. The mutant DNA polymerase has a *ts* phenotype, both in initiation and elongation, as well as in pyrophosphorolytic activity. The *ts*2 (98) mutant DNA polymerase is rather insensitive to temperature, but its initiation and elongation activities are activated by Mn^{2+} ions to a greater extent than the wild-type ϕ29 DNA polymerase. The *ts*2 (98) mutation is located in region 4.

Gene 6 product

Purified protein p6, a dimer of 24 000 Da, stimulates the formation of the TP–dAMP initiation complex, decreasing the K_m value for dATP. Protein p6 also stimulates the transition from the initiation complex to the first elongation products TP–$(dAMP)_2$ and TP–$(dAMP)_3$. DNAase I footprinting shows that protein p6 binds to double-stranded DNA fragments containing the left or right terminal sequences of ϕ29 DNA, producing a pattern of hypersensitive bands located 24 nucleotides apart along 200–300 bp, flanking protected regions. By hydroxyl radical footprinting, protein p6 bound to ϕ29 DNA terminal fragments gives rise to a specific pattern of protected regions of 3–4 nucleotides regularly spaced every 12 nucleotides, also spanning 200–300 bp, and shifted four nucleotides from each other in the two DNA strands. These regions define the contact sites that are flanked by strong DNaseI hypersensitive sites on alternative strands. The repeated motif, from a strong DNaseI hypersensitive site to the next one in the same DNA strand, contains two contact sites. The fact that the repeated unit is formed by two contact sites suggests that p6 binds as a dimer every 24 nucleotides. Protein p6 interacts with DNA through the minor groove; in agreement with this, distamycin A, which binds in the minor groove, displaces p6 from DNA and inhibits the p6 stimulation of ϕ29 DNA replication. These results and the fact that binding of protein p6 to circular DNA restrains positive supercoiling, support a model in which a DNA right-handed superhelix tightly wraps around a multimeric protein p6 core. The compaction of the DNA in the complex was shown to be 4.2-fold. The parameters that define the path followed by the DNA in the protein p6 complex have been determined: one superhelical turn has 63 bp with a pitch of 5.1 nm and a diameter of 6.6 nm. Consequently, the DNA should be slightly bent (66° every 12 bp) and underwound (11.5 bp/turn).

Main protein p6-recognition sequences are located between nucleotides 62 and 125 at the right ϕ29 DNA end and between nucleotides 46 and 68 at the left end. Since no sequence similarity exists in these regions, protein p6 does not seem to recognize a specific sequence in the DNA but rather a structural feature,

which could be the ability of the DNA to be bent every 12 bp. The activity of protein p6 in the initiation of replication requires the correct positioning of the complex relative to the ϕ29 DNA ends, as shown by insertion of 4 bp, 12 bp or 24 bp between the minimal replication origin and the main protein p6 binding determinants at the right ϕ29 DNA end. As expected, the distance of the p6 molecules with respect to the DNA end is increased in the +4 and +12 mutants by 4 bp and 12 bp, respectively, whereas it is conserved in the +24 mutant. No stimulation of the initiation reaction by p6 is observed in the +4 and +12 mutants, whereas the same stimulation as in the wild-type DNA is observed in the +24 mutant. These results suggest that other proteins involved in the initiation of replication recognize, at a precise position, either the p6 core or the conformational change induced by p6. By using the protein p6 counterparts of phages Nf and GA-1, as well as the TP and DNA polymerase from these phages, in addition to the TP–DNA complexes of ϕ29, Nf and GA-1, it has been shown that the activation of the initiation of replication requires not only the formation of a specific nucleoprotein complex but also its specific recognition by the proteins involved in the initiation of DNA replication.

Deletion mutagenesis has shown that the 14 C-terminal amino acids of protein p6 are dispensable; the activity of the protein decreases with deletions from 23 to 39 amino acids and is undetectable when 44 amino acids are removed. Deletion of 5 amino acids at the N-end greatly decreases the activity of protein p6, that is undetectable after removal of 13 amino acids. These two mutant proteins cannot interact specifically with the ϕ29 DNA ends, but they interfere with the binding of the wild-type protein p6, suggesting that the N-terminal region of protein p6, predicted to form an amphipatic α-helix, is involved in DNA binding. Indeed, site-directed mutants at the polar side of the putative α-helix showed impaired DNA binding and activation of initiation of ϕ29 DNA replication.

Protein p6 is among the most abundant viral proteins in ϕ29-infected *B. subtilis* cells, constituting about 700 000 copies/cell at late infection. By electron microscopy it has been shown that, *in vitro*, protein p6 forms heterogeneously-sized complexes all along ϕ29 DNA, suggesting that p6 may have a role in genome organization, besides its role in replication and transcription (see later).

Gene 5 product

The gene 5 product, essential for *in vivo* ϕ29 DNA replication, is one of the most abundant proteins in ϕ29-infected *B. subtilis* cells, constituting about 1.5×10^6 molecules/cell at late infection times. Purified protein p5 binds to single-stranded DNA, producing a twofold reduction in the DNA length as shown by electron microscopy. No effect of protein p5 on the formation of the TP–dAMP complex has been found. Protein p5 greatly stimulates TP–DNA replication at incubation times when the replication in the absence of protein p5 levels off. Protein p5 has helix-destabilizing activity and stimulates the rate of elongation in ϕ29 DNA replication when ϕ29 DNA polymerase mutants impaired in strand-displacement activity are used. The replication of primed M13-ssDNA is also stimulated by protein p5. Other SSB proteins such as *E. coli* SSB, phage T4 gp32, adenovirus DBP and human RF-A, as well as phage PRD1 protein p12, can functionally substitute for protein p5. Protein p5 binds to single-stranded DNA in the ϕ29 replicative intermediates produced *in vitro*, which are similar in structure to those observed *in vivo*. Altogether, the data available indicate that protein p5 is the ϕ29 SSB protein active during ϕ29 DNA replication.

Interaction of ϕ29 SSB with ssDNA leads to a quenching of its tyrosine-dependent intrinsic fluorescence, with a maximal value of 95%. The stoichiometry (n) of p5 binding to ssDNA is 3–4 nucleotides per ϕ29 SSB monomer. The effective binding constant ($K_{eff} = K_w$) to poly(dT) was 2×10^5 M^{-1}, the intrinsic binding constant (k) and the cooperativity parameter (w) being 4×10^3 M^{-1} and 50–70, respectively. Based on its translational frictional coefficient and its rotational correlation time, ϕ29 SSB has been modeled as a nearly spherical ellipsoid of revolution. The fluorescence properties of the ϕ29 SSB tyrosines (Tyr50, Tyr57 and Tyr76) are consistent with a direct participation in complex formation with single-stranded DNA. Site-directed mutagenesis indicated that, indeed, the three tyrosine residues of ϕ29 SSB, particularly Tyr76, are involved in single-stranded DNA binding.

In vitro amplification of ϕ29 DNA

By using appropriate amounts of four purified ϕ29 replication proteins – TP, DNA polymerase, protein p6 (double-stranded DNA-binding protein) and protein p5 (SSB) – it has been possible to amplify *in vitro* small amounts (0.5 ng) of the 19 285 bp long ϕ29 DNA by three orders of magnitude after 1 h of incubation at 30°C. The quality of the amplified material was demonstrated by transfection experiments, in which the infectivity of the amplified DNA, measured as the ability to produce phage particles,

was identical to that of the natural ϕ29 DNA obtained from virions.

In vitro ϕ29 DNA amplification in the absence of the ϕ29 SSB protein gives rise to the production of short DNA molecules that originate preferentially from the left ϕ29 DNA end. These short molecules consist in an inverted duplication of one of the DNA ends and they are produced by a template-switching mechanism. After their formation they would replicate by means of hairpin intermediates. Amplification of these short DNA molecules requires TP, DNA polymerase and protein p6, but ϕ29 SSB is no longer needed.

A sliding-back mechanism for protein-primed DNA replication

By using as templates synthetic oligonucleotides with either the wild-type sequence at the right or left ϕ29 DNA ends or with mutations at one of the three terminal Ts, it was shown that ϕ29 DNA replication does not start at the 3′ terminal nucleotide, but occurs opposite the second nucleotide at the 3′ end of the template. Nonetheless, all the nucleotides of the template, including the 3′ terminal one, are replicated. Taking these results into account, as well as the need of a terminal repetition of at least two nucleotides for an efficient elongation, a sliding-back model has been proposed with a special transition step from initiation to elongation. After formation of the TP–dAMP initiation complex directed by the second 3′ terminal nucleotide, the TP–dAMP complex would slide backwards to locate the dAMP residue in front of the 3′ terminal T, the second T acting again as a director for the incorporation of the second A residue. A sliding-back mechanism has been also shown to occur in the protein-primed replication of phage GA-1.

The DNA polymerase-primer TP heterodimer is not dissociated immediately after initiation nor in the sliding-back step. There is a transition stage in which the DNA polymerase synthesizes a five nucleotide-long DNA molecule while complexed with the primer TP, undergoes some structural change during replication of nucleotides 6–9, and finally dissociates from the primer protein when nucleotide 10 is inserted into the nascent DNA chain. This behavior probably reflects the polymerase requirement for a DNA primer of a minimum length to efficiently catalyze DNA elongation. The significance of such a limiting transition stage is supported by the finding of abortive replication products consisting of the primer TP linked up to eight nucleotides, detected during *in vitro* replication of TP–DNA, particularly under conditions that decrease the strand displacement capacity of ϕ29 DNA polymerase.

Transcription of the Phage ϕ29 Genome

Transcription map

Transcription of the early genes, located at the two ϕ29 DNA ends, occurs from right to left and is initiated at eight promoters named A1, A2a, A2b, A2c, B1, B2, C1 and C2, recognized by the *B. subtilis* σ^A RNA polymerase. Transcription of the late genes, clustered in the midle of the genome, takes place from left to right from a single promoter named A3 and requires, in addition to the σ^A RNA polymerase, the product of the viral early gene 4. Transcription starting at the A2a, A2b and A2c promoters, at the left part of the genome, gives rise to the viral proteins p6, p5, p4, p3, p2 and p1. The A1 promoter, located at the left DNA end, is involved in the production of a 174 nucleotides-long RNA, involved in the packaging of ϕ29 DNA into the proheads. The weak promoters B1 and B2 are located at the central part of the genome in a region coding for late genes, in which no ORF of significant length coding for early genes is present. The transcripts originating from the B1 and B2 promoters may act as antisense RNAs that would downregulate the expression of particular genes of the late polycistronic mRNA. Transcription from the C2 promoter, at the right ϕ29 DNA end, gives rise to protein p17. No function has been proposed for transcripts initiated at the C1 promoter, also located at the right part of the genome, although they contain the sequence for two small ORFs that are conserved among phages ϕ29, PZA, ϕ15 and B103. Transcription initiated at the late A3 promoter gives rise to the late proteins p7, p8, p8.5, p9, p10, p11, p12, p13, p14, p15 and p16, and does not require synthesis of the viral DNA. The eight early promoters have consensus sequences that correspond to the ones recognized by the *B. subtilis* σ^A RNA polymerase (TTGACA and TATAAT at the −35 and −10 regions, respectively). The late promoter has the TATAAT sequence at the −10 region, but no consensus sequence is present at the −35 region.

Termination of transcription of the early genes located at the left end of the genome occurs both at the very end of the DNA and at the beginning of gene 4, at the ρ-independent terminator named TA1. Therefore, a significant number of transcripts terminate at TA1 and code only for proteins p6 and p5, that are synthesized in large amounts in ϕ29-infected cells. Transcription termination of the early gene(s) located at the right ϕ29 DNA end, initiated at the C1 and C2 promoters, occurs at the ρ-independent bidirectional

terminator named TD1. Transcripts starting at the B2 promoter terminate at terminators TB1 and TB2 whereas those starting at promoter B1 do not terminate at a defined position. Transcription starting at the late promoter A3 terminates at the bidirectional terminator TD1.

Transcription regulation

***In vivo* transcription** The abundance of the transcripts produced from each viral promoter throughout the infection cycle has been analyzed. The early promoters A1, A2b, A2c and to a lesser extent, C2, and the late promoter A3 are the strongest ϕ29 promoters. Interestingly, protein p4, in addition to activate the late A3 promoter, is a repressor of the early promoters A2b and A2c, although repression is not complete and both promoters are still active at late times of infection.

***In vitro* transcription** The gene 4 product, required for *in vivo* transcription from the late A3 promoter and acting also as a repressor of the A2b and A2c promoters, has been cloned in *E. coli* under the control of the P_L promoter of phage lambda, and protein p4 has been overproduced. The purified protein p4 promotes the *in vitro* transcription from the late promoter when added to the *B. subtilis* σ^A RNA polymerase. Protein p4 has been characterized as a transcriptional activator required for the binding of σ^A RNA polymerase to the A3 promoter. By DNAaseI footprint analysis protein p4 was shown to bind to a region from nucleotides -58 to -104 from the transcription start site. The sequences specifically recognized by protein p4 are included in two 8 bp inverted sequences, as shown by hydroxyl-radical interference and methylation interference experiments. The protein p4-binding region in the A3 promoter has a sequence-dependent curvature of about 45°. Binding of protein p4 increases bending of the above DNA region to 80–85°.

Protein p4, 125 amino acids long, is a dimer in solution, and binds to DNA as a tetramer. The analysis of protein p4 mutants with deletions and substitutions at the carboxyl end, which is positively charged, have indicated that DNA bending induced by protein p4 has a role in the activation of transcription. Bending would be induced in two steps: first, binding of two dimers of protein p4 to the inverted recognition sequences and interaction between them, would bend the DNA between the inverted repeats; then, non-specific electrostatic interaction between the positively charged carboxyl-end of the protein and the negatively charged DNA backbone would produce bending at both sides of the protein p4 binding sites. Deletion of the first five amino acids at the amino-end of protein p4 gives rise to a stable protein that does not bind to the promoter.

Binding of protein p4 to its recognition region induces the binding of σ^A RNA polymerase to the late promoter through the formation of a closed complex. There is evidence indicating that direct contacts between protein p4 and σ^A RNA polymerase do exist. Interestingly, protein p4 deletion mutants at the carboxyl-end that do not induce a complete DNA bending are not active as transcriptional activators and do not favor the binding of σ^A RNA polymerase to the promoter. The curvature increase induced by protein p4 is likely to correctly orient protein p4 towards the σ^A RNA polymerase so that adequate contacts can be made. Activation at a distance by protein p4 can occur, and it seems to involve a DNA loop held by the interaction of protein p4 and σ^A RNA polymerase. Site-directed mutants at the carboxyl-end of protein p4 have been obtained. The results showed that Arg120 is the most critical residue for activation, probably mediating the interaction with RNA polymerase. Several basic residues have been identified, including Arg120, that contribute to maintain the DNA bending, probably via electrostatic interactions with the DNA backbone. The degree or stability of the induced bend apparently relies on the additive contribution of all basic residues of the carboxyl end of the protein. Therefore, the activation and DNA bending surfaces overlap, and Arg120 should interact with both DNA and RNA polymerase. Since protein p4 is a dimer in solution and binds to DNA as a tetramer, a model has been proposed in which two of the p4 subunits interact with the DNA, bending it, while the other two subunits remain accessible to interact with the RNA polymerase.

Protein p4 promotes the binding of purified *B. subtilis* RNA polymerase α subunit to the A3 promoter in a cooperative way. Binding is abolished by deletion of the last 15 amino acids of the α subunit. RNA polymerase reconstituted with a deleted α subunit lacking 15 amino acids at the carboxyl end could transcribe viral promoters not activated by protein p4, but lost the ability to recognize the A3 promoter in the presence of p4. In addition, the reconstituted RNA polymerase could not interact with protein p4. Thus, protein p4 activation of the A3 promoter requires an interaction between the carboxyl end of p4 and the carboxyl end of the α subunit of *B. subtilis* RNA polymerase to stabilize the RNA polymerase at the promoter.

Protein p4 does not interact with the *E. coli* RNA polymerase and cannot activate transcription with this enzyme. A chimerical α subunit containing the amino-terminal domain of the *E. coli* α subunit and

the carboxy-terminal domain (CTD) of the *B. subtilis* α subunit was reconstituted with the *E. coli* β and β' subunits and the vegetative σ factor from either *E. coli* (σ^{70}) or *B. subtilis* (σ^{A}). Both hybrid enzymes were responsive to protein p4 and efficiently supported activation at the A3 promoter through the same activation surface used with *B. subtilis* RNA polymerase. Therefore, the *B. subtilis* α-CTD allows activation by p4 even when the rest of the RNA polymerase subunits belong to *E. coli,* a distantly related bacteria.

Protein p4 binding site at the late A3 promoter overlaps with part of the main early promoter A2b. *In vitro* studies have shown that protein p4 represses the A2b promoter by excluding σ^{A} RNA polymerase from the latter directing it to the late promoter. In addition, the curvature induced by p4 binding impairs transcription from the A2b promoter.

Protein p4 also represses the early A2c promoter by binding to DNA immediately upstream from RNA polymerase in a way that does not hinder RNA polymerase binding; rather, the two proteins bind cooperatively to DNA. In the presence of p4, RNA polymerase can form an initiated complex at the A2c promoter that generates short abortive transcripts, but cannot leave the promoter. Mutation of protein p4 residue Arg120, which relieves the contact between the two proteins, leads to a loss of repression. Protein p4 could form a complex at the A2c promoter with wild-type α subunit, but not with a deletion mutant lacking the 15 carboxy-terminal amino acids. In addition, promoter repression by p4 was impaired when a reconstituted RNA polymerase lacking the 15 carboxy-terminal amino acids of the α subunit was used. Protein p4 could not interact with this mutant RNA polymerase at promoter A2c. Thus, the carboxy-terminal domain of the α subunit can receive regulatory signals, not only from transcriptional activators but also from repressors. On the other hand, the contact between protein p4 and RNA polymerase through the p4 domain containing Arg120 can activate or repress transcription, depending on the promoter. The position of protein p4 relative to that of RNA polymerase, which is different at the A3 and A2c promoters does not dictate the outcome of the interaction. Rather, it is the absence or presence of a -35 consensus box for the vegetative RNA polymerase that determines whether activation or repression occurs. Thus, stabilization of RNA polymerase at the promoter over a threshold level leads to repression.

The early protein p6, which stimulates DNA replication, modulates the expression of the right early genes repressing transcription from the C1 and C2 promoters. In addition, protein p6 represses transcription from the early A2c promoter, but only in the presence of protein p4. Thus, early transcripts from the left ϕ29 DNA end are repressed at two levels. On the one hand by repression of the A2b and A2c promoters by p4. On the other, by repression of the A2c promoter by protein p6 when p4 is present. Nonetheless, since *in vivo* repression of the early genes transcribed from the A2b and A2c promoters is not complete, it is likely that protein p4 is produced in limiting amounts, not being bound to all viral DNA molecules present in the cell at a given time.

Studies of the consequences on replication of collisions between ϕ29 DNA polymerase and the transcription machinery showed that codirectional collisions with stalled transcription ternary complexes at the early ϕ29 promoters C2, C1, B2 and B1 blocked replication fork progression. Upon collision, the DNA polymerase remained on the template and was able to resume elongation once the RNA polymerase was allowed to move. Collisions with RNA molecules moving in the same direction also interfered with replication, causing a decrease in the replication rate. It is likely that temporal regulation of transcription and the low probability that the replication and transcription processes colocalize *in vivo* contribute to achieving minimal interference between the two events.

Morphogenesis of Phage ϕ29

Phage ϕ29 assembly occurs by a single pathway in which the neck and tail proteins are incorporated into the DNA-containing head.

As a first step in ϕ29 morphogenesis the major head protein p8 and the nonstructural protein p7 (scaffolding protein) interact to get head assembly. The fiber protein p8.5 is not needed since phage ϕ29 mutants lacking this protein produce fiberless infective phage particles. To get a prolate head, the connector protein p10 must be functional since mutants in cistron 10 produce isometric heads; these heads have an electron-dense internal core and they contain, in addition to proteins p8 and p8.5, the nonstructural protein p7. In addition, the *in vivo* interaction of only proteins p7 and p8 produces particles with variable size and shape. However, in the presence of the connector protein p10, particles with uniform size and shape are produced. The scaffolding protein p7 and the connector protein p10 have been shown to interact *in vitro*. Proteolytic cleavage of p10 that results in the loss of 16 and 19 amino acids from the amino- and carboxy-termini, respectively, abolish the DNA-binding activity of the protein, but the general shape and size of the connector is not changed, as revealed by electron microscopy. Mutation of the three acidic

amino acids at the carboxyl end of p7 prevented it from interacting with p8.

In the absence of DNA synthesis or of protein p16 a prolate head more rounded at the corners than complete phage particles, and with an internal core is accumulated, that contains the head proteins p8 and p8.5, the connector protein p10 and the nonstructural protein p7. These particles seem to be intermediates in phage morphogenesis.

In order to get DNA encapsidation the tail protein p9 must be functional since infection with gene 9 mutants gives rise to prolate heads similar to the ones described above but containing, in addition to proteins p8, p8.5, p10 and p7, the nonstructural proteins p15 and p16. The latter is a DNA-packaging ATPase.

Once DNA encapsidation occurs, mediated by protein p16, the nonstructural proteins p7, p15 and p16 are no longer present in the assembly intermediates. The lower collar protein p11 is then assembled into the DNA-containing heads, followed by the tail protein p9, that requires the nonstructural protein p13 for functional assembly. Finally, the precursor of the neck appendages, p12*, once proteolytically processed into the mature protein p12, is assembled into the tail-containing particles.

A highly sensitive system for the *in vitro* assembly of phage ϕ29 has been developed. The system consists of: (1) recombinant procapsids obtained from *E. coli* extracts; (2) TP–DNA; (3) protein p16; (4) packaging RNA (pRNA; see below); (5) tail protein expressed in *E. coli* alone or coexpressed with the structural proteins of recombinant procapsids in *E. coli;* (6) extracts containing the coexpressed lower collar protein p11, the appendage protein p12 and the morphogenetic protein p13; (7) ATP.

DNA Encapsidation

In vitro encapsidation of TP–DNA has been obtained using extracts containing ϕ29 proheads and protein p16. Proteinase K-treated ϕ29 DNA is not packaged. Restriction nuclease digestion of TP–DNA shows that left end fragments of sizes ranging from 0.9×10^6 to 8×10^6 Da are selectively packaged. The packaging of a left end fragment of 4.1×10^6 Da (one-third of the genome) results in the exit of the scaffolding protein p7 from the proheads and the transition to the angular morphology characteristic of the complete ϕ29 particles. The oriented packaging from the left end is in agreement with the finding that this DNA end is the last to be released from the phage particles. When a system consisting of purified proheads and protein p16 is used, both left and right end TP–DNA fragments are packaged, although the efficiency of right end fragment packaging is about threefold lower than that of the left end. Internal fragments are also packaged, with efficiencies of 10–15%. Selective packaging of TP–DNA left ends is restored by the addition of bacterial cell extracts or glycerol.

The steps in *in vitro* packaging of TP–DNA in the purified system have been determined. Protein p16 first binds to and is modified by the prohead. The prohead–p16 complex then binds to TP–DNA and gives rise to a second modification of protein p16 that allows binding of ATP. The hydrolysis of ATP occurs concomitant with TP–DNA packaging. Binding and hydrolysis of ATP are dependent on both prohead and TP–DNA. Approximately one molecule of ATP has been reported to be used in the packaging of 2 bp of DNA. Protein p16 contains both the 'A-type' and the 'B-type' ATP-binding consensus sequences and the predicted secondary structure for ATP binding.

A small RNA 174 nucleotides long has been found in the proheads that are active for DNA packaging. Similar RNA has been isolated from the proheads of the ϕ29-related phages ϕ15 and SF5. Such RNA is not present in the mature virion. This small RNA is transcribed from the early A1 promoter, located at the left ϕ29 DNA end. During prohead purification 54 residues are removed from the 3′ end by nucleases giving rise to a 120 nucleotides-long pRNA fully active in prohead binding and DNA packaging. A conformational change of pRNA seems to occur in the presence of Mg^{2+} that leads to prohead binding. On the other hand, TP–DNA has a maturation pathway in which TP and the packaging protein p16 supercoil the DNA ends, probably as a prerequisite for efficient interaction with the prohead.

Both 5′ and 3′ ends of the 120 nucleotides-long pRNA are essential for DNA packaging and they become proximate in the formation of secondary structure. A deletion analysis showed that bases 6–113 comprised the minimum sequence required for full connector binding activity; connectors are the structural component in the procapsids to which pRNA binds. Bases 1–117 comprised the minimum sequence needed for full packaging activity. These data indicate that the helical region composed of bases 1–6 and 113–117 plays a crucial role in DNA translocation, but is dispensable for connector binding.

Mutant pRNA that prevents the formation of secondary structure at the 5′ and 3′ ends completely blocked *in vivo* phage assembly in cells expressing such a mutant pRNA. Phage ϕ29 assembly was also severely reduced *in vitro* in the presence of the mutant pRNA. On the other hand, mutation of the DNA packaging domain resulted in a pRNA with no DNA

packaging activity but intact procapsid binding capacity.

Six copies of the pRNA are present in the procapsids isolated from phage-infected cells. The pRNA associates with the procapsid during the DNA translocation process and leaves the procapsid after the DNA packaging reaction is complete. Evidence has been obtained that suggests that the six pRNAs per procapsid that are required for DNA packaging act consecutively. Breaking of any one of the individual links would ressult in the termination of the process.

Proheads of bacteriophage lambda, that contain the ϕ29 connector instead of that of lambda, have been obtained *in vitro*. The chimeric proheads can package both lambda and ϕ29 DNA. Thus, the connector domains involved in both head assembly and DNA packaging seem to be functionally similar in phages ϕ29 and lambda. Moreover, either the ϕ29 protein p16 or the lambda terminase complex can be used in the *in vitro* packaging system. Treatment of the proheads with RNAase A abolish packaging activity, that is restored by the addition of *E. coli* ribosomal RNAs. The DNA-containing λ–ϕ29 proheads can be complemented *in vitro* with ϕ29 proteins to yield infective particles capable of DNA transfer.

See also: ***Bacillus subtilis* phages.**

Further Reading

Anderson DL and Reilly BE (1976) Analysis of gene function in *Bacillus subtilis* bacteriophage ϕ29. In: Schlesinger D (ed.) *Microbiology 1976*, p. 254. Washington: American Society for Microbiology.

Anderson DL and Reilly BE (1993) Bacteriophage ϕ29 morphogenesis. In: Sonenstein AL, Hoch JA and Losick R (eds) Bacillus subtilis *and Other Gram-positive Bacteria: Biochemistry, Physiology and Molecular Genetics*, p. 859. Washington: American Society for Microbiology.

Blanco L and Salas M (1995) Bacteriophage ϕ29 DNA polymerase. In: Eckskein F and Lilley DMJ (eds) *Nucleic Acids and Molecular Biology*, vol. 9, p. 328. Berlin: Springer-Verlag.

Blanco L and Salas M (1996) Relating structure to function in ϕ29 DNA polymerase. *J. Biol. Chem.* 271: 8509.

Garver K and Guo P (1997) Boundary of pRNA functional domains and minimum pRNA sequence requirement for specific connector binding and DNA packaging of phage ϕ29. *RNA* 3: 1068.

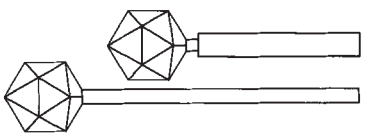
Geiduscheck EP and Ito J (1982) Regulatory mechanisms in the development of lytic bacteriophages in *Bacillus subtilis*. In: Dubnau DA (ed.) *The Molecular Biology of the Bacilli*, vol. I, p. 203. New York: Academic Press.

Salas M (1988) Phages with protein attached to the DNA ends. In: Calendar R (ed.) *The Bacteriophages*, p. 169. New York: Plenum Press.

Salas M (1991) Protein-priming of DNA replication. *Annu. Rev. Biochem.* 60: 39.

Salas M and Rojo F (1993) Replication and transcription of bacteriophage ϕ29 DNA. In: Sonensheim AL, Hoch JA and Losick R (eds) Bacillus subtilis *and Other Gram-positive Bacteria: Biochemistry, Physiology and Molecular Genetics*, p. 843. Washington: American Society for Microbiology.

BACILLUS SUBTILIS PHAGES



H. Ernest Hemphill, Department of Biology, Syracuse University, Syracuse, New York, USA

Introduction

Bacillus subtilis and related species of bacilli are common inhabitants of the soil. This same environment has been the source of a large variety of phages which infect these bacteria. Most have been little studied and are poorly characterized, but there are a few generalities. All known *B. subtilis* phages contain double-stranded (ds) DNA; neither single-stranded DNA nor RNA phages have been reported. The virions of *B. subtilis* phages have icosohedral heads and are tailed; helical forms have not been discovered. *B. subtilis* phages are commonly grouped into virulent phages which infect cells and complete their life cycles within a well-defined period of time, and temperate phages which are able to lysogenize their host. A third category of convenience, 'pseudotemperate phages', includes virulent phages with an extended and irregular latent period that mimics lysogeny, but does not involve a stable prophage.

packaging activity but intact procapsid binding capacity.

Six copies of the pRNA are present in the procapsids isolated from phage-infected cells. The pRNA associates with the procapsid during the DNA translocation process and leaves the procapsid after the DNA packaging reaction is complete. Evidence has been obtained that suggests that the six pRNAs per procapsid that are required for DNA packaging act consecutively. Breaking of any one of the individual links would ressult in the termination of the process.

Proheads of bacteriophage lambda, that contain the ϕ29 connector instead of that of lambda, have been obtained *in vitro*. The chimeric proheads can package both lambda and ϕ29 DNA. Thus, the connector domains involved in both head assembly and DNA packaging seem to be functionally similar in phages ϕ29 and lambda. Moreover, either the ϕ29 protein p16 or the lambda terminase complex can be used in the *in vitro* packaging system. Treatment of the proheads with RNAase A abolish packaging activity, that is restored by the addition of *E. coli* ribosomal RNAs. The DNA-containing λ–ϕ29 proheads can be complemented *in vitro* with ϕ29 proteins to yield infective particles capable of DNA transfer.

See also: ***Bacillus subtilis* phages.**

Further Reading

Anderson DL and Reilly BE (1976) Analysis of gene function in *Bacillus subtilis* bacteriophage ϕ29. In: Schlesinger D (ed.) *Microbiology 1976*, p. 254. Washington: American Society for Microbiology.

Anderson DL and Reilly BE (1993) Bacteriophage ϕ29 morphogenesis. In: Sonenstein AL, Hoch JA and Losick R (eds) Bacillus subtilis *and Other Gram-positive Bacteria: Biochemistry, Physiology and Molecular Genetics*, p. 859. Washington: American Society for Microbiology.

Blanco L and Salas M (1995) Bacteriophage ϕ29 DNA polymerase. In: Eckskein F and Lilley DMJ (eds) *Nucleic Acids and Molecular Biology*, vol. 9, p. 328. Berlin: Springer-Verlag.

Blanco L and Salas M (1996) Relating structure to function in ϕ29 DNA polymerase. *J. Biol. Chem.* 271: 8509.

Garver K and Guo P (1997) Boundary of pRNA functional domains and minimum pRNA sequence requirement for specific connector binding and DNA packaging of phage ϕ29. *RNA* 3: 1068.

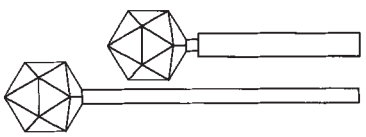
Geiduscheck EP and Ito J (1982) Regulatory mechanisms in the development of lytic bacteriophages in *Bacillus subtilis*. In: Dubnau DA (ed.) *The Molecular Biology of the Bacilli*, vol. I, p. 203. New York: Academic Press.

Salas M (1988) Phages with protein attached to the DNA ends. In: Calendar R (ed.) *The Bacteriophages*, p. 169. New York: Plenum Press.

Salas M (1991) Protein-priming of DNA replication. *Annu. Rev. Biochem.* 60: 39.

Salas M and Rojo F (1993) Replication and transcription of bacteriophage ϕ29 DNA. In: Sonensheim AL, Hoch JA and Losick R (eds) Bacillus subtilis *and Other Gram-positive Bacteria: Biochemistry, Physiology and Molecular Genetics*, p. 843. Washington: American Society for Microbiology.

BACILLUS SUBTILIS PHAGES



H. Ernest Hemphill, Department of Biology, Syracuse University, Syracuse, New York, USA

Introduction

Bacillus subtilis and related species of bacilli are common inhabitants of the soil. This same environment has been the source of a large variety of phages which infect these bacteria. Most have been little studied and are poorly characterized, but there are a few generalities. All known *B. subtilis* phages contain double-stranded (ds) DNA; neither single-stranded DNA nor RNA phages have been reported. The virions of *B. subtilis* phages have icosohedral heads and are tailed; helical forms have not been discovered. *B. subtilis* phages are commonly grouped into virulent phages which infect cells and complete their life cycles within a well-defined period of time, and temperate phages which are able to lysogenize their host. A third category of convenience, 'pseudotemperate phages', includes virulent phages with an extended and irregular latent period that mimics lysogeny, but does not involve a stable prophage.

Table 1 Representative *B. subtilis* phages

Phage	Size (nm) Head	Tail	DNA size (kb)	Related phages	Comments
Virulent phages					
SPO1	87	19 × 140 plus 25 × 60 baseplate	145	SP82, ϕ25, ϕe, SP8	Hydroxymethyluracil replaces thymine in DNA
SP50	80	25 × 70	140	ϕ1	
SPP1	45	6.5 × 140	41	SF6	
ϕ29	31.5 × 41.5	6 × 32.5	1.9	ϕ15, NF	Virion DNA covalently linked to a terminal protein
Pseudotemperate phages					
PBS1	120	22 × 240	290	PMB12	Uracil replaces thymine in DNA
SP15	120	15 × 250	385		5-dihydroxypentyl uracil partially replaces thymine; DNA glucosylated
SP10	90	17 × 160	120	PMB1	DNA contains some α-glutamylthymine
Temperate phages					
ϕ105	52	10 × 220	40	ρ6, ρ10, ρ14	
SPO2	50	10 × 180	40		
SPβ	72 × 82	12 × 358	126	ρ11, ϕ3, Z, SPR, I61, I63, I64, H1	
PBSX	45	20 × 200	13	PBSZ	Defective phage, head contains bacterial DNA

The species *B. subtilis* includes strains which show considerable variation from one another, and the distinction between *B. subtilis* and related species such as *B. pumilus*, *B. amyloliquefaciens* and *B. licheniformis* is sometimes ambiguous. A particular phage may be capable of infecting strains labeled as different species, but unable to infect all isolates of *B. subtilis*. Most of the phages discussed here are able to productively infect *B. subtilis* strain 168. This bacterium is John Spizizen's original transformable *Bacillus*, and is thought to be derived from the type species *B. subtilis* Marburg. ϕ29 and SP16 which are included in this survey infect strain 168, but grow equally well or better on other species.

Table 1 lists some *B. subtilis* phages of current interest. Those in which the molecular biology and genetics have been analyzed in detail are considered below.

Virulent Phages

SPO1

Phage SPO1 is representative of a group of common phages distinguished by the fact that their DNA contains a base substitution of hydroxymethyluracil (hmUra) in place of thymine. The virion has an icosahedral head and a complex 140 nm tail; the latter includes a central tube and contractile sheath and terminates in an elaborate baseplate 25 × 60 nm. The head contains 145 kilobase-pairs (kb) of DNA, which includes a 12.6 kb region of terminal redundancy as a direct repeat.

Phage SPO1 (and relatives SP82, 2C and ϕe) contain a group I, self-splicing intron within the structural gene for the DNA polymerase essential for phage replication. The intron contains an open reading frame encoding an endonuclease, although one with unique cleavage and DNA preferences compared to 'homing' endonucleases known in other introns. The intron in SPO1 was the first reported in Gram-positive bacteria. Subsequently, another group I self-splicing intron was reported in the thymidylate synthetase gene of the unrelated *B. subtilis* phage β22.

RNA transcription in SPO1 is conveniently divided into three temporally distinguishable phases called early, middle and late, which are regulated by a cascade of 'sigma factors' that modify the bacterial RNA polymerase. Viral RNA synthesis begins about 1 min after infection from promoters of early genes located predominantly in the regions of terminal redundancy. The synthesis of early RNA is catalyzed by unmodified host RNA polymerase. As expected, the early promoters have consensus nucleotide sequences (allowing for the hmUra substitution) similar to those of *B. subtilis* chromosomal genes read by core polymerase in conjunction with σ^A, the primary host sigma factor. Among the early genes is one (gene *28*)

which encodes a 220 amino acid protein (gp28) that binds to the bacterial core RNA polymerase, partially replacing σ^A. Beginning 4 min after infection, the modified enzyme initiates transcription of middle genes by recognizing novel 'middle' promoters which precede these genes. These promoters have a consensus sequence quite different from those associated with early SPO1 genes. Two middle genes, *33* and *34*, encode proteins gp33 (101 amino acids) and gp34 (197 amino acids) which further modify the host RNA polymerase allowing it to initiate transcription from a third class of promoters preceding late genes. These, too, have a unique consensus sequence. As the SPO1 latent period progresses, not only are new transcripts synthesized, but early and middle genes are shut-off. The repression of middle genes appears to be at least partially associated with SPO1-encoded DNA-binding protein TF1, because mutations in the *TF1* gene continue to synthesize some middle gene products. TF1 protein binds preferentially to specific sites within the hmUra-containing SPO1 DNA, causing bending of the DNA. It is possible that this bending interferes with transcription of middle genes.

Host DNA, RNA and protein syntheses are almost completely suppressed by 6–8 min after SPO1 infection, although there is no detectable degradation of the bacterial genome. The inhibition of RNA synthesis is selective, with transcription of rRNA and the mRNA for ribosomal proteins continuing late into infection. It is likely the reduction of general protein synthesis results from the failure to produce associated mRNAs. At least some of the suppression of RNA synthesis is associated with the product of SPO1 gene *e3*, which appears to target the β subunit of RNA polymerase, possibly causing premature termination of transcription. Since early phage RNAs are synthesized using unmodified host RNA polymerase, it is possible E3 protein may also play a role in suppression of transcription of early phage genes as the infection progresses.

ϕ29

ϕ29 with a genome size of 19 285 base pairs is among the smallest dsDNA viruses. A total of 18 genes are known. Seven genes controlling early functions expressed immediately after infection are located at both ends of the genome, and are transcribed in the same polarity. Eleven late genes are located near the center of the genome, and are transcribed in opposite polarity on the complementary DNA strand from the early genes. The ϕ29 virion contains only seven types of structural proteins, but is surprisingly complex, with intricate head projections and tail appendages originating near the collar.

Synthesis of both early and late ϕ29 RNA is catalyzed by unmodified host RNA polymerase. The initiation of late transcription requires protein p4, the product of early gene 4. This protein apparently binds to ϕ29 DNA in the region of the single late promoter A3, and acts as a transcription activator of late genes. However, promoter A3 is located within a complex of early promoters A2a, A2b and A2c, and binding of p4 represses the transcription from A2b and A2c even as it activates late functions. Whether this repression has a regulatory role during infection is not clear, because p4 apparently simultaneously increases transcription from A3 and early promoter A2a. The latter is thought to serve as a promoter for the same genes as does A2b.

Several transcription terminators have been localized on the ϕ29 genome. All have stem–loop structures followed by uridine-rich sequences, suggesting transcription termination in ϕ29 occurs by a rho-independent mechanism. Variations in the efficiency of terminators may play a role in regulating the quantities of specific transcripts and their associated proteins made during the infection. Two promoters give rise to transcripts that do not contain open reading frames, and it has been proposed that these are antisense RNAs which downregulate certain late genes.

ϕ29 DNA molecules isolated from viral particles have a 31 kDa terminal protein (TP) covalently linked at the two 5′ ends, and are in the form of circles and concatamers. Proteolytic cleavage converts the DNA into unit-length linear molecules, but simultaneously results in loss of transfecting activity. This suggests the ϕ29 DNA–TP complex is required for DNA synthesis. In a purified system, synthesis of new DNA strands is initiated at either DNA end when free molecules of TP, in the presence of an already existing ϕ29 DNA–protein TP complex as template, react with dATP to form a phosphodiester bond between the hydroxyl group of a serine at position 232 on the protein and 5′-dAMP. The generation of this protein–nucleotide complex is one of two reactions catalyzed by ϕ29 DNA polymerase. Subsequently, the newly synthesized TP–dAMP covalent complex serves as a primer to provide the 3′-OH group needed for elongation of a new strand of DNA, a reaction also catalyzed by the ϕ29 DNA polymerase. *In vivo*, as many as six phage genes appear to be involved in DNA synthesis, although the functions of some are not clear. The product of viral gene *6* (p6) stimulates formation of the TP–dAMP initiation complex, and the product of viral gene *5* is probably a single-stranded DNA-binding protein.

ϕ29 has been the focus of extensive studies of virion assembly and morphogenesis. *In vitro*, a precursor

prohead composed largely of a single capsid protein and a portal protein packages the phage DNA when it is complexed to virus protein gp3. The packaging requires an ATPase (virus protein gp16) and a 120 base virus-encoded RNA. The latter binds to the portal vertex of the procapsid as an initial step in DNA packaging, but is not detected in the mature virion. Once the DNA is encapsidated in the head, the three proteins of the neck and tail can be assembled to yield the mature virion.

Pseudotemperate Phages

Pseudotemperate phages are a diverse group of essentially virulent phages sharing in common a life cycle somewhat resembling that of temperate phages. They produce turbid plaques; bacteria cultured from these plaques appear to be immune to superinfection with the same phage and release infectious virions into the surrounding medium. However, there is no evidence that the phage genome becomes integrated into the bacterial chromosome as a prophage, nor does the viral DNA appear to replicate as a plasmid in the manner of *Escherichia coli* phage P1. Indeed, given that many pseudotemperate phages have DNA base substitutions, neither form of stable prophage seems likely. Apparently, 'pseudolysogeny' results from a delayed or very extended latent period following penetration. Host macromolecular syntheses are not suppressed, cells continue to divide, and a high percentage of spores produced from infected *B. subtilis* cultures are cured of the virus. At any given time, however, some portion of the phage complete a lytic cycle, lyse the host bacteria and are released into the environment. Phage titers as high as 10^{10} ml^{-1} have been reported in cultures of SP10-infected bacteria. Cured cells which occur in a growing culture are probably quickly reinfected by these free virions, thus perpetuating the pseudolysogen. Many pseudotemperate phages also carry out generalized transduction.

There are great differences in size, morphology and DNA composition (including base substitutions) among pseudotemperate *B. subtilis* phages. This may suggest that pseudolysogeny is an incidental and unprogrammed variation on lytic infection. However, there are some intriguing suggestions that the relationship between host and parasite is more complex. Clear-plaque mutants with a reduced capacity to establish a carrier stage have been reported. Several pseudotemperate phages have been found to enhance wild-type sporulation, increase the sporulation frequencies of various oligosporogenic or sporulation-negative mutants, and/or relieve glucose-mediated catabolite repression of sporulation in some *Bacillus* species. Genetic analysis of *B. subtilis* strain W23 phage PMB12 indicates at least three genes are required for it to enhance host cell sporulation. *B. thuringiensis* phage TP-13 converts oligosporogenic, acrystalliferous mutants into a spore-positive phenotype which also produces the insecticidal crystal protein typical of this species.

PBS1

The most widely used pseudotemperate *B. subtilis* phage is PBS1, although this attention reflects its importance in mediating generalized transduction more than an interest in its life cycle. PBS1 is a very large phage (see **Table 1**), and its 190 megadalton genome contains a total base substitution of uracil in place of thymine. In contrast to most *B. subtilis* phages which adsorb to the cell wall, PBS1 attaches to the flagellum and will efficiently infect only highly motile strains of its host. The virion possesses helical tail fibers (8 × 125 nm) which probably fasten to the bacterial flagellum, but the mechanism by which the viral DNA penetrates the cell is unclear. Following infection, PBS1, possibly using a virion protein, apparently modifies some of the host RNA polymerase to a form which is resistant to rifampin and lipiarmycin, drugs which ordinarily inhibit this enzyme. Still later a completely new RNA polymerase is synthesized and used to transcribe late functions. What, if any, role these polymerase changes have in pseudolyogeny is unknown.

Temperate Phages

B. subtilis 168 is naturally lysogenic for the prophage of SPβ, and many other temperate phages for this bacterium have been isolated from soil. Although there is indirect evidence suggesting SP16 could exist in a plasmid state, the three temperate phages which have been studied in detail (ϕ105, SPO2, SPβ) have life cycles similar to that of *E. coli* phage lambda. Following adsorption, linear DNA is injected, cyclizes and then is apparently inserted into the bacterial chromosome by a reciprocal recombination between attachment sites *att*P and *att*B on the phage and bacterial genomes, respectively. The site of incorporation into the bacterial chromosome is specific for each phage; no transposon-like phages with multiple insertion sites similar to *E. coli* phage Mu have been found among *B. subtilis* phages.

ϕ105

Phage ϕ105 is representative of a group of small temperate phages that have been isolated by several investigators. The 26 MDa viral genome has complementary single-stranded cohesive ends ($cos_{\phi 105}$) of

seven bases. The linear DNA cyclizes following infection and the prophage inserts at 244° on the 360° circular *B. subtilis* chromosome map. The ϕ105 repressor gene *cI* encodes a 144 amino acid polypeptide, which in its active tetrameric form binds to six apparent operators, five of which are located near two promoters, P_M and P_R, that are proximal to gene *cI*. The repressor stimulates transcription from P_M which is the promoter for the repressor gene. The repressor inhibits transcription from P_R and thereby probably blocks synthesis of functions required to initiate a lytic cycle. The prophage can be induced by ultraviolet light or mitomycin C; temperature-inducible mutants with heat-sensitive repressors have been isolated. Induction begins with several rounds of prophage DNA replication before the prophage is excised from the bacterial chromosome. This DNA replication actually extends into nearby bacterial markers. Replicating ϕ105 DNA is concatenated, and appears to be replicated by host enzymes.

SPO2

Phage SPO2 is approximately the same size as ϕ105, and the two viruses share a 5.7 kb region of partial homology near the centers of their genomes. However, SPO2 prophage inserts at 15° on the circular *B. subtilis* 168 chromosome. The linear virion DNA has cohesive ends and apparently circularizes following injection. The SPO2 prophage is a circular permutation of the virion DNA. Unlike ϕ105 which uses the host's DNA polymerase III during lytic growth, SPO2 encodes its own DNA polymerase similar to bacterial DNA polymerase I. Although there is no evidence that SPO2 alters or replaces the host RNA polymerase, the lytic cycle involves several temporally distinguishable classes of mRNA.

SPβ

As already noted, *B. subtilis* 168 carries the prophage of a virus called SPβ. Small numbers of infectious virions are present in the supernatants of strain 168 cultures, and the phage can be induced with UV or mitomycin C. The 126 kb prophage extends from about 183° to 192° on the *B. subtilis* map, a region close to the terminus of chromosomal replication. The gene order in SPβ prophage is a circular permutation of the order of markers in the linear virion DNA. A 27 kb region of nonessential DNA overlaps the phage attachment site $attP_{SP\beta}$ and is the site of numerous deletion mutations. Lysogens of SPβ contain rare specialized transducing particles in which a portion of this nonessential region may be substituted by bacterial DNA.

SPβ is closely related to temperate phages ρ11, ϕ3T and Z. Collectively, phages of this group are noted for their converting properties. With the notable exception of SPβ itself, all carry a structural gene for thymidylate synthetase (*thy*P) that is expressed in the prophage state. Thymine auxotrophs of *B. subtilis* lacking this enzyme are converted into prototrophy following lysogenization with any of these phages except SPβ. Bacteria lysogenic for SPβ or Z release a bacteriocin (betacin) which kills nonlysogens of the same strain. The presence of SPβ prophage makes cells refractile to infection with unrelated virulent phage ϕ1*m*. This inhibition is mediated by the product of the prophage *mpi* gene.

During lytic growth, most SPβ-related phages produce DNA methyltransferases that methylate cytidylic acids within the palindromes recognized by restriction endonucleases *Hae*III, *Fnu*4HI and others. Isoschizomers of these enzymes are made by some strains of *Bacillus;* thus the methylations could protect the viral DNA when infecting new hosts. However, it has not been established that the phages themselves produce corresponding restriction endonucleases.

Defective prophages

When *B. subtilis* strains are exposed to UV or mitomycin C, virtually all release phage particles with notably small heads and large, complex tails. These phages are defective and cannot productively infect new hosts. The best studied is PBSX which is carried as a prophage by *B. subtilis* 168. (As noted above, strain 168 is also lysogenic for nondefective phage SPβ.) Virions of PBSX contain a random assortment of 13 kb fragments of bacterial rather that viral DNA. The phage particles adsorb to and kill *B. subtilis* strains not lysogenic for the homologous prophage. However, the method of killing is unknown; the apparent contractile tail of PBSX does not inject even the bacterial DNA contained in the head. Indeed, the tails alone are reported to act as bacteriocins. Immunity and sensitivity to the defective phages is probably related to differences in cell wall components, since there is no infecting phage DNA that might be the target of endogenous repressors. For example, PBSX binds to erythritol teichoic acid found in *B. subtilis* W23, and it kills this strain. The homologous defective phage PBSZ released by *B. subtilis* W23 kills *B. subtilis* 168, which contains glycerol teichoic acid in its wall.

The prophage of PBSX is located at 112° on the *B. subtilis* map, between the markers *met*A and *pho*S. More than 33 kb of PBSX DNA fragments have been cloned, an amount greater than could be contained in

the virion head. The fragments include the presumptive PBSX repressor gene *xre*, a replication origin, 21 kb of what appears to be a single 'late operon', and various probable 'early' genes. Expression of the defective prophage is precisely regulated. The repressor binds to four operators, two of which block putative promoters associated with genes involved in lytic functions, and two which may be involved in autoregulation of *xre*. Transcription of all late functions is apparently initiated from one promoter, P_L, and involves positive control factor, Pcf. The latter protein is the product of an early or middle gene, and has an amino acid sequence similar to known *B. subtilis* sigma factors.

As already noted, defective phages similar to PBSX are widespread in *B. subtilis* strains and related species of this genus. This may imply these prophages are maintained because they offer some benefit to their hosts, since the viruses themselves cannot establish new lysogens. It has been suggested that the bacteriocin activity associated with these viruses provides a competitive advantage against related species of bacilli. However, this presumed advantage requires altruism on the part of some bacteria in the deme or clone, because those cells which release the phage die. Others argue that a phage gene or a bacterial gene transposed into the prophage serves some vital function. However, if the latter is the case, it is difficult to understand why so much of the prophage has been maintained, given its lethal potential.

Generalized Transduction

The premier generalized transducing phage for *B. subtilis* is PBS1. A single virion of this virus can incorporate 5–10% of the bacterial chromosome, allowing linkage of quite distant markers. This was instrumental in constructing the circular map of the *B. subtilis* chromosome. PBS1 appears to encapsidate bacterial DNA randomly, and there is no evidence of preferred packaging sites (*pac* sites) such as occur in *Salmonella* phage p22. Transducing particles of PBS1 contain only bacterial DNA and differ in buoyant density from infectious virions; the two can be separated on CsC1 density gradients. Small DNA molecules such as plasmids are not efficiently transduced by PBS1, but can be transduced by the small virulent phage SPP1.

Generalized transduction has been observed with many pseudotemperate phages including SP10 and SP15. However, PBS1 remains the phage of choice both for consistency with previous studies and because linkage and genetic distances are better calibrated in this virus.

Specialized Transduction

Specialized transduction has been carefully studied in SPβ and related phages, but it is also mediated by ϕ105. Lysates of the SPβ contain small numbers of transducing phages carrying bacterial markers proximal to the normal attachment site $att\mathrm{B}_{\mathrm{SP}\beta}$. In addition, SP$\beta$ prophage occasionally becomes inserted at aberrant positions in *B. subtilis* deletion mutants lacking $att\mathrm{B}_{\mathrm{SP}\beta}$. Induction of these lysogens gives rise to specialized transducing particles carrying markers close to the novel sites of prophage integration. SPβ variants containing transposon Tn*917* have been constructed. During lysogenization such phages insert into Tn*917* transposons located throughout the *B. subtilis* chromosome, and subsequently can transduce nearby bacterial markers.

SPβ specialized transducing particles probably originate from errors of prophage excision in which a portion of the viral DNA is replaced by bacterial DNA. As a result, virtually all transducing particles are defective and cannot produce plaques on new hosts. Two types of transduction have been recognized in the SPβ system. In 'replacement transduction' the bacterial portion of the infecting DNA undergoes recombination with and replaces the homologous region of the genome of the recipient. In 'addition transduction' the infecting phage-bacterial DNA becomes incorporated as a prophage, resulting in a bacterium diploid for the *B. subtilis* markers carried on the prophage. Addition transduction apparently occurs more readily if the recipient is already lysogenic for SPβ.

Transfection

When grown in a minimal salts medium, *B. subtilis* 168 becomes competent for the uptake of exogenous DNA, including DNA of bacteriophages (transfection). However, the process by which DNA enters the cell has been explored most thoroughly in transformation. In the latter, one strand of transforming DNA is hydrolyzed in the process of penetrating the cell wall and membrane, resulting in a single-stranded product in the cytoplasm. This DNA then displaces the homologous strand of the chromosomal DNA while hydrogen-bonding to the complementary strand.

It is assumed that transfecting viral DNA is also subject to degradation by nucleases during entry into the competent bacterium. Even when very high molecular weight SPO1 or SP82 DNA is used, completion of a lytic cycle and formation of new virions occurs only if the target cell incorporates several intact copies of the viral DNA. These

presumably undergo recombination with each other to form at least one functional genome which eventually yields progeny phage. Fragmented phage DNA can be used in marker rescue experiments in which competent cells are first exposed to phage DNA and then infected with mutant phage lacking the gene function to be rescued.

Transfection of ϕ29 DNA may proceed differently. DNA of this phage treated with proteolytic enzymes loses transfecting activity. This suggests the terminal protein on the ϕ29 genome enters the competent bacterium to continue its role in DNA synthesis.

Protoplasts of *B. subtilis* can be transfected with phage DNA in the presence of polyethylene glycol. In this situation, the viral DNA is apparently not degraded, and protoplast transfection is used in recovering viable phage following incorporation of cloned DNA into phage vector systems.

Phage Vectors for Gene Cloning

A number of *B. subtilis* phages have been examined as potential cloning vectors, but most useful are ϕ105 and SPβ. The genomes of both these phages have regions not required for lytic growth which can be replaced by cloned DNA.

Several strategies have been used in cloning DNA in *B. subtilis* phages; three are considered here. In 'direct transfection', ϕ105J119 DNA (ϕ105J119 is a variant of ϕ105) is circularized *in vitro* by ligating its complementary single-stranded cohesive ends. The vector is then again linearized by digesting with a restriction endonuclease such as *Sal*I, which cuts a unique site in the DNA. The target DNA is restricted with *Mbo*I. To prevent the DNA of vector and target from self-ligating, the unique cohesive ends of each are partially filled-in using the Klenow fragment of DNA polymerase I and, respectively, dCTP and dTTP or dATP and dGTP. The target DNA and ϕ105J119 vector are then ligated together and transfected into protoplasts of *B. subtilis*. The progeny phages that arise from transfected cells constitute a library of recombinant DNAs which subsequently must be screened to locate the desired clone.

A second cloning strategy called 'prophage transformation' has been used with a number of phages including ρ11 and ϕ105. The phage DNA and (usually) wild-type *B. subtilis* chromosomal DNA are digested with the same restriction enzyme. The two are then mixed together and ligated to form a mixture of phage–bacterial DNA hybrids. The latter is then used to transform an auxotrophic strain of *B. subtilis* already lysogenic for the temperate phage being used as a cloning vector. Protrophic transformants are selected on an appropriate medium; many of these transformants have the bacterial DNA inserted into the prophage. A small fraction of the latter can be induced to release nondefective specialized transducing phages carrying the selected marker. Lysates of such phage are a potential source of the cloned DNA.

A third strategy involves plasmid pCV1, a vector which contains a cloning site between two regions of homology to transposon Tn*917*. The plasmid is treated with both *Bam*HI and *Sst*I to produce equal amounts of two vector arms, which have Tn*917* sequences on their respective left or right ends. These are ligated to target DNA digested with *Sau*3A to form a mixture which includes some Tn*917*–left vector arm–target DNA–right vector arm–Tn*917* concatemers. These are then transformed into *B. subtilis* lysogenic for SPβ*c*2 *del*2::Tn*917*, a heat-inducible deletion mutant of SPβ which also contains transposon Tn*917*. The latter acts as a region of homology for the transforming DNA, some of which becomes inserted into the prophage. The transformants are selected for resistance to chloramphenicol which is conferred by a gene on plasmid pCV1.

Future Perspectives

Historically, interest in *B. subtilis* phages centered on using these viruses in genetic studies of their host. PBS1 was vital in creating a linkage map of the *B. subtilis* chromosome. Later SPO1 and SP82 were prototypes for understanding control of genes through manipulation of sigma factors, and continue to be useful in studies of how phages alter host functions. Interest continues in ϕ29 gene regulation, transcription activators and the terminal protein involved in DNA replication in this phage. ϕ29 and SPP1 are important models for the study of how DNA is encapsidated in phage heads. The discovery of specialized transduction and the development of techniques for cloning in phage have been and are likely to continue to be important in determining how *B. subtilis* regulates transcription, particularly of genes associated with sporulation.

Included in the genus *Bacillus* are bacteria which produce useful enzymes, some of which synthesize insecticides or are themselves insect pathogens, and a few capable of infecting vertebrates. All of these are parasitized by phages, including transducing phages, similar to those studied in *B. subtilis* 168. Attempts are being made to genetically manipulate these bacteria to increase production, insect pathogenicity, host ranges of insecticides, and to understand the nature of pathogenicity. Both phage and plasmid vectors are likely to play an important role in these studies.

See also: **Bacillus phage ϕ29 (*Podoviridae*); Lysogeny and prophage; Phage taxonomy and classification; Phage transduction; Phages as cloning vehicles; SPO1 phage (*Myoviridae*).**

Further Reading

Anderson D and Reilly B (1993) Morphogenesis of bacteriophage ϕ29. In: Sonenshein AL, Hoch JA and Losick R (eds) *Bacillus subtilis and Other Gram-Positive Bacteria*, p. 859. Washington, DC: American Society for Microbiology.

Errington J (1990) Gene cloning techniques. In: Harwood CR and Cutting SM (eds) *Molecular Biological Methods for Bacillus*, p. 175. New York: John Wiley.

Hemphill HE and Whiteley HR (1975) Bacteriophages of *B. subtilis*. *Bacteriol. Rev.* 39: 257.

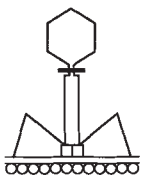
McDonnell GA, Wood H, Devine KM and McConnell DJ (1994) Genetic control of bacterial suicide: regulation of the induction of PBSX in *Bacillus subtilis*. *J. Bacteriol.* 176: 5820.

Salas M and Rojo F (1993) Replication and transcription of bacteriophage ϕ29 DNA. In: Sonenshein AL, Hoch JA and Losick R (eds) *Bacillus subtilis and Other Gram-Positive Bacteria*, p. 843. Washington, DC: American Society for Microbiology.

Stewart C (1993) SPO1 and related bacteriophages. In: Sonenshein AL, Hoch JA and Losick R (eds) *Bacillus subtilis and Other Gram-Positive Bacteria*, p. 813. Washington, DC: American Society for Microbiology.

Zahler SA (1993) Temperate bacteriophages. In: Sonenshein AL, Hoch JA and Losick R (eds) *Bacillus subtilis and Other Gram-Positive Bacteria*, p. 831. Washington, DC: American Society for Microbiology.

BACTERIAL IDENTIFICATION — USE OF PHAGES



Michael S. DuBow, McGill University, Montreal, Quebec, Canada

Introduction

Bacteriophages have been used in bacterial identification and classification since the pioneering work of Sonnenschein in 1925. The specificity of phage adsorption and lytic propagation on bacterial species and strains made them ideal agents for bacterial identification and the process, known as 'phage typing', is still an important use of bacteriophages. Between 1987 and 1991, at least 45 papers per year using phage typing were published.

In the middle twentieth century, phage typing was as popular as other means of bacterial identification, including biochemical/physiological markers and serological tests, but biotechnological advances have led to its diminished importance over the last decade. However, phage typing is frequently used in the 'battery of tests' approach to bacterial identification.

The main advantages of phage typing are relative ease of use and lack of necessity for sophisticated equipment. Many of the sets of typing phages are extremely stable, readily available, and can be stored for long periods. Additionally, automated phage typing allows rapid performance of multiple tests, a particular necessity in clinical situations. Phage typing also has industrial and environmental applications where it is desirable to keep track of a limited number of bacterial species and/or strains. Bacterial strains causing a particular industrial or epidemiological problem may differ from other strains in only a limited number of characteristics, and phages can be used to track these. Such characteristics include the presence of a particular surface antigen which the phage may use as an adsorption site, a particular restriction and modification system of the host, or even the presence of a particular temperate phage. These host characteristics may play a role in the complete lytic development of the phage after infection, and their absence or presence can be rapidly detected by the growth (or lack thereof) of a particular set of typing phages. As knowledge of bacterial characteristics improves, phage typing may also develop through the use of new and/or mutant phages for the characterization and identification of bacterial species and subspecies.

History

Throughout the 1920s and 1930s, many phages were isolated for use in the identification and classification of bacterial species and strains. It was found that few phages were capable of lysing all strains of a bacterial species, and also that many enterobacterial phages

See also: **Bacillus phage ϕ29 (*Podoviridae*); Lysogeny and prophage; Phage taxonomy and classification; Phage transduction; Phages as cloning vehicles; SPO1 phage (*Myoviridae*).**

Further Reading

Anderson D and Reilly B (1993) Morphogenesis of bacteriophage ϕ29. In: Sonenshein AL, Hoch JA and Losick R (eds) *Bacillus subtilis and Other Gram-Positive Bacteria*, p. 859. Washington, DC: American Society for Microbiology.

Errington J (1990) Gene cloning techniques. In: Harwood CR and Cutting SM (eds) *Molecular Biological Methods for Bacillus*, p. 175. New York: John Wiley.

Hemphill HE and Whiteley HR (1975) Bacteriophages of *B. subtilis*. *Bacteriol. Rev.* 39: 257.

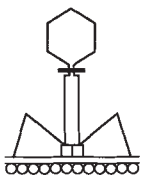
McDonnell GA, Wood H, Devine KM and McConnell DJ (1994) Genetic control of bacterial suicide: regulation of the induction of PBSX in *Bacillus subtilis*. *J. Bacteriol.* 176: 5820.

Salas M and Rojo F (1993) Replication and transcription of bacteriophage ϕ29 DNA. In: Sonenshein AL, Hoch JA and Losick R (eds) *Bacillus subtilis and Other Gram-Positive Bacteria*, p. 843. Washington, DC: American Society for Microbiology.

Stewart C (1993) SPO1 and related bacteriophages. In: Sonenshein AL, Hoch JA and Losick R (eds) *Bacillus subtilis and Other Gram-Positive Bacteria*, p. 813. Washington, DC: American Society for Microbiology.

Zahler SA (1993) Temperate bacteriophages. In: Sonenshein AL, Hoch JA and Losick R (eds) *Bacillus subtilis and Other Gram-Positive Bacteria*, p. 831. Washington, DC: American Society for Microbiology.

BACTERIAL IDENTIFICATION — USE OF PHAGES



Michael S. DuBow, McGill University, Montreal, Quebec, Canada

Introduction

Bacteriophages have been used in bacterial identification and classification since the pioneering work of Sonnenschein in 1925. The specificity of phage adsorption and lytic propagation on bacterial species and strains made them ideal agents for bacterial identification and the process, known as 'phage typing', is still an important use of bacteriophages. Between 1987 and 1991, at least 45 papers per year using phage typing were published.

In the middle twentieth century, phage typing was as popular as other means of bacterial identification, including biochemical/physiological markers and serological tests, but biotechnological advances have led to its diminished importance over the last decade. However, phage typing is frequently used in the 'battery of tests' approach to bacterial identification.

The main advantages of phage typing are relative ease of use and lack of necessity for sophisticated equipment. Many of the sets of typing phages are extremely stable, readily available, and can be stored for long periods. Additionally, automated phage typing allows rapid performance of multiple tests, a particular necessity in clinical situations. Phage typing also has industrial and environmental applications where it is desirable to keep track of a limited number of bacterial species and/or strains. Bacterial strains causing a particular industrial or epidemiological problem may differ from other strains in only a limited number of characteristics, and phages can be used to track these. Such characteristics include the presence of a particular surface antigen which the phage may use as an adsorption site, a particular restriction and modification system of the host, or even the presence of a particular temperate phage. These host characteristics may play a role in the complete lytic development of the phage after infection, and their absence or presence can be rapidly detected by the growth (or lack thereof) of a particular set of typing phages. As knowledge of bacterial characteristics improves, phage typing may also develop through the use of new and/or mutant phages for the characterization and identification of bacterial species and subspecies.

History

Throughout the 1920s and 1930s, many phages were isolated for use in the identification and classification of bacterial species and strains. It was found that few phages were capable of lysing all strains of a bacterial species, and also that many enterobacterial phages

were capable of lytically infecting more than one bacterial species. It was also discovered that surface antigens of the bacterium (identified serologically) could be correlated with phage sensitivity or resistance. Different colony-specific morphologies of bacterial strains were shown to correlate with growth of a particular phage, and these characteristics were useful in understanding microbial pathogenicity as well as virus–host interactions.

The discovery in 1934 of the Vi antigen on the surface of the typhoid bacillus *Salmonella typhi* led to the isolation of Vi phages capable of lysing the typhoid bacillus, but only if it expressed the Vi antigen. These phages made possible the differentiation and typing of this medically important bacterial species. In 1938, Craigie and Yen isolated a phage called ViII that would only grow on the previous strain from which it was propagated. This 'adaptation' allowed them to recognize and differentiate 11 types of the typhoid bacillus. This method of phage typing was soon standardized and achieved wide acceptance in the field.

Many other phages were isolated for phage typing schemes for both gram negative and gram positive bacterial strains. Most phage typing schemes are for bacteria of medical significance. However, there are phage typing schemes for bacteria involved in industrial fermentations and cheese formation. Although not phage typing *per se*, bacteriophages can be used as indicators of contamination by their hosts, for example the use of coliphages to detect fecal contamination of water.

The Technique

The first requirement for phage typing is phages that can discriminate between the bacteria under study. The phages are generally stored and used as sterile lysates. However, some are stable when dried, and can be placed on filter paper discs where they may be spotted using dispensers similar to those used in antibiotic sensitivity testing. The bacterial culture is spread as a lawn on the surface of a nutrient agar petri dish and, after drying, phages are spotted on to particular areas of the petri dish in a recognizable pattern. After incubation for 5–48 h, the lawn of bacteria is scored for areas of lytic growth of phages, and the particular phages that cause or do not cause lysis are noted. The degree of lysis and the size and morphology of the plaque can be important criteria for differentiating bacterial species and strains.

Automation of phage typing includes a variety of machines for the withdrawal and placement of the phages on to the bacterial lawn. In recent years, several machines have been designed that use trans-illuminant light to examine the petri dishes via computer scanners. Cluster analysis of the different lytic reactions is then carried out, and a computer printout giving probable bacterial identification is provided.

The development and use of the routine test dilution (RTD) concept was crucial for the establishment of phage typing. RTD is generally defined as the lowest dilution of the phage lysate that yields complete lysis of the particular test strain and/or bacterial strain used for phage propagation. It is important in preventing 'lysis from without' which can occur at high multiplicities of infection giving rise to false positives (or negatives) in phage typing. RTD avoids the problem of contaminating bacteriophages in the phage lysate lysing the bacteria and so obscuring the results. The use of the RTD prevents interference by any phage host-range mutants arising.

Isolation of Typing Phages

Phages are generally isolated from the environments in which the host bacterial strain under study ordinarily grows and lives, e.g. from sewage, feces, soil, water or, in the case of temperate phages, from the bacterium itself. The phages to be used in a typing scheme can have several properties, not all of which need to be common to all of the phages. For example, one may want to have a phage that can lytically grow on all strains of a particular species and thus provide positive identification for that species, in addition to phages that can discriminate between the subspecies or strains of a particular bacterial species. The levels of specificity and discrimination required will depend upon the needs of the particular phage typing scheme to be developed.

Lytic phages form clear plaques due to the complete lysis of the host, and these plaques are easier to score than the turbid ones of temperate phages. Nonetheless, temperate phages can discriminate between strains that are lysogenic for homoimmune phages and those that are not. If lytic phages are unavailable for the particular strain to be identified, then the culture may either be irradiated to induce any prophages that may be present, or the bacterial cell-free supernatant may be examined for any spontaneously induced temperate phages.

The host strain that will be used for propagation of the typing phages is also of importance. The term 'adapted phages' refers to the process of propagating a particular phage on a particular bacterial host. This can be used to select for host-range variants of a phage that will allow for increased discrimination by the typing scheme. It may also be useful in isolating phages whose DNA is modified according to the

pattern of DNA modification enzymes present in a particular host strain. The numerous restriction and modification systems of species and subspecies of bacteria make adapted phages useful and extend their powers of discrimination and specificity.

Many plasmids encode functions that can inhibit or enhance the growth of particular bacterial phages. For example, the F plasmid inhibits the lytic growth of phage T7, yet is necessary (due to the F pilus as the adsorption organelle) for the growth of male specific phages (e.g. Qβ, M13) in the bacterium *E. coli*. If a phage can or cannot be propagated on a particular strain, then this characteristic may be used to detect the presence or absence of a plasmid that encodes a particular determinant.

Phages used in phage typing must be stable, grow quickly enough and yield reproducible lysis and plaque morphology; there must be a sufficiently large number of different phages for adequate classification and identification for the bacteria under study; and the results of the typing scheme should be reproducible.

Host Characteristics and Typing Phages

The experimental end-point of phage typing schemes is the determination of which phages can and which cannot undergo a productive lytic infection of a particular bacterial isolate. These typing schemes are not only useful for bacterial identification but, with sufficient knowledge, can yield important information about the underlying genetics and physiology of the particular bacterial strain.

If one is using a typing scheme that includes temperate phages, then the capacity of these phages to grow may indicate the presence of a particular prophage which displays immunity (or pseudoimmunity) to superinfection. In *E. coli*, the presence of a λ prophage can be tested by the incapacity of λ phage to grow on that strain, as well as the incapacity of a mutant of the lytic phage T4 (T4rII) to grow due to the expression of the *rex* genes from the λ prophage. Many phages require bacterial appendages encoded by plasmids for their adsorption to the host cell, and inclusion of these phages in a typing scheme can allow discrimination between strains that contain these plasmids and those that do not. As many bacterial pathogenicity determinants are encoded by plasmids, this may be of clinical significance.

Lytic phage growth is dependent upon the presence of particular surface antigens (receptors) for phage adsorption. These surface antigens may include not only proteins located on the outer surface of the bacterium, but also lipopolysaccharides and capsules. Thus, phage typing schemes can track important surface properties of the bacterium that may be crucial in understanding bacterial spread and pathogenesis as well as the origins of a particular strain. Phages are dependent upon host cell physiology to complete their lytic cycle, and many parameters that are important epidemiologically can be discriminated and selected with the appropriate phage type. Examples of this include host-controlled restriction and modification systems, the presence of particular plasmids and plasmid-encoded gene products, as well as the enzyme systems required for the transcription, translation and replication of the viral genome and its assembly into a mature capsid followed by lysis of the host cell.

Rapid and automated tests that measure bacterial fermentation and growth on particular substrates, the use of monoclonal antibodies, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) patterns of bacterial proteins, as well as restriction fragment patterns and DNA probes for bacterial and gene identification are some of the technologies that are increasingly popular in microbiology. Also important is the use of the polymerase chain reaction (PCR) where a particular gene target whose DNA has been sequenced is the focus of study. Nonetheless, phage typing will continue to be an important tool for the rapid and accurate identification of a particular microorganism. The continued popularity of phage typing rests with its ease of use, capacity for automation, low cost and long history, with many typing phages and schemes present in the literature and widely available. Where one does not have access to sophisticated technology, phage typing has proved to be a method of choice for bacterial identification and tracing.

See also: **Phages in industrial fermentations.**

Further Reading

Ackermann H-W and DuBow MS (1987) *Viruses of Procaryotes*, vol. 1. Boca Raton: CRC Press.

Kasatiya SS and Nicolle P (1973) Phage typing. In: Laskin AI and Lechevalier HA (eds) *Handbook of Microbiology*, p. 632. Boca Raton: CRC Press.

[This article is reproduced from the 1st edn (1994).]

synthesis occurs during infection, due in part to the accumulation of large amounts of RNA 2 which is a very active messenger.

A small fraction of cultured *Drosophila* line 1 cells routinely survives infection with FHV and on examination the cells are found to be persistently infected. Viruses isolated from persistently infected cultures replicate poorly even in fresh cells, because their coat protein genes have accumulated multiple mutations. However, the establishment of persistence has been attributed to cellular changes rather than the mutations in the viral capsid gene which arise later. Defective viral RNAs that have suffered substantial deletions and rearrangements can be isolated from viruses harvested from persistently infected cultures, and the structures of these RNAs may provide insight into the *cis*-acting sequences necessary for RNA replication and encapsidation.

Epidemiology and Pathogenesis

The readiness with which alpha nodaviruses establish persistent infections in cell culture probably reflects their propensity to establish inapparent and persistent infections in their insect hosts. These infections can reduce egg viability and slow the development of the host without causing significant mortality. Under other conditions, such as overcrowding of the host, however, nodaviruses cause epizootic infections with high mortality. Such infections can be economically damaging in the case of SJNNV disease among hatchery-reared fish larvae. Large numbers of SJNNV particles are found in the brain and other cells of the central nervous system, and the infected larvae show abnormal swimming behavior and nervous necrosis.

Intraperitoneal inoculation of NOV into mice less than about 14 days old causes severe hind limb paralysis due to replication of the virus in the hind limb musculature and degeneration of spinal cord neurons. Mice inoculated when they are older than about 21 days show no signs of disease. It is not clear whether this change is due solely to the development of a responsive immune system, but 7-day-old mice born to or suckled by immunized mothers are completely resistant to intraperitoneal challenge with NOV. In wax moth larvae, NOV replicates predominantly in muscle cells, but cells of many other tissues are also susceptible to infection which kills the larvae.

See also: **Fish viruses; Persistent viral infection; Tetraviruses (*Tetraviridae*); Virus structure: Atomic structure, Principles of virus structure.**

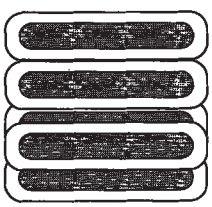
Further Reading

Ball LA and Johnson KL (1998) Nodaviruses of insects. In: Miller LK and Ball LA (eds) *The Insect Viruses*, New York: Plenum Press (in press).

Garzon S and Charpentier G (1992) Nodaviridae. In: Adams JR and Bonami JR (eds) *Atlas of Invertebrate Viruses*, p. 351. Boca Raton, FL: CRC Press.

Schneemann A, Reddy V and Johnson JE (1998) The structure and function of nodavirus particles: a paradigm for understanding chemical biology. *Adv. Virus Res.* 50: 381.

NONOCCLUDED BACULOVIRUSES



John P Burand, University of Massachusetts – Amherst, Amherst, Massachusetts, USA

History

The nonoccluded baculoviruses (NOB) are a loosely associated group of enveloped, rod-shaped viruses which, in many respects resemble the occluded baculoviruses except that they are not found associated with a protein crystal occlusion body. Only three NOBs have been studied to any significant extent. The first NOB to be identified was the *Oryctes* virus (Or-1V). The isolation of this virus resulted from a field study conducted throughout Southeast Asia in 1963, to find an agent that could be used to control the rhinoceros beetle, *Oryctes rhinoceros*, a pest on tropical palm plantations in the South Pacific. The virus was not naturally occurring in areas of the South Pacific where *O. rhinoceros* had been accidentally introduced and had become a pest. Since its initial discovery the virus has been found in insect populations in the Philippines, Sumatra, West Kalimantan, Thailand and India. Today, the virus is used to control beetle populations and serves as the best model for the use of a virus for insect pest control.

The newest addition to the NOB group is the Hz-2 virus (Hz-2V) or gonad-specific virus (GSV) which was identified in 1995 in electron micrographs of reproductive tissues from *Helicoverpa zea* moths, originating from a bollworm colony that was established at the USDA-ARS Southern Insect Management Laboratory in Stoneville, Mississippi. Adults from this colony showed lower than normal oviposition rates, and the symptoms of Hz-2V infection in these insects were described as atrophy of ovarian and testicular organs. These abnormalities could be transmitted to progeny insects by both diseased males and females and occurred in 63–78% of the individuals throughout five successive generations of insects.

The best characterized NOB is the Hz-1 virus (Hz-1V). Hz-1V was first identified in 1978 as a persistent agent in IMC-Hz-1 cells. This cell line was originally established from adult ovarian tissues of *H. zea* and attempts to infect these cells with other baculoviruses resulted in death of the cells without occlusion body formation. In retrospect, this was probably due to induction of the persistent Hz-1 virus. The first indication that IMC-Hz-1 cells were persistently infected with a virus came from observations made in 1972, that long, virus-like particles were visible in electron micrographs of these cells. In addition, these cells were found to periodically undergo a crisis phase where a large number of the cells became granulated and died. The virus persisting in these cells was eventually recovery and propagation in *Spodoptera frugiperda* (IPLB-SF-21) and *Trichoplusia ni* (TN-368) cells by Granados in 1978.

Properties of the Virion and Genome

All of the NOBs are rod-shaped enveloped particles with supercoiled, double-stranded DNA genomes ranging in size from 130 kilobase pairs (kb) for Or-1V to 220 and 230 kb for Hz-2V and Hz-1V, respectively. The genomes of both Or-1V and Hz-1V have been physically mapped and six regions of reiterated sequences were identified on the Or-1V genome. No such regions have been identified for either the Hz-1V or Hz-2V genome.

The Or-1V enveloped virus particle is 220 × 120 nm and has been reported to have a tail-like structure at one end.

Virion Proteins

The virus is composed of 27 structural proteins ranging in size from 9.5 to 215 kDa. A total of 14 of these proteins are thought to be envelope proteins. Hz-1V is longer and narrower than the Or-1V measuring 414 × 80 nm and is composed of 28 structural proteins ranging from 150 to 14 kDa in size. Of these proteins 14 are thought to be envelope glycoproteins since they were found to be radioactivity labeled in virus grown in the presence of N-[^{3}H]acetylglucosamine and [^{3}H]mannose. Hz-2V is also about 415 × 80 nm in size and is composed of only about 12 structural proteins. The envelope of Hz-2V is distinctive in that it appears to be slightly irregular with folds or ridges associated with individual filaments of approximately 5 nm in diameter located between the envelope and the nucleocapsid.

Replication

Or-1V replication has been studied *in vitro* using a cell line derived from the black beetle, *Heteronychus arator* (DSIR-HA-1179). Virus entry into these cells is thought to occur by pinocytosis and the first sign of viral cytopathic effect (CPE) is the rounding of these normally fibroblast-like cells, with complete cell lysis occurring by 3–4 days postinoculation (dpi). The first signs of virus replication, which include nuclear hypertrophy and migration of nuclear chromatin occur by about 7 hours postinfection (pi). Envelope material then accumulates in chromatin-free areas in the nucleus. Virus assembly begins with the formation of envelopes and nucleocapsid shells in the chromatin-free areas. The shells are then filled with an electron-dense core containing the viral DNA. Virus replication peaks at between 12 and 36 h pi. Virus particles enter the cytoplasm during this time, and are ultimately released from the cells by budding from the cytoplasmic membrane.

Eight of the reported 27 Or-1V structural proteins were detected in virus-infected, HA cells pulse labeled with [^{35}S]methionine. The two structural proteins, p4.6 and p10 were first detected at 4 hpi whereas the p11.5 protein was first detected at 10 hpi. The remaining six viral proteins p46.5, p40, p27, p25, p22 and p13 were all first detected at 6 hpi. Since Or-1V does not actively shut off HA cell protein synthesis during replication, the inability to detect the synthesis of the remaining virus structural proteins or any additional virus-induced intracellular proteins may be due to the presence of a large number of labeled cell proteins found in these samples. It is also possible that the remaining proteins contained a low level or were completely lacking methionine residues.

The CPE of Hz-1V replication in TN-368 cells begins with the rounding of the cells followed by nuclear hypertrophy. Virus replication and assembly takes place in the nucleus of infected cells in a manner similar to that observed for Or-1V. First, membrane vesicles appear in the nucleus, then these vesicles are filled with electron-dense capsids containing viral

DNA, giving rise to mature enveloped nucleocapsids. Virus replication culminates with release of infectious virus through the lysis of both the nuclear and cytoplasmic membranes. Enveloped virus particles inside the cell are complete and infectious prior to release by lysis.

The replication cycle of Hz-1V has been divided into three stages; an early stage, from 0 to 4 hpi, which is prior to the onset of viral DNA replication, an intermediate phase, from 4 to 8 hpi and a late phase, beyond 8 hpi. Pulse labeling of Hz-1V-infected cells with [^{35}S]methionine revealed a total of 37 virus-specific intracellular proteins. The sequential expression of these viral-induced proteins appears to follow a cascade pattern very reminiscent of the pattern of viral protein synthesis found in insect cells infected with occluded baculoviruses. One of the Hz-1V-induced proteins was first detected at 2 hpi and 25 others were detected first by 7 hpi. All remaining 11 viral-specific proteins were first detected after 8 hpi. Although not clearly demonstrated, it appears likely that the viral-specific proteins synthesized early during virus replication are required for the expression of proteins made later during Hz-1V replication. The pattern of expression of the viral-specific intracellular proteins differs in cells infected with defective interfering particles (DIPs), compared to the pattern of protein synthesis found in cells infected with standard virus particles. Infection of cells with DIPs has been shown to lead to the establishment of cell lines persistently infected with Hz-1V. At the present time it is unclear what this difference in the pattern of expression of viral proteins means or how it is related to the establishment of persistently infected cell lines by Hz-1V.

During productive Hz-1V replication, a total of 101 viral-specific transcripts ranging in size from 0.8 to over 9.5 kb in length have been detected. These transcripts map to dispersed regions along the viral genome and follow a pattern of temporal expression similar to that of virus-specific intracellular proteins. Of the 24 viral transcripts detected at 2 hpi, only three are highly expressed and one, a 2.9 kb transcript, is constitutively expressed (CE 2.90) throughout virus replication. An additional 30, 21 and 16 viral transcripts are made at 4, 6 and 8 hpi, respectively, and no new viral transcripts are made at later times after infection.

Two Hz-1V late genes have been characterized. The first was the p34 gene which is located between 50.3 and 52.5 map units on the viral genome, and which contains a 765 nucleotide (nt) open reading frame (ORF) capable of encoding a 34 kDa polypeptide. The second, is the p51 gene which maps between 40 and 44.6 map units, and contains a 1152 nt ORF potentially coding for a 51 kDa protein. The upstream, 5′ regulatory regions of both p34 and p51 have a conserved nine base sequence TTATAGTAT which is thought to contain the Hz-1V late promoter. Transcripts from both these genes (two transcripts of p34 and the major transcript of p51) were found to be initiated at sites within this nonanucleotide sequence. This putative, late, promoter sequence identified for Hz-1V late genes, is significantly different from the late baculovirus promoter motif NTAAG, suggesting that the transcription of Hz-1V late genes occurs differently from that of late, occluded baculovirus genes and likely via a different viral RNA polymerase.

Pathology and Transmission

Infection of *O. rhinoceros* larvae by Or-1V occurs primarily through the consumption of virus contaminated food, whereas adult insects can become infected when visiting contaminated breeding sites. The virus can also be transmitted by adults during mating, most likely through oral contact with virus from an infected partner. Midgut epithelial cells of both larvae and adults are the primary site of replication and from there the virus spreads to other tissues including the fat body. In larvae, virus replication in the midgut epithelium leads to the appearance of chalky white bodies under the abdominal integument just prior to death of the insect. The abdomen of infected larvae may become turgid and glassy or pearly in appearance. Eventually the fat body disintegrates and the larvae appear translucent.

The disease is commonly fatal in larvae and pupae as well as in adult beetles, however, in adults the infection is often more chronic with infected insects showing no external symptoms of the disease. Virus replication in the adult results in a tumor-like growth of densely packed, virus-filled cells in the midgut which appears swollen and whitish in color. The gut of infected beetles then, becomes filled with virus particles which are eventually defecated. Soon after infection, feeding and egg laying ceases, however, infected adults are capable of flying and mating. Infected beetles usually die within 30 days of infection. During this time these insects can serve as reservoirs and are capable of spreading the virus, via contaminated feces, when visiting breeding sites. Once it was determined that the virus was carried to breeding sites by infected adults, the virus was introduced as a control agent, into pest populations, simply by releasing virus-infected beetles. These releases led to dissemination of the virus in the population and long-term suppression of the rhinoceros beetle on islands in the South Pacific and in Tanzania. Today, a natural equilibrium appears to be

established between the level of the virus and the beetle population in these release sites with virus prevalence fluctuating between 30 and 50%.

The agonadal condition caused by Hz-2V results in female moths having enlarged common and lateral oviducts, a deformed bursa copulatrix and malformed or missing ovaries, accessory glands and/or spermatheca. Affected males have very small testes and are lacking seminal vesicles, vasa deferentia and/or accessory glands. These individuals are sterile and most infected females have a white, 'waxy plug' protruding from the vulva. This plug contains a high concentration of virus particles located inside vesicles which resemble baculovirus occlusion bodies but which lack the dense protein matrix characteristic of occlusion bodies. The replication of Hz-2V appears to be confined to reproductive tissues since the virus is readily observed in the nucleus of cells in these tissues and has not been found in any other tissues of infected animals.

Although most adult moths infected with Hz-2V exhibit agonadal pathology and are sterile, some infected individuals are fertile and can transmit the virus during mating. Both males and females can be asymptomatic carriers of the virus and are capable of transmitting the virus to progeny insects. Larvae and pupae from matings between Hz-2V carriers and uninfected moths appear normal, however, some emerge as sterile, agonadal, adults. Male and female moths injected or fed Hz-2V prior to mating can also transmit the virus to progeny insects. Moreover, it is also possible for the virus to be transmitted on the surface of eggs and by feeding or injecting it into larvae.

Persistence

Without the protection provided by a protein crystal occlusion body, typical of occluded baculoviruses, NOBs are rapidly inactivated outside their insect host. The limited survival of these viruses outside the host has led to the evolution of modes of transmission involving a closer association between them and their host which often involves the virus becoming latent or persistent. Or-1V, for example, is transmitted primarily through fecal contamination of nesting sites, whereas Hz-2V is sexually transmitted during matings with infected male or female moths. Not only is Hz-1V capable of productive replication in insect cells, but it is also able to establish persistent infections in cell culture. The persistence of Hz-1V is mediated by DIPs and involves the expression of a single 2.9 kb viral transcript known as the persistently associated transcript (PAT1). PAT1 is constitutively expressed in cells persistently infected with the virus and its expression is required for the establishment and maintenance of Hz-1V persistent infections. The unique biology of Hz-2V also involves a close association between the virus and its host with the virus remaining in a latent state throughout a major portion of the life cycle of the infected insect. The successful use of Or-1V for insect control is due largely to the close association it has with its host and its persistence in rhinoceros beetle populations. This success also suggests that other NOBs have potential for future use in insect pest control programs. Since the NOBs Hz-1V and Hz-2V can establish persistent associations with their hosts, these viruses also have potential for use as models not only for use in gaining information on the nature of viral persistence but also for use as tools for altering the genetic make-up of insects.

See also: **Baculoviruses (*Baculoviridae*): Nucleopolyhedrovirus; Insect pest control by viruses; Latency; Persistent viral infection.**

Further Reading

Bedford GO (1980) Biology, ecology, and control of palm rhinoceros beetles. *Annu Rev. Entomol.* 25: 309.

Burand JP (1998) Nudiviruses. In: Miller LK and Ball A (eds) *The Insect Viruses*. New York: Plenum Press, pp. 69–90.

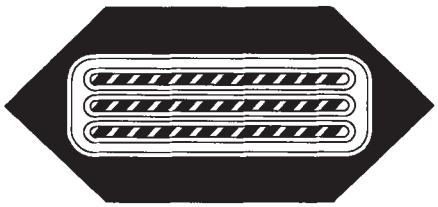
Burand JP, Kawanishi CY and Huang Y-S (1986) Persistent baculovirus infections. In: Granados RR and Federici BA (eds) *The Biology of Baculoviruses*, vol. 1, p. 159. Boca Raton: CRC Press.

Hamm JJ, Carpenter JE and Styer EL (1996) Oviposition day effect on incidence of agonadal progeny of *Helicoverpa zea* (Lepidoptera: Noctuidae) infected with a virus. *Annu. Entomol. Soc. Am.* 89: 266.

Huger AM and Kreig (1991) Baculoviridae. Nonoccluded baculoviruses. In: Adams JR and Bonami JR (eds) *Atlas of Invertebrate Viruses*, p. 287. Boca Raton: CRC Press.

Wood HA and Burand JP (1986) Persistent and productive infections with the Hz-1 baculovirus. *Curr. Top. Microbiol. Immunol.* 131: 119.

BACULOVIRUSES (*BACULOVIRIDAE*)



Contents

Granuloviruses

Doreen Winstanley, Horticulture Research International, Wellesbourne, Warwick, UK

David O'Reilly, Imperial College of Science, Technology and Medicine, London, UK

Introduction

The *Baculoviridae* are a family of large DNA viruses found only in invertebrates. They are characterized by the possession of a rod-shaped nucleocapsid (hence 'baculovirus', from *baculum*, meaning rod), a circular double-stranded DNA genome, and the ability to form proteinaceous occlusion bodies within infected cells. The family is subdivided into two genera, the genus *Granulovirus* (GVs) and the genus *Nucleopolyhedrovirus* (NPVs). The granuloviruses (or granulosis viruses) have only been isolated from the larvae of lepidopteran insects (butterflies and moths). The term 'granulosis' derives from the characteristic granular appearance of cells infected by these viruses, due to the presence of large numbers of occlusion bodies within the cells.

Taxonomy and Classification

The subdivision of the family *Baculoviridae* into genera is based on occlusion body morphology. NPVs form large polyhedral occlusion bodies that contain multiple virus particles, whereas GVs form a much smaller, ovoid occlusion body that generally only contains a single virion. Below the genus level, GVs have traditionally been assigned a species name solely on the basis of the host species from which they were first isolated. This is rather unsatisfactory from a taxonomic viewpoint because examples both of individual GVs infecting multiple hosts, and several distinct GVs infecting the same host, are not uncommon. In practice, it is necessary to confirm the identity of a GV isolate by restriction endonuclease mapping of its genome. In recent years, the increase in the amount of sequence data available for baculoviruses has enabled preliminary studies of the phylogeny of this virus family. Such studies have now been carried out using the sequences of several different genes (e.g. polyhedrin/granulin, **Fig. 1**). These studies uniformly confirm that the NPVs and GVs represent two well-separated clades, suggesting that the divergence of baculoviruses into NPVs and GVs took place early in the evolution of this group. As yet, the number of GVs for which suitable sequence data are available is too small, but in the future phylogenetic studies of this type should provide a more rational means of classifying GVs below the genus level.

Structure

GV virions comprise rod-shaped enveloped nucleocapsids (**Fig. 2A**) that contain a single molecule of supercoiled double-stranded DNA ranging in size

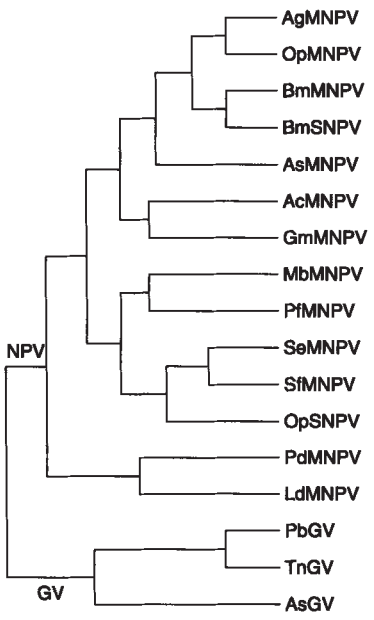


Figure 1 Baculovirus phylogeny based on polyhedrin and granulin protein sequences. The deep division between the NPV and GV clades is clear. (Modified with permission from Zanotto *et al* (1994) *J. Invertebr. Pathol.* 62: 147–164.)

from approximately 100 to 180 kb. The nucleocapsids resemble those of the NPVs and are typically 30–60 nm in diameter and 200–300 nm long. The cylindrical body of the capsid appears to be constructed as a helical stacked ring structure that repeats every third ring. The ends of the capsid are different, both from each other and from the body of the capsid. Like the NPVs, it seems probable that GV virions occur in two morphologically distinct forms, a budded form that buds from infected cells (**Fig. 2B**), giving rise to extracellular virus, and an occluded form found within occlusion bodies formed inside infected cells. SDS-polyacrylamide gel electrophoresis analysis of GV virions suggest they contain in excess of 18 different polypeptides, with budded virions and occluded virions displaying different protein profiles.

GV occlusion bodies; often referred to as capsules or granules, have a roughly oval morphology, with a major axis diameter of 300–500 nm and a minor axis diameter of 120–300 nm. They typically contain a single nucleocapsid (**Fig. 2C, D**), although occasionally capsules with several nucleocapsids have been observed. Electron micrographs of GV capsules suggest they are surrounded by an outer layer which is likely to be similar to the calyx surrounding NPV occlusion bodies. The major protein of GV capsules is known as granulin. Granulin proteins have a size of 28–30 kDa and are related at the amino acid sequence level to the polyhedrin proteins of NPVs. High-resolution visualization of the matrix of the capsule reveals that it is highly crystalline (**Fig. 2C, D**). It is thought that multimers of granulin form the subunits of this crystalline lattice.

Genome Organization and Expression

Field isolates of many GVs show considerable genomic heterogeneity. It is likely that a typical GV infection in the field often comprises multiple genomic variants coinfecting the host insects. For several GVs, detailed restriction endonuclease maps of individual GV genotypes have now been prepared. These maps will provide the framework for future characterization of the precise differences between genomic variants present in field isolates. At least some of the variation is due to the insertion of transposable elements from the lepidopteran host genome into the viral DNA. The horizontal transfer of transposable elements from the genomes of *Cryptophlebia leucotreta* (false codling moth) and *Cydia pomonella* (codling moth) into the *C. pomonella* GV genome has been demonstrated. These transposable elements belong to the family of Tc1-like transposons found in *Drosophila* and *Caenorhabditis* species. Such transposition events are likely to play a significant role in the evolution of GV diversity.

Typical GV genomes are likely to contain between 150 and 200 genes, based on comparison with the gene density observed in completely sequenced NPV genomes. A number of large scale sequencing projects of GV genomes are currently underway, so that the number of sequenced GV genes is increasing rapidly. It is likely that the first complete sequence of a GV genome will be available in the near future. Presently, approximately 35 GV genes with homologues in the databases have been identified. Most of these represent genes previously identified in NPV genomes. However, in many cases, these identifications are very tentative, often being based on incomplete sequence data. It is likely that GVs will encode a significant number of genes not found in NPVs. Characterization of such genes should provide fascinating insights into the molecular basis for the remarkable biology of these viruses (see below).

Of the potential GV genes identified to date, only a small number have been subjected to any form of functional characterization. Currently these are the *granulin, enhancin, iap, odvp-6E, egt* and *cathepsin* genes. All of these have NPV homologues. Several *granulin* gene sequences are now available from different GVs. The *granulin* gene encodes the major protein of the GV occlusion body, as described above. It is homologous to the *polyhedrin* genes of the NPVs (**Fig. 1**), in agreement with their similar function. Recent experiments, in which the *polyhedrin* gene of *Bombyx mori* (silk moth) NPV was replaced by the *granulin* gene of *Trichoplusia ni* (cabbage looper) GV (TnGV), show that the different morphology of NPV and GV occlusion bodies is not an intrinsic property of the polyhedrin/granulin gene alone, The BmNPV recombinant expressing the TnGV *granulin* gene formed large cuboid occlusion bodies, more similar to those normally formed by BmNPV and quite different from the small, ovoid occlusion bodies formed by TnGV. Gene expression studies in cell culture following CpGV infection suggest that there is only a single hyperexpressed viral gene product (granulin) present late in infection. A GV homologue of the NPV p10 gene has yet to be identified.

Enhancin genes have been identified in a range of GVs. The enhancin protein is a metalloproteinase whose function appears to be to digest the peritrophic membrane lining the midgut of the insect host to facilitate the initiation of the infection process. Until recently, enhancin was thought to be unique to GVs but it is now known that the *Lymantria dispar* (gypsy moth) NPV encodes an enhancin. The protein encoded by the *iap* gene acts to block apoptosis (programmed cell death) in infected cells (*i*nhibitor of

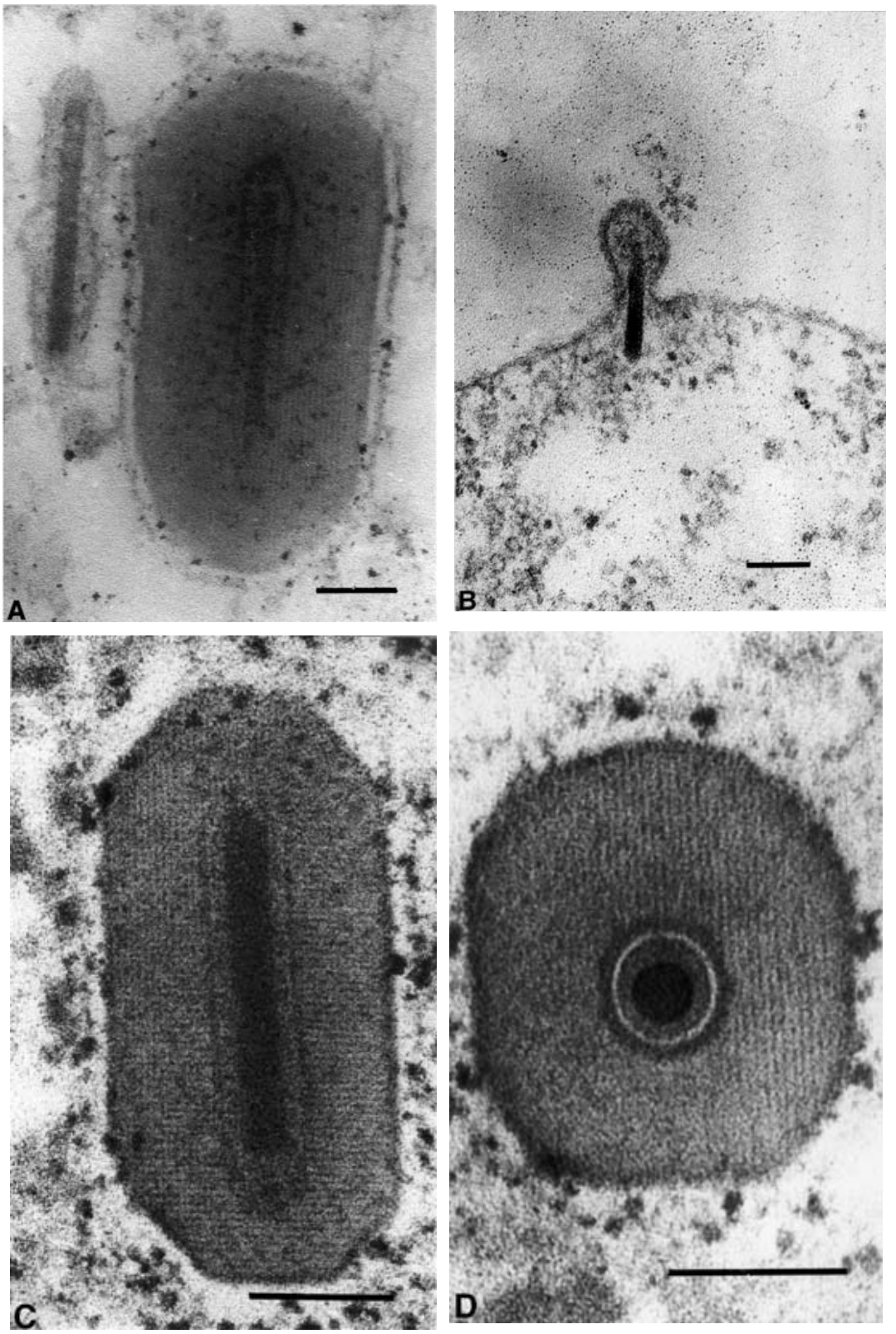


Figure 2 GV morphology. (**A**) Enveloped GV virion and adjacent occluded virion; (**B**) virus budding from plasma membrane; (**C**) longitudinal section of occlusion body; (**D**) transverse section of occlusion body. Bar = 100 nm. ((A) and (B) reprinted with permission from Winstanley D and Crook NE (1993) *J. Gen. Virol.* 74: 1599–1609.)

*ap*optosis). This was first identified in the CpGV genome, but it is now known that most baculoviruses possess one or several *iap* genes. ODVP-6E is a structural protein found in the envelope of virions contained within occlusion bodies. The *odvp-6E* gene has been identified in several NPVs and GVs. After polyhedrin/granulin, *egt* represents the baculovirus gene that has been sequenced most frequently. It appears to be present in a wide range of, if not all, baculoviruses. The EGT protein enables the baculovirus to interfere with the development of the infected host by inactivating the insects' ecdysteroid molting hormones. Finally, cathepsin is a cysteine protease involved in degradation of the host tissues and cuticle to facilitate release of the progeny virus.

Little is known about gene order in GVs, but available evidence suggests that there are regions in which the gene order is conserved between GVs and NPVs, and regions where extensive gene rearrangements have taken place. Similar to the NPVs, GV genes generally seem to be closely spaced, but without much overlap between them. A number of homologous repeated sequences have been described in the genome of TnGV. Homologous repeats (*hrs*) are a prominent feature of NPV genomes, where they are believed to act as enhancers of gene expression and origins of DNA replication. However, a characteristic feature of *hr* elements in NPVs is that each *hr* is composed of numerous short direct and inverted repeats and palindromic sequences. Such an internal structure is not observed in the repeat sequences identified in TnGV, so it remains to be seen whether the TnGV repeats are functionally equivalent to the NPV *hrs*.

Studies of the control of NPV gene expression have shown that NPV genes are expressed in a cascade fashion. Three broad transcriptional classes can be recognized – early, late and very late – based on the time in the viral infection process a gene is expressed. Late and very late NPV genes are transcribed by a novel, virus-induced RNA polymerase that recognizes the motif TAAG at the transcription start site. Temporal studies on viral gene expression in GV infections are limited because of the lack of highly permissive cell lines. *In vivo* studies that have been carried out are complicated by the involvement of different tissues, as well as by the lack of synchronous infection. However, GV genes that one would expect to be expressed late in infection generally have one or more TAAG sequences upstream of the start codon. In a few cases, transcription has been shown to initiate from this motif. Furthermore, a recombinant BmNPV has been constructed that includes the CpGV *cathepsin* gene and promoter. Transcription of the *cathepsin* initiates from the TAAG motif in the GV promoter, suggesting there are significant similarities between the transcription control mechanisms of GVs and NPVs.

Replication

Difficulties in achieving growth of GVs in cell culture have prevented the detailed studies of replication that have been possible with some NPVs. As a result, our understanding of GV replication is based primarily on *in vivo* studies using electron microscopy. Although there appear to be differences in detail between the various *in vivo* GV studies, depending on the virus, host and tissue, the overall cytopathology is similar. The infection process initiates when an insect consumes food contaminated with GV granules. The alkaline pH of the lepidopteran midgut promotes the dissociation of the occlusion bodies, with the consequent release of the virus particles into the lumen of the midgut. For many GVs, enhancin protein associated with the granules is believed to catalyze the partial disruption of the peritrophic membrane lining the midgut. This allows the virus particles easier access to the midgut epithelial cells. Virus entry is thought to be by fusion between the viral membrane and the midgut microvilli. The nucleocapsids migrate to the nuclear membrane. There is some evidence to suggest that the nucleocapsid uncoats at the nuclear pore (TnGV and *Estigmene acrea* (saltmarsh caterpillar) GV) and/or in the nucleus (*Scotogramma trifollii* (clover cutworm) GV). Once in the nucleus, there are major differences between the cytopathology of GV and NPV infections. Unlike NPVs, in GV infections the nuclear membrane breaks down before occlusion body formation. There appear to be two phases of nucleocapsid production, one prior to the fragmentation of the nuclear membrane and one postfragmentation. The early stages of GV infection resemble the prophase stage of mitosis. There is nuclear 'clearing' as a result of the chromatin and nucleolar complex becoming marginated. The granular and fibrillar components of the nucleolus separate and the nuclear pores become larger. Empty capsids form in the clear area of the nucleus and fill with nucleoprotein; the nucleocapsids appear scattered separately throughout the nucleus (**Fig. 3A**). The nuclear membrane then fragments, liberating the nucleocapsids into the cytoplasm. The nucleocapsids migrate to the periphery of the cell and align themselves with their conical end perpendicular to the thickened area of the plasma membrane, where budding occurs. While budding is occurring there is further proliferation of nucleocapsids at the virogenic stroma in the portion of the cell once occupied by the nucleus. The progeny nucleo-

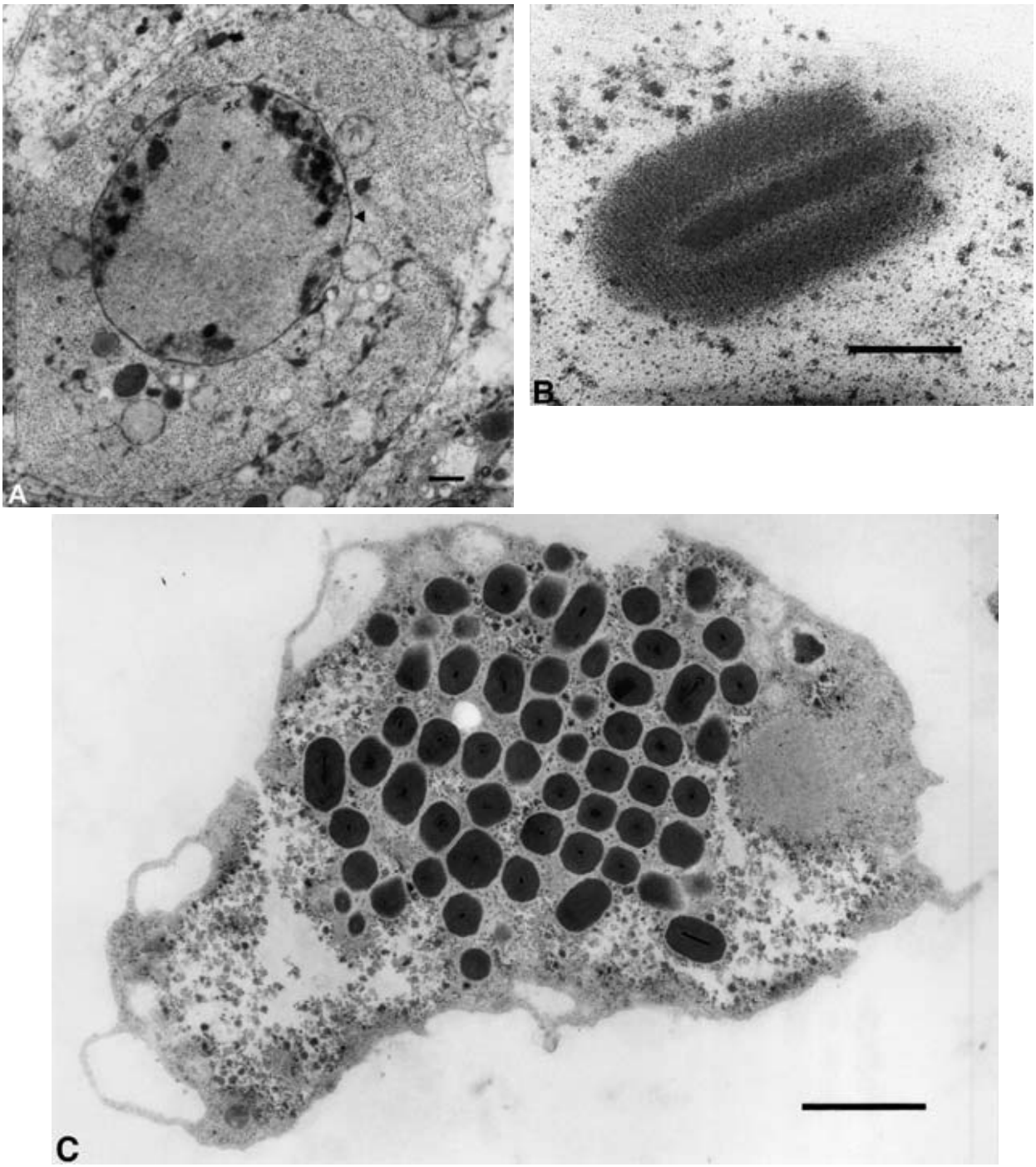


Figure 3 (**A**) Early stages of GV replication. Nucleocapsids throughout the nucleus prior to disintegration of the nuclear membrane. The nuclear membrane is indicated by an arrowhead. Bar = 1 μm. (Reprinted with permission from Winstanley D and Crook NE (1993) *J. Gen. Virol.* 74: 1599–1609.) (**B**) Occlusion from one end of the virion. Bar = 100 nm. (**C**) *In vitro* replication of CpGV. *C. pomonella* cell late in infection containing occluded virus particles (capsules). Bar = 1 μm.

capsids migrate to the periphery of the cell, where they either bud or accumulate in areas rich in vesicular membranes, where envelopment occurs. The nucleocapsids align perpendicularly on membrane structures, where they become enveloped. The deposition of granulin on the virion envelope initiates occlusion, which usually progresses by the crystallization of granulin from one end of the rod-shaped virion (**Fig. 3B**). During occlusion all stages in viral replication are evident in the cell. The capsules form

in the periphery of the cell. In cell culture the cells generally round up and fill with occlusion bodies (**Fig. 3C**); *in vivo* cells detach from the basal laminar and from adjacent cells. In infected insects membrane-bound vesicles enclosing occlusion bodies may be observed in some cells, historically named 'boules hyaline'. These may be hemocytes which phagocytose capsules released into the hemolymph. This phenomenon has also been observed in a *C. pomonella* cell line. Capsules are released from the cells by the rupture of the plasma membrane and accumulate in the center of the fat body, the main site of occlusion body production, and in the hemolymph. As a result, just before death, the infected larva becomes milky/paler due to the accumulation of capsules. After death the cadaver will melanize and either liquefy or dessicate, depending on the type of GV infection.

Host Range and Pathology

The host range of most GVs is relatively narrow, with infection being confined to one or more species within the same family as the original host. The few crosstransmission studies where the progeny virus has been identified have shown that in some cases the resulting virus is in fact the homologous virus of the host and probably resulted from activation of an inapparent virus or a contaminant in the inoculum. However, at least four GVs are known to have a relatively wide host range, CpGV, *Heliothis armigera* (cotton bollworm) GV (HaGV), *Spodoptera frugiperda* (fall armyworm) GV and *Xestia c-nigrum* (spotted cutworm) GV (XcGV).

Tissue tropism is an important factor in determining the development of disease in a GV-infected host. The pathogenicity of different GVs is correlated with the extent of infection of various organs in larvae. GVs have recently been grouped into three types based on their tissue tropism. Type 1 have a relatively narrow tissue tropism with infection occurring only in the midgut and fat body; type 2 exhibit wider tissue tropism involving midgut, fat body, epidermis and possibly tracheal matrix and malpighian tubules; type 3 shows stringent tissue tropism, replicating only in the midgut. *Harrisina brillians* (Western grapeleaf skeletonizer) GV is the only recorded lepidopteran baculovirus where GV replication is confined to the midgut epithelium. The infection spreads from cell to cell, with or without the production of budded virus. It is one of the few examples where the virus is more pathogenic to older instars, with first instars dying after approximately 17 days and third instars at 8 days. The larvae die of dehydration due to chronic diarrhea when the occlusion body-filled cells slough off from the gut. However, in general, the speed of kill of a GV depends on several parameters such as dose and susceptibility of the host as well as the tissue tropism. In most GV infections the fat body is the main site of virus replication and occlusion body production. GVs have also been described as 'slow' or 'fast' GVs. The 'slow' GVs (which include many noctuid GVs, e.g. TnGV, HaGV, *Lacanobia oleracea* (tomato moth) GV, *Agrotis segetum* (cutworm) GV, XcGV) have high LD_{50} values, with death being prolonged, as long as 10–20 days, far beyond the normal larval stage. Some of the slow noctuid GVs have much larger genomes in the region of 170 kb, e.g. TnGV, HaGV, XcGV. The 'fast' GVs (e.g. *Artogeia rapae* (small white butterfly) GV (ArGV), CpGV and *Plutella xylostella* (diamond back moth) GV) are highly infectious, have low LD_{50} values <5 capsules per neonate and rapid speeds of kill. The epidermal cells are not infected in the slow/type 1 GVs and the infected insects do not liquefy on death but melanize, shrink and develop a leathery epidermis.

Ecology

GVs are known to be responsible for epizootics in gregarious lepidoptera where the larvae sporadically reach high populations (e.g. *A. rapae*) as a result of contamination of feeding surfaces by virus released from the remains of infected larvae. GV-infected larvae generally climb late in infection and hang by their prolegs. Liquefaction of the dead larvae releases infectious capsules on to surfaces below and aids dissemination of the virus. Epizootics contribute significantly to the natural control of lepidopteran pests, e.g. *A. rapae* and *S. trifolii*. There are reports of the interaction of parasitoids in conjunction with GV-infected hosts and in the transmission of GVs to healthy larvae. Parasitoids that develop in GV-infected larvae are able subsequently to transmit the disease to healthy hosts. Predators produce virus-contaminated excrement after feeding on infected larvae and it appears that several predators actually prefer virus-infected to healthy prey. Among predators, birds are probably more efficient in spreading disease because of the large numbers of insects they consume and the great distances they travel. Occluded virus can remain infectious in the soil for many years, protected from UV damage. Factors such as rain or seed germination are most likely responsible for movement of virus from the soil reservoir on to plants.

GVs as Insecticides

GVs have been successfully used for the control of many pests in small-scale field trials; however, only a

few viruses have been used on a larger scale, e.g. ArGV to control *A. rapae* in China, and *Erinnyis ello* GV to control cassava hornworm in Brazil. Up to 18 GV products have been, or are being, registered and produced, e.g. CpGV is marketed in Europe and has provided effective control of *C. pomonella*, a major pest of apples and a lesser pest of pears and several other fruits. GVs have an important role to play in the control of insecticide resistant pests such as *P. xylostella*, which is a major pest of Cruciferae crops, particularly in countries like Malaysia.

See also: **Baculoviruses (*Baculoviridae*): Nucleopolyhedrovirus.**

Further Reading

Crook NE (1991) Baculoviridae: subgroup B, comparative aspects of granulosis viruses. In: Kurstak E (ed.) *Viruses of Invertebrates*, p. 73. New York: Marcel Dekker.

Doerfler W and Bohm P (eds) (1986) *The molecular biology of baculoviruses. Curr. Top. Microbiol. Immunol.* 131.

Granados RR and Federici B (eds) (1986) *The Biology of Baculoviruses*. Boca Raton: CRC Press.

Hess RT and Falcon LA (1987) Temporal events in the invasion of the codling moth *Cydia pomonella* by granulosis virus: an electron microscope study. *J. Invertebr. Pathol.* 50: 85.

Miller LK (ed.) (1997) *The Baculoviruses*. New York: Plenum Press.

Tanada Y and Hess RA (1991) *Baculoviridae*. Granulosis viruses. In: Adams JR and Bonami JR (eds) *Atlas of Invertebrate Viruses*, p. 227. Boca Raton: CRC Press.

Nucleopolyhedrovirus

George F Rohrmann, Department of Microbiology, Oregon State University, Corvallis, USA

History

As early as 4000 BC, corresponding to the advent of silk production in Asia, came an awareness of a variety of silkworm diseases that posed a constant threat to this emerging industry. One of the major diseases affecting silkworms was likely caused by nucleopolyhedrovirus infection. Protocols for the identification and elimination of infected silkworms were established well before the agents of silkworm diseases were identified. In the mid-nineteenth century, following the development of microscopy, a silkworm disease was identified that was characterized by the presence of massive numbers of polyhedron-shaped, highly refractile, crystalline occlusion bodies present in the infected insect. The disease was called 'polyhedrosis' and by the early twentieth century, polyhedrosis disease was shown to be transmitted by a virus. In the mid-1930s, two distinct polyhedrosis diseases were differentiated by the nuclear or cytoplasmic location of the occlusion bodies, or polyhedra. Subsequently the nuclear polyhedrosis viruses (nucleopolyhedroviruses) were classified as a genus in a new family of viruses, the *Baculoviridae*, whereas the cytoplasmic polyhedrosis viruses were found to have double-stranded, segmented, RNA genomes and were therefore placed in the *Reoviridae*. Concurrent with the finding that these viruses cause a disease of beneficial insects, was the observation that certain insect pests were also afflicted with a similar disease. Therefore, the early impetus for studying nucleopolyhedroviruses was for their potential in insect control programs. More recently nucleopolyhedroviruses have achieved widespread use in both basic and applied biomedical research because of their ability to produce large quantities of proteins from foreign genes that have been inserted into the viral genome under the control of the occlusion body protein promoter.

Taxonomy and Host Distribution

Nucleopolyhedroviruses (NPVs) of the genus *Nucleopolyhedrovirus* are members of the *Baculoviridae*, a family of DNA viruses, characterized by the occlusion of the virions in a crystalline protein matrix. NPV virions contain a single molecule of circular, supercoiled, double stranded (ds) DNA that ranges in size from approximately 90 to over 170 kb in different species of the virus. The genome is packaged in enveloped, rod-shaped (the name baculovirus is derived from the Latin word, *baculum* meaning rod) nucleocapsids, 30–60 nm in diameter and 250–300 nm in length. The two genera of baculoviruses (*Nucleopolyhedrovirus* and *Granulovirus*) are distinguished by the size and morphology of the occlusion body.

NPVs replicate in cell nuclei and are characterized by the occlusion of virions in large (1–15 μm) polyhedron-shaped occlusion bodies comprised predominantly of a single protein called polyhedrin. The occlusion body is surrounded by a structure called the polyhedron envelope or polyhedron calyx (**Fig. 1**). Virions may be present as either single (SNPV) or multiple (MNPV) nucleocapsids within a viral envelope (**Fig. 1**). Although this differentiation is commonly used in describing NPVs, the significance of these morphotypes is unclear and does not appear to be a definitive phylogenetic feature. NPVs are named after the insect host from which they were first described. Such terminology is confusing because the

few viruses have been used on a larger scale, e.g. ArGV to control *A. rapae* in China, and *Erinnyis ello* GV to control cassava hornworm in Brazil. Up to 18 GV products have been, or are being, registered and produced, e.g. CpGV is marketed in Europe and has provided effective control of *C. pomonella*, a major pest of apples and a lesser pest of pears and several other fruits. GVs have an important role to play in the control of insecticide resistant pests such as *P. xylostella*, which is a major pest of Cruciferae crops, particularly in countries like Malaysia.

See also: **Baculoviruses (*Baculoviridae*): Nucleopolyhedrovirus.**

Further Reading

Crook NE (1991) Baculoviridae: subgroup B, comparative aspects of granulosis viruses. In: Kurstak E (ed.) *Viruses of Invertebrates*, p. 73. New York: Marcel Dekker.

Doerfler W and Bohm P (eds) (1986) *The molecular biology of baculoviruses. Curr. Top. Microbiol. Immunol.* 131.

Granados RR and Federici B (eds) (1986) *The Biology of Baculoviruses*. Boca Raton: CRC Press.

Hess RT and Falcon LA (1987) Temporal events in the invasion of the codling moth *Cydia pomonella* by granulosis virus: an electron microscope study. *J. Invertebr. Pathol.* 50: 85.

Miller LK (ed.) (1997) *The Baculoviruses*. New York: Plenum Press.

Tanada Y and Hess RA (1991) *Baculoviridae*. Granulosis viruses. In: Adams JR and Bonami JR (eds) *Atlas of Invertebrate Viruses*, p. 227. Boca Raton: CRC Press.

Nucleopolyhedrovirus

George F Rohrmann, Department of Microbiology, Oregon State University, Corvallis, USA

History

As early as 4000 BC, corresponding to the advent of silk production in Asia, came an awareness of a variety of silkworm diseases that posed a constant threat to this emerging industry. One of the major diseases affecting silkworms was likely caused by nucleopolyhedrovirus infection. Protocols for the identification and elimination of infected silkworms were established well before the agents of silkworm diseases were identified. In the mid-nineteenth century, following the development of microscopy, a silkworm disease was identified that was characterized by the presence of massive numbers of polyhedron-shaped, highly refractile, crystalline occlusion bodies present in the infected insect. The disease was called 'polyhedrosis' and by the early twentieth century, polyhedrosis disease was shown to be transmitted by a virus. In the mid-1930s, two distinct polyhedrosis diseases were differentiated by the nuclear or cytoplasmic location of the occlusion bodies, or polyhedra. Subsequently the nuclear polyhedrosis viruses (nucleopolyhedroviruses) were classified as a genus in a new family of viruses, the *Baculoviridae*, whereas the cytoplasmic polyhedrosis viruses were found to have double-stranded, segmented, RNA genomes and were therefore placed in the *Reoviridae*. Concurrent with the finding that these viruses cause a disease of beneficial insects, was the observation that certain insect pests were also afflicted with a similar disease. Therefore, the early impetus for studying nucleopolyhedroviruses was for their potential in insect control programs. More recently nucleopolyhedroviruses have achieved widespread use in both basic and applied biomedical research because of their ability to produce large quantities of proteins from foreign genes that have been inserted into the viral genome under the control of the occlusion body protein promoter.

Taxonomy and Host Distribution

Nucleopolyhedroviruses (NPVs) of the genus *Nucleopolyhedrovirus* are members of the *Baculoviridae*, a family of DNA viruses, characterized by the occlusion of the virions in a crystalline protein matrix. NPV virions contain a single molecule of circular, supercoiled, double stranded (ds) DNA that ranges in size from approximately 90 to over 170 kb in different species of the virus. The genome is packaged in enveloped, rod-shaped (the name baculovirus is derived from the Latin word, *baculum* meaning rod) nucleocapsids, 30–60 nm in diameter and 250–300 nm in length. The two genera of baculoviruses (*Nucleopolyhedrovirus* and *Granulovirus*) are distinguished by the size and morphology of the occlusion body.

NPVs replicate in cell nuclei and are characterized by the occlusion of virions in large (1–15 μm) polyhedron-shaped occlusion bodies comprised predominantly of a single protein called polyhedrin. The occlusion body is surrounded by a structure called the polyhedron envelope or polyhedron calyx (**Fig. 1**). Virions may be present as either single (SNPV) or multiple (MNPV) nucleocapsids within a viral envelope (**Fig. 1**). Although this differentiation is commonly used in describing NPVs, the significance of these morphotypes is unclear and does not appear to be a definitive phylogenetic feature. NPVs are named after the insect host from which they were first described. Such terminology is confusing because the

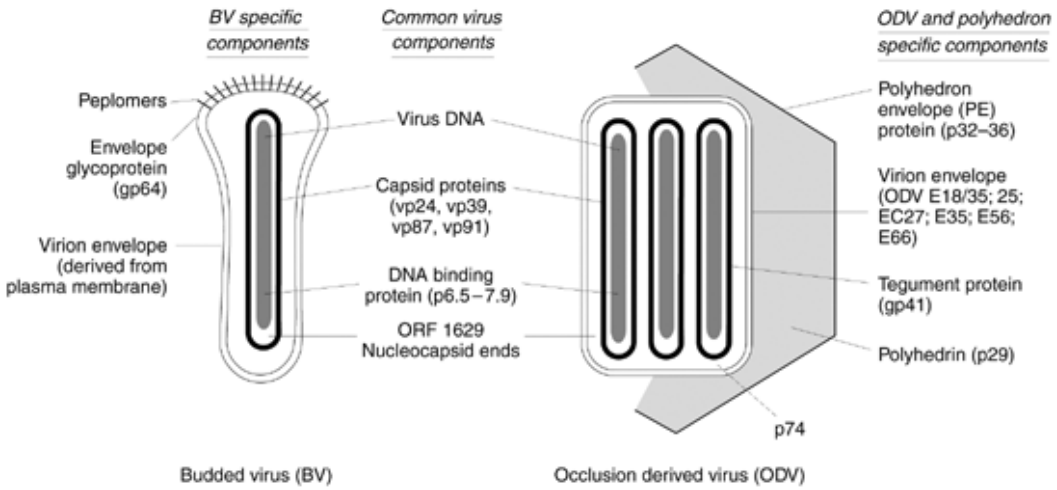


Figure 1 Location of baculovirus structural components. The two baculovirus phenotypes are shown with shared and phenotype-specific components indicated. The dashed line indicates that the location of p74 has not been determined. (Modified with permission from Blissard GW and Rohrmann GF (1990) Baculovirus diversity and molecular biology. *Annu. Rev. Entomol.* 35: 125, by Annual Reviews Inc.)

same virus may infect several insect species and therefore may be called by a variety of names. Some of the most commonly studied baculoviruses are *Autographa californica* MNPV (AcMNPV), *Bombyx mori* NPV (BmNPV), *Lymantria dispar* MNPV (LdMNPV), and *Orgyia pseudotsugata* MNPV (OpMNPV). Their hosts are the alfalfa looper, silkworm, gypsy moth and Douglas-fir tussock moth, respectively.

The granuloviruses (GVs) are occluded in small (0.15–0.5 μm) ellipsoidal-shaped occlusion bodies composed predominantly of a single protein called granulin that is related in amino acid sequence to polyhedrin. The GV virions are normally present as single nucleocapsids within an envelope.

Several nonoccluded rod-shaped DNA viruses pathogenic for insects have been described. These have previously been assigned to a nonoccluded genus of the *Baculoviridae*. However, due to the lack of definitive information on their phylogenetic status, they have been moved to a nonassigned category.

Host Range

Infection by baculoviruses is limited exclusively to arthropods, predominantly holometabolous insects (those undergoing complete metamorphosis). Whereas GVs are pathogenic only for members of the order Lepidoptera (butterflies and moths), NPVs are not only infectious for Lepidoptera, but also for Diptera (flies), Hymenoptera (sawflies) and Trichoptera (caddis flies). In addition, NPVs are infectious for several species of the crustacean order, Decapoda (shrimp), and are a major problem in the shrimp farming industry in Asia. GVs reportedly infect about 150 species of Lepidoptera. NPVs have been reported to infect about 300 species of Lepidoptera, 20 species each of Diptera and Hymenoptera, and a single species of Trichoptera. Individual viruses have a very limited host range and normally only infect closely related insects within a single order.

Genome Structure, Replication and Transposable Elements

Baculoviruses have double-stranded, circular, supercoiled DNA genomes that vary greatly in size from about 100 kb to over 170 kb. The AcMNPV, BmNPV, and OpMNPV genomes have been sequenced and are of similar size (134, 128 and 132 kb, respectively) and are predicted to encode for about 150 proteins. Whereas the AcMNPV and BmNPV genomes are closely related in organization and gene content, the genome of OpMNPV lacks 28 putative genes found in AcMNPV, but contains 26 genes not present in the AcMNPV genome. In addition, the average amino acid sequence identity between predicted open reading frames (ORFs) in AcMNPV and OpMNPV is 56%. In contrast, the *Lymantria dispar* NPV (LdMNPV) genome is much larger (161 kb) than those from AcMNPV and OpMNPV. Furthermore, the pattern of organization for many LdMNPV genes appears to be unrelated to the pattern observed in AcMNPV and OpMNPV. The AcMNPV genome contains a series of eight homologous repeated (*hr*) sequences each containing a series of repeated

imperfect palindromes centered around an EcoR1 site. These repeated sequences have been shown to act as enhancer elements for both early and late gene expression. Related *hr* sequences have been found in a number of other baculoviruses.

The replication of the baculovirus genome is not well-characterized. Recent evidence suggests that the *hr*-enhancer sequences may also function as origins of DNA replication. Three ORFs with sequence similarity to genes encoding proteins involved in DNA replication in other organisms have been identified in the AcMNPV genome. These include genes with homology to DNA polymerase, proliferating cell nuclear antigen (PCNA, that stimulates DNA polymerase δ in other organisms), and helicase (helicase is likely to be involved in the unwinding of duplex DNA during replication). Transient replication assays have identified six genes that are essential for DNA replication. Two of these genes, *dna polymerase* and *p143(helicase)* have homology to genes involved in replication in other organisms. In addition, one of the essential genes, *late expression factor-3 (lef-3)* has the properties of a single-stranded (SS) DNA binding protein, whereas another (*lef-2*) has a primase-like domain that is essential for its function. The viral transactivator, *ie-1* is also required for DNA replication and may function as an origin binding protein as it has been shown to bind to *hr* sequences. Although a role has not been determined for the sixth essential replication gene, *lef-1,* it has been shown to interact with *lef-2*. The mechanism of DNA replication is poorly understood. However, it has been shown that *hr*-containing plasmids replicated in the transient assay appear to be present as high-molecular-weight concatemers suggesting that they may replicate via a 'rolling circle' mechanism. In addition, high-molecular-weight putative concatemers of viral genomic DNA have been observed in DNA extracted from infected cells.

Transposable elements are often reported to be present in baculovirus genomes and likely play an important role in baculovirus evolution. The transposable elements that have been identified include a retrotransposon and a number of different apparently nonautonomous transposable elements. Both these types of transposable elements appear to originate from multicopy DNA in the host cell genome indicating that exchange of genetic information between host cells and viral genomes occurs.

Regulation of Gene Expression

Progression through the viral infection cycle is governed by a cascade of early, late and very late gene transcription. Early transcription begins before the initiation of replication of the viral genome and is inhibited by α-amanitin indicating that early transcription is RNA polymerase II dependent. Investigations using reporter gene constructs transfected into insect cells suggest that the level of transcription from early gene promoters is modulated by at least two viral transactivating factors and *hr* enhancer sequences. Both transactivation and enhancement probably contribute to the speed and facility with which the NPVs are capable of directing the host cell metabolic machinery to selectively transcribe genes necessary for viral replication. Following initiation of viral DNA replication, late gene expression is initiated and the transcription of some, if not all, host genes is repressed. Very late in infection, two genes involved in occlusion body formation (polyhedrin and p10), are hyperexpressed. The transcription of late genes is dependent on the presence of an α-amanitin-resistant RNA polymerase with a unique subunit composition that is distinct from that of the three host RNA polymerases. This polymerase recognizes baculovirus late promoters that normally contain the sequence motif A/G/T TAAG. The late mRNAs normally initiate within the promoter sequence. Eighteen baculovirus genes have been identified that are essential or stimulatory for late gene expression. Because of the nature of the assay used for the identification of these genes, they include the complement of genes also required for DNA replication. However, in addition to the gene required for DNA replication are two genes encoding proteins that have limited homology to the two largest subunits of RNA polymerase II. At least two additional genes have been implicated in very late transcription.

Baculovirus genes are frequently clustered into transcription units that produce overlapping RNAs both in the same or opposite orientation. The differential expression of baculovirus genes may be influenced by these overlapping transcripts. Although splicing does not appear to play a major role in the regulation of most baculovirus genes, the mRNA encoding the AcMNPV transactivating factor gene (IE1) appears to be spliced and this may create several differentially expressed forms of the IE1 protein.

The NPV Life Cycle and Pathogenesis

In the NPVs that have been most intensively studied (e.g. AcMNPV and OpMNPV), the life cycle involves the production of two structurally distinct viral phenotypes (**Fig. 1**). Each phenotype has a specific role during the baculovirus life cycle (**Fig. 2**). The budded virus (BV) phenotype (also known as extracellular virus, ECV or EV) is produced during the early stages of infection and is responsible for the

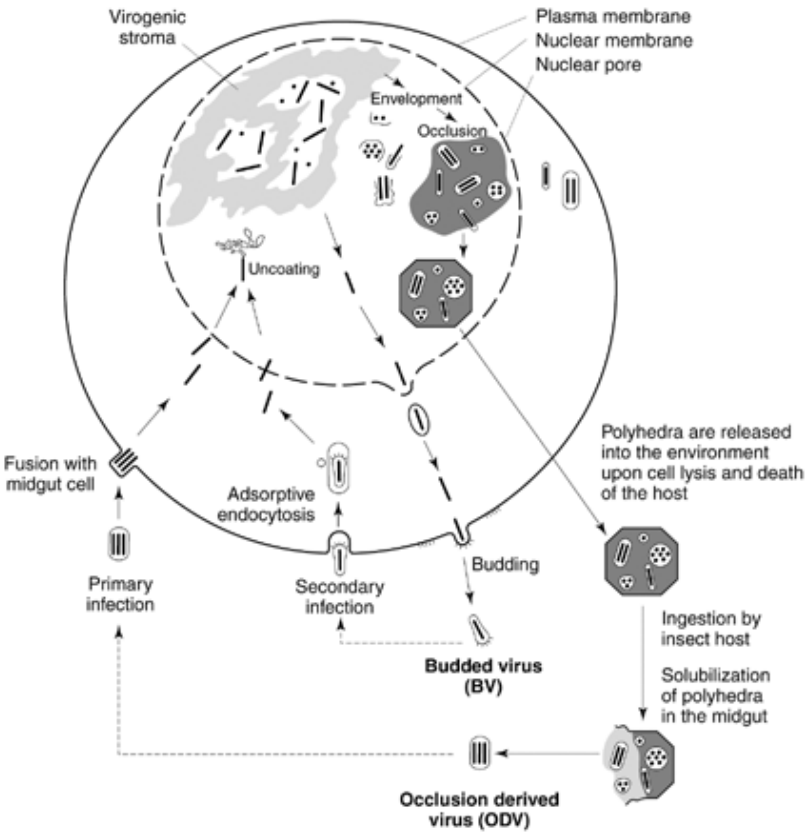


Figure 2 Diagram of the infection cycle of a nucleopolyhedrovirus. See text for description. (Modified with permission from Blissard GW and Rohrmann GF (1990) Baculovirus diversity and molecular biology. *Annu. Rev. Entomol.* 35: 125, by Annual Reviews Inc.)

systemic spread of the virus within the insect. It is also highly infectious for cultured cells. In contrast, the occlusion-derived virion (ODV) phenotype (also known as polyhedron-derived virus, PDV, or occluded virus, OV) is produced during the terminal stages of the viral infection and is occluded within polyhedra. Occluded ODV are capable of persisting for extended periods outside the host insect. Although the nucleocapsids of the two phenotypes appear to be identical, their envelopes are derived from different sources.

The initiation of infection by different NPVs requires from less than 50 to several thousand occlusion bodies per insect and fatal infections take from four days to three weeks. The duration of NPV infections is dependent on factors governing virulence, that are not well characterized. In addition, the species of insect, the larval instar, viral dose and the ambient temperature also influence the length of time of the infection.

Studies with AcMNPV indicate that after ingestion of polyhedra by a susceptible insect, the polyhedra pass through the insect foregut and dissolve when they encounter the alkaline pH of the midgut. ODV are released from the dissolved occlusion bodies and infect both the columnar epithelial cells (that border on the gut lumen) and regenerative cells (that are not exposed to the lumen). The virus appears initially to replicate in the epithelial cells and bud from the cells in a polar manner such that the virus moves away from the gut lumen. The virus has been shown to infect tracheolar cells which apparently act as a conduit for the spread of the infection throughout the insect. In contrast to the pathology outlined above, the replication of the hymenopteran virus, *Neodiprion sertifer* SNPV, appears to be confined to gut cells.

At the cellular level, infection causes cells to become round, and the nuclei enlarge such that they appear to fill the majority of the cell. A distinctive structure called the virogenic stroma develops in the nucleus. This structure is thought to be the major site of viral replication and assembly. Once assembled within the nucleus, nucleocapsids destined to become BV are transported through the cytoplasm and obtain an envelope as they bud through the plasma mem-

brane that has been modified by at least one virally encoded protein. In contrast, ODV become surrounded by an envelope that assembles within the nucleus. Subsequently, they become occluded and are released on the death of the cell.

NPV infection initially results in few symptoms and infected larvae continue feeding. In some insect cells, AcMNPV infection initiates an apoptotic response. However, AcMNPV is capable of blocking apoptosis with a unique protein, p35, which has only been found in AcMNPV and the closely related BmNPV. Other baculoviruses lack p35, but contain an unrelated gene called inhibitor of apoptosis (*iap*) that is also capable of blocking apoptosis. Since their discovery in baculoviruses, *iap* homologues have been identified in a variety of eukaryotes.

As the viral infection progresses, molting of the larval instars is blocked by the production of a virally encoded enzyme, UDP-glucosyltransferase. This enzyme inactivates ecdysteroids that are involved in larval molts and pupation. This block may facilitate increased levels of viral production by keeping the insects in a feeding phase. The late stages of infection are characterized by the production of massive numbers of polyhedron-shaped occlusion bodies within the infected cell nucleus. Near the end of the infection cycle the insects become sluggish and cease feeding. Their epidermis becomes fragile and may rupture, and frequently they seek out the highest nearby location and die in an inverted position. Two virus-encoded proteins, a cathepsin-like protease and a chitinase, appear to contribute to the disintegration of the insect during the final stages of infection. This is thought to facilitate the dissemination of the virus.

Viral Structures and Composition

Structural proteins of polyhedra

Polyhedra contain all the structural components of occluded virions (ODV), as well as two well-characterized components: polyhedrin and the polyhedron envelope protein (**Fig. 1**). Another protein, p10, appears to be associated with the formation of polyhedra. Polyhedrin is a protein composed of about 245 amino acids (29 kDa) and is the major component of polyhedra. Polyhedrin has received considerable attention because it is hyperexpressed and comprises up to 18% or more of the total cellular alkali-soluble protein late in infection.

Polyhedra are surrounded by an electron-dense envelope that has been called the polyhedron envelope, polyhedron membrane or polyhedron calyx. When polyhedra are subjected to alkaline dissolution in the laboratory, the bag-like polyhedron envelope remains with many virions trapped inside. A protein of 34–36 kDa appears to be a major component of the polyhedron envelope of AcMNPV and OpMNPV. In addition to polyhedrin, another baculovirus protein, p10, is also highly expressed late in infection. It forms extensive fibrillar structures that are found in both the nucleus and cytoplasm of infected cells. The p10-containing fibrillar structures show a close association with the developing polyhedron envelope.

Structural proteins of the nucleocapsid

The nucleocapsids of BV and ODV appear to be very similar in composition. The nucleocapsid is composed of at least one small putative DNA binding protein, a protein apparently specific to the basal end structure, and a number of capsid proteins, and the viral genome (**Fig. 1**).

Baculoviruses have large genomes that must be highly condensed to be efficiently packaged within a nucleocapsid. Histones do not appear to be associated with DNA packaging within nucleocapsids. In AcMNPV, a small gene has been identified that encodes an arginine/serine–threonine rich protein of 54 amino acids (termed p6.9) that is thought to be a DNA-binding protein. Homologues of the AcMNPV p6.9 gene have been isolated from other NPVs. These proteins consist of more than 40% arginine and approximately 30% serine or threonine residues. It has been suggested that the basic arginine residues of the DNA-binding protein neutralize the acidic residues of the viral DNA to enable condensation of the viral genome. Upon entry into an insect cell, serine and threonine residues on the DNA binding protein may become phosphorylated by a protein kinase. This would result in the unpackaging of the viral DNA. This hypothesis is supported by the observation that protein kinase activity is associated with purified capsids of granulosis viruses and with both BV and ODV of AcMNPV.

In addition to a DNA-binding protein, a protein of approximately 39 kDa (vp39) has been identified that appears to be a major component of the nucleocapsid of NPVs. This protein is present in both BV and ODV virions at a relatively high concentration. Immunoelectron microscopy confirmed that the vp39 protein is a component of the nucleocapsid and showed that it was randomly distributed over the entire surface of the nucleocapsid. In addition to vp39, vp80 (p87 in OpMNPV), p24 (OpMNPV) and vp91 (OpMNPV) have been found associated with nucleocapsids (**Fig. 1**). A phosphoprotein called ORF 1629 or pp78/83 has been shown to be associated with capsid basal end structures.

Proteins of the budded virus envelope

The major virally encoded protein found associated with the BV envelope is called gp64 (or gp67) and has been characterized in both AcMNPV and OpMNPV (**Fig. 1**). Both immunoelectron and immunofluorescence microscopy indicate that gp64 is concentrated at the plasma membrane. Nucleocapsids become enveloped with the gp64-modified plasma membrane (**Fig. 2**) during their exit from cells. Budded virus primarily enter cells by endocytosis. In this process, the entire virion is endocytosed into an intracellular vesicle called an endosome. It is thought that gp64 may be involved in the fusion of the viral envelope with the endosomal membrane.

The gp64 protein contains a signal peptide sequence at its N-terminus that appears to be cleaved to form the mature protein. Signal peptides are associated with nascent secretory and membrane proteins and are involved in protein trafficking across the membrane of rough endoplasmic reticulum (ER). Signal peptides are normally cleaved off during this process. Once within the ER, secretory proteins move to the cell surface via the Golgi complex where glycosylation may occur. The gp64 amino acid sequence contains five to seven sites for N-linked glycosylation and has been shown to be highly glycosylated (as much as 10 kDa of the molecular weight is contributed by sugar residues). A putative C-terminal anchor domain has also been identified in gp64. The anchor domain is a membrane-spanning domain (that is hydrophobic) followed by a hydrophilic domain that remains on the cytosol side of the membrane. Such domains serve to anchor a protein into the plasma membrane. In addition, palmitic acid is ester-linked to the AcMNPV gp64 peptide making it the major acylated protein associated with BV.

Other viral-specific components of the BV envelope have not yet been reported.

The ODV envelope and other structures

ODVs display a classic bilayer envelope structure that may play an important role in the initiation of infection in the insect gut and could also be essential for the association of the ODV with polyhedrin in the nucleus. The source of the ODV envelope has not been clearly defined but it may be derived from invaginations of the inner nuclear membrane that result in the formation of microvesicles in which a number of ODV envelope-specific proteins are associated. Virally encoded proteins associated with the ODV envelope include E18/35, E25, EC27, E35, E56 and E66. EC27 is found both in the ODV envelope and capsid. In addition, a glycoprotein, gp41, appears to be localized in the space between the nucleocapsids and the envelope (or tegument) in ODV.

A gene, essential for insect infection, called p74, has been identified. AcMNPV mutants, in which the p74 gene has been inactivated, are capable of replicating in cultured cells. However, occluded virus produced in cell culture is noninfectious when fed to insects. This indicates that p74 is required for infectivity when ingested by insects and also suggests that the protein must be associated with ODV.

Ecology

Baculoviruses play a major role in regulating the level of insect populations in the environment. Many insect populations are characterized by cycles of expansion and subsequent collapse. As the number of insects increases, occlusion bodies that contaminate soil or vegetation from past baculovirus epidemics are ingested by susceptible insects and initiate an infectious cycle. When the insects die they disintegrate and contaminate surrounding vegetation. This leads to the virus being disseminated by wind, birds and insects. Thus the virus can spread over vast areas and can significantly contribute to the eventual collapse of an insect population. For example, in forest ecosystems, insect infestations can encompass several million hectares and baculoviruses often play a prominent role in the eventual collapse of these large populations.

Economic Importance

Baculovirus Insecticides

NPVs are highly virulent for insect pests and have been shown to be effective in insect control programs. Currently an NPV pathogenic for the velvetbean caterpillar is used to treat approximately one million hectares of soybeans per year in Brazil. Despite such instances, the use of NPVs for pest control is not widespread. This can be attributed to both the narrow host range of individual NPVs, and the fact that insects may survive for several weeks after infection. During this time they continue feeding and therefore can inflict extensive crop damage prior to death. This limits the commercial potential of NPVs compared to chemical insecticides that are often rapidly toxic to a large number of different insect pests. However, because broad-spectrum chemical insecticides often destroy beneficial insects, and can be acutely toxic to vertebrates, alternative means of controlling insects have become increasingly attractive. With the advent of genetic engineering, insect-specific toxin genes have been inserted into the AcMNPV genome and have been shown to signifi-

cantly accelerate the speed that the virus kills a target insect. Such recombinants may also have an extended host range because insect mortality is not dependent on a completed viral infection cycle. Therefore, NPVs may become more extensively used in future pest control programs.

Baculovirus expression vectors

For baculoviruses to efficiently occlude large numbers of virions, massive amounts of polyhedrin must be produced during the infectious cycle. Although polyhedrin is a hyperexpressed gene product, it is not necessary for growth of the virus in cell culture. This has been exploited in the development of the baculovirus expression system that utilizes recombinant NPVs for the production of foreign gene products. A number of transfer plasmids have been constructed that permit the insertion of foreign genes between polyhedrin flanking sequences and under the control of the polyhedrin promoter. The engineered plasmid DNA is then cotransfected along with wild-type viral DNA into insect cells. Homologous recombination between the flanking sequences of the transfer plasmid and the identical sequences in the viral genome occurs at a relatively high frequency. In the resulting recombinant viruses, the polyhedrin gene is replaced by the foreign gene. These recombinants can then be identified by their polyhedrin negative phenotype. This system has become widely used in biomedical research laboratories in recent years because of the relative simplicity of engineering baculovirus expression vectors and their ability to produce high levels of proteins that are post-transcriptionally modified in a manner similar to higher eukaryotes.

See also: **Vectors: Animal viruses, Plant viruses; Baculoviruses (*Baculoviridae*): Granuloviruses.**

Further Reading

Blissard GW (1996) Baculovirus–insect cell interactions. *Cytotechnology* 20: 73.

Granados RR and Federici BA (eds) (1986) *The Biology of Baculoviruses*, Vols I and II. Boca Raton: CRC Press.

King LA and Possee RD (1992) *The Baculovirus Expression System. A Laboratory Guide*. London: Chapman and Hall.

Miller LK (ed.) (1997) *The Baculoviruses*. New York: Plenum Press.

O'Reilly DR, Miller LK and Luckow VA (1992) *Baculovirus Expression Vectors: A Laboratory Manual*. New York: WH Freeman.

Volkman LE (1997) Nucleopolyhedrovirus interactions with their insect hosts. *Adv. Virus Res.* 48: 313.

BARNAVIRUSES (*BARNAVIRIDAE*)

C Peter Romaine, Department of Plant Pathology, The Pennsylvania State University, University Park, USA

History and Classification

Our appreciation for viral infection of fungi originated in 1962 with the discovery by M. Hollings of several morphologically distinct virus-like particles (VLPs) in cultivated mushrooms (*Agaricus bisporus*) affected by a serious malady known as La France disease. Among the VLPs observed was a 19 × 50 nm bacilliform particle bearing a striking resemblance to a plant virus, alfalfa mosaic virus (AIMV). Mushroom bacilliform virus (MBV) was isolated in the early 1980s and found to be a unique fungal virus, in that it possessed a positive polarity, single-stranded (ss) RNA genome, a feature common to most plant viruses, rather than a double-stranded (ds) RNA genome typifying the vast majority of fungal viruses. Interestingly, amino acid sequence analysis of the putative replicase and the coat suggests an evolutionary relationship between MBV and plant viruses, particularly luteoviruses and carmoviruses.

MBV is the sole member of the genus *Barnavirus* belonging to the family *Barnaviridae* (*Barna* derived from *ba*cilliform-shaped *RNA* virus). Thus, the key features distinguishing this genus in the family *Barnaviridae* from other taxa of fungal viruses is a ssRNA genome and a bacilliform-shaped virion. Viruses in the other genus of the *Barnaviridae* family infect plants and the genus is exemplified by maize rayado fino virus.

Virus Structure and Composition

Virions of MBV are bacilliform, 18–20 nm wide and 48–53 nm long, and lack prominent surface projec-

cantly accelerate the speed that the virus kills a target insect. Such recombinants may also have an extended host range because insect mortality is not dependent on a completed viral infection cycle. Therefore, NPVs may become more extensively used in future pest control programs.

Baculovirus expression vectors

For baculoviruses to efficiently occlude large numbers of virions, massive amounts of polyhedrin must be produced during the infectious cycle. Although polyhedrin is a hyperexpressed gene product, it is not necessary for growth of the virus in cell culture. This has been exploited in the development of the baculovirus expression system that utilizes recombinant NPVs for the production of foreign gene products. A number of transfer plasmids have been constructed that permit the insertion of foreign genes between polyhedrin flanking sequences and under the control of the polyhedrin promoter. The engineered plasmid DNA is then cotransfected along with wild-type viral DNA into insect cells. Homologous recombination between the flanking sequences of the transfer plasmid and the identical sequences in the viral genome occurs at a relatively high frequency. In the resulting recombinant viruses, the polyhedrin gene is replaced by the foreign gene. These recombinants can then be identified by their polyhedrin negative phenotype. This system has become widely used in biomedical research laboratories in recent years because of the relative simplicity of engineering baculovirus expression vectors and their ability to produce high levels of proteins that are post-transcriptionally modified in a manner similar to higher eukaryotes.

See also: **Vectors: Animal viruses, Plant viruses; Baculoviruses (*Baculoviridae*): Granuloviruses.**

Further Reading

Blissard GW (1996) Baculovirus–insect cell interactions. *Cytotechnology* 20: 73.

Granados RR and Federici BA (eds) (1986) *The Biology of Baculoviruses*, Vols I and II. Boca Raton: CRC Press.

King LA and Possee RD (1992) *The Baculovirus Expression System. A Laboratory Guide*. London: Chapman and Hall.

Miller LK (ed.) (1997) *The Baculoviruses*. New York: Plenum Press.

O'Reilly DR, Miller LK and Luckow VA (1992) *Baculovirus Expression Vectors: A Laboratory Manual*. New York: WH Freeman.

Volkman LE (1997) Nucleopolyhedrovirus interactions with their insect hosts. *Adv. Virus Res.* 48: 313.

BARNAVIRUSES (*BARNAVIRIDAE*)

C Peter Romaine, Department of Plant Pathology, The Pennsylvania State University, University Park, USA

History and Classification

Our appreciation for viral infection of fungi originated in 1962 with the discovery by M. Hollings of several morphologically distinct virus-like particles (VLPs) in cultivated mushrooms (*Agaricus bisporus*) affected by a serious malady known as La France disease. Among the VLPs observed was a 19 × 50 nm bacilliform particle bearing a striking resemblance to a plant virus, alfalfa mosaic virus (AIMV). Mushroom bacilliform virus (MBV) was isolated in the early 1980s and found to be a unique fungal virus, in that it possessed a positive polarity, single-stranded (ss) RNA genome, a feature common to most plant viruses, rather than a double-stranded (ds) RNA genome typifying the vast majority of fungal viruses. Interestingly, amino acid sequence analysis of the putative replicase and the coat suggests an evolutionary relationship between MBV and plant viruses, particularly luteoviruses and carmoviruses.

MBV is the sole member of the genus *Barnavirus* belonging to the family *Barnaviridae* (*Barna* derived from *ba*cilliform-shaped *RNA* virus). Thus, the key features distinguishing this genus in the family *Barnaviridae* from other taxa of fungal viruses is a ssRNA genome and a bacilliform-shaped virion. Viruses in the other genus of the *Barnaviridae* family infect plants and the genus is exemplified by maize rayado fino virus.

Virus Structure and Composition

Virions of MBV are bacilliform, 18–20 nm wide and 48–53 nm long, and lack prominent surface projec-

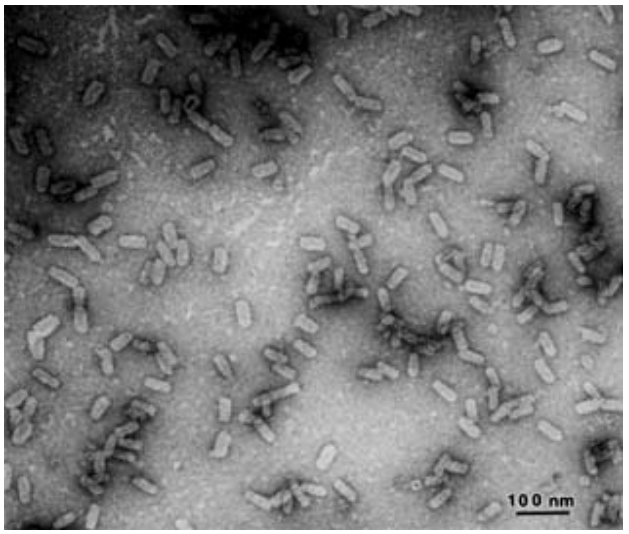


Figure 1 Electron micrograph depicting the 19 × 50 nm bacilliform-shaped virions of MBV.

tions and an envelope (**Fig. 1**). Optical diffraction patterns of the virions resemble those of A1MV, suggesting a morphological subunit diameter of approximately 10 nm and a $T = 1$ icosahedral symmetry.

MBV is stable between pH 6 and 8 and an ionic strength of 0.01 to 0.1 mol L^{-1} phosphate, and is insensitive to chloroform. Purified virus preparations show a single UV-absorbing component following rate-zonal centrifugation in sucrose density gradients and equilibrium centrifugation in cesium sulphate gradients. Virions have a molecular weight of *c.* 7.1 × 10^6 Da and a buoyant density in cesium sulphate of 1.32 g mL^{-1}.

A single molecule of a linear ssRNA of 4 kb constitutes *c.* 20% of the virion by weight. There are an estimated 240 molecules of a single major polypeptide of 24.4 kDa (based on electrophoretic mobility under denaturing conditions) forming the capsid.

Genome Structure

The complete nucleotide sequence of the MBV genome has been determined. The genome is composed of a linear, positive-sense, ssRNA, which is 4009 nucleotides in length. The genomic RNA contains four major open reading frames (ORFs) and has 5′ terminus and 3′ terminus noncoding regions of 60 nucleotides and 250 nucleotides, respectively (**Fig. 2**). The RNA is neither capped at the 5′ terminus nor polyadenylated at the 3′ terminus.

ORF 1 commences at the first AUG codon at nucleotide 61 and ends with UAA at nucleotide 598. ORF 2 is in the +1 reading frame, commencing at nucleotide 68 and terminating at nucleotide 2039 with UAG. ORF 3 is in the same reading frame as ORF 1 (−1 relative to ORF 2), beginning at nucleotide 1882 and ending with a UAA codon at nucleotide 3144. An overlap of 259 nucleotides exists between ORF 2 and ORF 3. ORF 4 initiates at nucleotide 3162, 18 nucleotides downstream of the ORF 3 termination codon in the same reading frame, and terminates with a UGA at nucleotide 3757.

ORFs 1–4 are capable of encoding polypeptides of 20, 73, 47 and 22 kDa, respectively. ORF 3 encodes a putative replicase and ORF 4 encodes the capsid protein. The deduced amino acid sequences of ORFs 2 and 3 both contain putative helicase-like motifs. Three minor ORFs (5–7) potentially encode polypeptides of 8, 6.5 and 6 kDa, respectively. The putative polypeptides encoded by ORFs 1, 5, 6 and 7 show no homology to known polypeptides. In a cell-free system, the RNA directs the synthesis of a major polypeptide (77 kDa), and possibly several minor polypeptides (21–37 kDa).

Host Range and Geographic Distribution

The known host range of MBV is limited to the cultivated button mushroom, *A. bisporus*; however, bacilliform particles that are morphologically similar to MBV have been observed in the field mushroom, *A.*

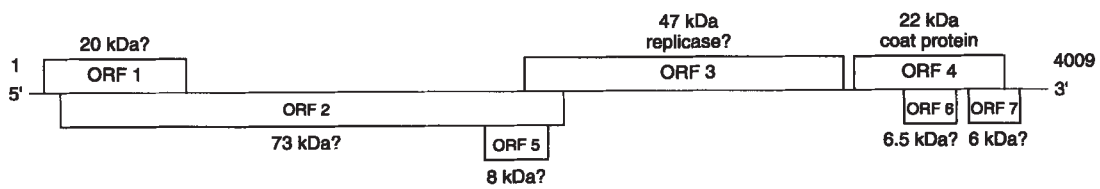


Figure 2 Genome organization of MBV. The genomic RNA (4009 nucleotides) is shown as a bold solid line with the four major opening reading frames (ORFs) 1–4 and the three minor ORFs 5–7 denoted by open rectangles. Numbers below or above the ORFs indicate the size of the polypeptide they may encode. ORF 4 encodes the major capsid protein of 22 kDa and ORF 3 encodes a putative RNA-dependent RNA polymerase (47 kDa).

campestris. The distribution of MBV coincides with that of the commercial cultivation of *A. bisporus*; the virus has been reported in most major mushroom-growing countries.

Transmission and Pathogenicity

Horizontal transmission of MBV is thought to occur through hyphal anastomosis between noninfected mycelium and infected mycelium and basidiospore germlings. MBV can infect *A. bisporus* singly, but is more commonly found as a double infection with La France isometric virus (LIV), a dsRNA genome virus implicated in the etiology of La France disease. MBV is not required in pathogenesis involving LIV, but it remains to be determined if it is a minor cause of La France disease, the causative agent of an unrecognized pathology, or benign. In support of a nonpathogenic nature, a line of *A. bisporus* infected by MBV did not show any overt phenotypic changes in the vegetative and reproductive stages.

Serology

MBV is highly immunogenic. Antibody titers of 1/1024 determined by Ouchterlony double diffusion test can be readily obtained to purified virus. Serotypes have been detected among isolates of MBV, but their possible biological significance is not known. Antibodies raised against MBV do not crossreact with LIV or AlMV.

Detection

Virions of MBV accumulate singly or as aggregates in the cytoplasm of stem and cap tissues of *A. bisporus*. The amount of virus that can be purified from mushrooms singly-infected by MBV is typically several orders of magnitude lower than that obtained from La France disease-affected mushrooms coinfected by LIV. MBV can be detected in apparently healthy and diseased mushrooms by reverse transcription–polymerase chain reaction using primers targeting a sequence in the putative replicase gene.

See also: **Totiviruses (*Totiviridae*): General features; Hypoviruses (*Hypoviridae*); Prions: Human and Animal, Yeast and Fungi.**

Further Reading

Hollings M (1962) Viruses associated with a die-back disease of cultivated mushroom. *Nature* 196: 962.

Revill PA, Davidson AD and Wright PJ (1994) The nucleotide sequence and genome organization of mushroom bacilliform virus: a single-stranded RNA virus of *Agaricus bisporus* (Lange) Imbach. *Virology* 202: 904.

Romaine CP and Schlagnhaufer B (1991) Hybridization analysis of the single-stranded RNA bacilliform virus associated with La France disease of *Agaricus bisporus*. *Phytopathology* 81: 1336.

Romaine CP and Schlagnhaufer B (1995) PCR analysis of the viral complex associated with La France disease of *Agaricus bisporus*. *Appl. Environ. Microbiol.* 61: 2322.

Romaine CP and Schlagnhaufer B (1996) PCR analysis of three RNA genetic elements in *Agaricus bisporus*. In: Royse DJ (ed.) *Mushroom Biology and Mushroom Products*, p. 449. University Park, PA: Pennsylvania State University Press.

Tavantzis SM, Romaine CP and Smith SH (1980) Purification and partial characterization of a bacilliform virus from *Agaricus bisporus:* a single-stranded RNA mycovirus. *Virology* 105: 94.

Tavantzis SM, Romaine CP and Smith SH (1983) Mechanism of genome expression in a single-stranded RNA virus from the cultivated mushroom *Agaricus bisporus*. *Phytopathology* 106: 45.

BENYVIRUSES

Tetsuo Tamada, Research Institute of Bioresources, Okayama University, Kurashiki, Japan

Taxonomy and Classification

The benyviruses (type species beet necrotic yellow vein virus, BNYVV) were formerly classified as possible members of the *Furovirus* genus, which have rigid rod-shaped virions, plus-strand RNA genomes, are generally bipartite and are transmitted by soil-borne fungi of the family Plasmodiophorales. Based on genome organization and sequence relatedness, these viruses have been recently reclassified in the following genera: *Furovirus* (type species soil-borne wheat mosaic virus), *Pecluvirus* (type species peanut

campestris. The distribution of MBV coincides with that of the commercial cultivation of *A. bisporus*; the virus has been reported in most major mushroom-growing countries.

Transmission and Pathogenicity

Horizontal transmission of MBV is thought to occur through hyphal anastomosis between noninfected mycelium and infected mycelium and basidiospore germlings. MBV can infect *A. bisporus* singly, but is more commonly found as a double infection with La France isometric virus (LIV), a dsRNA genome virus implicated in the etiology of La France disease. MBV is not required in pathogenesis involving LIV, but it remains to be determined if it is a minor cause of La France disease, the causative agent of an unrecognized pathology, or benign. In support of a nonpathogenic nature, a line of *A. bisporus* infected by MBV did not show any overt phenotypic changes in the vegetative and reproductive stages.

Serology

MBV is highly immunogenic. Antibody titers of 1/1024 determined by Ouchterlony double diffusion test can be readily obtained to purified virus. Serotypes have been detected among isolates of MBV, but their possible biological significance is not known. Antibodies raised against MBV do not crossreact with LIV or AlMV.

Detection

Virions of MBV accumulate singly or as aggregates in the cytoplasm of stem and cap tissues of *A. bisporus*. The amount of virus that can be purified from mushrooms singly-infected by MBV is typically several orders of magnitude lower than that obtained from La France disease-affected mushrooms coinfected by LIV. MBV can be detected in apparently healthy and diseased mushrooms by reverse transcription–polymerase chain reaction using primers targeting a sequence in the putative replicase gene.

See also: **Totiviruses (*Totiviridae*): General features; Hypoviruses (*Hypoviridae*); Prions: Human and Animal, Yeast and Fungi.**

Further Reading

Hollings M (1962) Viruses associated with a die-back disease of cultivated mushroom. *Nature* 196: 962.

Revill PA, Davidson AD and Wright PJ (1994) The nucleotide sequence and genome organization of mushroom bacilliform virus: a single-stranded RNA virus of *Agaricus bisporus* (Lange) Imbach. *Virology* 202: 904.

Romaine CP and Schlagnhaufer B (1991) Hybridization analysis of the single-stranded RNA bacilliform virus associated with La France disease of *Agaricus bisporus*. *Phytopathology* 81: 1336.

Romaine CP and Schlagnhaufer B (1995) PCR analysis of the viral complex associated with La France disease of *Agaricus bisporus*. *Appl. Environ. Microbiol.* 61: 2322.

Romaine CP and Schlagnhaufer B (1996) PCR analysis of three RNA genetic elements in *Agaricus bisporus*. In: Royse DJ (ed.) *Mushroom Biology and Mushroom Products*, p. 449. University Park, PA: Pennsylvania State University Press.

Tavantzis SM, Romaine CP and Smith SH (1980) Purification and partial characterization of a bacilliform virus from *Agaricus bisporus:* a single-stranded RNA mycovirus. *Virology* 105: 94.

Tavantzis SM, Romaine CP and Smith SH (1983) Mechanism of genome expression in a single-stranded RNA virus from the cultivated mushroom *Agaricus bisporus*. *Phytopathology* 106: 45.

BENYVIRUSES

Tetsuo Tamada, Research Institute of Bioresources, Okayama University, Kurashiki, Japan

Taxonomy and Classification

The benyviruses (type species beet necrotic yellow vein virus, BNYVV) were formerly classified as possible members of the *Furovirus* genus, which have rigid rod-shaped virions, plus-strand RNA genomes, are generally bipartite and are transmitted by soil-borne fungi of the family Plasmodiophorales. Based on genome organization and sequence relatedness, these viruses have been recently reclassified in the following genera: *Furovirus* (type species soil-borne wheat mosaic virus), *Pecluvirus* (type species peanut

Table 1 First occurrences of rhizomania (BNYVV)

Year	*Country (main region)*
1949	Italy (northern regions)
1965	Japan (Hokkaido)
1971	Yugoslavia (Serem)
1972	Greece (Thessaly and Yannouli area)
1973	France (Erstein region, Alsace)
1974	Germany (southern parts of Hesse near Frankfurt)
1978	Czechoslovakia, China (Inner Mongolia)
1979	Austria (Danube Valley), Romania, former USSR
1982	Hungary
1983	USA (California), Switzerland (Seeland, regions of Chietres and Anet), Bulgaria, The Netherlands
1984	Belgium
1987	United Kingdom (East Anglia), Turkey
1993	Spain

BNYVV is also found in Poland, Kazakstan, Kirgizia and Mongolia. Based on Asher (1993).

clump virus), *Pomovirus* (type species potato mop-top virus) and *Benyvirus* (type species BNYVV). These genera have not been assigned to a family or families.

Benyvirus (BNYVV) differs from these and other viruses with rod-shaped particles, including *Tobamovirus*, *Tobravirus* and *Hordeivirus*, in polymerase phylogeny, genome organization and expression strategy. For example, BNYVV RNA is 3′-polyadenylated, whereas the 3′ ends of other virus RNAs are not polyadenylated but can be folded into a tRNA-like structure. The putative replication-associated protein of BNYVV RNA is encoded by a single long open reading frame (ORF) producing a large product, which is autocatalytically processed to give the two species of products. In contrast, other viruses contain motifs for these functions on two ORFs separated by a leaky stop codon or by two respective genomes. Based on the polymerase phylogeny, BNYVV is included in a cluster together with animal *Togavirus* in the alphavirus-like superfamily.

The other *Benyvirus* members include beet soil-borne mosaic virus (BSBMV) and burdock mottle virus (BdMV), both of which are of similar genome organization and sequence homology to BNYVV. BNYVV and BSBMV are more closely related to each other than to BdMV.

Geographic Distribution

BNYVV causes the rhizomania disease of sugarbeet (*Beta vulgaris* var. *saccharifera*). The disease was first found in Italy during the 1950s, in the Po Plain and Adige Valley, but the causal virus was not identified until the 1970s, although the soil-inhabiting fungus *Polymyxa betae* had been found to be associated with the disease. Subsequent first reports of rhizomania in other countries from around the world are shown in **Table 1.** From 1971 to 1983 it was observed in an increasing number of countries, from central and southern Europe to eastern and northern Europe. In 1987, a single focus was discovered in eastern England, since when several more foci have been found in the same area of the UK. BNYVV is presently considered to occur in most sugarbeet-growing countries in Europe, but has not yet been recorded in the Republic of Ireland or in the Scandinavian countries – Denmark, Sweden and Finland.

In Japan, BNYVV was first found in 1965 and spread in sugarbeet-growing areas in Hokkaido. In China, since the first recorded finding in 1987, BNYVV has spread throughout the districts along the Yellow River and Xinjiang Uygur. In the USA, BNYVV was first recorded in California in 1983 and in Texas in 1987. Between 1992 and 1994, the disease was found in Colorado, Idaho, Nebraska and Wyoming.

BSBMV is widely distributed in the USA, but has not been identified from other countries. BdMV was first found in burdock (*Arctium lappa* L.) in Japan in 1970.

Economic Importance

BNYVV causes severe damage in sugarbeet. Yield losses depend greatly on the inoculum level in the soil, the weather conditions and the time of infection. Severe infection leads to reduction in yield of 50% or more; in particular, the sugar content is reduced from

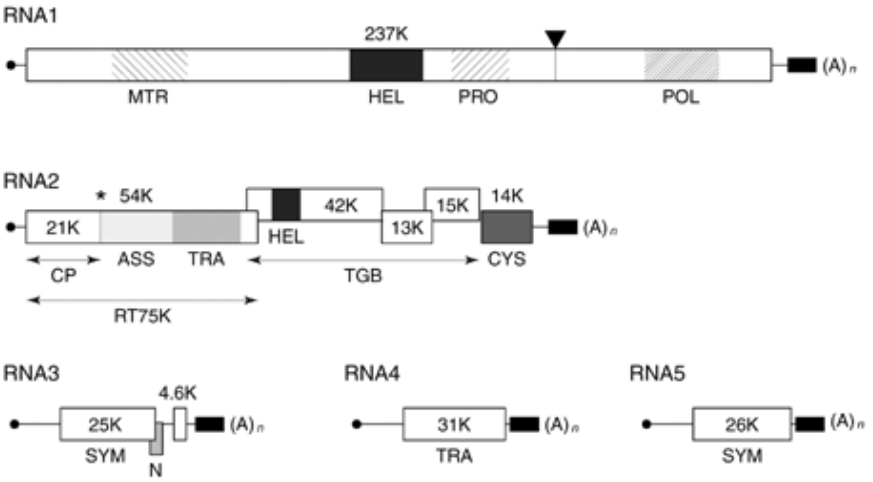


Figure 1 Genome organization of beet necrotic yellow vein virus. The filled circles indicate the cap structures, the boxes indicate ORFs, and the black rectangles indicate conserved common sequences preceding the 3′ poly(A) tail. The sizes of polypeptide products are given as K (kDa). The black triangle indicates the location of the predicted cleavage site and the asterisk indicates a readthrough amber terminal codon. The coat protein (CP), the readthrough protein (RT) and the triple gene block (TGB) are shown by arrows. The methyltransferase domain (MTR), the NTP-binding/helicase domain (HEL), the papain-like proteinase domain (PRO), the GDD replicase domain (POL), the cystein-rich (CYS) region and those involved in virus assembly (ASS), transmission (TRA), symptom expression (SYM) and necrosis (N) are indicated.

16–18% to less than 10%. Since BNYVV survives in the soil for many years without any decrease in infectivity, its presence makes it necessary to avoid growing sugarbeet in heavily infested soils.

BNYVV shows a great capacity for local dispersal from infested fields and for spread to regions previously uninfested. For example, although the exact origin of the epidemic since the 1970s is obscure, it is certain that large areas of beet cultivation in Europe, the USA and China were free from BNYVV until recently, but there has been considerable spread within Europe and elsewhere (**Table 1**). Its absence from certain countries and regions has been confirmed by intensive surveying. It is thus only in the last 15–20 years that BNYVV has developed into a serious problem in many sugarbeet-growing regions of the world.

Virus Structure

BNYVV particles are hollow, rigid nucleoprotein rods around 20 nm in diameter, with essentially two dominant particle lengths of 390 nm (RNA1) and 270 nm (RNA2). BNYVV has shorter particles of 105, 90 and 80 nm, corresponding to smaller RNA species of RNA3, -4 and -5, respectively. For BNYVV, the single-stranded right-handed helix has a 2.6 nm pitch with an axial repeat of four turns, involving 49 subunits of a 21 kDa protein, which encapsidate each single-stranded genomic RNA component separately. RNA contains about 5% of the particle weight.

Genome Structure and Molecular Biology

The genome of BNYVV consists of five plus-strand RNA components designated RNA1 to RNA5 in order of decreasing size. A genetic map of the viral RNAs is shown in **Fig. 1.** All five viral RNAs have cap structures at their 5′-termini and terminate in a 3′-poly(A) tail. Close sequence homology among the five RNAs is limited to the 5′-terminal 8–9 nucleotides and the last 70 nucleotides preceding the 3′-poly(A) tail.

RNA1 contains a single long ORF encoding a polypeptide of 237 kDa which contains the information necessary for replication of the viral genome. This ORF contains three distinct replication-associated domains: a methyltransferase domain, an NTP-binding/helicase domain and a polymerase domain. The primary translation product of RNA1 can be cleaved autocatalytically into two species of 150 kDa and 66 kDa by a papain-like protease motif between the helicase domain and the polymerase domain. The 66 kDa protein which contains the entire polymerase domain is detected immunologically in virus-infected protoplasts.

RNA2 consists of six ORFs. The 5′-proximal ORF of RNA2 encodes the 21 kDa coat protein. The coat protein cistron is separated from a long (54 kDa) in-phase ORF by a single amber termination codon which is suppressed about 10% of the time to produce a coat protein-54 kDa fusion protein of 75 kDa (read-

through protein). The 75 kDa readthrough protein is involved in virus assembly and fungus transmission.

The central portion of RNA2 contains a cluster of three slightly overlapping genes known as the triple gene block (TGB), encoding proteins of 42 kDa, 13 kDa and 15 kDa. Synthesis of 42 kDa protein is directed by subgenomic RNA2, whereas synthesis of both 13 kDa and 15 kDa proteins is probably directed by a dicistronic subgenomic RNA2. The TGB has amino acid sequence homology and similar hydrophobicity to equivalent proteins involved in cell-to-cell movement of a number of other plant viruses, including the potex-, carla-, hordei- pomo- and pecluviruses. The first TGB protein (42 kDa) has sequence motifs characteristic of a superfamily 1 DNA or RNA helicase, including a 'P-loop' ATP/GTP-binding domain. This protein possesses nucleic acid-binding activity. The binding site is situated near the N-terminus, but the P-loop motif is not required for nucleic acid-binding. The second TGB protein (13 kDa) has two potentially membrane-spanning hydrophobic domains separated by a hydrophilic sequence that contains a highly conserved peptide motif of unknown significance. The N-terminal portion of the third TGB protein (15 kDa) is hydrophobic. The 42 kDa and 13 kDa proteins are detected in a membrane-enriched subcellular fraction.

The 3′-proximal ORF of RNA2 encodes a cystein-rich 14 kDa protein which is expressed from a subgenomic RNA. The 14 kDa protein is soluble and binds zinc ions *in vitro*, suggesting that it may bind RNA and/or DNA to regulate host genome expression.

RNA3 encodes a 25 kDa protein. The 3′-terminal 600 nucleotides of RNA3 are easily detected *in vivo* as an unencapsidated, subgenomic mRNA which encodes a 4.6 kDa polypeptide of unknown function. A short ORF N (necrosis) overlaps the 3′-terminal portion of the 25 kDa ORF. The 25 kDa protein is soluble *in vivo* and localized in both cytoplasm and nuclei of infected leaves. RNA3 is involved in symptom expression. RNA4 has a longer ORF for a 31 kDa protein which is soluble *in vivo*. The RNA4 is important for fungus transmission. RNA5 contains a single ORF encoding a 26 kDa protein which is involved in symptom expression. RNA3, -4 and -5 have unusually long 5′-noncoding regions of 445, 379 and 443 nucleotides, respectively.

Genetics

BNYVV RNAs 1 and 2 have 'housekeeping' genes involved in replication, assembly and cell-to-cell movement, whereas RNA3, -4 and -5 are associated in vector-mediated infection of sugarbeet roots. RNA1 is able to replicate by itself in protoplasts, indicating that it contains all the information needed for replication.

RNA2 encodes the coat protein at its 5′ end. This ORF must be expressed very efficiently. The readthrough protein of the coat protein is a minor component of virions and is located at the ends of the viral particles. The readthrough domain consists of two subdomains, in which a region within the N-terminal half of the domain is involved in virus assembly, whereas the C-terminal portion contains sequences important for vector transmission. Alanine scanning mutagenesis of the C-terminal region shows a KTER motif which is important for fungal transmission.

Cell-to-cell movement is controlled by the three slightly overlapping TGB genes (42 kDa, 13 kDa and 15 kDa). Expression of the first two proteins can complement independently, but overexpression of 15 kDa inhibits cell-to-cell movement of the virus infection. The cystein-rich 14 kDa protein may bind RNA and/or DNA to regulate host genome expression. Indeed, there is evidence that the 14 kDa protein acts *in cis* to stimulate the accumulation of RNA2, but also acts independently and *in trans* as an enhancer of viral coat protein synthesis.

Despite the essential functions of RNA1 and RNA2 for virus multiplication, natural field isolates always include RNA3 and RNA4, or in addition RNA5. The 25 kDa protein encoded by RNA3 increases virus multiplication in roots and is responsible for rhizomania symptoms, and the RNA4-encoded 31 kDa protein facilitates fungus transmission. RNA5, encoding the 26 kDa protein, is associated with the severity of symptom expression in roots. Synergistic interactions are found between RNA3 and RNA4 or RNA5 and RNA4.

When leaves of laboratory hosts such as *Tetragonia expansa* are mechanically inoculated with BNYVV, RNA3, -4 and -5 can be eliminated intentionally from the isolate, or if present at the outset, they may disappear spontaneously or undergo extensive internal deletion during virus propagation. RNA3 has dramatic effects on symptoms on leaves. Virus isolates containing RNA3 produce bright yellow local lesions on *T. expansa* whereas isolates lacking RNA3 produce much milder symptoms. Mutagenic analysis reveals that the 25 kDa protein is responsible for these yellow spot lesion phenotypes. In addition, there is a short ORF, called N (necrosis), which overlaps the C-terminus of the 25 kDa ORF. The N gene is not detectably expressed from full-length RNA3, but can induce necrotic local lesions when activated by deletion of upstream sequences, or when inserted into another plant virus replicon.

The smaller RNAs of BNYVV are a useful material for the identification of sequences that are recognized *in cis* by the viral replication process, because they do not encode factors required for their own replication. At the 3′-terminus the *cis*-active domain is thus located within the last 70 residues preceding the 3′-poly(A) tail, in which the region can be folded into a double hairpin secondary structure which is common to the five viral RNAs. This structure probably comprises the promoter for initiation of minus-strand RNA synthesis. At the 5′-terminus, *cis*-active sequence elements are located in the first 292 residues of RNA3, in which at least two of the essential subdomains appear to be important in the formation of secondary structure. A sequence about 200 nucleotides from the 5′-terminus contains a *cis*-active encapsidation signal for RNA3. For RNA4, the 5′-proximal *cis*-essential elements are limited to the first 400 residues, but essential subdomains within this region have not been mapped. These sequences are presumably important for initiation of synthesis of the RNA plus-strand.

Serological Relationships and Variability

BNYVV and BSBMV are serologically related but distinct. BNYVV and BdMV are not serologically related. No serological differences are found among BNYVV isolates. However, the majority of the virus isolates may be classified into two major groups (A and B types) by single-strand conformation polymorphisms of immunocapture reverse transcriptase–polymerase chain reaction (RT-PCR) products. The nucleotide sequences of A and B types are about 97% identical when averaged over all four RNAs. The A type is found in most European countries, USA, China and Japan, whereas the B type is detected in Germany and France. It is not known whether the A and B types differ in pathogenicity. In a region of France, a third type has been found, called P, which contains RNA5. RNA5 is also detected in BNYVV isolates from Japan and China. The isolates containing RNA5 show a much stronger virulence than isolates lacking RNA5. Considerable sequence variation is also found between RNA5 molecules from French and Japanese isolates.

Host Range, Symptoms and Diseases Caused

Benyviruses (BNYVV, BSBMV and BdMV) have a narrow host range: Chenopodiaceae plant species such as the genera *Beta* and *Chenopodium,* and *T. expansa* are especially susceptible. BNYVV infects most species of the family Chenopodiaceae and several species belonging to the Aizoaceae, the Amarathaceae, the Caryophyllaceae and the Solanaceae. The virus tends to be restricted to the inoculated leaves of most host plants, when inoculated mechanically by sap. Symptoms appear as chlorotic or yellow spots which spread along the veins. The virus systemically infects *Beta macrocarpa* and spinach, causing stunting and conspicuous leaf mottling.

In nature, BNYVV infects sugarbeet, fodder beet, Swiss chard and spinach. The disease is usually distributed as foci (patches) in the sugarbeet field. The most useful leaf symptom is visible at the end of the growing season. Leaves become pale yellow in color, with long petioles and upright growth. Symptoms are characterized by root stunting and proliferation on lateral rootlets on the main taproot, and yellow-brown vascular bundles. These are typical root symptoms of BNYVV, called rhizomania. In early and severe infection, the plants are stunted, wilted and eventually die. The taproots are very small and lateral roots and rootlets proliferate. In this condition, the bright yellow color followed by necrosis along veins (giving the virus the name necrotic yellow vein) is very rarely seen. This symptom only results from movement of virus to leaves. Slight and later infection may produce no obvious symptoms.

BSBMV shows milder symptoms in sugarbeet than BNYVV in greenhouse tests. Systemic foliar symptoms caused by BSBMV appear more frequently than those caused by BNYVV. The symptoms are slight leaf distortion, mottling and yellow vein banding. Although BSBMV often causes no obvious symptoms in sugarbeet roots, some plants systemically infected with BSBMV may show similar symptoms to rhizomania. BSBMV and BNYVV are also found in the same field and even in the same plant in rhizomania-infested areas.

BdMV causes faint leaf chlorosis in burdock plants.

Detection and Diagnosis

Because foliage and root symptoms of benyviruses are obscure, as described above, ELISA is the best method for the efficient and accurate diagnosis of BNYVV. Samples should be extracted from lateral roots or from the tip of the taproot. Nucleic acid hybridization and RT-PCR assays have been developed for the detection of BNYVV. These techniques are specific, but not yet suitable for routine tests of large numbers of samples. They are used for distinguishing BNYVV strains or variants which contain different smaller RNAs. In soil or adherent soil, a biological test is possible. Beet bait plants are grown in suspected soil, and their rootlets are tested by ELISA. Bait plant tests to estimate soil infestation with BNYVV using

pregrown sugarbeet seedlings can be used to estimate the level of infestation as well as to calculate potential yield losses.

Transmission by Fungus

BNYVV and BSBMV are transmitted by the soil-inhabiting fungus *Polymyxa betae*. No vectors are known for BdMV, but its genome structure suggests that it is fungus-transmissible. *P. betae* belongs to the family Plasmodiophoraceae and is an obligate parasite of root tissue of mostly Chenopodiaceae plant species, including sugarbeet. The genus *Polymyxa* includes *P. graminis*, which is a vector of many viruses belonging to *Furovirus*, *Pecluvirus* and *Bymovirus*. *P. betae* and *P. graminis* are morphologically indistinguishable, but have different host ranges. The life cycle of these fungi consists of biflagellate zoospores, multinucleate plasmodia, zoosporangia and thick-walled resting spores in clusters or cystosori, but all lack a mycelial stage.

BNYVV can survive in soil within the long-lived resting spores of the vector. There is no evidence for virus replication inside the fungal vector. The virus is acquired by *P. betae* only *in vivo* from infected root cells. Virus particles are internal, because transmission cannot be reduced by treating viruliferous zoospores with virus-specific antiserum or by washing resting spores with acid or alkali. When a viruliferous zoospore comes into contact with a healthy plant root, it becomes encysted and the contents, including virus particles, enter the epidermal cell. During growth of the plasmodium, the virus is probably released into the host cell cytoplasm. The plasmodium becomes either a zoosporangium or a cystosorus with clusters of resting spores, from which viruliferous zoospores are eventually released for infection of further host plants.

Molecular studies for BNYVV transmission show that the C-terminal region of readthrough proteins of coat protein encoded by RNA2 is essential for fungal transmission. In addition, other genome components have an indirect effect on fungal transmission: the 31 kDa protein (RNA4) is important for transmission efficiency. Although RNA3 and RNA5 are not involved in fungal transmission, the 25 kDa protein (RNA3) is responsible for rhizomania of sugarbeet, and the abnormal proliferation of fine rootlets seems to be favorable for the multiplication of *P. betae*. Thus, there may be a symbiotic interaction between BNYVV and *P. betae*. In the case of BNYVV, RNA3, -4 and -5 play important, but different, roles in the vector-mediated infection of sugarbeet roots.

Epidemiology

BNYVV is not transmitted by seed or pollen; however, the spread of the virus can result from soil contamination of seeds which have been produced in infested areas. Dried infected roots or air-dried soils have been shown to retain the infectivity for more than 15 years. A similar longevity of the fungus is observed in field conditions. Thus the virus disease has occurred when sugarbeet has been grown in fields in which no crops have been cultivated for 10–15 years.

The spread of BNYVV (with resting spores of viruliferous *P. betae*) is brought about by the use of machinery on contaminated land, transportation of infested soil (beet roots, potatoes, possible beet seed or any vegetables grown on infested land) and application of irrigation. Factory waste, washing water and agricultural equipment are potentially significant in the spread of the disease. Stable manure can play a role in the dispersal of BNYVV, because *P. betae* is capable of passing undamaged through the digestive tracts of animals. Fungal resting spores can be dispersed by floods or wind.

The most important factors affecting the development of disease in infected fields are the level of inoculum, the temperature of and the moisture in soil. As a zoosporic fungus, *P. betae* requires a high soil moisture level for maximum activity: water is essential to enable cysts to germinate and zoospores to swim to roots. Thus, the development of the disease becomes severe where there is poor soil structure, inadequate drainage, frequent heavy rainfall or the use of (excessive) irrigation. A relatively high temperature of about 25°C is suitable for *P. betae* activity. Soil temperature in the spring and early summer may therefore be of particular importance because the earlier that the plants are infected, the more severe the damage. *P. betae* infects most rapidly and actively in neutral or alkaline soils.

Control

Control of diseased fields is not likely to be achieved by management of cultural or agronomic practices. Some chemicals, especially soil fumigants, have been partially effective in reducing yield losses but are generally not economic. The search for tolerant or resistant cultivars has been actively carried out since 1978: the results obtained have been very encouraging. Forecasting or estimating the level of infestation which corresponds to losses in yield would be helpful for adaptation of resistant varieties and other control measures.

In moderately or heavily infested areas, agronomic factors and cultural practices appear to be of minor, if any, importance in disease management. However, in areas where BNYVV is still absent or the inoculum potential is low, measures such as wider crop rotation, controlled irrigation and effective drainage can delay the spread and incidence of the disease.

In general, soil fumigants such as dichloropropene, dichloropropene and dichloropropane, chloropicrin and methyl bromide have been proved to be effective. Treatments with such fumigants may be economically worthwhile where the disease is seriously widespread and no other control measures are available; however, they are only partially effective because, although resting spores of the fungus in the upper layers of the soil may be killed, reinfestation from lower layers occurs during the growing season and treatments must be repeated each time a crop is grown.

Several resistant cultivars such as Rizor have been developed and are presently grown in rhizomania-infested regions of continental Europe and other countries. The resistance of such cultivars is caused by a restriction of virus multiplication and/or translocation in the roots, but not by resistance to infection by the vector *P. betae*. In general, resistant and tolerant cultivars that have been so far developed yield about 10% less than standard cultivars in normal conditions. The continuing efforts of plant breeders should ensure that new varieties that are higher yielding and more resistant will be developed, either by conventional breeding or by biotechnological methods, in the future.

See also: **Furoviruses; Plant virus disease – economic aspects.**

Further Reading

Asher MJC (1993) Rhizomania. In: Cooke DA and Scott RK (eds) *The Sugar Beet Crop: Science into Practice.* p. 311. London: Chapmann and Hall.

Jupin I, Tamada T and Richards K (1991) Pathogenesis of beet necrotic yellow vein virus. *Semin. Virol.* 2: 112.

Richards KE and Tamada T (1992) Mapping functions on the multipartite genome of beet necrotic yellow vein virus. *Annu. Rev. Phytopathol.* 30: 291.

Rush CM and Heidel GB (1995) Furovirus diseases of sugar beets in the United States. *Plant Dis.* 79: 868.

Torrance L and Mayo MA (1997) Proposed re-classification of furoviruses. *Arch. Virol.* 142: 435.

BF23 Phage *see* **T5-Like Phage and Related Phages**

BIRNAVIRUSES – ANIMAL (*BIRNAVIRIDAE*)

Hermann Becht, Linden, Germany

History and Definition

The family *Birnaviridae* was established in 1986 to describe and classify a group of viruses which carry a bisegmented double-stranded RNA (dsRNA) genome as their prominent characteristic. Further unique features can be noted for this family: the genomic segments are circularized by a genome-linked protein; segment A of the genome is structurally and functionally bicistronic, and the main translation product is a polyprotein cleaved by a virus-coded protease. The two main representatives of this virus family are the infectious pancreatic necrosis virus of fish (IPNV) and the causative agent of infectious bursal disease of chickens (IBDV). Infectious pancreatic necrosis was described for the first time in Canada in 1940; the viral nature of the causative agent was defined in 1960, and in subsequent years the structure and molecular biology of this virus has been studied extensively.

In 1962 an outbreak of a novel disease in a chicken flock was described in Gumboro, Delaware, USA, and according to this location of the first description further outbreaks have subsequently been referred to as 'Gumboro disease'. The most prominent lesion of the preferentially affected organ coined the designation 'infectious bursal disease' and IBDV as the causative virus. The first attempts to clarify the etiological agent mostly depended on electron micro-

In moderately or heavily infested areas, agronomic factors and cultural practices appear to be of minor, if any, importance in disease management. However, in areas where BNYVV is still absent or the inoculum potential is low, measures such as wider crop rotation, controlled irrigation and effective drainage can delay the spread and incidence of the disease.

In general, soil fumigants such as dichloropropene, dichloropropene and dichloropropane, chloropicrin and methyl bromide have been proved to be effective. Treatments with such fumigants may be economically worthwhile where the disease is seriously widespread and no other control measures are available; however, they are only partially effective because, although resting spores of the fungus in the upper layers of the soil may be killed, reinfestation from lower layers occurs during the growing season and treatments must be repeated each time a crop is grown.

Several resistant cultivars such as Rizor have been developed and are presently grown in rhizomania-infested regions of continental Europe and other countries. The resistance of such cultivars is caused by a restriction of virus multiplication and/or translocation in the roots, but not by resistance to infection by the vector *P. betae*. In general, resistant and tolerant cultivars that have been so far developed yield about 10% less than standard cultivars in normal conditions. The continuing efforts of plant breeders should ensure that new varieties that are higher yielding and more resistant will be developed, either by conventional breeding or by biotechnological methods, in the future.

See also: **Furoviruses; Plant virus disease – economic aspects.**

Further Reading

Asher MJC (1993) Rhizomania. In: Cooke DA and Scott RK (eds) *The Sugar Beet Crop: Science into Practice.* p. 311. London: Chapmann and Hall.

Jupin I, Tamada T and Richards K (1991) Pathogenesis of beet necrotic yellow vein virus. *Semin. Virol.* 2: 112.

Richards KE and Tamada T (1992) Mapping functions on the multipartite genome of beet necrotic yellow vein virus. *Annu. Rev. Phytopathol.* 30: 291.

Rush CM and Heidel GB (1995) Furovirus diseases of sugar beets in the United States. *Plant Dis.* 79: 868.

Torrance L and Mayo MA (1997) Proposed re-classification of furoviruses. *Arch. Virol.* 142: 435.

BF23 Phage *see* **T5-Like Phage and Related Phages**

BIRNAVIRUSES – ANIMAL (*BIRNAVIRIDAE*)

Hermann Becht, Linden, Germany

History and Definition

The family *Birnaviridae* was established in 1986 to describe and classify a group of viruses which carry a bisegmented double-stranded RNA (dsRNA) genome as their prominent characteristic. Further unique features can be noted for this family: the genomic segments are circularized by a genome-linked protein; segment A of the genome is structurally and functionally bicistronic, and the main translation product is a polyprotein cleaved by a virus-coded protease. The two main representatives of this virus family are the infectious pancreatic necrosis virus of fish (IPNV) and the causative agent of infectious bursal disease of chickens (IBDV). Infectious pancreatic necrosis was described for the first time in Canada in 1940; the viral nature of the causative agent was defined in 1960, and in subsequent years the structure and molecular biology of this virus has been studied extensively.

In 1962 an outbreak of a novel disease in a chicken flock was described in Gumboro, Delaware, USA, and according to this location of the first description further outbreaks have subsequently been referred to as 'Gumboro disease'. The most prominent lesion of the preferentially affected organ coined the designation 'infectious bursal disease' and IBDV as the causative virus. The first attempts to clarify the etiological agent mostly depended on electron micro-

scopy of virus particles which are found abundantly in the target organ, the bursa of Fabricius (BF). From these pictures IBDV was tentatively classified as a picornavirus, adenovirus, reovirus or orbivirus, until the essential structural features of a plaque-purified isolate were determined and showed remarkable similarities with the previously defined IPNV.

Besides these two prominent representatives other virus strains have been identified which possess the structural criteria of a birnavirus: Drosophila X virus (DXV) isolated accidentally from the fruit fly, Tellina virus (TV) and Oyster virus (OV) from bivalve molluscs. A virus with birnavirus characteristics has been isolated from European eels (EV) affected by stomatopapillomas. Tumors, however, could not be induced by isolated virus, although it has been claimed to cause 50% mortality in young eels. Considerable damage can be caused by yellowtail ascitic virus (YAV) in cultured yellowtail fingerlings with signs of ascites and hemorrhage in the liver.

In mammals, birnavirus-like agents have only been observed in fecal samples of humans, rats, guineapigs and pigs. Differences from true birnaviruses have been noted in size, length of genome segments, buoyant density; the designation 'picobirnaviruses' has therefore been proposed. Association of these agents with outbreaks of diarrhea is still uncertain.

Since infectious bursitis in chickens and infectious pancreatic necrosis in salmids cause considerable economic losses, only these viruses have been studied in detail and will be considered here.

Taxonomy and Classification

Since confirmation of the occurrence of a true birnavirus in mammals is still missing, classification of this family, Birnaviridae, contains three genera: genus *Avibirnavirus* (type species infectious bursal disease virus, IBDV); genus *Aquabirnavirus* (type species infectious pancreatic necrosis virus, IPNV); genus *Entomobirnavirus* (type species Drosophila X virus, DXV).

Properties of the Virion, Genome and Proteins

The prominent characteristic of a birnavirus is the genomic bisegmented dsRNA (**Fig. 1**). It is enclosed in an icosahedral capsid, about 60 nm in diameter, which is arranged as a single shell (**Fig. 2**). For the fine architecture of the capsid a symmetry of $T = 13$ has been determined. The handedness for the strains examined was dextro for IBDV and EV and laevo for IPNV. Four structural proteins could be identified in purified virus particles, two of which constitute the

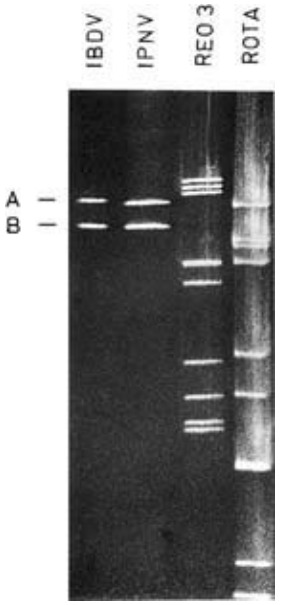


Figure 1 The two genomic segments of dsRNA of infectious bursal disease virus and infectious pancreatic necrosis virus in comparison to the genomic segments of reovirus 3 and rotavirus after polyacrylamide gel electrophoresis.

major structural components. In IBDV the two major proteins (VP2, mol. wt 40 kDa; VP3, 32 kDa) are present in almost equal quantities, whereas in IPNV VP2 (60 kDa) amounts to more than 50% of the total protein mass. The minor proteins comprise the large component VP1 (90 kDa) and VP4 (28–29 kDa). The latter is incorporated into IPNV particles only in minute amounts, whereas the infected cell carries this protein abundantly; hence the designation NS for nonstructural in the IPNV nomenclature. Structural similarities among aquatic birnaviruses can be detected by serological crossreactions. Subgroups have been established by crossneutralization of different isolates.

Host Range and Epidemiology

IPNV has been isolated from brook trout and rainbow trout, where considerable economic losses can occur mainly in hatchery-reared young fish not older than 6 months. Surviving or subclinically infected fish may become life-long carriers. Recent epizootics have been reported with mortality rates of 10–20%. In other reports mortality exceeded 50%. The virus is shed with urine, feces and sex products and is easily transmitted. The virus has also been isolated from Atlantic salmon, but its pathogenic significance for this fish remains uncertain. Other subclinically infected fish species are considered as virus carriers

and as a potential source of infection. Although IPNV is distributed worldwide, preferential habitats seem to exist for individual serotypes. In Norway serotypes 2 and 3 have been isolated from farmed rainbow trout and Atlantic salmon, whereas serotype 1 has not so far been detected.

IBDV has also a worldwide distribution and can cause considerable damage to the poultry industry. The serotypes determine the host range of this virus. Serotype I is exclusively pathogenic for chickens; infections in turkeys are subclinical. Serotype II, on the other hand, has been isolated from turkeys affected with coryza and diarrhea, but it remains unclear whether this serotype has any true pathogenic significance. Infections of chickens with serotype II do not cause clinical manifestations or noticeable lesions, and natural infections are uncommon. Transfer of the virus usually takes place via the fecal–oral route.

Stability of Viruses

Both viruses are highly stable under different environmental conditions. IPNV remains fully infective for months in freshwater or seawater. Progressive inactivation occurs after drying or heating at 60°C or storage at extreme pH conditions (pH 2 or pH 9). It is rapidly inactivated by exposure to chlorine, iodine and ultraviolet irradiation. IBDV can persist in poultry houses even after thorough cleaning and disinfection. This complicates attempts to control infections by hygienic means and makes vaccination inevitable. This virus is more resistant to heat and ultraviolet light than reovirus. It is inactivated at pH 12.0, but remains infective at pH 2.0. Infectivity is markedly reduced by exposure to 1% formalin for 1 h; 1% phenol or cresol inactivates the virus within 1 h.

Clinical Signs and Pathology

Fish infected by IPNV usually darken; they may move in spirals around their long axis, may develop exophthalmia, abdominal dilatation and ventral hemorrhages. Livers and spleens are pale, the viscera may be covered with multiple petechia. A clear or milky mucus in the stomach and anterior intestine is taken as indication of IPNV. Histologic examination reveals necrotic foci in acinar and islet tissue of the pancreas. As high virus titers are usually present in kidney tissue, this organ is a preferred site for virus isolation.

Chickens infected by a pathogenic strain of IBDV show symptoms of general distress and apathy after an incubation period of 2–3 days. Severity of these general manifestations progresses rapidly, and the birds usually die within 1–3 days. The most conspicuous lesions concern the BF. The organ is enlarged, edematous and in some cases hemorrhagic. Petechial or diffuse hemorrhages may be found in muscular tissue. Lymphoid follicles of the BF are totally necrotic, and in surviving birds the follicles are devoid of lymphoid cells.

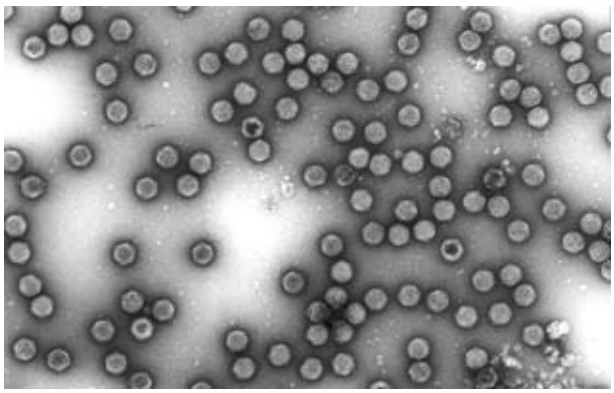


Figure 2 Electron micrograph of purified particles of infectious bursal disease virus; average diameter about 60 nm.

Pathogenic Properties

A single passage of a virus sample derived from a BF infected with IBDV is sufficient to reduce pathogenic properties of the isolate. Although continuous animal-to-animal passages of a virulent strain are lethal to almost 100% of birds, *in vitro* cultivation in chick embryo cells (CEC) abrogates the lethal outcome of an infection with this type of material. In that case, birds hardly show any clinical signs. However, the BF is still severely affected and all follicles are almost completely depleted of lymphoid tissue. In the small plaque variant, obtained after undiluted passages or by selection of a small plaque from the plaque population, the pathogenic properties are even more reduced. Infection of chickens with a small plaque variant is tolerated by the animals without exhibiting any clinical signs, and only limited necrotic foci are visible in lymphoid tissue of the BF. The isolated lesions are repaired so that immunodeficiencies do not result from such an infection, which stimulates the production of virus-specific antibodies.

For IPNV this correlation between plaque size and virulence has not been established. A high variability among pathogenic properties of different isolates has been reported, and continuous passage in cell culture did not invariably lead to attenuation of the virus.

Cell Tropism of IBDV

The pivotal position of the BF for virus replication in the natural disease, which becomes decisive for the

outcome of an infection, has been underlined by experiments in which bursectomized birds survived a lethal infection without any clinical manifestation. Lymphoid cells from the BF obviously represent optimal host cells for rapid production of maximal numbers of infectious virus particles. The bursal stage of differentiation of B cells is decisive for virus replication, because stem cells or peripheral B cells are completely refractory and do not replicate the virus. Among lymphoid cells isolated from the BF only a fraction can be infected *in vitro*. This is in contrast to the situation *in vivo* where in thin sections of the BF viral antigen can be demonstrated by immunofluorescence in virtually all cells of a follicle. This means that besides the stage of differentiation of B cells the microenvironment of the BF has a considerable impact on the functioning of B lymphoid cells as ideal host cells for IBDV. Expression of Ig-receptors at the surface of bursal cells does not seem to influence their sensitivity towards IBDV infections. This exquisite tropism for bursal cells determines the age-dependent sensitivity of chicks towards IBDV infections. Lethal infections are mostly restricted to 3–6 weeks of age when the BF has reached its maximal stage of development.

In Vitro Replication

IPNV replicates to high titers of 10^8–10^9 PFU ml^{-1} in a number of cell lines derived from fishes. The RTG-2 cell has been most commonly used for *in vitro* studies. Various cell lines of amphibian, avian or mammalian origin do not support replication of the virus. The virus replicates well at 10–26°C, poorly at 4°C and not at all at 30°C. Cell-associated virus can be demonstrated at about 4 h postinfection, virus starts to be released at about 7 h after infection and reaches maximal levels at about 16 h postinfection.

IBDV strains can be propagated in CEC where plaque formation is helpful for virus titration. It has been a common observation, however, that highly pathogenic strains can be isolated in cell culture only with great difficulty or not at all. After an eclipse period of about 4 h virus titers increase to reach a maximum of about 10^7 PFU ml^{-1} at 12–16 h after infection. Virus replication has also been noted in Vero cells and other continuous cell lines, where prolonged replication cycles have been observedI.

The consecutive steps involved in virus replication have not been traced individually. The most serious obstacle encountered in biochemical analysis has been the failure of birnaviruses to block synthesis of cellular constituents. It has been particularly difficult to direct sufficient label into newly synthesized IBDV RNA. Cellular background synthesis could not be repressed by actinomycin D which can normally be used for selective inhibition of cellular RNA synthesis, because these viruses proved to be highly sensitive towards low doses of this drug. Some differences in the dose of actinomycin D inhibiting IPNV replication has been noted in dependence on the host cells used. No nuclear phase is involved in birnavirus replication. Virus particles become transcriptionally active without pretreatment of the virion with chymotrypsin. Precursor molecules can reach the polymerase–template complex through the single shell of the capsid and can initiate transcription and RNA replication. For the intracellular replication cycle these *in vitro* findings mean that there is no definite need for uncoating or degradation of the capsid. *In vitro* polymerase activity yielded two species of RNA sedimenting at 24S and 14S which hybridized with the two segments of genomic RNA. During the first 10 min after the *in vitro* reaction had been started, newly synthesized RNA remained restrained to a complex containing intermediate products which were partly RNase sensitive and barely entered the polyacrylamide gel. The newly synthesized 14S component could be identified as the two segments of the genomic RNA, since their migration rate in polyacrylamide gels and the RNase resistance was identical to the genomic form of RNA (**Fig. 3**). The 24S RNA species consisted of single-stranded (ss)RNA which did not self-anneal, was RNase sensitive and supported synthesis of virus-specific proteins in an *in vitro* translation system. All structural viral components could be precipitated with virus-specific antibodies from the reaction mixture.

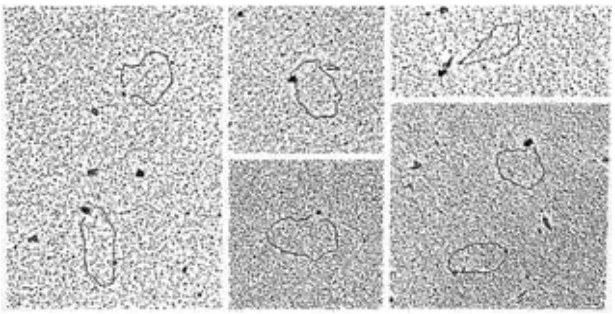


Figure 3 Electron micrograph of circular dsRNA–protein complexes released from infectious bursal disease virus particles by heating in the presence of 1.5% sodium dodecyl sulfate. (Courtesy H. Müller.).

Protein synthesis is followed by processing of the polyprotein coded by segment A. The autocatalytic cleavage of this precursor attributed to the activity of VP4 is only indirectly deduced from the activity of truncated regions of segment A expressed in *Escher-*

ichia coli; there is no proof whether the second level of processing, which involves the transit of the precursor VP2a to the main structural element VP2, might also be a function of VP4 or whether the trimming of this precursor is carried out by cellular proteases. In extracts from IBDV-infected cells only the 50 kDa precursor could be demonstrated in substantial amounts; the final major structural component VP2 does not accumulate in the cell but is assembled into the virion immediately before virus particles are released into the medium. Since virus particles could be isolated from the BF which had a high specific infectivity in spite of their lower density due to the 50 kDa precursor, cleavage could take place on the assembled particles, at least in some types of host cells. Truly 'incomplete' particles with greatly reduced infectivity, reduced density and excessive amounts of the 50 kDa precursor besides a series of aberrant proteins, are preferentially formed during undiluted passages. Stable small plaques are formed under these von Magnus conditions.

This is in contrast to findings with IPNV where undiluted passages did not increase the yield of defective interfering (DI) particles, but nevertheless their interference was held responsible for the survival of host cells. Incomplete particles could not be separated from standard virus by isopycnic centrifugation.

Organization of the Genome, Processing and Functional Significance of Gene Products

When routine methods for RNA sequencing became available, the coding region of birnavirus genomes were defined, nucleotide sequence of various isolates and serotypes were compared, and the genomic organization of both segments could be delineated. On segment A one open reading frame (ORF 1) coding for a polyprotein of 110 kDa is arranged in the following order: NH_2-VP2-VP4(NS for IPNV)-VP3-COOH. This primary translation product is rapidly cleaved into the precursor protein of VP2 (pVP2 or VP2a), VP4 and VP3. There is good evidence that this first step of processing is autocatalytically carried out by VP4. A cellular protease is most likely responsible for the following step of the VP2 precursor cleavage which proceeds more slowly and less efficiently, so VP2a can regularly be found in virus particles in proportions of a minor structural protein. It has been firmly established now that VP2 is the structural protein responsible for the induction of neutralizing antibodies, whereas VP3, the other major structural component, does not have this capacity.

ORF 2, which precedes and partially overlaps ORF 1, encodes a protein (VP5) of 145 amino acids in IBDV and a 17 kDa protein in IPNV, which is not incorporated into virus particles. This nonstructural protein was demonstrated in the cytoplasm of infected cells. Very recently a VP5-defective mutant was generated by site-directed mutagenesis. Although this mutant replicated at a slower rate in permissive cells, virus yields finally reached the levels of the original strain. This means that VP5 is not essential for virus replication. Its deletion may be useful as a marker, if tracing a particular virus strain is desired.

Figure 4 outlines the entire genome of IBDV including the terminal noncoding regions which were determined only recently. The 5′-NCR of segment A consists of 96 nucleotides adjacent to the start codon of the *VP5* gene; the terminal region of segment B spans 111 nucleotides to the start codon of the *VP1* gene. There is extensive homology between the 5′ noncoding region of segments A and B, particularly along the 32 5′ nucleotides. No homologies exist, however, in the 3′ terminal sequences of the two

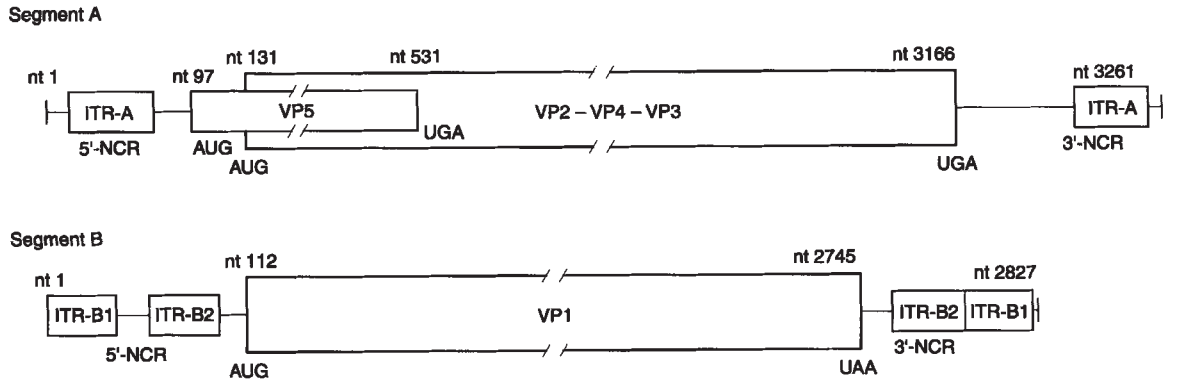


Figure 4 Model of the genomic organization of segments A and B of the IBDV genome. Number of nucleotides (nt), the startcodons and stopcodons of the ORFs, the different inverted terminal repeats (ITR) and the alignment of the codons for the viral proteins VP1–VP5 are indicated. (Courtesy E. Mundt and H. Müller.).

segments. Different inverted repeats were noted in both segments. This type of structure also appears to hold true for IPNV. Some interesting speculations have been put forward with respect to the functional significance of some of these sites. A sequence of 13 nucleotides in the 5′ terminus of both segments corresponds to the binding site of chicken 18S rRNA. An exchange of one nucleotide in the 5′ end of segment A of an attenuated strain might be indicative for the genetic constellation determining the pathogenic property of a particular strain. Secondary structures, derived from these terminal sequences, might be involved in virus replication, packaging of viral elements and possibly in determining the range of susceptible host cells.

Elegant proof that the sequences presented for segments A and B comprise the entire viral genome was obtained when a full yield of infectious virus resulted from cells which had been transfected with RNA transcribed from cloned DNA. Fidelity of replication induced by this synthetic positive-stranded RNA was ascertained by the introduction of tagged sequences into the transfecting RNA which could be recovered in the genome of the newly generated virus.

Comparison of the nucleotide sequence of ORF 1 of three European isolates and an Australian strain has established a close relatedness, the greatest difference being 7.8% among nucleotides and 2.7% at the amino acid level. VP4 was found to be fully conserved, some amino acid changes are scattered throughout VP3 and VP2 except for a tight cluster of exchanges in protein VP2. These variable regions provide the structural basis for the formation of the epitope which is responsible for the induction of neutralizing antibodies. It has now been established with certainty that VP2 is the reaction partner for neutralizing antibodies.

Genome segment B codes for a single protein, the structural component VP1. This protein has been found to be the stabilizing link between the ends of the dsRNA segments to form ring-like structures. During the first attempts to determine the size of the RNA segments no protease or fully denaturing conditions had been applied for the isolation of genomic RNA. Conservation of intact RNA circles, which remained closed by the protein under these conditions, explains why considerably higher molecular weights had initially been determined for both genomic RNA segments.

The close association of this protein with viral RNA had insinuated a polymerase function. Indeed, VP1 turned out to be a multifunctional enzyme. Its role as a replicase and transcriptase became evident by the *in vitro* demonstration of dsRNA products which had the size of the genomic segments, and by the production of mRNA which could code for the *in vitro* synthesis of virus-specific proteins. Formation of a VP1–GMP complex shows how this protein is indispensable for capping of mRNA. This means that besides polymerase activities this protein functions as a guanylyltransferase and most likely as a methyltransferase. It has even been postulated that VP1 serves as a primer for *in vitro* transcription which is carried on in a semiconservative strand displacement mechanism. This concept has to be reconsidered in view of the recent finding that plus strand RNA from both segments is sufficient to initiate virus replication.

Antigenic Structure

Neutralization has been the most sensitive and widely used method for serotyping birnavirus isolates. Among most IPNV strains some crossneutralization has been observed, but three different groups have been established with the following reference strains: VR 299 for serotype 1, Ab for serotype 2 and Sp for serotype 3. A further strain N1 with a distinct antigenic structure has recently been isolated from Atlantic salmon in Norway. All aquatic birnaviruses crossreact serologically to some extent. A high degree of crossneutralization has been noted for OV and TV; fluorescent antibodies crossreacted with IPNV and TV.

For IBDV two definite serotypes could be established which show some residual crossneutralization. Serotype I encompasses the classical strains causing bursal disease in chicks, whereas serotype II differentiates the nonpathogenic strains originally isolated from turkeys.

Neutralizing antibodies raised against either serotype of IBDV precipitated VP2 in extracts prepared from infected CEC; these antibodies did not bind in immunoblots. It can be concluded, therefore, that the antigenic site responsible for the induction of neutralizing antibodies is highly conformation-dependent. The isolation of escape-mutants with neutralizing antibodies as selection barriers clearly showed that at least three independent types of epitope exist in the domain responsible for the induction of neutralizing antibodies. The structural basis for the formation of this conformation-dependent epitope is provided by two hydrophilic regions in the central part of the VP2 peptide. Exchange of one amino acid in one of the hypdrophilic parts is sufficient for altering the neutralizing specificity. Towards the C-terminal end of VP2, adjacent to this conformational site, identical amino acid sequences form a sequence-dependent epitope crossreacting with both serotypes. Considerable variation in the degree of neutralization has been noted among

isolates in America, and vaccination failures have been attributed to these 'variants'. Such an antigenic drift could not be confirmed for strains isolated in Europe or in Africa.

Antigenic differences between serotypes I and II not only reside in VP2; structural protein VP3 can also be used for differentiating the two serotypes. Monoclonal antibodies could be prepared which were directed against VP3 of one serotype only, whereas other antibodies recognized a common epitope. The serotype-specific and the common epitopes are arranged on VP3 in a nonoverlapping manner. Formation of the common and type-specific epitopes is in agreement with identical and mismatching amino acid sequences yielding hydrophilic segments on the VP3 polypeptide. Amino acid exchanges clustered in hydrophobic parts did not interfere with the antigenic specificity. There is no serologic crossreactivity between IBDV and other birnaviruses.

Immune Response

Antibodies against IBDV appear rapidly; 3 days after infection neutralization assays are positive. A protective immunity fully depends on neutralization. Accordingly, antibodies to serotype II do not protect chicks against infections with virulent type I viruses. Birds that survive an acute infection, or animals that become infected during the insensitive age shortly after hatching, are likely to suffer from a severe general immunosuppression. The tropism of IBDV for B cells in the BF destroys all lymphoid follicles in this organ and blocks further supply of immunocompetent mature peripheral B cells. Frequent consequences of this immunosuppressive viral infection are opportunistic infections by various agents which are mostly lethal for the animals during the weeks which follow an infection.

Laboratory Diagnosis

Infections by IPNV are confirmed by virus isolation in permanent fish cells which are preferentially inoculated with samples of kidney tissue. Virus can be titrated in these cells by plaque assay. Direct diagnosis can be established by immunofluorescence with thin sections from internal organs.

Agar gel precipitation is the most feasible and economic method to demonstrate IBDV-specific antigens in extracts from bursal tissue. Antigen containing cells can be easily identified by immunofluorescence with impression smears of bursal lymphoid tissue or frozen sections of this organ. Virus can be isolated in embryonated eggs or CEC under the previously mentioned restrictions. Neutralization or ELISA are efficient and feasible methods for serodiagnosis. Whole purified virus produced *in vitro* should be used for coating ELISA plates. Use has been made of standard polymerase chain reaction (PCR) techniques. Since the nucleotide sequence of the entire genome is now known, primers can be selected according to conserved or variable regions to diagnose infections and differentiate virus strains.

Prevention and Control

Live and killed vaccines are available to control IBDV. Recombinant strains have been generated and their use in the field is being evaluated. Attenuated strains mostly have a reduced replication rate *in vitro* and only produce limited foci in the BF which regenerate without any significant loss of lymphoid tissue. For one of these attenuated strains formation of small plaques could be used as an *in vitro* marker. Vaccination protocols try to guarantee protective antibody levels during the first weeks of life by passive transfer of maternal antibodies. Killed vaccines can, therefore, be used to boost high antibody levels in laying hens. Application of attenuated virus should try to fill the gap between the fading passive immunity and the production of own antibodies.

Prophylaxis of IPNV infections is mainly based on hygienic measures, such as exchange of contaminated water and addition of disinfecting iodophores during the fertilization period. Immunoprophylaxis is hampered by the circumstance that during the sensitive period fry are not protected by maternal antibodies, and about 30 days elapse before young fish acquire immunocompetence. Furthermore, the lack of cross-protection between serotypes makes the requirements for a protective immunity uncertain.

Future Perspectives

At the molecular level a number of steps have yet to be elucidated for a full description of the replication cycle of birnaviruses. A paradox phenomenon in the state of our current knowledge is the cytopathic consequence of birnavirus infections in the absence of a shutdown of cellular synthetic events. The successful establishment of a reverse genetics system will greatly facilitate future studies about replication and gene expression. The fascinating aspect of infectious bursal disease, the exquisite tropism of the virus for B cells at the bursal stage of development with deleterious effects for lymphoid tissue, is far from being resolved and will require profound description of B cell differentiation in the BF. Finally, nothing is yet known about the immediate cause of death during an acute infection. Since removal of the primary

target organ has no immediate consequences on the health of the affected bird, other vital organ systems must be expected to fail by the fatal attack of the heavy load of virus which has been produced in the BF and is flooding the organism. Attempts have hardly been successful to define the genomic constellation responsible for pathogenic properties of IBDV by comparing nucleotide sequences of strains with widely differing pathogenicity. Since the entire viral genome is known now, the potential contribution of the noncoding regions to the pathogenic property of a virus strain will be amenable to experimental trials. The recent development of a reverse genetics system will certainly be helpful in this respect, and besides other questions about virus replication at the molecular level, this type of approach will be useful to design a new generation of vaccines according to current needs. The occurrence and pathogenic properties of birna-like viruses in mammals will merit special attention.

See also: **Fish viruses.**

Further Reading

Dobos P (1995) The molecular biology of infectious pancreatic necrosis virus (IPNV). *Annu. Rev. Fish Dis.* 5: 25.

Dobos P, Hill BJ, Hallet R *et al* (1979) Biophysical and biochemical characterization of five animal viruses with bisegmented double-stranded RNA genomes. *J. Virol.* 32: 593.

Gatti MSV, Pestana de Castro AF and Ferras MMG (1989) Viruses with bisegmented double-stranded RNA in pig faeces. *Res. Vet. Sci.* 47: 397.

Mahardika GNK and Becht H (1995) Mapping of cross-reacting and serotype-specific epitopes on the VP3 structural protein of the infectious bursal disease virus (IBDV). *Arch. Virol.* 140: 765.

Mundt E and Vakharia VN (1996) Synthetic transcripts of double-stranded birnavirus genome are infectious. *Proc. Natl. Acad. Sci. USA* 93: 11131.

BK Virus *see* **JC and BK Viruses**

Border Disease Virus *see* **Bovine Diarrhea Virus and Border Disease Virus**

BORNA DISEASE VIRUS (*BORNAVIRIDAE*)

Lothar Stitz and **Rudolf Rott**, Bundesforschungsanstalt für Viruskrankheiten der Tiere, Tübingen, Justus-Liebig-Universität Giessen, Giessen, Germany

History

Disease symptoms comparable to those observed nowadays after natural infection with Borna disease virus (BDV) in horses and sheep have been reported in the literature of veterinary science for more than 200 years. Synonyms such as disease of the head (Kopfkrankheit), brain fever, subacute meningitis or hypersomnia of horses reflect the restriction of the disease to the central nervous system. The current name of this disease was adopted in 1895 after an epidemic among horses of a cavalry regiment in the town of Borna in Saxony near Leipzig (Borna disease, BD). Many important studies, which have greatly contributed to the understanding of the disease in its natural host but also in experimental animals, were performed in the 1920s. Viral etiology was established as early as 1925 by Zwick and Seyfried. In the course of these studies histological examination of the brain revealed extensive pathological changes as a prominent sign of this central nervous system disease. Since then detailed studies have been performed on the spectrum of susceptible host species and on manifestations of the disease. Unusual features such as RNA splicing, overlap of transcription units and transcription signals, aspects of genome organization and

target organ has no immediate consequences on the health of the affected bird, other vital organ systems must be expected to fail by the fatal attack of the heavy load of virus which has been produced in the BF and is flooding the organism. Attempts have hardly been successful to define the genomic constellation responsible for pathogenic properties of IBDV by comparing nucleotide sequences of strains with widely differing pathogenicity. Since the entire viral genome is known now, the potential contribution of the noncoding regions to the pathogenic property of a virus strain will be amenable to experimental trials. The recent development of a reverse genetics system will certainly be helpful in this respect, and besides other questions about virus replication at the molecular level, this type of approach will be useful to design a new generation of vaccines according to current needs. The occurrence and pathogenic properties of birna-like viruses in mammals will merit special attention.

See also: **Fish viruses.**

Further Reading

Dobos P (1995) The molecular biology of infectious pancreatic necrosis virus (IPNV). *Annu. Rev. Fish Dis.* 5: 25.

Dobos P, Hill BJ, Hallet R *et al* (1979) Biophysical and biochemical characterization of five animal viruses with bisegmented double-stranded RNA genomes. *J. Virol.* 32: 593.

Gatti MSV, Pestana de Castro AF and Ferras MMG (1989) Viruses with bisegmented double-stranded RNA in pig faeces. *Res. Vet. Sci.* 47: 397.

Mahardika GNK and Becht H (1995) Mapping of cross-reacting and serotype-specific epitopes on the VP3 structural protein of the infectious bursal disease virus (IBDV). *Arch. Virol.* 140: 765.

Mundt E and Vakharia VN (1996) Synthetic transcripts of double-stranded birnavirus genome are infectious. *Proc. Natl. Acad. Sci. USA* 93: 11131.

BK Virus *see* **JC and BK Viruses**

Border Disease Virus *see* **Bovine Diarrhea Virus and Border Disease Virus**

BORNA DISEASE VIRUS (*BORNAVIRIDAE*)

Lothar Stitz and **Rudolf Rott**, Bundesforschungsanstalt für Viruskrankheiten der Tiere, Tübingen, Justus-Liebig-Universität Giessen, Giessen, Germany

History

Disease symptoms comparable to those observed nowadays after natural infection with Borna disease virus (BDV) in horses and sheep have been reported in the literature of veterinary science for more than 200 years. Synonyms such as disease of the head (Kopfkrankheit), brain fever, subacute meningitis or hypersomnia of horses reflect the restriction of the disease to the central nervous system. The current name of this disease was adopted in 1895 after an epidemic among horses of a cavalry regiment in the town of Borna in Saxony near Leipzig (Borna disease, BD). Many important studies, which have greatly contributed to the understanding of the disease in its natural host but also in experimental animals, were performed in the 1920s. Viral etiology was established as early as 1925 by Zwick and Seyfried. In the course of these studies histological examination of the brain revealed extensive pathological changes as a prominent sign of this central nervous system disease. Since then detailed studies have been performed on the spectrum of susceptible host species and on manifestations of the disease. Unusual features such as RNA splicing, overlap of transcription units and transcription signals, aspects of genome organization and

deduced protein sequence led to the classification of BDV in the genus *Bornavirus* of the family *Bornaviridae*.

Properties of the Virion

In agreement with earlier filtration experiments, electron microscopic studies of thin sections of infected cells and on partially purified virus revealed the presence of spherical enveloped virus particles with a diameter of 50–100 nm. The presence of an envelope was demonstrated by the sensitivity of virus infectivity to lipid solvents as well as the recent characterization of virus-specific glycoproteins. Earlier experiments with inhibitors of nucleic acid synthesis suggested that BDV is an RNA virus, a finding that has recently been substantiated by molecular biological techniques.

Properties of the Genome

Data on the nature of the BDV genome have been obtained from complementary DNA clones using libraries prepared either from the brain of BDV-infected rats or from cell cultures. The genomic single-stranded (ss) RNA of 9.5 kb with complementary 3′ and 5′ termini has been shown to be of negative polarity. Investigations of the organization of the genome have revealed five major open reading frames (ORFs) on the antiviral genome. Several unusual aspects for an ssRNA virus have been recognized in molecular biological studies, in particular an overlap of reading frames, a post-translational processing of subgenomic RNAs (RNA splicing) and nuclear phases indispensable for virus replication. Results of sequence analyses of viruses obtained from various animal species, human or tissue culture, revealed a remarkable conservation of coding sequences.

Properties of the Viral Proteins

Immunoprecipitation with polyclonal and monoclonal antibodies can detect several proteins in infected brains and in tissue cultures, with approximate relative molecular masses of 60, 38–39, 24 and 14 kDa. The most abundant viral proteins in infected cells as well as in viral particles have a molecular mass of 38–39 and 24 kDa. The expression of the identified ORFs in various expression systems permitted the closer characterization of the major virus-specific proteins. So far, five proteins have been identified and in part characterized. ORF 1, which is the prominent 3′ ORF, encodes the 38–39 kDa protein (also known as p40), the putative nucleoprotein (NP). ORF 2 codes for a 24 kDa protein (also known as p23), a protein phosphorylated in its serine residues, representing the putative phosphoprotein (P). Recently, an additional open reading frame (ORF X1) has been identified, greatly overlapping with ORF 2, encoding a non-glycosylated BDV protein designated p10. The third open reading frame (ORF 3) encodes the p16 protein which is glycosylated post-translationally, resulting in gp18 (also known as the 14 kDa protein), representing the putative membrane protein (M). ORF 4 encodes p57 that is found in its *N*-glycosylated form at approximately 90 kDa (described as gp84 and gp94) and represents the putative precursor glycoprotein of the virus (GP); cleavage products of this GP have been reported to result in molecular masses of approximately 40 kDa by the subtilisin-like cellular protease furin. The products of ORF 1 and ORF 2 (p40 and p24) have been shown to form a complex which has been deduced from coprecipitation by monoclonal antibodies as well as from one-dimensional peptide digestion. Finally, the gene product of ORF 5, which is localized at the 5′ end of the viral antigenome, has not so far been identified. This ORF predicts a protein of 180–190 kDa and is expected to represent the RNA-dependent RNA polymerase of BDV.

With respect to the intracellular localization of these virus-specific proteins, extensive analyses have been performed. It appears that proteins p10, p24 and p40 are found mainly in the nucleus of infected cells. A colocalization of p24 and p10 on the one hand and p40 and p10 on the other, might be explained by the fact that sequences within the p10 sequence have been identified which are typical for nuclear export trafficking. The p10 itself has been demonstrated both in the nucleus and the cytoplasm. In addition, evidence has been presented that indicates the presence of a nuclear targeting sequence in the 39 kDa protein which is not present in the 38 kDa protein. This results in accumulation of the 39 kDa protein in the nucleus, whereas the 38 kDa protein is found in both the nucleus and the cytoplasm. The heavily *N*-glycosylated gp94 has been seen both intracytoplasmically in the endoplasmic reticulum as well as on the cell surface, and its function is seen in context with viral entry, assembly and budding of BDV. The gp94 as well as the gp18 apparently contain epitopes inducing the production of neutralizing antibodies, which can be found late after infection.

Physical Properties

BDV does not differ in its physical properties from any other known enveloped virus. It has been shown to be sensitive to lipid solvents, such as chloroform or acetone, and to detergents. At low temperature and at

pH 5–12 BDV is rather stable, whereas infectivity is destroyed by high temperatures and pH lower than 5.

Replication

BDV replicates to relatively low titers of approximately 10^6 $TCID_{50}$ ml^{-1} in embryonic brain cells of various provenance, including human. Besides neural cells, other cell types isolated from the brain, such as astrocytes and oligodendrocytes, support virus production. A variety of different cell lines not derived from the central nervous system have been found to be susceptible to infection. Such cell lines, supporting the growth of BDV, e.g. MDCK or Vero cells, readily become persistently infected. It was estimated that a single cell produces only one infectious virus particle, although a large amount of viral antigen can be demonstrated immunohistologically.

Little is known of the consecutive steps involved in virus replication. There is some evidence that the major glycoprotein (gp94) mediates penetration by membrane fusion after the fusion peptide becomes exposed by proteolytic cleavage of the glycoprotein. In contrast to other nonsegmented RNA viruses, the BDV genome is replicated and transcribed in the nucleus of infected cells. The cellular splicing machinery is required to process some of the primary RNA transcripts. A variety of mechanisms are used to regulate gene expression, including the use of overlapping reading frames, overlapping transcription units, alternate RNA splicing and leaking scanning of ribosomes during protein translation. The replication cycle seems to be completed within 24 h. Only a little of infectious virus, if any, is released from infected cells.

In situ hybridization was successful in various cell types of the nervous system, such as neurons of various brain regions, in Purkinje cells and in ventral motor neurons of the spinal cord in accordance with the detection of BDV-specific proteins by immunocytochemical methods. Astrocytes, oligodendroglia, ependymal cells and Schwann cells have also been identified as target cells for BDV. In addition, the normally strictly neurotropic virus can also replicate in cells of peripheral organs under defined circumstances, such as after infection of newborns or in long-term immunosuppressed animals.

Geographic Distribution

BD was originally described as a disease restricted to certain endemic areas in eastern and southern Germany and Switzerland. Recent seroepidemiological studies, however, have shown that natural infection is much more widely distributed than previously anticipated. These investigations revealed seropositive horses throughout Germany, but also in other European countries, North America and Japan, findings which strongly support the notion that BD might have a worldwide distribution.

Host Range and Virus Propagation

Natural infection with BDV has originally been shown to occur in horses, sheep, cattle and rabbits. Recently, cats, ostriches and various zoo ruminants have been identified as natural hosts. Reports on infection of other species, such as goat, deer and donkey, are rare, and BDV etiology has not been proven unequivocally, although such cases seem likely in view of the extremely broad experimental host spectrum of the virus. During recent years it has been proposed that BDV or a closely related virus might also be involved in infections of humans. There was a prevalence of seropositive reactions in patients with psychiatric disorders from Germany, the USA and Japan. The specificity of this reaction could be substantiated when a BDV-specific protein translated by RNAs which had been derived from a BDV-specific cDNA clone was used for the assay. Recently, the presence of virus-specific nucleic acid has been demonstrated in postmortem brain samples of schizophrenic patients and patients with bipolar disorders, and possibly even in normal human brain tissue, but not in samples from patients with multiple sclerosis, Alzheimer's disease or Parkinson's disease. Similar results were obtained in some postmortem samples that had been investigated immunocytochemically. There are no confirmed reports of virus isolation from humans and thus final proof as to whether BDV can be implicated in human disease must await further verification.

Experimental hosts comprise a wide variety of species ranging from birds to nonhuman primates. The most thoroughly investigated experimental BDV infection in rats has contributed considerably to the elucidation of the disease process. As disease symptoms of horses and rats do not differ significantly from each other, and as cell types and location of cells involved in the inflammatory reaction in both species are fully comparable, experimental BDV infection of rats can be regarded as a model system for natural infection. Efforts to establish a mouse model of BD have only recently been successful. Interestingly, adult infected mice show infectious virus persisting in the brain, but only very rarely does clinical disease become apparent and most animals appear to be resistant to the disease. In contrast, the infection of suckling mice results in neurological symptoms with

an incidence of 20–100%, depending on the mouse strain.

The virus readily replicates in the brain of infected animals. After adaptation the virus grows to titers of approximately 10^6 $TCID_{50}$ g^{-1} tissue. In contrast to intracerebrally infected experimental animals, where the virus can be detected throughout the brain, in naturally infected hosts the presence of virus is found to be restricted to certain or single areas in the brain without evidence for virus-specific antigen in other brain regions.

Serological Relationship and Variability

Serologically, no distinction can be made between different virus isolates by using polyclonal antibodies. Variable detectability of viral antigens in brain specimens by monoclonal antibodies indicates, however, that some strain variation may exist. No conclusive data are available on the occurrence of biologically different virus strains in natural hosts. It remains to be seen whether inapparent infections in horses and sheep might be due to variant strains. All virus strains used for infection of experimental animals originate from brain tissue of naturally infected diseased horses. These isolates mostly underwent serial passages in rabbits and were adapted thereafter to different animals. Different biological properties of virus isolated at various times after infection from rats could also point to the existence of variants. In this context it should be emphasized that virus isolates exist that cause extreme obesity syndrome or behavioral alterations in infected rats.

Sequence analyses confirmed the general view of highly conserved regions in the BDV genome.

Epidemiology

Originally BD was considered an endemic disease occurring sporadically in central Europe. Recent seroepidemiological investigations in horses have revealed virus-specific antibodies in 12% of randomly collected horse sera from Germany, Switzerland and other European countries. Most of these animals were without clinical signs of BD, but some exhibited disease symptoms within an observation period of 1 year. Since the chance of contact between horses has increased tremendously during recent years as a result of worldwide horse trading and international sporting activities, worldwide distribution of BD cannot be excluded. No data are available on the reservoir of the virus. There is some evidence that virus is shed during inapparent infection of horses and sheep, which also could lead to further propagation of the infection. If one takes into account that persistent infections can be established in tolerant infected rats and that these animals continuously shed virus, this mode of infection appears to be a possiblity for maintaining BDV in the horse population. Whether persistently infected tolerant rats or other rodents could represent a virus reservoir has not been investigated.

Transmission and Tissue Tropism

Intranasal infection seems to be the most likely route of natural infection, since nerve endings in the nasal mucosa are readily accessible to the virus. Furthermore, certain pathological peculiarities observed in naturally infected horses, such as edema of the bulbus olfactorius, can be found in experimentally infected animals only after intranasal infection. Another possible route of infection might be orally, via the trigeminal nerve, which has been found to contain BDV-specific antigen in horses; however, the possibility still exists that positive reactions in the nerve are rather due to early centrifugal spread of BDV via this nerve. In its natural hosts and also in adult experimentally infected animals the virus shows a strict neurotropism. After the virus has entered the nerves it migrates axonally to the central nervous system, where it replicates in neurons and astrocytes, especially in neurons of the hypothalamus. With time the virus spreads throughout the central nervous system. Various routes of infection have been shown to result in clinical disease in susceptible animals. Experimentally, the most common is the intracerebral infection, but it has also been demonstrated that hosts get sick after intranasal, intraperitoneal or even intravenous inoculation of the virus. In rats infected as adults the virus shows the same strict neurotropism as in the natural hosts. Infectious virus is found in the brain as early as 7 days after infection and reaches titers of 10^6 $TCID_{50}$ ml^{-1}. Infectious virus can also be detected in the spinal cord, in ganglia of the autonomic nervous system, in peripheral nerves, in the adrenal glands and neural cells of the retina, although at considerably lower titers or by immunocytochemistry only. No infectivity can be found in the eyes of rats late during the course of infection, which coincides with severe degeneration of neurons in the retina. In all neuronal tissues mentioned above, infectivity can be found throughout the life of the infected animal, i.e. they develop a persistent productive infection. In sharp contrast to rats infected as adults, neonatally infected rats harbored infectious virus not only in the central nervous system but also in cells of peripheral organs. Since in neonatally infected rats infectious virus was also found in salivary glands, lacrimal fluid and urine, those animals have to be regarded as infectious chronic carriers of BDV. A similar pattern of

distribution of BDV was found in long-term immunosuppressed adult rats. Loss of immunocompetence in athymic rats or in animals treated with a single application of cyclophosphamide does not result in virus replication in organs outside the central nervous system. This phenomenon remains unexplained.

Pathogenicity

Virus strains isolated from naturally infected animals are equally pathogenic. This comparable degree of pathogenicity, however, might not represent the entire picture *in vivo*, as BDV has been exclusively isolated from diseased animals. Besides these highly pathogenic strains, variants with reduced pathogenicity may exist in inapparently infected animals. The potential existence of such virus variants has been documented in rats, where strains of different origin could cause different clinical manifestations or inapparent infections. All of the BDV strains originating from brain tissue of diseased horses or sheep induced disease in rabbits as well as in rats. Infection with the same virus did not induce disease in adult mice and hamsters but replicated in the brains of these experimental animals. This type of infection led to behavioral disorders and neurological symptoms in rats, or it only resulted in behavioral alterations and premature senility in tree shrews. The question of why in some species infection remains subclinical cannot yet be answered. Similarly, no conclusive data exist to explain why certain experimentally infected rats develop obesity; different pathogenicity associated with virus variants is one of the options.

Immune Response

The immune response to BDV has been shown to represent the basis of the pathogenesis of the disease. Whereas infection of adult immunocompetent animals regularly results in disease, infection of newborn or athymic rats or animals rendered immunodeficient by drugs leads neither to encephalitis nor disease, despite the persistence of the virus. These findings, in conjunction with the property of BDV being a noncytolytic virus that persists in the brain of immunocompromised hosts at titers comparable to those in immunocompetent animals, provide all the characteristics of an immunopathological disease. In rats infected as adults a vigorous antibody response to BDV antigens can be observed, which obviously does not participate in the immunopathogenesis of the disease because BDV-specific antibodies adoptively transferred to immunoincompetent recipients do not induce pathological changes or disease; the same is true for the appearance of antibodies in rats infected as newborns. Furthermore, rats immunosuppressed by cyclosporin A and still capable of mounting a cellular immune response under experimental conditions do not synthesize virus-specific antibodies but develop BD. The cellular basis of the immune-mediated disease was strengthened by the fact that adoptive transfer of immune cells induces BD in infected immunosuppressed recipients. The role of virus-specific T cells in the pathogenesis of BD was demonstrated by the induction of disease after passive transfer of *in vitro* established homogeneous BDV-specific CD4+ T cell lines. Those recipients regularly develop clinical symptoms characteristic for BD in rats. However, there is convincing evidence that CD4+ T cells do not cause disease by themselves but seem to induce the activity of cytolytic CD8+ T cells. In the absence of CD8+ T cells no disease or immunopathology is seen, even if CD4+ T cells can be detected in the brain. Furthermore, CD8+ T cells have been shown to synthesize mRNA for perforin, the major molecule involved in cytolysis of target cells by CD8+ T cells, in parallel to the occurrence of destruction of cells in the brain resulting in brain atrophy. Other mechanisms are still under investigation, such as proinflammatory cytokines or radicals which may contribute to cytotoxicity in this CD4+ T cell-dependent disease, with CD8+ T cells acting as effector cells. Interestingly, virus-specific major histocompatibility complex (MHC) class II restricted T cells, when applied before infection, prevent the manifestation of clinical signs in rats; again, functional CD8+ T cells can be found in the brains of those animals. Antibodies to virus-specific antigens are synthesized early after infection in all BDV-infected animals but neutralizing activity becomes effective after long and variable periods postinfection and only reaches very low titers.

Clinical Features of Infection

After incubation times varying from weeks to months, and possibly even longer, the disease occurs sporadically in natural hosts. If the infection becomes manifest, the disease is characterized during the early phase by disturbances of sensory functions, impaired posture of the limbs, temporary immobility and excitations, ataxia, hyperesthesia, vision disorders and nystagmus, together with anorexia, fever and colics. The early neurological symptoms are mainly disorders of functions governed by the limbic system, whereas during the later stages of the disease dysfunctions of the motor system, such as paralysis and pareses, predominate. Most naturally infected animals die 1–2 weeks after onset of the disease, but recoveries or recurrence of disease were also ob-

served. Essentially the same sequence and comparable disease symptoms can be observed after infection of experimental animals with virus obtained from naturally infected horses or sheep, namely rats, rabbits, chicken and monkeys infected with virus strains that produce neurological disease. The disease starts with alertness and loss of fear. Later, rats show movements of the head even at rest and incoordination with increasing hyperactivity and aggressiveness. Thereafter, most rats enter a stage of disease where passiveness and hypersomnia dominate. These symptoms are at least in part attributable to developing blindness. In the chronic stage of the disease, signs of dementia, chronic debility and behavioral abnormalities, including severe learning deficiencies, prevail. In individual animals, and dependent on the virus strain used, paralysis and even paresis can be observed; however, only very few rats succumb to the disease in general, as a consequence of wasting disease due to the inability to take food and water.

Pathology and Histopathology

Pathological changes after BDV infection are restricted to the central nervous system, i.e. the brain, spinal cord and the retina. In naturally infected hosts a massive, perivascular as well as parenchymal inflammation is found. The sequence and development of pathological changes have been extensively studied in rats, and appear to be very similar to the situation in naturally infected hosts. BDV-infected rats develop a severe nonpurulent disseminated mononuclear meningoencephalitis, with the most intense inflammatory reaction restricted to the grey matter of cortex and diencephalon. Macrophages and T lymphocytes are the most numerous cell populations present in the inflammatory lesions during the earlier phases of the local reaction in both experimentally infected rats and in naturally infected horses and sheep, and B lymphocytes predominate in the histological picture at later stages. The initial reaction is characterized by a focal accumulation of mononuclear cells in the leptomeninges at day 8–10. Thereafter, perivascular and parenchymal infiltrations are observed, which intensify during the course of the disease and consist of massive accumulations of inflammatory cells in perivascular spaces and the neuropil between days 20 and 30 postinfection, when the maximal extent of the lesions is seen. Beyond day 30 postinfection the intensity of the inflammatory reaction decreases, and late after infection (> 60 days) the number and intensity of the inflammatory infiltrates is significantly reduced, although the level of infectious virus remains constant. Simultaneously with the development of the encephalitis, degenerative lesions can be found in hippocampal and cortical regions. One of the consequences of the necrotic process is a dilatation of the lateral ventricles resulting in a marked hydrocephalus accompanied by a severe cortical brain atrophy, most probably due to a progressive loss of neurons. Reactive astrogliosis is found in all areas with inflammatory lesions which is a common feature in brain inflammation. In infected neurons intranuclear acidophilic inclusion bodies (Joest–Degen inclusion bodies) can be found and have been regarded as pathognomonic of BD. In the brain the virus is predominantly found in neurons, but viral antigen is also detected in astrocytes, oligodendrocytes and ependymal cells. Histopathological lesions in the eye of infected rats and rabbits are seen almost exclusively in the retina, resulting in a degeneration of rods and cones and ultimately in a progressive disappearance of neurons from the inner and outer nuclear layers. The complete loss of neurons is the reason for the increasing blindness of infected animals, and late after infection 'burned-out' eyes are found, devoid of any inflammatory reaction. This finding explains the absence of the virus from the eye. A peculiarity of natural BDV infection in horses, and also found in rats infected intranasally, is the edema of the bulbus olfactorius which lends support to the hypothesis that natural infection occurs via the nasal route.

Prevention and Control

No vaccine is available which could mediate protection against the disease. Since prevention and control of virus diseases greatly depend on knowledge of the route of natural infection and the epidemiological situation, seroepidemiological studies in countries with intensive horse breeding and trading is an urgent need and have been initiated only recently. Therefore, during recent years the laborious demonstration of antiviral antibodies by immunofluorescence on persistently infected indicator cells has been complemented by the development of more feasible and sensitive serological assays such as enzyme-linked immunosorbent assay (ELISA) and immune blotting, which facilitates the procedure for the determination of BDV-specific antibodies. The diagnosis can be confirmed by virus isolation in sensitive tissue-culture cells. With the advent of viral sequence information, new diagnostic reagents for BDV infection have been introduced for serology as well as oligonucleotide primers for molecular studies. More recently *in situ* hybridization and polymerase chain reactions have become valuable tools for diagnosis. The control of the disease has to focus on the identification of potential carrier animals which spread the virus and

are responsible for its distribution in a given population. These animals should be eliminated. Data from studies in humans with psychiatric disorders provide an increasing body of evidence that BDV is able to infect humans and may represent a human pathogen. It is not yet known how humans get infected, as no convincing correlation between human patients and contact with infected individual animals or herds has been found. The potential role of BDV as a human pathogen requires substantiation. There is a controversal discussion about the stipulation that blood monocytes of psychiatric patients contain BDV nucleic acid. Several researchers reported that they were able to detect the presence of viral RNA components in those cells, whereas others were not successful.

Future Perspectives

Of great and urgent importance is the refinement of reliable methods for routine diagnosis. This includes assay systems for antiviral antibodies as well as the virus itself. These methods should be used in seroepidemiological studies in natural hosts and in humans with various psychiatric disorders. Considerable efforts have been made in this respect but generally-accepted assay systems have not been developed so far. When available, the test methods will not only be employed in studies in humans, but will be used in studying the dissemination of BDV in animals in general. Furthermore, the molecular characterization and the classification of BDV needs further effort. In particular, all virus-specific antigens, including the glycoproteins against which antibodies in other virus diseases have been shown to be protective, have to be analyzed in more detail. Finally, although progress on the pathogenetic pathways of this immune-mediated disease after persistent virus infection has been made, many questions concerning the immunopathogenesis remain to be solved for both BD as well as for the model of a virus-induced immune-mediated inflammatory reaction in the brain.

See also: **Autoimmunity; Diagnostic techniques: Detection of viral antigens, nucleic acids and specific antibodies, isolation and identification by culture and microscopy; Immune response: Cell mediated immune response, General features; Pathogenesis: Animal viruses.**

Further Reading

Bilzer T and Stitz L (1997) Immunopathology and neurodegeneration in Borna disease. *Brain Pathol.* 7: 1317.

Hatalski G, Lewis AJ and Lipkin WI (1997) Borna disease. *Emerg. Infect. Dis.* 9: 129.

Koprowski H and Lipkin WI (eds) (1995) Borna disease. *Curr. Top. Microbiol. Immunol.* 190.

Pringle C and Easton AJ (1997) Monopartite negative strand RNA genomes. *Semin. Virol.* 8: 49.

Richt JA, Pfeuffer I, Christ M *et al* (1997) Borna disease infection in animals and humans. *Emerg. Infect. Dis.* 3: 343.

BOVINE DIARRHEA VIRUS AND BORDER DISEASE VIRUS (*FLAVIVIRIDAE*)

Edward J Dubovi, Department of Population Medicine and Diagnostic Science, College of Veterinary Medicine, Cornell University, Ithaca, New York, USA

Introduction

For the purposes of this entry, bovine viral diarrhea virus (BVDV) and border disease virus (BDV) will be considered a single viral entity, with different sources of the isolates – either bovine or ovine; there are, however, data indicating that BDV can be separated from BVDV at the molecular level. A closely related virus, classical swine fever virus (CSFV) (also referred to as hog cholera virus), does have consistent antigenic differences from BVDV and BDV. Accordingly, CSFV will be addressed separately. Some confusion regarding BVDV may be encountered because of a lack of uniformity in the designation of this virus. Early reports described the agent as virus diarrhea (VD) virus while others working with isolates from a different clinical syndrome coined the term mucosal disease (MD) virus. When it became evident that the

are responsible for its distribution in a given population. These animals should be eliminated. Data from studies in humans with psychiatric disorders provide an increasing body of evidence that BDV is able to infect humans and may represent a human pathogen. It is not yet known how humans get infected, as no convincing correlation between human patients and contact with infected individual animals or herds has been found. The potential role of BDV as a human pathogen requires substantiation. There is a controversal discussion about the stipulation that blood monocytes of psychiatric patients contain BDV nucleic acid. Several researchers reported that they were able to detect the presence of viral RNA components in those cells, whereas others were not successful.

Future Perspectives

Of great and urgent importance is the refinement of reliable methods for routine diagnosis. This includes assay systems for antiviral antibodies as well as the virus itself. These methods should be used in seroepidemiological studies in natural hosts and in humans with various psychiatric disorders. Considerable efforts have been made in this respect but generally-accepted assay systems have not been developed so far. When available, the test methods will not only be employed in studies in humans, but will be used in studying the dissemination of BDV in animals in general. Furthermore, the molecular characterization and the classification of BDV needs further effort. In particular, all virus-specific antigens, including the glycoproteins against which antibodies in other virus diseases have been shown to be protective, have to be analyzed in more detail. Finally, although progress on the pathogenetic pathways of this immune-mediated disease after persistent virus infection has been made, many questions concerning the immunopathogenesis remain to be solved for both BD as well as for the model of a virus-induced immune-mediated inflammatory reaction in the brain.

See also: **Autoimmunity; Diagnostic techniques: Detection of viral antigens, nucleic acids and specific antibodies, isolation and identification by culture and microscopy; Immune response: Cell mediated immune response, General features; Pathogenesis: Animal viruses.**

Further Reading

Bilzer T and Stitz L (1997) Immunopathology and neurodegeneration in Borna disease. *Brain Pathol.* 7: 1317.

Hatalski G, Lewis AJ and Lipkin WI (1997) Borna disease. *Emerg. Infect. Dis.* 9: 129.

Koprowski H and Lipkin WI (eds) (1995) Borna disease. *Curr. Top. Microbiol. Immunol.* 190.

Pringle C and Easton AJ (1997) Monopartite negative strand RNA genomes. *Semin. Virol.* 8: 49.

Richt JA, Pfeuffer I, Christ M *et al* (1997) Borna disease infection in animals and humans. *Emerg. Infect. Dis.* 3: 343.

BOVINE DIARRHEA VIRUS AND BORDER DISEASE VIRUS (*FLAVIVIRIDAE*)

Edward J Dubovi, Department of Population Medicine and Diagnostic Science, College of Veterinary Medicine, Cornell University, Ithaca, New York, USA

Introduction

For the purposes of this entry, bovine viral diarrhea virus (BVDV) and border disease virus (BDV) will be considered a single viral entity, with different sources of the isolates – either bovine or ovine; there are, however, data indicating that BDV can be separated from BVDV at the molecular level. A closely related virus, classical swine fever virus (CSFV) (also referred to as hog cholera virus), does have consistent antigenic differences from BVDV and BDV. Accordingly, CSFV will be addressed separately. Some confusion regarding BVDV may be encountered because of a lack of uniformity in the designation of this virus. Early reports described the agent as virus diarrhea (VD) virus while others working with isolates from a different clinical syndrome coined the term mucosal disease (MD) virus. When it became evident that the

agents causing both clinical syndromes were similar, attempts were made to reconcile the terminology with bovine viral diarrhea–mucosal disease (BVD-MD) virus. With the advent of genotyping, designations such as BVDV type 1 and BVDV type 2 have appeared. Consistency of terminology has yet to be achieved.

History

The generally recognized beginning of the literature history of BVDV occurred in 1946 with the clinical description of an apparently new disease in cattle in New York State. A series of astute observations and experiments suggested a viral etiology, and the term virus diarrhea was applied to the clinical syndrome. Several other reports described disease syndromes similar to virus diarrhea but no attempt was made to define the etiological agent. In the early to mid-1950s, another clinical entity, which became known as mucosal disease, was described. While similar to virus diarrhea in some respects, mucosal disease was clearly different in the severity of the individual cases and in the small number of animals affected in a given herd. It was not until 1957 that an etiological agent was isolated in tissue culture from both virus diarrhea cases and mucosal disease cases. The initial isolate from the virus diarrhea-affected animals did not produce any cytopathic effects in cell culture, whereas the agent from the mucosal disease animals did. In 1960 a plaque assay was developed for a cytopathic isolate of BVDV. This test permitted a comparison by virus neutralization of the viruses isolated from cases of virus diarrhea and mucosal disease. The results of the comparison indicated that the agents involved in the two syndromes were essentially indistinguishable. The relationship between the cytopathic isolates and the noncytopathic isolates was not defined until the advent of molecular biology.

The descriptive history of BDV began about the same time as BVDV, in the late 1940s, but was more delayed in its development due to the lower incidence of severe clinical disease. The initial focus of interest was along the border between Wales and England, thus the name border disease. A viral etiology for the disease was suggested in the early 1970s through transmission studies using cell-free filtrates from affected animals. About the same time, a link to BVDV was indicated by the ability of ovine sera from experimentally infected animals to recognize BVDV antigens. It took several years longer to isolate a viral agent in cell culture and to reproduce the clinical disease seen in natural cases. As will be discussed later, BDV is closely related to BVDV, not only in its molecular characteristics but also in the clinical disease that it can produce.

Taxonomy and Classification

Early structure analyses of BVDV and CSFV indicated a virion architecture similar to the classic togaviruses. Since there was no apparent insect vector for these viruses, the term 'nonarthropod-borne togaviruses' was created to include BVDV, BDV, CSFV, equine arteritis virus, lactate dehydrogenase virus and rubella. With the establishment of the antigenic relationship of BVDV, BDV and CSFV, a new genus, *Pestivirus,* was created within the family *Togaviridae* in 1982. Data on the genomic structure of the pestiviruses indicated a closer relationship to viruses such as yellow fever virus and West Nile virus, and in 1991 the pestiviruses were reclassified in the genus *Pestivirus* within the *Flaviviridae* family. Yellow fever virus is in the genus *Flavivirus*. The hepatitis C group of viruses are also included within this family under the genus 'Hepatitis C-like viruses'.

Properties of the Virion

As indicated earlier, BVDV and BDV were considered togaviruses. As such, the virion is composed of an icosahedral core with a lipid envelope. The protein composition of the virion has been difficult to determine because of problems in purifying the virus. Recent work, mainly with CSFV, has provided substantial data on the virion structural proteins. These data, along with data on proteins expressed from genomic clones, provide the following picture. The envelope contains three glycoproteins, E^{RNS}, E1 and E2. The glycoproteins may exist in the virion as hetero- and homodimers. Most neutralizing monoclonal antibodies (MAbs) are directed against E2, while several have been reported to bind to E^{RNS}. The nucleocapsid protein C is the second protein of the open reading frame (ORF) rather than the first protein N^{PRO}, as assumed initially.

Properties of the Genome

The genome of pestiviruses is an RNA molecule of positive strand polarity (**Fig. 1A**). Sequence analysis of the genome indicates a size of about 12.3 kb, but some variation may be found for those isolates with host cell insertions and sequence duplications. The genomic RNA lacks polyadenylation at the 3′ end (3′ untranslated region, 3′ UTR) and the 5′ end lacks a cap structure. The 5′ UTR contains a complex stem–loop structure with secondary structure consistent with an internal ribosome entry site (IRES). The genomic RNA has significant secondary structure, as

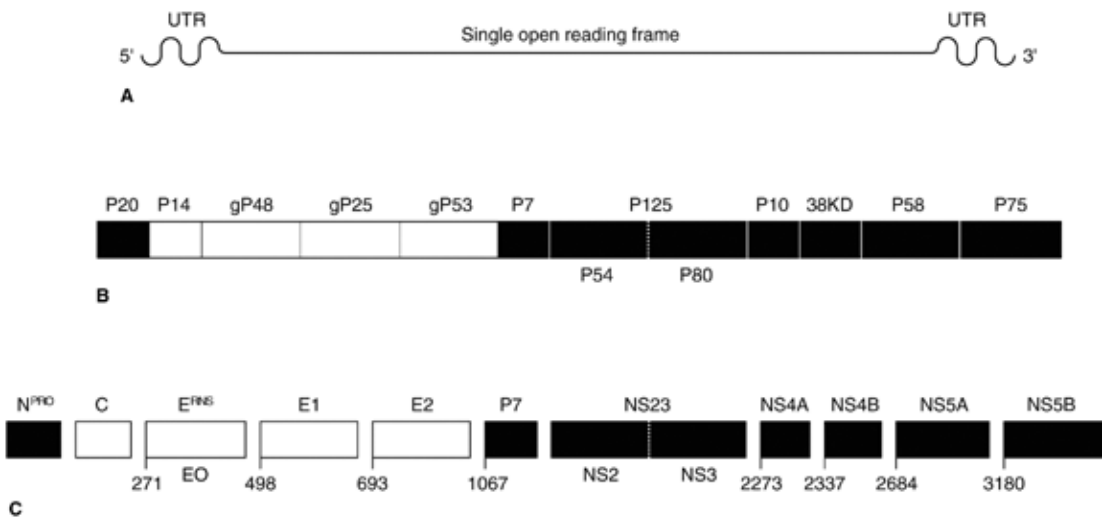


Figure 1 BVD virus genome and translation products. (**A**) Single-stranded genome of positive polarity with untranslated regions (UTR) at the 5′ and 3′ ends. (**B**) Polyprotein with the old designations for the processed proteins. (**C**) Processed proteins of BVD virus with the currently accepted nomenclature. The numbers at the left end of proteins are the N-terminal amino acids for that protein based on the SD-1 sequence. The white areas in (**B**) and (**C**) indicate structural proteins while the black areas are nonstructural proteins.

indicated by its sedimentation coefficient, resistance to low concentrations of RNase, and its solubility in high salt.

Properties of Proteins

Studies on the structure/function of the BVDV and BDV proteins are yet to be completed. The first protein at the 5′ end of the genome, N^{PRO} (N-terminal proteinase), possesses an autoprotease activity consistent with a serine protease specificity (**Fig. 1C**). The function of this protein is unknown, other than to cleave itself from the nascent polypeptide chain. The nucleocapsid protein C is located next to N^{PRO}. The three glycoproteins E^{RNS}, E1, and E2 are next in the coding sequence. As indicated earlier, these proteins are in the virion envelope, with E2 containing the majority of the neutralization sites. E^{RNS} (soluble RNA) is somewhat unusual in that it contains RNase activity, the significance of which is unknown.

The nonstructural proteins comprise approximately 75% of the coding capacity of the genome (**Fig. 1C**). The NS2 protein contains a zinc-finger-like domain, but its function has not been determined. The NS3 protein contains a serine proteinase activity which is responsible, along with NS4A, for cleavage of the polyprotein encompassing the rest of the nonstructural region. The NS3 protein also contains an NTPase activity as well as an RNA helicase activity. It has been suggested that NS5A and NS5B form the viral replicase complex. NS4B protein has been identified in infected cells, but its function is unknown. While most of the data on the proteins of pestiviruses have been done with BVDV, the limited data on BDV are consistent with those from BVDV.

Physical Properties

The physical properties of the virion are consistent with those of flaviviruses. The viral particle is 40–60 nm in diameter with a density of 1.15 g ml^{-1}. Estimates of sedimentation coefficients range from 100 to 150 S. The infectivity of the virus particle is sensitive to lipid solvents such as ether and chloroform. High temperature or low pH are also detrimental to infectivity.

Replication

Very little is known concerning the various aspects of the replication cycle of BVDV or BDV. Replication of the virus is not sensitive to inhibitors of DNA-dependent RNA synthesis such as actinomycin D. There is no evidence for the involvement of the cell nucleus in the replication cycle. The replication cycle time of the virus is 12–16 h for both the cytopathic and noncytopathic strains. In general, there are larger quantities of viral products in cells infected with the cytopathic strains of BVDV than with the noncytopathic strains. Immunofluorescent signals from cytopathic strain-infected cells are much stronger than those from noncytopathic strain-infected cells. In addition, detection of viral RNA in noncytopathic strain infected cells is difficult, whereas it is readily

detectable in cytopathic strain-infected cells. The replication complex of BVDV and BDV has not been characterized.

Characterization of Transcription, Translation, and Post-Translational Processing

All available evidence indicates that there is no subgenomic RNA produced in the infected cell as a part of the normal replication pattern. As will be discussed, defective interfering (DI) particle RNA does exist and can be translated into BVDV proteins.

Sequence analyses of BVDV and CSFV show a single ORF which spans the entire genome (**Fig. 1**). At the 5′ end of the genome are approximately 400 nucleotides that do not appear to code for a protein product. Sequence analysis of this region suggests an internal ribosome entry site consistent with translation initiation in a cap-independent manner. Experimental data support the use of this IRES for translation initiation.

With a single ORF spanning 12 kb of RNA, one could anticipate post-translational protein processing, and indeed it has been found. As indicated earlier, N^{PRO} is an autoprotease which results in its removal from the nascent polypeptide chain. The three virion glycoproteins can be found as a single glycoprotein gp116. Further processing of this protein produces the mature E2 and a precursor protein gp62. This protein is processed to E^{RNS} and E1. The protease(s) responsible for these cleavage reactions are presumably of cellular origin. Support for this comes from the expression of a portion of E1 and E2 in a vaccinia recombinant construct. In the absence of other BVDV proteins, the cleavage of this protein occurred such that all neutralizing sites on E2 as defined by MAbs were expressed in infected cells. A minor amount of E2 can be found in infected cells attached to p7. The significance of this structure is unknown, as is the function of p7.

The NS23 nonstructural protein contains the NS2 and NS3 proteins. The mechanisms for generating NS2 and NS3 may be different, depending on the virus used to initiate the infection. With cytopathic viruses, more accessible cleavage sites within the NS23 protein may be created to generate NS3. With NCP viruses, processing of NS23 to yield NS2 and NS3 may occur at a level that is undetectable. As discussed previously, NS3 possesses proteinase activity which processes the precursor protein for the other nonstructural proteins. p165 and p133 proteins have been detected, and p133 has been shown to be processed to NS5A and NS5B. With the exception of glycosylation, there are no reports of other modifications to the pestivirus proteins.

Assembly

The pestiviruses appear to mature by budding through intracellular membranes. Electron microscopic examination of infected cells shows virus-like particles in cytoplasmic vacuoles. Additionally, BVDV antigens have not been demonstrated on the surface of infected cells, thus supporting an internal maturation site for the virus.

Geographic and Seasonal Distribution

There is no geographic restriction for BVDV and BDV. These viruses exist wherever ruminants are found. There also does not appear to be any seasonal pattern to the disease outbreaks caused by these viruses. Some clustering may occur at those times of the year when animals are herded together, such as when range cattle are assembled into feedlots or dairy cattle are confined to sheltered areas in winter. For BDV, a greater awareness of the disease will usually occur at lambing when affected animals are detected. However, seasonality is not a factor in the epidemiology of these infections.

Host Range and Virus Propagation

The host range of BVDV (and BDV) may involve all of the cloven-hoofed ungulates of the order Artiodactyla. The pestiviruses can produce productive infections in cattle, sheep, swine and domestic goats. BVDV has been isolated from deer, giraffe, antelope of various species, American bison, musk ox and wildebeest. The ability of BVDV to infect sheep and produce clinical disease raised the question as to whether isolates from sheep could be considered a separate pestivirus. The significance of BVDV in the free-ranging ruminants is largely unknown, but the risk for these animals in zoological parks may be considerable. Evidence for a broad host range in ruminants is largely based on serological data from zoological parks. Experimentally, the rabbit can be infected with BVDV, but there is no evidence of natural infection.

BVDV can be propagated in cells originating from cattle, sheep, goats and swine. Cells from other sources have been found to be infected with BVDV, but these infections have arisen through the propagation of cells with fetal bovine serum contaminated with BVDV. Virtually all commercial lots of fetal bovine serum are contaminated with BVDV. Cell lines from rabbit, cat, deer, antelope, cattle, sheep, goat, pig, bison and dolphin have been shown to be

susceptible to BVDV. There is still no convincing evidence for BVDV propagation in human and nonhuman primate cells.

Genetics

The amount of data on the genetic relationships among the pestiviruses has increased dramatically in the 1990s. The most striking heritable difference found with BVDV and BDV is the existence of noncytopathic and cytopathic isolates. While the extent of cytopathic changes in cell culture is dependent on the cell type and culture conditions, noncytopathic isolates do not produce significant cytopathic changes in cell culture. The vast majority of field isolates of BVDV and BDV are noncytopathic and this biotype is responsible for most of the episodes of acute disease. The genetic basis for the biotypic differences is known for selected pairs of isolates. When virus-encoded proteins from infected cells are compared, the cytopathic isolates show a protein (NS3) not usually found in cells infected with noncytopathic isolates. An NS23 protein found with both biotypes contains all of the amino acids sequence found in NS3. With noncytopathic viruses, the NS3 protein is not produced, or is done so with such low efficiency as to go undetected.

When the nucleotide sequences of several cytopathic BVDV isolates were compared to the CSFV, it was found that the cytopathic BVDV isolates contained host cell sequences inserted into the NS23 region of the genome. One common host cell insertion is ubiquitin. Other insertions have been found in the NS23 gene, which were identified as duplicated regions of the viral genome. Insertions are often associated with viral gene duplications and/or rearrangements. The phenotypic result of these insertions is the enhanced production of NS3 and the development of cytopathology in cell culture. Similar results have been found for the cytopathic isolates of BDV.

Insertions and gene duplications produce viral genomes that are larger than the NCP viruses. However, it was found that subgenomic RNA was present with some of the cytopathic isolates. Analysis of these RNAs revealed the presence of DI particles with some unique attributes. One such DI had a deletion that included the entire structure gene complex along with the NS2 gene. The phenotypic result of this deletion is the enhanced production of NS3 if the cell is also co-infected with an NCP helper virus. Enhanced production of NS3 again produces a cytopathic effect in cell culture, but in this case the system is unstable, i.e. a cytopathic virus cannot be biologically cloned because of the need for the helper virus. While enhanced production of NS3 appears to be necessary for the development of cytopathology in cell culture, the mechanism for this process is unknown.

Evolution

The genetic relatedness of the pestiviruses was initially determined by analysis using polyclonal antibodies. While differences were noted among the viruses, the extent of the differences could not be accurately assessed. With the use of MAbs, it became evident that CSFV could be separated from BVDV and BDV. Sequence analyses of CSFV (Alfort) and BVDV (NADL) show 66% nucleotide and 85% amino acid homologies. Clearly, the viruses are closely related. At present, there are insufficient data to speculate on the origin of the pestiviruses, i.e. whether pestiviruses originated in ruminants with a spillover into swine, or the reverse.

As the comparative studies of the pestiviruses moved from MAb analyses to sequence analyses, the focus of attention centered on the 5′ untranslated region (UTR) of the genome. This region is highly conserved among the pestiviruses, but the data from it have been used to segregate pestiviruses into four major groups: CSFV-like viruses, 'true' border disease viruses, BVDV type 1 and BVDV type 2. A proposal has been made to use these groups as the basis of a new nomenclature for the pestiviruses, but this has not so far been accepted by the International Committee on the Taxonony of Viruses.

Serologic Relationships and Variability

As stated previously, BVDV, BDV and CSFV are antigenically related and the identification of etiological agents for their respective diseases was aided by their serological relationships. BVDV infection of pigs induces the production of antibodies which can recognize CSFV, but the neutralizing antibody titer against CSFV is very low, if detectable. These results suggested that some antigenic differences existed between CSFV and BVDV. The production of MAbs against CSFV and BVDV allowed a more thorough analysis of these differences. Some MAbs recognize all pestiviruses, while others are specific for CSFV and not BVDV or BDV. MAb analyses of ovine and bovine isolates suggested that ovine isolates might be different from the bovine isolates. Nucleic acid sequence data support the separation of bovine and ovine virus groups.

Antigenic variation within the BVDV isolates was not initially considered significant enough to warrant the designation of unique serotypes. However, there is

significant variation which impacts on both the biology of the disease and measures devised to control spread of the infection. Crossneutralization tests using serum specific for given isolates revealed 100–1000-fold differences in neutralizing titers. These differences are consistently found when comparisons are made using BVDV type 1 and type 2 viruses. Glycoprotein E2 appears to be the major antigen for the induction of neutralizing antibodies. MAbs specific for neutralizing sites on this protein show a wide range of reactivity with field isolates, indicating a high degree of variation. Amino acid sequence comparisons for this protein between strains Osloss and NADL show only a 78% homology. Variations in other areas of the genome are indicated by the fact that DNA probes have not been successful in detecting all strains tested.

Epidemiology

The key factor in the epidemiology of BVDV and BDV is the persistently infected animal. Infection of the bovine fetus with noncytopathic strains of BVDV prior to the development of immunological competence (approximately 120 days gestation) can result in the production of an animal that is persistently infected with BVDV throughout its life. The animal is immunologically tolerant to the strain of BVDV that initiated the infection. Virus can be cultured from all mucosal surfaces and blood can contain up to 10^6 infectious units per millilitre. The virus is maintained in the population through the persistently infected animal. Females that survive long enough to reproduce give birth to persistently infected offspring. Persistently infected males shed virus in semen, and through either natural or artificial breeding can transmit the virus to susceptible females. Acutely infected females can begin the cycle anew if they are carrying a fetus at the critical stage of gestation.

Transmission and Tissue Tropism

The main mode of transmission of BVDV and BDV is animal to animal through the oral route. Both acutely and persistently infected animals shed virus in all secretions or excretions, although urine and feces are not considered useful diagnostic samples. Venereal transmission through virus-containing semen or through virus-contaminated biologics used in embryo transfer procedures does occur, but is a minor consideration in the overall picture.

In most instances, virus initially replicates in the oronasal mucosa. There is some evidence of strain variation in the ability to replicate in the upper respiratory tract. Following replication at the initial site of infection, the virus can then spread systemically either as free virus in the blood or in circulating lymphoid cells. Acutely infected animals will generally show a transient leukopenia which resolves by 2 weeks postinfection. It is during this viremic stage that virus gains access to the fetus and establishes the persistent infection cycle. In the persistently infected animal, virus can be isolated from all tissue samples.

Pathogenicity

Many acute infections with BVDV and BDV are subclinical or inapparent, as indicated by a high seroprevalence of antibodies to BVDV or BDV without a corresponding amount of disease. The initial clinical description of BVDV infections included diarrhea as a major symptom, thus the original designation virus diarrhea virus. Recently, a more severe acute form of BVDV has been described. In many cases the initial signs were of a severe respiratory problem, although no lung pathology was noted. Mortality rates were high in unvaccinated herds and abortions were common in marginally vaccinated herds. The severity of the disease signs and the extent of the lesions in infected animals made it very difficult to distinguish these acute infections from classic cases of mucosal disease as described below (high fever, oral erosions and severe diarrhea). The viruses associated with this severe form of BVDV were more commonly the genotype 2 viruses. However, genotype as defined by the 5′ UTR is not linked to virulence.

A unique aspect of BVDV, and to some extent BDV, is the production of the disease complex known as mucosal disease. Mucosal disease was identified as a disease entity separate from virus diarrhea mainly because of the severity of the clinical symptoms and the low prevalence. The recognition that both forms of clinical expression were caused by the same virus did nothing to explain why mucosal disease animals invariably died. Current information indicates that mucosal disease starts with the persistently infected animal. These animals carrying a noncytopathic strain of BVDV or BDV become infected with a cytopathic strain which can arise as a *de novo* mutation or through challenge with a heterologous BVDV. The cytopathic virus appears to replicate unchecked in the persistently infected animal because of the tolerance to the noncytopathic virus. Severe clinical symptoms can develop, with subsequent death of the animal. Mucosal disease can only be reproduced experimentally if one uses a persistently infected animal in the challenge model.

Clinical Features of Infection

Experimentally infected animals show a biphasic fever response which might not be detected under field conditions. A consistent feature of BVDV infections is the production of a transient leukopenia. Both T and B cells may be reduced, along with a large reduction in neutrophils. Platelet counts can be affected to the extent that petechial hemorrhages develop on mucosal surfaces. Virus can be recovered from the blood for a highly variable period of time. In most cases, virus is cleared by 14 days postinfection, but it has been isolated for up to 7 weeks in experimental settings. Strain of virus and the age of the host may be factors generating the highly variable response. Infected animals may show a mild nasal discharge and/or mild-to-severe diarrhea by day 8–12 postinfection. Again, depending on the strain of virus, oral erosions, high fever, respiratory signs, severe diarrhea and petechial hemorrhages may be present.

The most significant aspect of BVDV and BDV infections relate to reproductive problems. Both viruses readily cross the placenta and infect the developing fetus. Infections early in gestation can result in the death of the embryo. The impact on the fetus at later stages of gestation is dependent upon the strain of the virus and the developmental age of the fetus. Infection of the fetus with noncytopathic strains before immunological competence can result in the production of persistently infected animals. Infection of the fetus can also result in abortion, stillbirths or mummification. If the fetus survives the infection, numerous congenital defects can result, such as cerebellar hypoplasia, hydranencephaly, retinal atrophy, cataracts, bone growth retardation, arthrogryposis, alopecia and hypotrichosis. The classic congenital anomaly produced by BDV is referred to as the 'hairy-shaker syndrome', in which the affected lambs have an abnormal hair coat and tremble or shake because of a demyelinating encephalopathy.

The clinical features of mucosal disease mirror the acute disease but with much greater severity. The onset of symptoms may be sudden or may extend over several weeks or months with recurrent overt symptoms. Animals generally show a high fever, anorexia and depression. Mucosal surfaces may show extensive erosions that can coalesce to form large areas of necrosis. A profuse watery diarrhea can develop and may turn bloody as the infection progresses. Under normal field conditions, all animals with mucosal disease die. For the chronic form of the disease the diarrhea will be intermittent with progressive weight loss and general unthriftiness. Chronic erosive lesions may be found in the mouth and on the skin. Death is also the inevitable outcome of this condition.

The persistently infected animals show a wide spectrum of clinical signs ranging from apparently normal to the unthrifty 'poor doer'. While the normal developing persistently infected animal exists, it is the exception rather than the rule. Such animals generally show a retarded growth rate and poor body condition. Many of these animals succumb to other pathogens in the environment that have little impact on their non-persistently infected herdmates. The deficit in the persistently infected animals' defense system which prevents clearance of agents not normally of clinical significance has not been defined.

Pathology and Histopathology

The lesions associated with acute BVDV and BDV infections may not be prominent. The lesions involving the oral mucosa are highly variable. If present, the lesion affects the stratified squamous epithelia with mild mononuclear cell infiltration of the lamina propria. The most significant histopathological finding involves the lymphoid tissue. Swelling and edema of mesenteric lymph nodes along with follicular lymphocytolysis has been observed following experimental BVDV infections. In addition, similar lesions were observed in the gut-associated lymphoid tissue. Lesions in the intestine were restricted to areas overlying the lymphoid areas. With mucosal disease and the severe forms of acute disease the oral erosions are much more severe and extensive, with necrosis and the formation of ulcers. The most significant and consistent lesion is the erosion of the gut-associated lymphoid tissue or Peyer's patches. The distinctive change in the Peyer's patches is necrosis of the centers of the lymph nodules. Similar changes may be seen in the mesenteric lymph nodes. Collapse of the intestinal mucosa overlying the lymphoid tissue gives rise to the characteristic erosions seen throughout the intestine.

Immune Response

While there is some belief that pestivirus infections are immunosuppressive, development of an antibody response to the viruses are as would be expected. Antibody may be detected as early as 10 days postinfection, although there are reports of very delayed responses. Antibody titers may continue to rise for several months following acute infection. Challenge of previously infected animals with the homologous virus results in no evidence of acute disease. Challenge with heterologous isolates may result in a transient infection such that fetal infection may occur before the viremia is checked. Persistently infected animals may also mount an antibody response to heterologous strains of BVDV, but this does not result in the

clearance of the virus which established the persistent infection. There are no data on cell-mediated immune reactions following BVDV infections.

Prevention and Control

The key factor in the control of BVDV and BDV is the prevention of the production of persistently infected animals. Various modified live and killed vaccines are available for BVDV. While these vaccines may prevent development of acute disease, they have not been shown consistently to prevent fetal infection, particularly when heterologous viruses are used as a challenge. Contamination of biologics with BVDV will continue to be a potential source of virus for susceptible animals.

In areas where the prevalence of the virus is very low and movements of cattle are controlled, eradication programs have been instituted. Detection of the persistently infected animals is essential to these efforts. The success of these programs may depend on the lack of a wildlife reservoir for the virus.

Future Perspectives

From the molecular biology perspective, efforts will continue to determine the structure/function relationships of the viral-encoded proteins. The construction of infectious clones of the pestiviruses will greatly accelerate these studies. In addition, the function of NS3 can be approached in a systematic fashion with designed mutants. Issues of virulence and the role of specific proteins can now be addressed. The mucosal disease complex is perhaps the most unique viral disease entity in virology. While the economic impact of this disease is not significant, the delineation of its pathogenesis will continue to intrigue researchers. The control of pestivirus infections will require the development of vaccines that can protect the developing fetus, and more data are needed on the significance of the antigenic variation that exists with BVDV and BDV.

See also: **Hog cholera virus (*Flaviviridae*); Hepatitis C virus (*Flaviviridae*); Persistent viral infection; Yellow fever virus (*Flaviviridae*).**

Further Reading

Baker JC and Houe H (eds) (1995) Bovine viral diarrhea virus. *Vet. Clin. North Am.* 11 (3).

Brownlie J and Clarke MC (eds) (1990) *Bovine Virus Diarrhoea: Scientific and Technical Review*. Paris: International Office of Epizootics.

Meyers G and Thiel H-T (1996) Molecular characterization of pestiviruses. *Adv. Virus Res.* 47: 53.

Terpstra C (1985) Border disease: a congenital infection of small ruminants. In: Pandey R (ed.) *Progress in Veterinary Microbiology and Immunology*, vol. I, p. 175. New York: Karger.

BOVINE HERPESVIRUS (*HERPESVIRIDAE*)

Michael J Studdert, School of Veterinary Science, The University of Melbourne, Parkville, Victoria, Australia

History

Clinical diseases caused by herpesviruses in members of the family *Bovidae* have been recognized for centuries. However, it was not until the first and probably most important virus now called bovine herpesvirus 1 (BHV1) was isolated from the genital disease coital exanthema also called in the female infectious pustular vulvovaginitis (IPV) and from the respiratory disease infectious bovine rhinotracheitis (IBR) in the late 1950s that any of these diseases was confirmed to be caused by a herpesvirus. Historically IPV and its male counterpart infectious pustular balanoposthitis (collectively the male and female diseases are termed coital exanthema or Blaschenausschlag) were commonly described diseases in central Europe throughout the 19th century. It was common for a single bull in a village to serve all the female cattle in that village and where distances were small also in nearby villages and Blaschenausschlag

clearance of the virus which established the persistent infection. There are no data on cell-mediated immune reactions following BVDV infections.

Prevention and Control

The key factor in the control of BVDV and BDV is the prevention of the production of persistently infected animals. Various modified live and killed vaccines are available for BVDV. While these vaccines may prevent development of acute disease, they have not been shown consistently to prevent fetal infection, particularly when heterologous viruses are used as a challenge. Contamination of biologics with BVDV will continue to be a potential source of virus for susceptible animals.

In areas where the prevalence of the virus is very low and movements of cattle are controlled, eradication programs have been instituted. Detection of the persistently infected animals is essential to these efforts. The success of these programs may depend on the lack of a wildlife reservoir for the virus.

Future Perspectives

From the molecular biology perspective, efforts will continue to determine the structure/function relationships of the viral-encoded proteins. The construction of infectious clones of the pestiviruses will greatly accelerate these studies. In addition, the function of NS3 can be approached in a systematic fashion with designed mutants. Issues of virulence and the role of specific proteins can now be addressed. The mucosal disease complex is perhaps the most unique viral disease entity in virology. While the economic impact of this disease is not significant, the delineation of its pathogenesis will continue to intrigue researchers. The control of pestivirus infections will require the development of vaccines that can protect the developing fetus, and more data are needed on the significance of the antigenic variation that exists with BVDV and BDV.

See also: **Hog cholera virus (*Flaviviridae*); Hepatitis C virus (*Flaviviridae*); Persistent viral infection; Yellow fever virus (*Flaviviridae*).**

Further Reading

Baker JC and Houe H (eds) (1995) Bovine viral diarrhea virus. *Vet. Clin. North Am.* 11 (3).

Brownlie J and Clarke MC (eds) (1990) *Bovine Virus Diarrhoea: Scientific and Technical Review*. Paris: International Office of Epizootics.

Meyers G and Thiel H-T (1996) Molecular characterization of pestiviruses. *Adv. Virus Res.* 47: 53.

Terpstra C (1985) Border disease: a congenital infection of small ruminants. In: Pandey R (ed.) *Progress in Veterinary Microbiology and Immunology*, vol. I, p. 175. New York: Karger.

BOVINE HERPESVIRUS (*HERPESVIRIDAE*)

Michael J Studdert, School of Veterinary Science, The University of Melbourne, Parkville, Victoria, Australia

History

Clinical diseases caused by herpesviruses in members of the family *Bovidae* have been recognized for centuries. However, it was not until the first and probably most important virus now called bovine herpesvirus 1 (BHV1) was isolated from the genital disease coital exanthema also called in the female infectious pustular vulvovaginitis (IPV) and from the respiratory disease infectious bovine rhinotracheitis (IBR) in the late 1950s that any of these diseases was confirmed to be caused by a herpesvirus. Historically IPV and its male counterpart infectious pustular balanoposthitis (collectively the male and female diseases are termed coital exanthema or Blaschenausschlag) were commonly described diseases in central Europe throughout the 19th century. It was common for a single bull in a village to serve all the female cattle in that village and where distances were small also in nearby villages and Blaschenausschlag

Table 1 Herpesviruses of members of the family *Bovidae*

Designation	Common name (synonyms)	Genome		
		Subfamily	G + C (mole %)	size kb
Bovine herpesvirus 1	Infectious bovine rhinotracheitis HV, coital exanthema	α	72	140
Bovine herpesvirus 2	Bovine mammillitis virus; Allerton virus, pseudolumpy skin disease HV	α	64	133
Bovine herpesvirus 4	Movar HV	γ	50	145
Bovine herpesvirus 5	Bovine encephalitis HV	α	72	140
Ovine herpesvirus 1	Sheep pulmonary adenomatosis associated HV	γ		137
Ovine herpesvirus 2	Sheep assoc. malignant catarrhal fever of cattle HV	γ		
Caprine herpesvirus 1	Goat HV	α		
Alcelaphine herpesvirus 1	Wildebeest HV, malignant catarrhal fever HV of European cattle	γ	61	160
Alcelaphine herpesvirus 2	Hartebeest HV	γ		
Cervid herpesvirus 1	Red deer HV	α		
Cervid herpesvirus 2	Reindeer HV	α		

was a frequently observed sequel to mating. The isolation of IBR virus in 1957 and IPV virus in 1958 and subsequent work that established that the two viruses were essentially identical led to the designation of BHV1.

A second bovine herpesvirus, BHV2, was recognized as a cause of pseudolumpy skin disease and, as an independent syndrome, mammillitis about 1960 although both diseases were known clinically well before this time. The disease known as bovine malignant catarrhal fever has also been described for at least a century and the causative herpesvirus of the African form of the disease, formerly designated BHV3 (sometimes BHV4), was first isolated in 1968. However, the natural host for the best characterized malignant catarrhal fever herpesvirus, acquired by European cattle as originally reported in Southern Africa, is the wildebeest (*Connochaetes gnu*) and this virus is now termed Alcelaphine herpesvirus 1. A slowly growing, highly cell-associated herpesvirus, uncertainly associated with a number of disease syndromes in cattle, is called BHV4. In European breeds of cattle a distinctly different virus causes encephalitis and this virus is tentatively designated BHV5 pending definition of its definitive host, which may not be European cattle. No alphaherpesviruses of sheep have been isolated. A poorly characterized gammaherpesvirus associated with ovine pulmonary adenomatosis has been described and a second ovine gammaherpesvirus is the cause of sheep-associated bovine malignant catarrhal fever.

Classification

A complete list of herpesviruses affecting members of the family *Bovidae* and their classification as to subfamily either *Alphaherpesvirinae* or *Gammaherpesvirinae* together with some other salient properties of each including guanine + cytosine content (moles%), and genome size in kilobases are shown in **Table 1.**

Structure

Each of the viruses listed in **Table 1** has a typical herpesvirus morphology. Virions are enveloped and about 150 nm in diameter. The double-stranded DNA genome is wrapped around a fibrous spool-like core. There is an icosahedral nucleocapsid 100 nm in diameter composed of 162 hollow capsomers: 150 hexamers and 12 pentamers. The nucleocapsid is surrounded by a layer of globular material called the tegument that is enclosed by a typical bilayer lipoprotein envelope within which are embedded glycoproteins some of which appear as projecting spikes in negatively stained electron micrographs. There are about 12 distinct glycoproteins associated with the envelope spikes. Though the size of the DNA genome varies (**Table 1**) there is evidence that there

are up to 76 open reading frames (genes) coding for a corresponding number of individual proteins. About 40 of these proteins are structural, i.e. associated with the virion, whereas the remainder are nonstructural, being found only in infected cells. Repeat DNA sequences are found in the genomes of all bovine herpesviruses. For the alphaherpesviruses a set of two indirect repeats brackets the so-called short region of the genome. The gammaherpesviruses have a set terminal repeat structure within each of which, a variable number of tandemly repeated sequence motifs is found.

Replication

Virus replication occurs in the nucleus of cells and typically results in the production of large intranuclear, eosinophilic inclusion bodies. The replication cycle involves at least three classes of genes termed α, β and γ or immediate early, early and late, the expression of which is coordinately regulated in a cascade manner during the replication cycle.

Geographic Distribution

In general, each of the bovine herpesviruses occurs worldwide paralleling the distribution of the host species. The bovine viruses, with minor exceptions, have a restricted host range. None is known to affect nonbovine species, most are restricted to the primary host species the notable exceptions are the viruses causing malignant catarrhal fever in European cattle that are acquired from wildebeest or sheep.

Antigenic Relationships

Bovine herpesviruses are genetically stable with only a single antigenic type described for each species and no observable changes in antigenicity over time have been recognized. Some intratypic (within species) differences are detectable using restriction endonuclease DNA fingerprinting but these differences have not been correlated with antigenic differences in the proteins coded for by the variable regions.

Epidemiology

In general, transmission requires close contact, particularly the kinds of physical contact that bring moist epithelial surfaces into apposition, e.g. coitus, or licking and nuzzling as between mother and offspring. In large closely confined populations such as cattle feedlots or zoo collections short distance aerosol is an important mode of transmission.

Pathogenesis

Alphaherpesviruses typically cause localized lesions, particularly of mucosal surfaces of the respiratory and genital tracts or the skin, that are characterized by the sequential production of vesicles, pustules and shallow ulcers that become covered by a pseudomembrane and heal after 10–14 days, usually without scar formation.

Generalized alphaherpesvirus infections may occur in very young calves or in a fetus prior to abortion. Encephalitis produced by bovine encephalitis herpesvirus occurs as a consequence of spread from the nasal cavity to the brain, via trigeminal nerve branches.

The gammaherpesvirus BHV4 is associated with low-grade clinical infection. Malignant catarrhal fever is a uniformly fatal disease associated with mucosal erosions, ophthalmia and encephalitis that appear to be immune mediated. Lesions are characterized by infiltration and proliferation of lymphocytes. It is still not known which lymphocyte population is targeted by the virus but it is probably B lymphocytes. Immune complexes involving viral antigens are probably also produced and contribute to pathology.

Latency, whereby the genome, probably as a circularized episome, persists in ganglion cells typically the trigeminal and sciatic in the case of alphaherpesviruses, and in white blood cells in the case of gammaherpesviruses, is a hallmark of bovine herpesviruses. From these sites of latency, virus is periodically shed to give rise to recurrent disease, shedding and transmission to incontact animals.

Clinical Diseases

Bovine herpesvirus 1 causes coital exanthema and infectious bovine rhinotracheitis. Both diseases are a consequence of lesions of the mucous membrane at the two sites. The extent and severity of the lesions may vary from mild subclinical disease to acute complicated severe clinical disease that is more likely to occur in the case of IBR in feedlot cattle where the disease is complicated by secondary bacterial infections. Complicated cases of IBR in feedlot cattle are not infrequently fatal.

BHV1 may occasionally cause enteritis in calves. Encephalitis caused by BHV1 has not been confirmed; all cases of alphaherpesvirus encephalitis in cattle have been caused by a distinctly different virus designated bovine encephalitis herpesvirus or BHV5. In groups of young calves the mortality caused by BHV5 encephalitis may approach 100%.

Mammillitis caused by BHV2 may be acute leading to loss of skin from the teats, udder and perineal

regions following vesicle and pustule formation. Pseudolumpy skin disease caused by BHV2 appears to be a consequence of viremic spread, possibly as a cell-associated viremia with localization of the virus in the skin resulting in large golf-ball-sized subcutaneous swellings. These eventually resolve after 3–4 weeks.

Caprine herpesvirus 1 disease is characterized by a variety of clinical signs including conjunctivitis and lesions of the respiratory, gastrointestinal or genital tracts. Abortion may occur.

The red deer and reindeer alphaherpesviruses probably cause clinical disease and have a similar natural history to BHV1.

Bovine malignant catarrhal fever, caused by either alcelaphine herpesvirus 1 or 2 or a putative sheep-associated herpesvirus, follows an incubation period of 3 weeks and is characterized by fever, depression, leucopenia, profuse nasal and ocular discharge, generalized lymphadenopathy, extensive mucosal erosions, central nervous system signs and bilateral ophthalmia, that begins as a keratoconjunctivitis and extends to a panophthalmitis. Death, which is invariable, occurs about 1 week after the onset of clinical signs.

Immune Response

Both antibody and cell-mediated immune responses are generated during herpesvirus infections. Neutralizing antibody primarily directed against envelope glycoproteins is probably important in long-term immunity. Viral antigens, some of which may be nonstructural, immediate early and early proteins, are incorporated into the cell membrane and serve as targets for cytotoxic T lymphocytes. The immune response does not prevent the establishment of latency and its role in regulating reactivation of latent virus and recurrent disease and shedding is debated. A central contradiction of herpesvirus immunity is that following natural infection, immune animals are also animals that are infected for life.

Inactivated, attenuated live and deletion mutant recombinant DNA live virus and DNA vaccines are available or in development for the control of BHV1. Vaccines are not generally available for the control of other bovine herpesvirus diseases.

Prevention and Control

BHV1 genital disease can be controlled by eliminating carrier cattle identified either serologically or by reactivation and isolation of virus following the administration of corticosteroids such as dexamethasone. Alternatively, where it is important to do so, such as for bulls in artificial breeding centers, a two herd system may be established. IBR is often associated with stress of transport, intercurrent disease, overcrowding and the mixing together of cattle from different sources all of which are typically associated with feedlot operations. Awareness and minimization of these predisposing factors can reduce the severity of clinical disease. In an increasing number of countries test and slaughter programs have achieved total eradication of BHV1 from national herds.

The epidemiology of BHV2 is not well understood and the only possible approach to prevention and control would be to consider removal of known infected cattle.

Malignant catarrhal fever, since it is acquired from a second, heterologous host (wildebeest, sheep), is clearly preventable by avoiding contact. The sporadic nature of the disease and the lack of detailed knowledge of the putative sheep-associated virus make avoidance difficult. In zoo collections bovid species known to harbor alcelaphine herpesviruses 1 and 2 should not be cohabited with those species known to be susceptible to malignant catarrhal fever.

Future

There is considerable interest in the molecular biology of the bovine herpesviruses; the entire nucleotide sequence of BHV1 has been determined, and substantial amounts of the genomes of BHV4 and alcelaphine herpesvirus 1 have also been sequenced. Characterization of the transcripts and proteins of the viruses continues. Progress in developing better vaccines and diagnostic reagents for BHV1 based on recombinant DNA technologies, including DNA vaccines, is continuing. The unusual epidemiologies of BHV2 and BHV5 are matters for future inquiry. The unusual pathogenesis of malignant catarrhal fever and the characterization of the sheep-associated virus responsible for most cases of malignant catarrhal fever in the western world are a part of ongoing work. There are many members of the family *Bovidae* for which herpesviruses have not been identified and over time it may be expected that more viruses will be isolated.

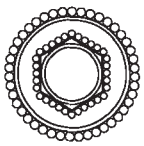
See also: **Herpes simplex viruses (*Herpesviridae*): General features, Molecular biology.**

Further Reading

Ackermann M, Edwards S, Keil G *et al* (1996) Infectious bovine rhinotracheitis and other ruminant herpesviruses. *Vet. Microbiol.* 53: 1.

Dinter Z and Morein B (1990) *Virus Infections of Ruminants*. Amsterdam: Elsevier.
Ludwig H Bovine herpesviruses. In: Roizman B (ed.) *The Herpesviruses*, vol. 1, p. 135. New York: Plenum Press.
Roizman B, Desrosiers RC, Fleckenstein B *et al* (1995) *Herpesviridae*. In: Murphy FA, Farquet CM, Bishop DHL (eds) *Sixth Report of the International Committee on Taxonomy of Viruses*, p. 114. New York: Springer-Verlag/Wien.

BOVINE IMMUNODEFICIENCY VIRUS (*RETROVIRIDAE*)



Matthew A Gonda, Program Resources, Inc./DynCorp,
National Cancer Institute – Frederick Cancer Research and Development Center,
Frederick, Maryland, USA

History

Retroviruses have been found in a variety of species from fish to man. They are causally associated with an assortment of pathologic states, including malignant and nonmalignant cytopathic diseases, such as leukemias and immunodeficiencies, respectively, while some infections are inapparent or without consequence. Animal retroviruses received a considerable amount of attention during the last several decades, especially after the discovery that they encoded a novel enzyme, reverse transcriptase (RT), and transduced oncogenes. Novel findings about their biology helped propel improvements in technology and investigations that led to the identification and isolation of the first human retroviruses.

In the late 1960s the intensive search for an infectious agent that might be the cause of enzootic bovine leukemia/lymphosarcoma led to the discovery and isolation of three morphologically and biologically distinct classes of retroviruses in cattle. One of these retroviruses was a bovine lentivirus, which recently was extensively characterized by Gonda and colleagues (1987); the name bovine immunodeficiency virus (BIV) was adopted for this unique lentivirus because of the striking morphologic, serologic and genetic features shared with the human and simian immunodeficiency viruses (HIV and SIV respectively). The other two retroviruses discovered were: bovine syncytial virus (BSV), a 'foamy virus' or spumavirus; and bovine leukemia virus (BLV), an oncovirus.

BIV was originally isolated by Van Der Maaten and colleagues (1972) from a Holstein cow obtained from a herd in Louisiana with persistent lymphocytosis, which is one of the associated diseases of BLV infection. The BIV-infected animal was purported to have clinical signs of disease that included a mild persistent lymphocytosis, generalized hyperplasia of lymph (hemolymph) nodes, central nervous system lesions, weakness and emaciation. BIV, isolated from this animal and propagated in tissue culture, was inoculated into specific-pathogen-free calves, where it caused a mild lymphocytosis and lymphadenopathy. Because this bovine lentivirus was not the intensively sought after viral agent of leukemia/lymphosarcoma, it was put into low-temperature storage and its biology went unstudied for nearly a decade and a half after its initial discovery. With the breakthrough that HIV-1 is a retrovirus and a member of the lentivirus subfamily, interest in BIV, and lentiviruses in general, has been renewed; and, much progress has been made in characterizing the microbiology and macrobiology of the retrovirus BIV.

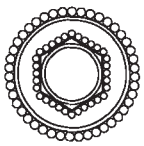
Taxonomy and Classification

The family *Retroviridae* is comprised of seven genera. BIV is the only member of the Lentivirus genus of retroviruses isolated from cattle, and because of this relationship, it shares many traits in common with members of this genus of retroviruses.

All retroviruses are protein-enveloped, positive-stranded RNA viruses that encode a unique enzyme, RT, capable of catalyzing the flow of genetic information from RNA to DNA, counter to that of most biologic systems. Thus, retroviruses have a DNA intermediate in their life cycle that can integrate into the host genome. Retroviruses can be further categorized based on whether they are horizontally or vertically (germline) transmitted. Classification into

Dinter Z and Morein B (1990) *Virus Infections of Ruminants*. Amsterdam: Elsevier.
Ludwig H Bovine herpesviruses. In: Roizman B (ed.) *The Herpesviruses*, vol. 1, p. 135. New York: Plenum Press.
Roizman B, Desrosiers RC, Fleckenstein B *et al* (1995) *Herpesviridae*. In: Murphy FA, Farquet CM, Bishop DHL (eds) *Sixth Report of the International Committee on Taxonomy of Viruses*, p. 114. New York: Springer-Verlag/Wien.

BOVINE IMMUNODEFICIENCY VIRUS (*RETROVIRIDAE*)



Matthew A Gonda, Program Resources, Inc./DynCorp,
National Cancer Institute – Frederick Cancer Research and Development Center,
Frederick, Maryland, USA

History

Retroviruses have been found in a variety of species from fish to man. They are causally associated with an assortment of pathologic states, including malignant and nonmalignant cytopathic diseases, such as leukemias and immunodeficiencies, respectively, while some infections are inapparent or without consequence. Animal retroviruses received a considerable amount of attention during the last several decades, especially after the discovery that they encoded a novel enzyme, reverse transcriptase (RT), and transduced oncogenes. Novel findings about their biology helped propel improvements in technology and investigations that led to the identification and isolation of the first human retroviruses.

In the late 1960s the intensive search for an infectious agent that might be the cause of enzootic bovine leukemia/lymphosarcoma led to the discovery and isolation of three morphologically and biologically distinct classes of retroviruses in cattle. One of these retroviruses was a bovine lentivirus, which recently was extensively characterized by Gonda and colleagues (1987); the name bovine immunodeficiency virus (BIV) was adopted for this unique lentivirus because of the striking morphologic, serologic and genetic features shared with the human and simian immunodeficiency viruses (HIV and SIV respectively). The other two retroviruses discovered were: bovine syncytial virus (BSV), a 'foamy virus' or spumavirus; and bovine leukemia virus (BLV), an oncovirus.

BIV was originally isolated by Van Der Maaten and colleagues (1972) from a Holstein cow obtained from a herd in Louisiana with persistent lymphocytosis, which is one of the associated diseases of BLV infection. The BIV-infected animal was purported to have clinical signs of disease that included a mild persistent lymphocytosis, generalized hyperplasia of lymph (hemolymph) nodes, central nervous system lesions, weakness and emaciation. BIV, isolated from this animal and propagated in tissue culture, was inoculated into specific-pathogen-free calves, where it caused a mild lymphocytosis and lymphadenopathy. Because this bovine lentivirus was not the intensively sought after viral agent of leukemia/lymphosarcoma, it was put into low-temperature storage and its biology went unstudied for nearly a decade and a half after its initial discovery. With the breakthrough that HIV-1 is a retrovirus and a member of the lentivirus subfamily, interest in BIV, and lentiviruses in general, has been renewed; and, much progress has been made in characterizing the microbiology and macrobiology of the retrovirus BIV.

Taxonomy and Classification

The family *Retroviridae* is comprised of seven genera. BIV is the only member of the Lentivirus genus of retroviruses isolated from cattle, and because of this relationship, it shares many traits in common with members of this genus of retroviruses.

All retroviruses are protein-enveloped, positive-stranded RNA viruses that encode a unique enzyme, RT, capable of catalyzing the flow of genetic information from RNA to DNA, counter to that of most biologic systems. Thus, retroviruses have a DNA intermediate in their life cycle that can integrate into the host genome. Retroviruses can be further categorized based on whether they are horizontally or vertically (germline) transmitted. Classification into

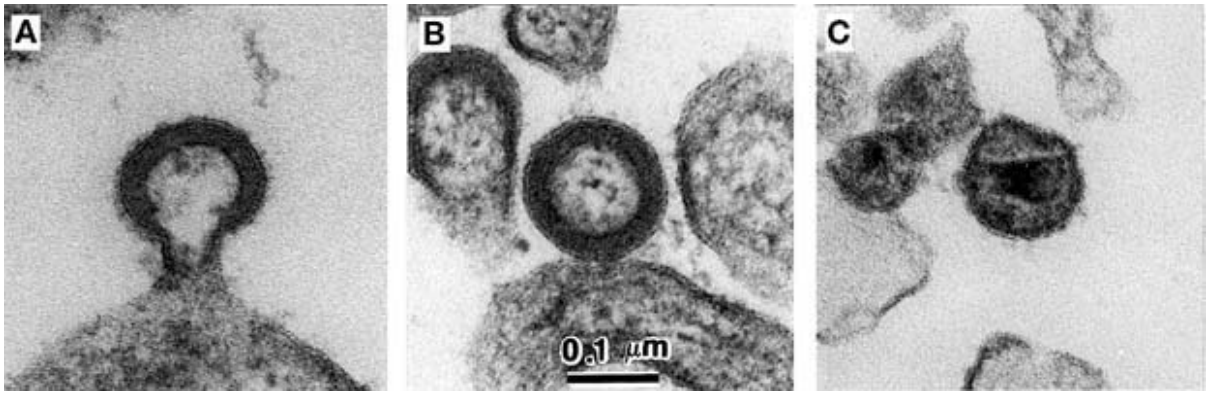


Figure 1 Morphogenesis of the bovine immunodeficiency virus (BIV) virion. (**A**) Cell-associated particle in the initial stage of budding. (**B**) Immature extracellular virus. (**C**) Mature extracellular particle with bar- or cone-shaped nucleoid.

one of the three retrovirus subfamilies most commonly is based on their morphology as observed in the electron microscope, select features of their biology and pathobiology, and the organization of their genome. The lentiviruses are exogenously acquired retroviruses that are often associated with slow, persistent and debilitating infections that may lead to death.

Properties of the Virion

The morphogenesis of the BIV virion observed by electron microscopy is typical of that of most retroviruses wherein particle assembly begins at the plasma membrane (**Fig. 1**). Initially, at the site of particle formation, a crescent-shaped, variably electron dense shell (virus core/nucleocapsid) forms beneath the plasma membrane and becomes more concentric as assembly proceeds. A distinct dark thin rim, which becomes a ring in the more advanced stages of morphogenesis, is visible at the innermost region of the electron dense shell. The electron dense shell is composed of Gag and Gag–Pol precursor proteins, although it has been demonstrated that the *gag* open reading frame (ORF) contains the minimal information necessary for particle formation. The outer envelope of cell-associated virus is studded with projections (10 nm) when observed in preparations specially stained with tannic acid; the projections are believed to be the surface (SU) envelope protein which is anchored to the viral envelope by the transmembrane (TM) envelope protein. Shedding of virus particles by the infected cell is accomplished by the process called budding, something similar to exocytosis, wherein a completely enveloped spherical virion, which has captured the genome-length, positive-stranded RNA, is released by a pinching off of the membrane at the base of the virus. These cell-free virions are still immature and are believed to be noninfectious. Morphogenesis of the immature virion is completed outside the cell when the viral protease (PR) cleaves the Gag and Gag–Pol precursors into the individual subunits; concomitantly, the spherical nucleocapsid condenses into a bar or cone shape. There is an electron lucent region between the denser cone-shaped core and amorphous material beneath the viral envelope in mature virions. The mature virions are 120–130 nm in diameter.

Properties of the Genome

The virus particle is believed to contain two copies of a single-stranded RNA genome as found in other retroviruses. The replication intermediates of lentiviruses can be double-stranded linear and/or circular DNA molecules. The genome-length, positive-sense RNA genome contains all the coding capacity of the DNA intermediate. The full-length RNA genome and all subgenomic messages have a common 5′ leader sequence and 3′ terminal poly(A) tracts. Lentivirus genomes are organizationally similar, but may differ in complexity. In the form of the linear DNA intermediate, the BIV genome (**Fig. 2**) contains the obligatory retrovirus structural genes, in the invariable order, *gag*, *pol* and *env*, flanked on the 5′ and 3′ ends by a complete copy of a long terminal repeat (LTR). The LTRs contain the promoters, enhancers and terminators of transcription. In addition, the genomes of lentiviruses are more complex than those of most oncoviruses in that they contain accessory (nonstructural/regulatory) genes in, and overlapping, the central region between the *pol* and *env* genes; and, in several viruses, post-*env*. The accessory genes appear to orchestrate virus expression, infectivity and replication.

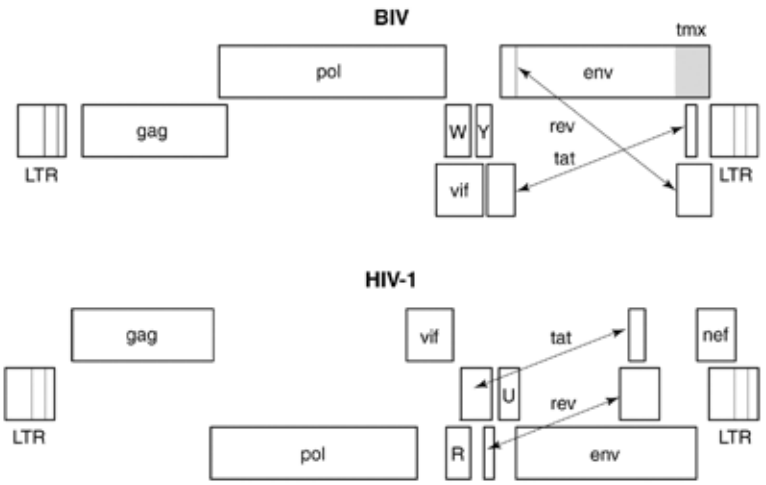


Figure 2 Comparative genome organization of BIV and HIV-I. The location of major open reading frames (ORFs) corresponding to the structural genes (*gag, pol* and *env*), flanked on the 5′ and 3′ ends by long terminal repeats (LTRs) are shown as well as those corresponding to the nonstructural/regulatory or accessory genes of BIV (*vif, tat, rev, tmx, W* and *Y*) or HIV-I (*vif, tat, rev, nef, R* and *U*) deduced from analysis of all three reading frames of their respective DNA sequence. Adapted from Gonda *et al.* (1990) In: *Animal Models in AIDS*, with permission of the editors and Elsevier Science Publishers, Inc.)

In terms of genome organization, BIV is the most complex nonprimate lentivirus characterized to date. It has several ORFs in, or overlapping, the central region that potentially encode at least five accessory genes that have been designated *tat, rev, vif, W* and Y, a single gene *tmx* is found post-*env* (**Fig. 2**). The predicted and/or known gene products of the accessory genes *vif* and *tat*, as well as those of the structural genes *gag*, *pol* and *env*, of BIV have some sequence similarity to their counterparts in HIV-1, which has aided in their identification. Nevertheless, the genomes of BIV and HIV-1 have diverged greatly overall, with the predicted products of the *gag* and *pol* ORFs having the greatest sequence similarity. The genome locations and other conserved traits in predicted products of *rev* and W and Y ORFs suggest that they are probably analogous to *rev* and *R, U,* and *X* genes, respectively, found in the primate lentiviruses. The function of the BIV *tmx* gene is not known, but it resides in the 3′ end of the genome in a position analogous to the *nef* gene of primate lentiviruses.

Characterization of Transcription and Translation

The transcriptional pattern of BIV is also very complex; there are at least five size classes (8.5, 4.1, 3.8, 1.7 and 1.4 kb) of mRNA related to the BIV infection, which supports the intricate nature of the genome (**Fig. 3**). The largest transcript (8.5 kb) contains sequences from all regions of the genome and, by analogy to other retroviruses, serves as the genomic RNA as well as the mRNA for expression of *gag* and *gag–pol* frameshift products. A number of splice donor and acceptor sites are strategically situated throughout the genome permitting the precise splicing out of introns to connect coding exons. The *env* and accessory gene mRNAs of BIV are derived from the primary transcript by single- or multiple-splicing events, respectively. In all singly or multiply spliced messages, an intron is removed between the end of the common leader sequence, which starts at the cap site in the 5′ LTR and ends at a splice-donor site before the initiation of the *gag* gene, and the beginning of the structural or accessory gene. The 4.1 kb RNA may encode *vif*, but it has not been unambiguously identified. The 3.8 and 1.7 kb RNAs are the *env* and *tat* spliced messages, respectively; the 1.4 kb RNA band contains at least the *rev* and *tmx* spliced messages. cDNA cloning experiments have shown that BIV's *tat* and *rev* genes contain two exons; the first is in or overlapping the central region and the second is in the 3′ end of the *env* region of the genome, but in a different reading frame from *env*. Thus, *tat* and *rev* mRNAs are translated from doubly spliced primary transcripts; the BIV *tmx* gene is singly spliced. The identity and structure of the W and Y mRNAs are not known. The function of the BIV *tat* product, to enhance transcription from the viral LTR, has been demonstrated; cellular factors also appear to play a role in activating or enhancing transcription from the viral LTR. At present, the functions of the *vif, rev, tmx* W and Y products in BIV are under investigation.

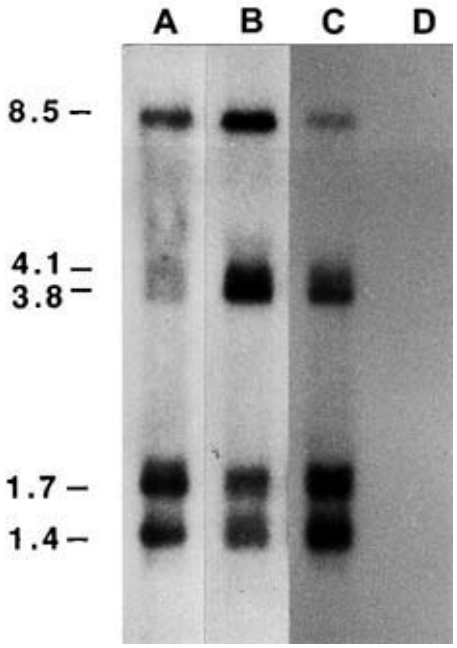


Figure 3 Northern blot analysis of BIV transcripts. (**A**) BIV 106-infected; (**B**) BIV 127-infected; (**C**) BIV parental (R-29)-infected; and (**D**) uninfected BLAC-20 cells. A ^{32}P-labeled probe representing the entire BIV proviral sequence was used to detect the viral RNA. Adapted from Oberste *et al.* (1991) *J. Virol.*, with permission of the American Society for Microbiology.

The primary Gag precursor (Pr53) and the Gag–Pol polyprotein (p174) are translated from the genome-length viral RNA. Since the *gag* and *pol* genes of BIV overlap and are in different reading frames, the synthesis of the Pol is via the Gag–Pol polyprotein and requires a −1 frameshift in the translation of this mRNA. The viral PR resides in the amino proximal region of the Pol precursor and is required for correct processing of Gag and Pol proteins into functional subunits. The viral PR becomes active after budding of the virus particle from the plasma membrane. It specifically cleaves sites between the Gag and Pol precursors in the polyprotein and within the Gag and Pol precursors. There are at least three functional Gag subunit proteins found in the Gag precursors, in the order, matrix (MA), capsid (CA) and nucleocapsid (NC), going from N- to C-terminus. The MA protein of BIV is a nonmyristylated 16 kD protein which inserts into the plasma membrane. The CA protein is 26 kD and is the major constituent of the cone-shaped core and contains the most commonly found cross-reactive epitopes of lentiviruses. The NC protein migrates as a 13 kD protein; however, amino acid sequencing has demonstrated that the BIV NC protein has a molecular weight of 7 kD. It is unclear whether BIV's NC protein migrates as a dimer or whether other physicochemical features cause it to migrate abnormally slow. The NC protein functions in binding the genomic RNA into the core during budding. By analogy to other retro-viruses, there are believed to be three Pol subunit proteins, PR, RT and endonuclease/integrase (IN); they have not been individually identified in BIV-infected cells or virions, but the existence of PR, RT and IN in virions has been demonstrated through functional studies.

The SU and TM envelope proteins of BIV are also synthesized as a precursor polyprotein. The SU, TM and precursor Env proteins of BIV are highly glycosylated. Carbohydrate accounts for up to 50% of the empirically determined molecular weight of the SU Env protein. The BIV Env precursor, gp 145, is a protein of 145 kD that is cleaved at the peptide sequence KPR$^{\downarrow}$AVG into SU (gp 100) and TM (gp 45) glycoproteins of 100 and 45 kD, respectively. The function of the SU protein is to act as the viral receptor and that of the TM protein is to anchor the SU to the exterior of the virus particle; both proteins are involved in the cell fusion activity of the virus.

Geographic Distribution

Our knowledge of the world-wide distribution of BIV is limited. Surveys have recently begun to reveal seroepidemiologic data on the prevalence of BIV in the US. Strikingly, BIV infections appear to be more prevalent in southern than in northern locales in the US. Within positive herds, the prevalence of BIV infection can be as high as 80%. Frequently, herds that are largely infected with BIV are also infected with the bovine retroviruses BLV and BSV; a high percentage of the animals in these herds found positive for BIV, and tested for BLV, are found to be dually infected. Recent serologic findings indicate that BIV may exist in Europe as well as Central and South America.

Host Range and Virus Propagation

The host range of lentiviruses is normally very restricted and is limited to the natural host and closely related species. *In vitro,* BIV is a cytopathic virus that induces syncytium (multinucleated giant cell) formation when an infected cell comes into contact with an uninfected cell. The formation of syncytia leads to cell death. Cell-to-cell transmission of the virus is the most effective means of infecting cells *in vitro. In vitro,* BIV infects primary embryonic bovine cells of lung, brain, thymus, and spleen. Virus can be recovered from bovine peripheral blood leukocytes by coculture, but peripheral blood leukocytes have proved difficult to grow and therefore

difficult to infect *in vitro*. Embryonic bovine spleen is the preferred tissue for the rescue and propagation of field isolates of BIV. In addition, BIV has been shown to infect epithelial bovine trachea (EBTr), canine fetal thymus (Cf2Th) and embryonic rabbit epithelial (EREp) established cell lines, but these cells are not as sensitive to virus infection as the embryonic bovine spleen cells. Nevertheless, EBTr, Cf2Th and EREp, since they are adapted to long-term passage, are the best vehicle for propagating BIV for antigen. BIV replication is best detected *in vitro* by immunofluorescence, RT, syncytia induction and electron microscopy assays and polymerase chain reaction (PCR) amplification of viral DNA sequences. BIV does not infect primary or established human cells and thus, it does not appear to be a threat to humans. The *in vivo* host range of BIV is also broader than that of most lentiviruses. In addition to cattle, BIV has been shown persistently to infect several breeds of rabbits. At least in the case of rabbits, the infection targets the immune system and viral sequences have been detected primarily in spleen, lymph nodes and bone marrow. Mice, rats, guinea pigs and sheep appear to be refractive to infection. Infected rabbits will prove important in the study of the biology of BIV.

Evolution

Lentiviruses are a genetically diverse group of retroviruses. Despite this genetic diversity, there are certain regions of the genome of all retroviruses that are highly conserved and upon which evolutionary relationships can be readily derived. One such region is the N-terminus of the RT domain of the *pol* gene (**Fig. 4A**). Phylogenetic analyses (derivation of phylogenetic trees) for this region of the genome have shown that BIV clusters with the retroviruses whose RTs require Mg^{2+} as cation, which is in keeping with known biological data (**Fig. 4B**). More importantly, BIV is a distinct member of the lentivirus subfamily of retroviruses and evolutionarily closer to the hypothetical ancestral virus that gave rise to all other lentiviruses. For this conserved segment of *pol*, BIV appears to be equidistant in its relationship to all other lentiviruses. Sequence comparisons from other ORFs of the genome give alternate protein-dependent tree topographies, often with BIV being closer to the primate lentiviruses.

Serologic Relationships and Variability

The serological relationship of BIV, other lentiviruses and retroviruses, can be assessed by various methods. In Western blots, BIV antiserum and CA antigen cross-react with corresponding CA antigen and antisera, respectively, from equine infectious anemia virus (EIAV), SIV and HIV-1. Cross-reactivity has also been found between the NC proteins of BIV and HIV-1. Heterologous competitive radioimmunoassays using radiolabeled HIVp24 (CA antigen) as competing antigen and a polyvalent BIV serum that contains reactivity to BIV CA antigen also have proved most informative in determining the serologic relationship of BIV to other lentiviruses (**Fig. 5**). In this assay, the lentiviruses, BIV, HIV-1, EIAV and SIV, all appear to compete completely; no other lentivirus or oncovirus gives appreciable competition. Thus, this assay indicates that the CA proteins of the competing lentiviruses share some common antigenic determinants that can be detected with precipitating anti-BIV serum.

Several functional proviral molecular clones of BIV have been sequenced, permitting an assessment of the amount of genetic variability which exists within a single isolate. Numerous point mutations are present within the genome; those which cause coding substitutions are most prevalent in the *env* coding region. The present data suggest that BIV displays significant genomic variability. It is not yet known whether coding substitutions in Env translate into antigenic variants, as has been shown for visna virus and EIAV.

Transmission and Tissue Tropism

Lentiviruses, in general, are spread by exchange of body fluids. Aerosols do not appear to play a role in the infectious transfer, although insect borne transmission has been demonstrated for EIAV. Cell-associated virus is the most efficient mechanism of transferring the infection. For HIV-1, the transmission issue has been carefully assessed; blood and sexual transmission appear to be the most common modes. In the case of BIV, the issue has not been thoroughly addressed; however, it appears that iatrogenic transmission of infected blood may be the main mode since very small amounts of infected blood are capable of passing the infection to an uninfected animal. The reuse of hypodermic needles, failure to cleanse between rectal examinations frequently used to assess fetal development during pregnancy, or failure to clean instruments used in de-horning are three probable mechanisms for introducing infectious material. It is also possible that common troughs containing waste milk from BIV-infected mothers that are used to feed calves may be a source of infectious virus.

The target cell and receptor for BIV are not known. BIV can readily be recovered from peripheral blood leukocytes of infected animals by coculturing with bovine embryonic spleen cells. There are a significant

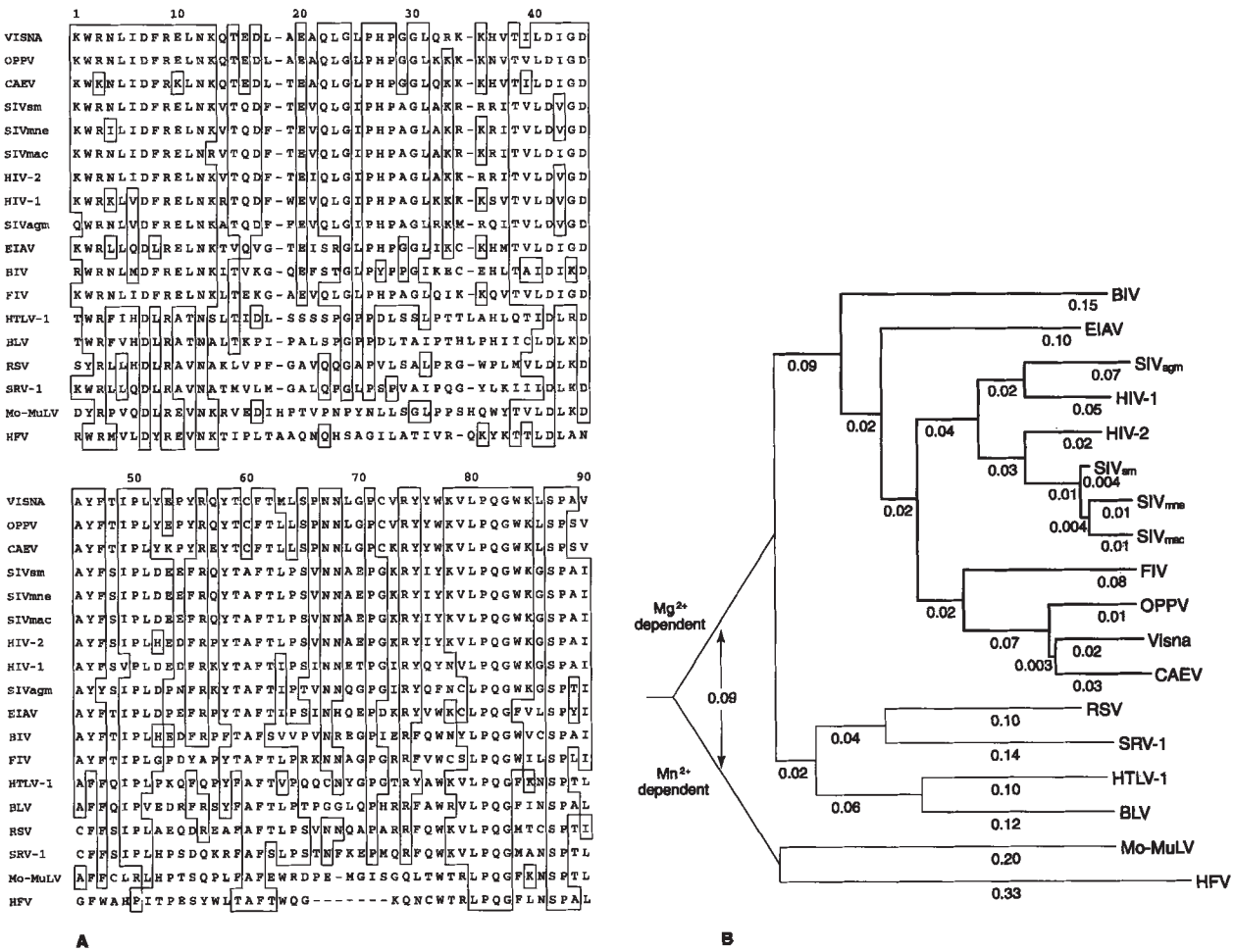


Figure 4 Evolutionary relationship of BIV. (**A**) Alignment of 90 consecutive amino acids in the conserved reverse transcriptase (RT) domain of the *pol* genes from lentiviruses with those of other retroviruses. Retroviruses used were visna virus, OPPV, CAEV, SIV_{sm}, SIV_{mne}, SIV_{mac}, HIV-2, HIV-1, SIV_{agm}, EIAV, BIV, FIV, HTLV-1, BLV, RSV, SRV-1, Mo-MuLV and HFV. The alignment shown is generally that found to be optimal with visna virus; slight improvements in the other pairwise alignment scores can be made by minor shifts in the placement of gaps. Shaded boxes are drawn around identical residues when seven or more lentiviruses share that residue. (**B**) Fitch–Margoliash phylogenetic tree based on analysis of 90 amino acids from the highly conserved RT domain of Pol of lentiviruses and, for comparison, those of other retroviruses, as presented in Garvey *et al.* (1990). Branch lengths are in units of $-\log M$ where M is the frequency of matching residues. The tree was rooted with HFV (spumavirus) and Mo-MuLV (oncovirus) as the outgroup taxons because they consistently had the lowest alignment scores and because their RTs preferentially use Mn^{2+} cations as cofactor. The average percent deviation of the tree was 5.17. Reprinted from Garvey *et al.* (1990) *Virology,* with permission of the editors and Academic Press, Inc.

number of monocytes in these preparations and, if BIV follows the trend of other lentiviruses, monocytes/macrophages are the most likely candidate target cells. Moreover, viral antigen has been demonstrated in monocytes/macrophages in the spleens of infected rabbits.

Pathogenicity

Short-term experimental studies in calves have demonstrated that BIV can cause a mild lymphadenopathy and lymphocytosis. This has been repeated with proviral molecular clones. Very little else is known of the pathobiology of BIV infections in its natural host.

Immune Response

The immune response of animals transfused with BIV-infected blood or experimentally infected with tissue culture-derived cell-associated virus has been studied in some detail. Transfused and experimentally inoculated animals initially appear to respond to the infection similarly. By 2 weeks post-transfusion or

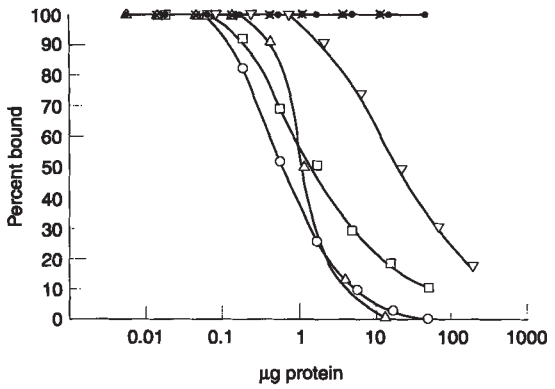


Figure 5 Immunological relatedness of major core or CA proteins of retroviruses using a heterologous competitive radioimmunoassay for the major Gag (p24) protein of HIV. The viruses (or mock virus preparations) used to compete were HIV (○), BIV (△), STLV-III (□), visna virus and CAEV (×), EIAV (▽) and FeLV, BLV, HTLV-I, HTLV-II and uninfected concentrated H9 cell supernates (•). The antiserum was a rabbit antibody to BIV and the radiolabeled antigen was HIV p24. Reprinted from Gonda *et al.* (1987) *Nature* (London), with permission of the editors and Macmillan Magazines Ltd.

post-inoculation, there is a measurable humoral immune response which increases with time and plateaus at about 2 months. ELISA titers of 1:12500 to 25000 are not uncommon. Virus rescue can be achieved early but becomes more difficult after three months postinfection. However, infection and humoral response appear to persist for the life of the animal. The same is the case for rabbits experimentally inoculated with tissue culture-derived virus. The cell mediated arm of the immune response has not yet been directly measured.

Prevention and Control

The epidemiology of BIV infection of cattle herds has only recently indicated the scope of infection in natural populations. Therefore, there is no current plan to attempt to eradicate BIV. However, since BIV is an infectious retrovirus, vaccination is the most likely scenario for its potential control. This may prove a difficult, although not impossible, task considering the experience encountered with other vaccination attempts with retroviruses.

Future Perspectives

Despite the fact that BIV has been recognized for over 20 years, the microbiology and macrobiology of BIV have only recently come under investigation. A great deal has been learned about the molecular biology of this virus. BIV does not appear to be a threat to humans. A better understanding of the pathogenesis and epidemiology of BIV is needed to assess further the need for control measures. An understanding of the pathogenesis, or lack thereof, may provide clues as to how lentiviruses cause disease. In this regard, the study of the biology of BIV in both rabbits and cattle may provide clues useful in understanding and preventing HIV-1 infections.

See also: **Bovine leukemia virus (*Retroviridae*); Caprine arthritis encephalitis virus (*Retroviridae*); Equine infectious anemia virus (*Retroviridae*); Feline immunodeficiency virus (*Retroviridae*); Genetics of animal viruses; Virus structure: Atomic structure; Human immunodeficiency viruses (*Retroviridae*): General features; Latency; Persistent viral infection; Simian immunodeficiency viruses (*Retroviridae*); Visna-Maedi viruses (*Retroviridae*).**

Further Reading

Battles JK, Hu MY, Rasmussen L, Tobin GJ and Gonda MA (1992) Immunological characterization of the *gag* gene products of bovine immunodeficiency virus. *J. Virol.* 66: 6868.

Garvey KJ, Oberste MS, Elser JE, Braun MJ and Gonda MA (1990) Nucleotide sequence and genome organization of biologically active proviruses of the bovine immunodeficiency-like virus. *Virology* 175: 391.

Gonda MA (1992) Bovine immunodeficiency virus. *AIDS* 6: 759.

Gonda MA and Oberste MS (1992) AIDS – The human immunodeficiency virus: Molecular and structural aspects of its biology. In: Kurstak E (ed.) *Control of Virus Diseases*. New York: Marcel Dekker, p. 3.

Gonda MA *et al.* (1987) Characterization and molecular cloning of a bovine lentivirus related to human immunodeficiency virus. *Nature* 330: 388.

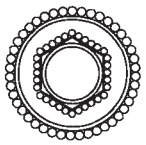
Gonda MA *et al.* (1990) Development of the bovine immunodeficiency-like virus as a model of lentivirus disease. In: Gaudry D and Hennessen W (eds) *Developments in Biological Standardization*. vol. 72, Basel: S. Karger p. 97.

Gonda MA *et al.* (1990) Contemporary developments in the biology of the bovine immunodeficiency-like virus. In: Schellekens H and Horzinek M (eds) *Animal Models in AIDS*. Amsterdam: Elsevier Science Publishers B.V./Biomedical Division, p. 233.

Snider TG, Hoyt PG *et al.* (1997) Natural and experimental bovine immunodeficiency virus infection in cattle. *Vet. Clin. North Am. Food Anim. Pract.* 13(1): 151.

[This article is reproduced from the 1st edn (1994).]

BOVINE LEUKEMIA VIRUS (*RETROVIRIDAE*)



Kathryn Radke, Animal Science Department, University of California at Davis, Davis, California, USA

History

Bovine leukemia virus (BLV) is the etiologic agent of enzootic bovine leukosis, a multicentric cancer of B lymphocytes. This disease of mature cattle was first described in Germany in 1878. By the early 1900s, it was known to be present continuously within herds of dairy cattle, but to affect only some of the animals. Hematologic observations subsequently revealed that more cattle had abnormally high numbers of blood lymphocytes (lymphocytosis) than actually developed leukosis. Patterns of natural and experimental transmission indicated that enzootic leukosis was caused by an infectious agent that appeared to be bloodborne. This was supported by the realization in the early 1960s that lymphocytosis and leukosis frequently occurred in Swedish dairy herds vaccinated against babesiosis with whole bovine blood. In 1969, C-type retroviral particles were identified in cultures of mitogen-stimulated lymphocytes obtained from peripheral blood of cattle with lymphocytosis or leukosis, and from blood of calves inoculated with tumor tissue or blood from such cattle. The virus was shown in 1972 to be infectious in cattle and sheep, and oncogenic in sheep. With the demonstration in 1974 that virions contained reverse transcriptase activity, BLV was unequivocally identified as a retrovirus. A close relationship between BLV and human T-cell leukemia virus was discovered in the early 1980s.

Taxonomy and Classification

BLV is a replication-competent, oncogenic retrovirus (family *Retroviridae*) that composes the genus *Deltaretrovirus* along with the human T-cell leukemia viruses (HTLV-1 and -2) and simian T-cell leukemia virus (STLV-1). These viruses have a unique mechanism of tumorigenesis. Their genomes lack cell-derived oncogenes that are present in rapidly transforming retroviruses such as Rous sarcoma virus. Tumors are clonal outgrowths of one virus-infected cell, but the proviral DNA genome is integrated at different sites in the host cell genome in tumors from separate individuals. Thus, BLV and HTLV are not integrated adjacent to a host cell oncogene such as c-*myc* that could be overexpressed by viral promoters and enhancers of transcription, as happens during tumor induction by avian leukosis virus and murine leukemia virus. Tumorigenesis by BLV and HTLV is thought to be initiated by alterations in host cell gene expression that are mediated by the viral Tax protein, a positive regulator of viral transcription. Consequent alterations in cell proliferation may render infected cells susceptible to mutations in host genes that are likely to be necessary for tumorigenesis. The long asymptomatic period between infection and development of clonal tumors, together with the low incidence of tumors among infected individuals, suggests that infection of a cell with BLV or HTLV is only one of several genetic alterations required for tumor formation.

Virion and Genome Structure

BLV virions are spherical particles 90–120 nm in diameter with a buoyant density of 1.15–1.17 g ml^{-1}. A membranous envelope encloses a centrally located, electron-dense icosahedral capsid or core of 60–80 nm in diameter, which contains the RNA genome. Both virions and virus-infected lymphocytes are heat-sensitive: pasteurization of milk by heating rapidly (15–20 s) to 72.5°C or slowly (30 min) to 62°C destroys infectivity. Disruption of the virion's lipid envelope by detergents or organic solvents also ablates infectivity, as envelope-associated viral proteins mediate binding and entry into cells.

The virion genome is a dimer of two identical, polyadenylated, plus strand RNAs of 8419 nucleotides. (Nucleotide numbers refer to a representative BLV genome sequence having NCBI Genbank Accession Number AF033818.) $tRNA^{Pro}$ is base-paired to genomic RNA where it primes minus strand DNA synthesis by reverse transcriptase. The 8.7 kb double-stranded DNA proviral genome (**Fig. 1**) is bounded by long terminal repeats (LTRs) formed during reverse transcription of terminal repeats (R) and sequences unique to the 5′ (U5) and 3′ (U3) ends of the RNA genome. Each LTR (533 nt) has the sequence U3 (211/213 nt)-R (234 nt)-U5 (88/86 nt). BLV and HTLV have unusually long R regions that separate the polyadenylation signal within U3 from the polyadenylation site at the end of R. From the 5′ end of the genome, BLV encodes the *gag*, *pro*, *pol* and *env* genes common to all replication-competent retro-

viruses. In addition, the 3′ end encodes a nontranslated region, *orf III* and *orf IV*, and *tax/rex* genes unique to BLV and HTLV. Open reading frames for *pro, pol* and *env* each overlap the preceding gene. A nontranslated region (>400 nt) following *env* and preceding *orf III* and *orf IV* is conserved in length among BLV isolates, suggesting that it serves some important function. Complete sequences of proviruses have been derived from tumors of Japanese, Belgian and Australian cattle.

Viral Proteins

Eight BLV-encoded proteins are found in virions. The RNA genome is coated by nucleocapsid protein (p12NC), a very basic, phosphorylated protein that binds RNA without sequence specificity. This protein contains two copies of a sequence (Cys-X_2-Cys-X_4-His-X_4-Cys) that resembles zinc-finger domains of DNA-binding proteins. The hydrophobic capsid protein (p24CA) forms the shell of the viral core, which contains the genome–NC complex as well as three enzymes: protease, reverse transcriptase and integrase. The pepstatin-sensitive aspartyl protease (p14PR) cleaves polyproteins containing Gag, Gag-Pro and probably Gag-Pro-Pol sequences into the proteins found in mature virions. The BLV reverse transcriptase (p70RT) is both an RNA-dependent and a DNA-dependent DNA polymerase that preferentially uses Mg^{2+} as a divalent cation. Although the BLV integrase (p32IN) has not been studied, it is most probably an endonuclease that mediates integration of the provirus into host DNA.

The interface between cores and the virion envelope is formed by the matrix protein (p15MA). The N-terminal glycine of this phosphoprotein is covalently linked to the fatty acid myristate, conferring affinity for membranes. MA binds specifically to BLV RNA in a region of U5-5′ *gag* and may assist in formation of RNA dimers during virion assembly. p15MA is processed to p10MA and p4, whose function is unknown.

The virion envelope, derived from the host cell plasma membrane, contains two viral glycoproteins. The surface (gp60SU) glycoprotein is anchored to the envelope by a labile association with the extracellular domain of the transmembrane glycoprotein (gp30TM). SU binds to cell-surface receptors and is the target of neutralizing antibodies. TM mediates membrane fusion via 28 hydrophobic, N-terminal amino acids (aa) and spans the lipid envelope by a more distal hydrophobic stretch of 20 aa. The 57 aa cytoplasmic domain of TM encodes three Tyr-x-x-Leu/Ile motifs, two of which can participate in signal transduction in a chimeric protein. SU has a 31 kDa protein backbone containing eight potential *N*-linked glycosylation sites and eight Cys residues. TM has a 24 kDa protein backbone containing two potential *N*-linked glycosylation sites and six Cys residues.

Two BLV-encoded regulatory proteins are present in BLV-expressing cells but not in virions. The nuclear Tax protein (34–38 kDa) *trans*-activates transcription from the BLV promoter. This activity requires an N-terminal, zinc-finger-like Cys-His motif. A central, leucine-rich segment functions as an activator domain when expressed as a chimera with the yeast GAL4 protein. The nuclear phosphoprotein Rex (16–19 kDa) acts post-transcriptionally to promote the cytoplasmic expression of unspliced and singly spliced viral mRNAs encoding the structural proteins and enzymes composing virions.

Sequences encoding novel proteins termed R3 and G4 are present in unusual spliced transcripts of the *orf III* and *orf IV* regions that lie upstream of *tax/rex*. These transcripts were cloned from infected lymphocytes obtained from calves early during infection (*orf III*) or from cattle with persistent lymphocytosis (*orf IV*). Roles for these proteins in the BLV life cycle are unknown. Deletion of *orf III* and *orf IV* restricts BLV propagation in animals and delays tumor development.

Replication

BLV binds to a cell-surface receptor and fuses with the host cell membrane upon exposure of the fusion domain of TM. Release of viral cores into the cytoplasm, reverse transcription of the viral genome, transport of proviral DNA into the nucleus, and integration into the host cell genome at essentially random sites presumably proceed similarly as for other oncogenic retroviruses. Stable integration of the provirus is thought to require mitotically active host cells. Like all known retroviruses and other retrotransposable elements, TG...CA residues form the boundaries of the integrated BLV provirus, which is flanked by 6 bp direct repeats in host cell DNA.

Transcription of the provirus by host cell RNA polymerase II begins at the start of R (+1; cap site) in the 5′ LTR, and is governed by *cis*-acting promoter and enhancer sequences in the U3 region. The TATA box is 44 nt upstream from the start site and a CAAT box is at −97. The viral Tax protein activates transcription from the viral promoter in *trans*. Tax response elements are three 21 bp imperfect direct repeats centered at −54, −129 and −154, each containing a cyclic AMP response element (CRE) and an AP-4 binding site. Bovine CRE-binding protein (CREB)-2 binds to these elements, as do activating transcription factors (ATF)-1 and -2. Tax facilitates dimerization of CREB/ATF factors by

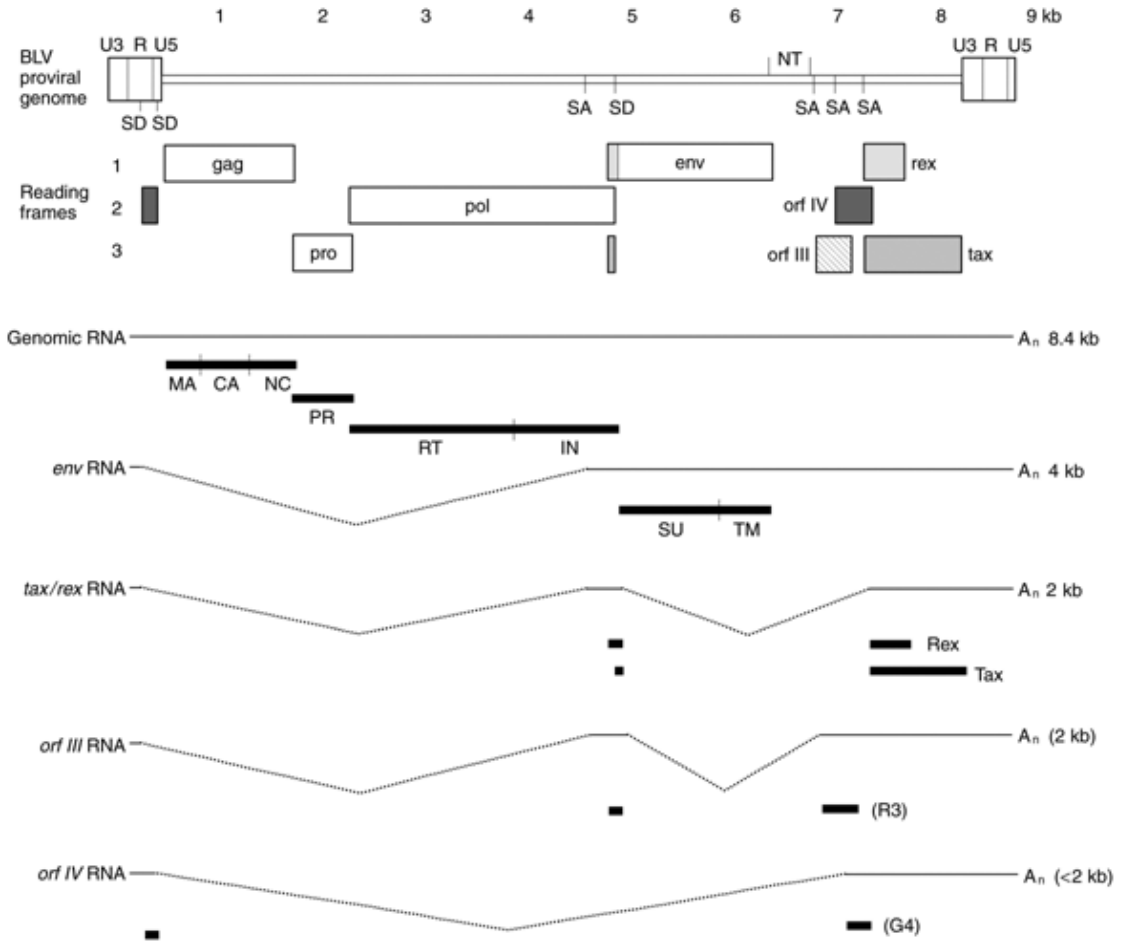


Figure 1 Bovine leukemia virus (BLV) genes, transcripts and proteins. The proviral genome is shown approximately to scale, with open reading frames indicated below it. NT is the putative nontranslated region. Splice donor (SD) and acceptor (SA) sites are marked on the proviral genome. Exons present in BLV transcripts are indicated with medium lines and introns with dotted lines. Proteins encoded by each transcript are designated with thick lines. See text for details. The transcripts shown for *orf III* and *orf IV* and their encoded proteins are provisional.

interacting with their basic-leucine zipper domains, thereby increasing their binding to the CREs. Transcription from the BLV LTR cannot entirely depend upon Tax, since this viral protein must be translated from newly transcribed RNA during early viral expression. NF-κB transcription factors bind between the two promoter-proximal CREs and increase BLV expression both in the presence and absence of Tax. Interferon regulatory factors (IRF)-1 and -2, for which a binding site is located in the U5 region of the LTR, may also affect Tax-independent transcription. A glucocorticoid response-like element is located just upstream of the promoter-proximal CRE. *Cis*-acting elements in the 5′ U3 region can act as positive or negative regulators of LTR expression, depending upon the cell type.

By analogy to HTLV-1, the 3′ *cis*-acting polyadenylation signal in U3 and poly(A) site at the end of R are brought together by a secondary structure formed at the 3′ ends of transcripts. This Rex response element acts in *cis* and in a position-dependent manner to confer post-transcriptional regulation of viral mRNAs by the BLV Rex protein.

Full-length 8.4 kb transcripts serve both as mRNA and as new genomes; all other BLV transcripts are spliced derivatives of this species (**Fig. 1**). The splice donor in R has been mapped to nucleotide 94, with an acceptor at 4443 for 4 kb *env* mRNA. A second donor at position 4665 has an acceptor at 7042 for 2 kb *tax/rex* mRNA and at 6619 or 6812 for 2 kb *orf III* mRNA. The splice donor for 2 kb *orf IV* mRNA is at 293 and the acceptor is at 6860.

Early transcription yields all classes of mRNA in the nucleus, but only 2 kb RNAs are cytoplasmic. During early expression, Tax and Rex proteins are translated from doubly spliced transcripts in

different, overlapping reading frames. Tax synthesis initiates at an AUG immediately preceding the second splice donor site. Rex translation begins at the *env* initiation codon in the second exon, so the N-terminus of Rex contains the first 17 aa of Env. When Rex accumulates, viral expression enters the late stage in which 8.4 and 4 kb transcripts encoding virion proteins are also transported into the cytoplasm and are translated.

Three polyprotein precursors, Pr45 Gag, Pr70 Gag-Pro, and Pr145 Gag-Pro-Pol, are translated on free ribosomes from unspliced, 8.4 kb RNA templates. Each protein domain is encoded by a different open reading frame, which is opened by a −1 frameshift that may be mediated by a $tRNA^{Asn}$ containing a highly modified queuine base in its anticodon loop. Frameshifting is inefficient, so the larger proteins are produced in correspondingly smaller amounts. The singly spliced, 4 kb *env* transcript is translated on membrane-bound ribosomes into the polyprotein precursor, gPr72 Env. This protein is glycosylated in the rough endoplasmic reticulum and Golgi apparatus, where it is cleaved into SU and TM by subtilisin/kexin-like host cell proteases. The viral glycoproteins are then transported to the plasma membrane.

All Gag polyproteins can associate with the inner face of the plasma membrane via their N-terminal, myristylated glycine residue. During virus assembly, MA protein may promote dimerization of full-length viral RNA. Each of two noncontiguous RNA encapsidation signals has a stable stem–loop structure. Assembly of capsids underneath the plasma membrane coincides with budding of immature virions from the plasma membrane where they acquire viral envelope proteins. Virions initially have an electron-lucent center that becomes electron-dense upon maturation, when Gag polyproteins are cleaved by the viral PR. Mature BLV virions are often associated with the surface of infected cells and can be found within intracellular vacuoles. BLV is not known to be lytic. Some types of infected cells form multinucleated syncytia in culture.

Evolution

The origin of BLV is unknown. It is not derived from an endogenous retrovirus of cattle. BLV is more closely related to HTLV than to other retroviruses, based on the degree of similarity in nucleotide and amino acid sequences of the viral Pol, NC and CA proteins, and on structural similarity of the envelope proteins. BLV and HTLV differ from mammalian C-type retroviruses by the preference of their reverse transcriptases for Mg^{2+} rather than Mn^{2+}, and by the presence of two copies rather than one of a Cys-His motif in the nucleocapsid protein. These features are also shared by avian and certain mammalian retroviruses that are clearly distinct from BLV and HTLV.

The *tax* and *rex* regulatory genes of BLV and HTLV are not related to any known genes or protein products. Further, little similarity is evident between BLV and HTLV for either gene, yet the strategy for expression is the same for both viruses and the products have analogous functions. Tax proteins of BLV and HTLV *trans*-activate transcription from the autologous viral promoter, but do not function reciprocally. In contrast, Rex proteins of BLV and HTLV function interchangeably in post-transcriptional regulation of viral expression.

Geographic Distribution and Epidemiology

BLV infection is worldwide, clustering within geographic areas as well as within herds in those areas. Higher percentages of dairy cattle are infected than beef cattle. Tumors occur in 1–10% of infected animals, a frequency that is probably lower than if cattle were allowed to live for their potential lifespan of 25–30 years. Beef cattle are usually slaughtered before 2 years, and dairy cattle by approximately 5 years. Infection is more likely to occur when cattle are in close confinement; the incidence of infection rises with age. Susceptibility to infection, to development of lymphocytosis and to formation of tumors is independently influenced by host genetic factors, some of which are encoded within the bovine major histocompatibility complex (MHC).

Host Range and Virus Propagation

Cattle are the predominant natural host for BLV infection, but domesticated zebu and water buffalo, sheep and wild capybara (a South American rodent) have also become infected. A number of animals can be infected experimentally. Sheep are widely used as experimental subjects because they are very sensitive to infection and a large percentage develop disease. Goats become infected, but very few develop tumors. Rabbits become persistently infected and immunosuppressed but do not develop tumors. Chimpanzees, rhesus monkeys, deer, pigs, cats and rats can be infected, but do not develop disease. There is no serological evidence that humans become infected with BLV and no consistent epidemiologic association between bovine leukosis and human leukemia.

A broad range of cells is susceptible to BLV infection in culture, including bovine, ovine, canine, feline, rodent and human cells. However, many infected cells do not express viral genes. The cellular

receptor for BLV is distinct from receptors for a large number of other retroviruses, including HTLV. Continuous lines of fetal lamb kidney cells and bat lung cells are the standard laboratory sources of BLV. Bioassay is by induction of syncytia following infection of early-passage embryonic bovine spleen or ovine cells, or of the CC81 line of feline cells transformed by murine sarcoma virus. Focal infectivity can also be scored using antibodies to the BLV CA protein.

Several infectious molecular clones of BLV exist; two are known to be pathogenic in sheep. Direct injection of provirus into sheep results in infection and tumorigenesis.

Genetic and Serologic Variability

BLV sequences are highly conserved among isolates from different parts of the world. Proviral sequences of three tumor isolates from Belgium, Japan and Australia differ by 3%, mainly in point mutations scattered throughout the genome. Variants display some restriction enzyme polymorphism. Envelope genes of a larger number of isolates show little variation; substitutions present at 6% of the nucleotides alter 4.3% of the encoded amino acids. HTLV-1 is similarly conserved in sequence.

Antibodies from BLV-infected animals are widely crossreactive. The N-terminal portion of the SU glycoprotein contains three conformational epitopes (F, G, H) that are dependent upon glycosylation and disulfide bonding. These epitopes are the targets of neutralizing antibodies; the H epitope plays a role in membrane fusion. Different isolates of BLV lack one or even two of these epitopes, but not all three.

Transmission

BLV-infected animals are rarely if ever viremic, so natural transmission is by transfer of infected lymphocytes in blood or perhaps secretions. Cell-free virus can establish experimental infections. Transfer of infected cells is facilitated by direct contact between cattle. The probability of infection depends on the frequency of virus-infected cells, which is elevated in animals with lymphocytosis. As little as 0.1 μl of blood can transmit an experimental infection. Milk and colostrum contain virus-infected cells but pasteurization inactivates BLV. Tracheal and bronchoalveolar washings, nasal secretions and saliva have proven at least once to contain infective cells. Urine, semen, plasma and serum do not transmit infection.

Transplacental infection occurs at a low rate, but transfer of embryos from infected to uninfected cows does not transmit BLV. Blood and tissues in common maternity pens are likely vehicles for transmission. Calves are susceptible to oral exposure by colostrum or milk from birth to 3 days, but colostral antibodies usually prevent infection and can persist for 6 months after birth. During dehorning, blood can be acquired from gouges and from other treated calves in the same pen. Sniffing, coughing and sneezing can aerosolize lymphocytes, but open wounds and the avid licking behavior of cattle are probably significant routes of transmission. Sheep do not transmit BLV horizontally.

Veterinarians and farmers can transfer blood between cattle by serially using the same obstetrical sleeve during rectal palpation to detect pregnancy, or by serially using needles and syringes for vaccination or blood sampling. Transmission by these routes is preventable. Cattle are highly sensitive to intradermal infection, so biting insects could be vectors, especially in tropical areas. However, insects have not yet transmitted BLV in experiments testing normal feeding conditions.

Cell Tropism

B lymphocytes are the predominant hosts for BLV *in vivo*. Peripheral blood mononuclear cells cultured for a few hours to induce viral expression, but not long enough to permit spread of infection, contain cells positive for cytoplasmic viral CA protein and surface immunoglobulin (Ig)M. Monocytes, CD8+ T cells and granulocytes have been found to contain provirus at very low levels *in vivo*. CD4+ and CD8+ T lymphocytes and endothelial cells can become infected in long-term cultures of peripheral blood mononuclear cells. BLV antigen has been demonstrated in bovine mammary epithelial cells.

Pathogenesis

A number of features of pathogenesis are shared by BLV and HTLV. Many individuals become infected and develop antibodies but never display overt clinical symptoms. Infected individuals are not viremic and most infected lymphocytes do not express the virus *in vivo* at any given time. However, expression can be induced by culturing blood mononuclear cells. Virus production is expected to occur intermittently or at very low levels in tissues because antibody synthesis continues throughout many years of infection. Numbers of infected host cells increase with time as well. A small percentage of infected individuals develop clonal tumors with long latency after infection. Tumor cells do not contain readily detectable viral gene products.

When a naive animal acquires BLV-infected lymphocytes, virus is produced and establishes a lifelong infection of the new host. The sites of BLV replication are not well defined, because few infected cells express viral genes. In experiments that tested the potential of tissues from experimentally infected calves to transmit BLV infection to sheep, spleen cells consistently did so. However, a persistent infection could be established in splenectomized calves. Cells from bone marrow (the source of blood monocytes) and lymph nodes were sometimes infectious for sheep. In another study of early infection in calves, BLV SU glycoprotein was present on a few mononuclear leukocytic cells in sections of intestinal mucosa, spleen and lymph nodes. Thus, BLV can replicate at multiple sites.

Overt viremia could occur very early after infection but has not been demonstrated. Antibodies specific for the viral SU glycoprotein and CA protein are evident within 2–4 weeks after experimental infection, but may take longer to develop in natural infections involving small amounts of virus. Virus replication is intense during the early weeks of experimental infections, but infected mononuclear blood cells contain few if any viral transcripts as they circulate. Infected cells express BLV in culture more readily at this time than a few months later. Early increases in B cells and CD8+ T cells have been demonstrated in experimentally infected calves and in sheep infected with large, but not small, numbers of virus-positive cells.

BLV is silently present in most circulating, infected mononuclear cells. This may result from repression of virus transcription as well as the lack of appropriate activation signals. Since BLV is adapted to the life cycle of lymphocytes, virus expression is likely to be regulated by the cellular pathways activated during an immune response. Productive expression of BLV *in vivo* would recruit new host cells into the population at risk for tumorigenesis. When cultured, infected cells rapidly transcribe BLV and gene expression is characterized by early regulatory and late productive stages. Polyclonal activators of lymphocytes such as lipopolysaccharide, anti-IgM, pokeweed mitogen, concanavalin A and phytohemagglutinin (PHA) increase transcription of BLV within a few hours. PHA greatly increases production of BLV proteins, either by an autonomous effect of the lectin on B-cell signaling pathways, or by a paracrine effect of lymphokines produced by T cells, or both. Signaling pathways mediated by protein kinase C and calmodulin participate in the induction of BLV expression by cultured lymphocytes.

Expression of the BLV Tax protein affects transcription of host cell genes as well as viral genes. Transcription of the c-*fos* and somatostatin promoters is activated threefold in the presence of Tax. However, other candidate genes whose expression might alter host cell proliferation remain unidentified. Very low levels of transcripts encoding Tax, Rex R3, G4 and structural proteins have been detected in circulating cells using the polymerase chain reaction (PCR). These transcripts could be distributed among cells in early stages of viral expression and in rare cells activated to full BLV expression. Upon overexpression, either Tax or G4 protein can cooperate with the Ras oncogene protein of Harvey murine sarcoma virus to immortalize and transform primary rat embryo fibroblasts in culture. The cells are tumorigenic in nude mice. By itself, Tax extends the lifespan of primary fibroblasts. For Tax to extend the lifespan of infected B cells would require its expression in these cells *in vivo*. In culture, infected B cells from peripheral blood of BLV-infected sheep exhibit delayed apoptosis.

About 30% of infected cattle develop persistent lymphocytosis in which the absolute lymphocyte count is increased significantly above the average for age and breed. Cattle can develop tumors without having had persistent lymphocytosis and not all cattle with persistent lymphocytosis go on to develop tumors within their productive lifespans. Some investigators report that sheep develop persistent lymphocytosis, but others find that lymphocyte increases signal the onset of tumor formation in this host. B cells are expanded in persistent lymphocytosis; some are polyclonally infected with BLV. These excess B cells are activated, having surface IgM, class II MHC proteins and integrins at high density; many are CD5-positive. Some are in the S and G_2/M phases of the cell cycle. B cells from these animals proliferate in culture in response to interleukin (IL)-2; their expansion may be stimulated *in vivo* by increased cytokine production. Blood mononuclear cells from cattle in persistent lymphocytosis show increased levels of IL-2 and IL-10 transcripts, whereas those from animals with normal lymphocyte counts have interferon-γ transcripts elevated as well. An extended lifespan could contribute to increased numbers of B cells; the ratio of Bcl-2 protein to Bax protein is increased in blood cells from cattle with persistent lymphocytosis and in tumors. Blood lymphocytes from cattle with persistent lymphocytosis have been used for many studies of virus expression, since the infected population is large and the cells readily express BLV in culture.

Tumors develop in 1–10% of infected cattle during their productive lives; most occur 4–8 years after infection. A large percentage of experimentally infected sheep develop tumors with latencies of less

than 1 to more than 6 years, depending on the size of the initial virus inoculum. Tumors, termed lymphomas or lymphosarcomas, are multicentric and infiltrating, with B cell characteristics including rearranged Ig genes and expression of class II MHC antigens, IgG or IgM and often CD5. The cells resemble lymphoblasts, prolymphocytes and mature lymphocytes. In addition, histiocytes and macrophages are often found in tumor tissue. When large numbers of neoplastic B cells are present in blood, this is termed leukemia.

With tumor growth, animals present a variety of symptoms, including decreased milk production, enlargement of lymph nodes, loss of appetite and weight, depression, heart problems, fever, diarrhea, bulging eyes and posterior paralysis, depending upon which organs have been affected by tumors or infiltrated with tumor cells. Lymph nodes, abomasum, heart, uterus, spinal canal and orbital cavity are commonly involved. Tumor masses can be as large as a person's head. Tumors are inevitably fatal, and animals usually live only a short time after diagnosis. BLV is not involved in three types of sporadic bovine leukosis: calf lymphosarcoma, thymic lymphosarcoma and skin leukosis.

Tumors within each animal are rare clonal outgrowths of a single, BLV-infected cell that contains 1–3 copies of the provirus. Many tumors contain full-length provirus, but deleted proviruses retaining the 3′ *tax/rex* region have also been found. Viral RNA is detected only at very low levels within tumor cells, indicating that overt BLV expression is not required to maintain the tumor state. Mutated host cell genes are thought to participate in tumorigenesis. Missense mutations in p53 have been identified in about half of BLV-induced tumors in cows, but not in sheep.

Immune Response

Titers of antibodies against the BLV SU glycoprotein and CA protein increase with the duration of infection. Neutralizing antibodies directed against the SU glycoprotein are evident within several weeks after infection and increase sharply at the time when the frequency of infected cells increases transiently in blood. Complement-dependent, cytotoxic antibodies specific for SU have been demonstrated. Antibodies against the intracellular Rex regulatory protein appear episodically throughout infection in sheep. Killing of BLV-infected cells by MHC-restricted cytotoxic T-cells or by natural killer cells has been difficult to characterize due to the lack of suitable target cells.

Infected animals are not obviously immunosuppressed or highly susceptible to infectious diseases. Subtle alterations in immune function have been reported, including lower levels of secretory IgM, diminished responsiveness to antigens, production of immunoglobulins with impaired reactivity, and persistence of ringworm infections. Levels of IgM increase in persistent lymphocytosis. BLV-infected milking cows are more likely to be culled from a herd, but no statistically significant association has been established with decreased milk production or reproductive capacity.

Prevention and Control

Original efforts to control enzootic bovine leukosis were based on identification and removal from herds of cattle with lymphosarcoma or with persistent lymphocytosis. Eradication programs implemented in Denmark and Germany succeeded in greatly reducing the number of herds with leukosis. Development of assays for viral antibodies enabled identification of infected animals with normal lymphocyte counts; these animals were then eliminated or segregated from the rest of the herd. Precipitating antibodies against the viral envelope proteins are easily detected using an inexpensive immunodiffusion assay, but enzyme-linked immunosorbent assays for envelope- or CA-specific antibodies are more sensitive. Since animals may be seronegative in the earliest stages of infection, the most sensitive diagnostic technique is amplification of BLV proviral DNA present in blood lymphocytes by PCR. A few copies of provirus can be detected in 10^5–10^6 cells. Prudent management can greatly reduce transfer of BLV-infected lymphocytes to uninfected animals. Vaccination to prevent infection has been attempted using inactivated virus, uninfected lymphoid cell lines and viral glycoprotein, either purified or expressed from a vaccinia virus vector. No approach has consistently prevented short-term infection and no effective vaccine exists at present.

Future Perspectives

BLV is a valuable animal model for understanding pathogenesis of HTLV. The isolation of molecular clones of BLV that are infectious and pathogenic has allowed testing of mutated proviruses. The sites at which BLV replicates *in vivo* await identification. Cellular and immunological factors controlling virus expression and production are being investigated. An understanding of early and late stages of abnormal host cell proliferation requires identification of the host cell genes whose expression is altered by the BLV Tax protein. Subsequent genetic changes that lead to tumor formation remain unidentified. BLV lends itself

to investigations of the role of co-infections in development of disease and of host genetic factors affecting infection and development of disease. Approaches to vaccination can be tested. The role of the cell-mediated immune response in protection from infection or from development of tumors remains a challenging area for investigation.

See also: **Bovine immunodeficiency virus (*Retroviridae*); Human T-cell leukemia viruses (*Retroviridae*): HTLV-1, HTLV-2; Immune response: Cell mediated immune response, General features; Immune escape mechanisms; Latency; Persistent viral infection.**

Further Reading

Burny A and Mammerickx M (eds) (1987) *Enzootic Bovine Leukosis and Bovine Leukemia Virus*. Boston: Martinus Nijhoff.

Kettmann R, Burny A, Callebaut I *et al* (1994) Bovine leukemia virus. In: Levy JA (ed.) *The Retroviridae*, vol. 3, p. 39. New York: Plenum Press.

Miller JM and Van Der Maaten MJ (1982) Bovine leukosis – its importance to the dairy industry in the United States. *J. Dairy Sci.* 65: 2194.

Petropoulos C (1997) Retroviral taxonomy, protein structures, sequences and genetic maps. In: Coffin JM, Hughes SH and Varmus HE (eds) *Retroviruses*, p. 757. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Theilen GH and Madewell BR (eds) (1987) Bovine hematopoietic neoplasms, sarcomas and related conditions. In: *Veterinary Cancer Medicine* 2nd edn, p. 408. Philadelphia: Lea & Feibiger.

Bovine Papilloma Virus *see* **Papillomaviruses – Animal**

Bovine Parvovirus *see* **Parvoviruses – Rodents, pigs, cattle and waterfowl**

Bovine Spongiform Encephalopathy *see* **Prions**

BROMOVIRUSES (*BROMOVIRIDAE*)

Paul Ahlquist, Howard Hughes Medical Institute,
University of Wisconsin–Madison, Madison, Wisconsin, USA

Introduction and Classification

The bromoviruses are a genus of icosahedral viruses in the *Bromoviridae*, a family of multipartite, positive-strand RNA viruses that infect plants. Although not a cause of major economic losses, bromoviruses have been extensively studied as experimental models for basic investigations of virus replication and gene expression, virus–host interactions, virion assembly, and general molecular biology.

The type and best-studied member of the bromoviruses is brome mosaic virus (BMV). BMV infects a number of cereal grains and other grasses and is distributed throughout most of the world with the exception of Australia. Other known bromoviruses include cowpea chlorotic mottle virus (CCMV), broad bean mottle virus (BBMV), melandrium yellow fleck virus, spring beauty latent virus and cassia yellow blotch virus. Of these latter viruses, the legume-infecting CCMV and BBMV are the best characterized.

Among the five genera presently classified in the *Bromoviridae*, bromoviruses are most closely related to the cucumoviruses, a genus of plant viruses of

to investigations of the role of co-infections in development of disease and of host genetic factors affecting infection and development of disease. Approaches to vaccination can be tested. The role of the cell-mediated immune response in protection from infection or from development of tumors remains a challenging area for investigation.

See also: **Bovine immunodeficiency virus (*Retroviridae*); Human T-cell leukemia viruses (*Retroviridae*): HTLV-1, HTLV-2; Immune response: Cell mediated immune response, General features; Immune escape mechanisms; Latency; Persistent viral infection.**

Further Reading

Burny A and Mammerickx M (eds) (1987) *Enzootic Bovine Leukosis and Bovine Leukemia Virus*. Boston: Martinus Nijhoff.

Kettmann R, Burny A, Callebaut I *et al* (1994) Bovine leukemia virus. In: Levy JA (ed.) *The Retroviridae*, vol. 3, p. 39. New York: Plenum Press.

Miller JM and Van Der Maaten MJ (1982) Bovine leukosis – its importance to the dairy industry in the United States. *J. Dairy Sci.* 65: 2194.

Petropoulos C (1997) Retroviral taxonomy, protein structures, sequences and genetic maps. In: Coffin JM, Hughes SH and Varmus HE (eds) *Retroviruses*, p. 757. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Theilen GH and Madewell BR (eds) (1987) Bovine hematopoietic neoplasms, sarcomas and related conditions. In: *Veterinary Cancer Medicine* 2nd edn, p. 408. Philadelphia: Lea & Feibiger.

Bovine Papilloma Virus *see* **Papillomaviruses – Animal**

Bovine Parvovirus *see* **Parvoviruses – Rodents, pigs, cattle and waterfowl**

Bovine Spongiform Encephalopathy *see* **Prions**

BROMOVIRUSES (*BROMOVIRIDAE*)

Paul Ahlquist, Howard Hughes Medical Institute,
University of Wisconsin–Madison, Madison, Wisconsin, USA

Introduction and Classification

The bromoviruses are a genus of icosahedral viruses in the *Bromoviridae*, a family of multipartite, positive-strand RNA viruses that infect plants. Although not a cause of major economic losses, bromoviruses have been extensively studied as experimental models for basic investigations of virus replication and gene expression, virus–host interactions, virion assembly, and general molecular biology.

The type and best-studied member of the bromoviruses is brome mosaic virus (BMV). BMV infects a number of cereal grains and other grasses and is distributed throughout most of the world with the exception of Australia. Other known bromoviruses include cowpea chlorotic mottle virus (CCMV), broad bean mottle virus (BBMV), melandrium yellow fleck virus, spring beauty latent virus and cassia yellow blotch virus. Of these latter viruses, the legume-infecting CCMV and BBMV are the best characterized.

Among the five genera presently classified in the *Bromoviridae*, bromoviruses are most closely related to the cucumoviruses, a genus of plant viruses of

substantial agricultural significance. These two genera share many properties, including similar virion structure and genome organization, similarities in the sequence of all three nonstructural proteins, and similar noncoding sequences. At a more distant level, bromovirus-encoded RNA replication factors share amino acid sequence similarity with proteins encoded by many morphologically and genetically diverse positive-strand RNA viruses of plants and animals, including tobacco mosaic virus, the animal alphaviruses, and others. Thus, bromoviruses are members of what has sometimes been called the alphavirus-like replicative superfamily of positive-strand RNA viruses.

Virion Structure

Bromovirus virions are nonenveloped particles approximately 28 nm in diameter. The outer capsid is composed of 180 copies of a single 20 kDa coat protein arranged with $T = 3$ quasi-icosahedral symmetry. Cryoelectron microscope reconstructions show that BMV and CCMV have extremely similar capsid structures. The capsid structure of CCMV has been solved to high resolution by x-ray crystallography (**Fig. 1**), and the detailed features cited below are derived from this structure. The coat protein subunits contain a core β-barrel fold similar to that found in the coat proteins of many other isometric virions. However, in contrast to most other virions, these barrels are oriented with their β-strands nearly perpendicular to the capsid surface, causing significant protuberance of the hexameric and pentameric capsomeres in which the subunits cluster. Adjacent coat protein subunits in these individual capsomeres interact primarily by hydrophobic residues. In addition, the hexameric capsomeres are further stabilized by the cooperative interaction of an N-proximal portion of each coat protein to form a hexameric β-strand ring or tubule at the quasi-sixfold axis.

The individual pentameric and hexameric capsomeres are linked into a comprehensive network by the coat protein C-termini. These C-termini extend radially from the capsomeres, allowing each coat protein subunit to participate in a reciprocal exchange of C-termini with a directly opposed partner subunit in a neighboring capsomere. Each extended C-terminus is anchored by being clamped between the β-barrel core and an N-proximal loop of its partner subunit. This reciprocal C-terminal exchange appears responsible for the existence of unassembled coat proteins as dimers, and thus may be one of the earliest interactions in capsid assembly. Coat protein linkage by C-terminal extensions has not been found to date in other RNA viruses but has been found in a similarly clamped form in the DNA papovaviruses, SV40 and polyoma.

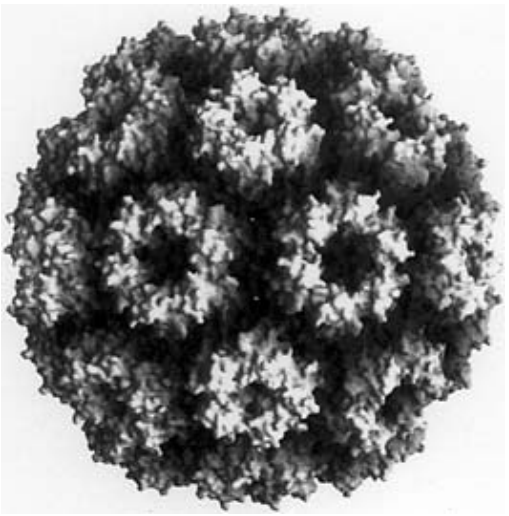


Figure 1 Surface structure of the CCMV capsid as determined by x-ray crystallography. The diameter of the capsid is approximately 28 nm. The hexameric and pentameric capsomeres generated by the arrangement of the 180 coat protein subunits in $T = 3$ quasi-icosahedral symmetry are visible. More detailed features of the capsid architecture are described in the text. (GRASP image produced by J.-Y. Sgro from the crystallography data of J. Speir, J. Johnson and colleagues.)

Inside the capsid, the single-stranded viral RNA is arranged as an interior subshell, leaving the virion center hollow. The N-terminal 26 amino acids of the coat protein, which are highly basic and required for RNA packaging, extend into the interior of the capsid in a crystallographically disordered state and interact with the RNA to neutralize phosphate charges. Additionally, the RNA interacts with 13 of the amino acids that are crystallographically ordered on the interior capsid surface. These include ionic interactions with a number of lysines and arginines and a stacking interaction between the bases of two consecutive RNA nucleotides and a tryptophan side chain.

Bromovirus capsids are stable at pH 5.0. At pH 7.0 in the absence of Ca^{2+} or Mg^{2+}, the capsid diameter swells approximately 10% due to repulsion among negatively charged residues at a calcium-binding site at the quasi-threefold axes. Although this repulsion creates large openings in the capsid at the quasi-threefold axes, the viral RNA is not spontaneously released, rendering the swelling reversible. Since some mutational results indicate that virion competence for swelling is not essential to initiate infection, the biological significance of this reaction is still under study.

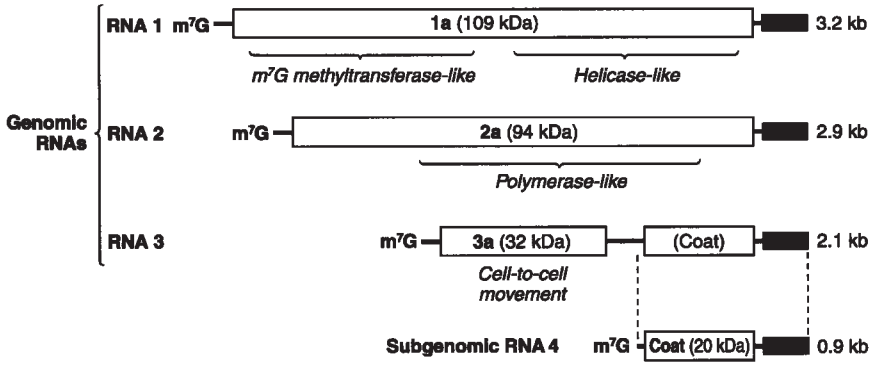


Figure 2 Map of a typical bromovirus genome, showing genomic RNAs 1, 2 and 3 and subgenomic RNA4. The four open reading frames that are known to be expressed are shown along with the sizes of the encoded 1a, 2a, 3a and coat proteins. Within the *1a* and *2a* genes, the bracketed sections encode the polymerase-like, helicase-like, and m^7G methyltransferase-like protein domains that show conservation with other viruses (see text). As indicated, all four RNAs are capped at the 5′ end with m^7Gppp and at the 3′ end bear conserved tRNA-like regions (dark boxes) with aminoacylable -CCA_{OH} termini. The RNA and protein sizes shown are those of BMV; these sizes vary slightly for other bromoviruses.

Genome Structure and Expression

High resolution density gradients separate the virions of each bromovirus into three classes with identical capsids but differing RNA contents. The highest density particles each contain a single copy of a 3.2 kb RNA designated RNA1, the lowest density particles each contain a single copy of 2.9 kb RNA2, and the intermediate density particles each contain one copy of 2.1–2.2 kb RNA3 plus one copy of 0.9 kb RNA4. Because the viral genetic information is divided among these RNA types and virion types as described below, all three virion types must infect a cell to initiate a productive bromovirus infection.

All four encapsidated bromovirus RNAs are 5′-capped, messenger-sense RNAs (**Fig. 2**). RNA1, RNA2 and RNA3 comprise the total genome. Each of these genomic RNAs serves as mRNA for a separate noncapsid protein: RNA1 and RNA2 encode the 1a and 2a proteins, respectively, which are involved in viral RNA replication and transcription. RNA3 serves as the mRNA for the 3a nonstructural protein, which is required for the cell-to-cell spread of infection in plants. RNA4 is a subgenomic coat protein mRNA derived from the 3′ half of RNA3.

Translation of bromovirus coat protein from subgenomic RNA4 ensures that significant coat protein synthesis only initiates in secondary stages of infection, after sufficient nonstructural protein has been produced to support RNA replication complex assembly and negative-strand RNA synthesis, and synthesis of positive-strand genomic and subgenomic mRNA has begun.

Conversely, when increasing amounts of BMV virion RNA are added to wheat germ translation extracts, the high translation efficiency of RNA4 competitively inhibits translation of nonstructural proteins, particularly 3a, from the genomic RNAs. Late in infection when viral RNAs have accumulated to high levels and translation factors may become limiting, this competitive inhibition could provide a simple mechanism to downregulate nonstructural protein expression while preferentially synthesizing the large amounts of coat protein necessary for encapsidation.

As for most positive-strand RNA viruses, the combined bromovirus genomic RNAs are infectious. Similarly, *in vitro* transcripts from complete cDNA clones of all three genomic RNAs are infectious in combination. The specific infectivity of such *in vitro* transcripts is greatly reduced unless their 5′ ends are capped and have no more than one additional nonviral residue. DNA containing bromovirus cDNA appropriately fused to a suitable DNA-dependent RNA polymerase promoter can also be directly infectious.

RNA Replication

Bromoviruses replicate in a completely RNA-dependent fashion, producing a negative-strand RNA intermediate for each genomic RNA. Negative- and positive-strand RNA accumulations are regulated independently, yielding an approximately 100-fold excess of positive-strand, i.e. virion sense, RNA late in infection.

As discussed further below, bromovirus host ranges for systemic infection are relatively limited. However, protoplast and other experiments show that bromoviruses replicate their RNA and assemble virions in directly inoculated cells from a wide variety of plants, including plants that are not hosts for systemic infection. Furthermore, BMV RNAs are replicated and direct subgenomic mRNA synthesis in 1a- and 2a-expressing cells of the well-studied yeast *Saccharomyces cerevisiae*. BMV RNA-dependent RNA synthesis in yeast duplicates the known features of BMV RNA synthesis in plant cells, including dependence on the 1a and 2a proteins and the same *cis*-acting RNA replication signals identified in plant cell studies, equivalent intracellular localization, similar ratios of positive- to negative-strand RNA, etc. Yeast therefore must contain BMV-compatible forms of all host factors essential for BMV RNA replication and subgenomic mRNA synthesis.

Bromovirus RNA replication and subgenomic RNA4 synthesis require the viral 1a and 2a proteins but not the 3a or coat proteins. The 1a and 2a proteins interact *in vitro* and, in plant and yeast cells, co-localize on membranes bearing endoplasmic reticulum markers. *In vivo* labeling shows that these sites of 1a and 2a accumulation are the sites of viral RNA synthesis.

Two large domains in protein 1a and one domain in protein 2a show sequence conservation with proteins encoded by other members of the alphavirus-like superfamily (**Fig. 2**). Mutations in any of these three conserved regions can block or alter bromovirus RNA replication. The C-terminal conserved domain in protein 1a contains conserved motifs of the DEAD box protein family, some of which have been shown to be RNA helicases. Mutations in this region interfere with genomic RNA replication and subgenomic RNA4 synthesis. The N-terminal conserved domain in protein 1a is related to alphavirus protein nsP1, which encodes methyltransferase and potential guanylyl transferase activities implicated in RNA capping. The conserved central domain in protein 2a is related in sequence to RNA-dependent RNA polymerases from the picornaviruses and some other viruses. The C-terminal 2a segment following this region is dispensable for RNA synthesis in protoplasts, whereas the N-terminal 2a segment preceding the polymerase-like domain interacts with the helicase-like domain of 1a.

Although the N- and C-terminal domains of 1a are related to the proteolytically processed alphavirus proteins nsP1 and nsP2, mutations in these two domains of 1a fail to genetically complement each other, suggesting that these domains may function cooperatively at one or more steps of RNA replication. Similarly, the known interaction of 1a and 2a may organize multiple steps and activities of RNA synthesis in a single, coordinated complex. Gene exchanges between bromoviruses indicate that positive-strand RNA synthesis depends on some aspect of protein 1a–2a interaction that is not required for negative-strand RNA synthesis.

For *in vitro* study, template-dependent RNA-dependent RNA polymerase activities can be isolated from bromovirus-infected cells. These infection-specific activities are initially membrane-associated but can be solubilized with appropriate detergents. Such extracts have high template specificity in negative-strand synthesis, copying bromovirus RNAs but not other viral or cellular RNAs. Immunoprecipitation and copurification results with such extracts suggest that host proteins may be complexed with proteins 1a and 2a, and may have roles in the conduct or regulation of RNA synthesis, as noted below.

Cis-Acting Sequences in Bromovirus RNA Synthesis

The regulatory signals for genomic RNA replication and RNA4 transcription have been mapped on several bromovirus RNAs. One interesting regulatory feature of bromovirus RNAs is their tRNA-like 3′ termini (**Fig. 2**). The last 200 bases of the three genomic RNAs are almost identical within each virus and similar between viruses, and form an extensive secondary and tertiary structure whose major features are conserved among bromoviruses and cucumoviruses. This 3′ structure contains the promoter sequences for negative-strand RNA synthesis and participates in several tRNA-specific reactions. Bromovirus RNAs lacking the terminal A of their normal, tRNA-like -CCA(3′-OH) end, e.g. can be adenylated by cellular tRNA nucleotidyltransferase. This adenylation reaction appears to be a normal step in the maturation of bromovirus RNA, since negative-strand synthesis initiates on the penultimate C of virion RNAs and therefore incorporation of the final 3′ A residue on progeny positive strands must involve nontemplated nucleotide addition. tRNA nucleotidyltransferase thus appears to perform a telomerase-like role in maintaining the ends of bromovirus genomic RNAs. The mature -CCA(3′-OH) end can be aminoacylated with tyrosine by the cellular tyrosyl tRNA synthetase. Though indicating highly specific tRNA mimicry, the functions of this aminoacylation, possibly including roles in RNA synthesis, stabilization, or both, are not yet well established.

5′-proximal sequences are also required in *cis* for bromovirus RNA amplification, presumably to mediate positive-strand initiation, to enhance RNA

stability, or both. In addition, for some RNAs, internal sequences are required in *cis* for efficient viral RNA accumulation. The 5′ noncoding regions of bromovirus genomic RNAs and an intercistronic segment required for efficient BMV RNA3 accumulation all contain exact copies or close approximations of the sequence motif GGUUCAAyyCC, where y = U or C. This is one of two consensus motifs in cellular RNA polymerase III promoters and also corresponds to the invariant residues of tRNA TΨC loops. Mutations in these consensus elements reduce bromovirus RNA accumulation in *cis*, showing that sequence elements recognized by host cell factors play significant roles in bromovirus RNA synthesis.

Experiments *in vitro* show that the subgenomic coat protein mRNA, RNA4, is produced by internal initiation of transcription on negative strand RNA3. The signals directing this transcription reside within a 100 base segment upstream from the BMV coat gene. Sequences within the first 20 bases upstream of the coat protein constitute a core promoter sufficient to direct site-specific transcription initiation at a low level. Additional upstream sequences, including a 16–22 base oligo(A) tract and partial repeats of core promoter sequences, stimulate the level of transcription. A similar core promoter sequence and 35–45 base oligo(A) tract are present upstream of the CCMV coat gene. Extensive studies on the effects of nucleotide changes in the core promoters for BMV subgenomic mRNA synthesis and negative-strand RNA synthesis have been conducted to identify key residues in each promoter.

Host Gene Dependence of BMV RNA Replication

Probable dependence of bromovirus RNA replication on host factors was suggested by the role of tRNA nucleotidyltransferase in viral RNA maturation, the presence of tRNA-like sequence features and functions in multiple *cis*-acting RNA replication signals, the association of viral RNA synthesis with cellular membranes, cofractionation of some cellular proteins with bromovirus RNA-dependent RNA polymerase activity, host-specific differences in viral RNA accumulation, and other results. More direct evidence for the dependence of bromovirus replication on host factors has been derived from the ability of BMV to replicate in yeast. By applying yeast genetic approaches to selectable and screenable marker genes expressed from BMV RNA replicons, mutant yeast strains have been selected that are unable to support normal levels of BMV RNA replication and subgenomic mRNA synthesis. Such BMV-inhibiting mutations occur in multiple yeast complementation groups with distinguishable effects on the virus. Thus, multiple host factors appear to contribute to a variety of steps in bromovirus replication.

Genetics

Both homologous and nonhomologous recombination between genomic RNAs can be observed in bromovirus infections when appropriate selection pressure is applied. Deletions introduced into the 3′ tRNA-like region of one genomic RNA, e.g. can be repaired by recombination with the wild-type tRNA-like region of another genomic RNA. In addition to helping maintain the conserved 3′ ends, recombination is presumed to have played an important evolutionary role in initially establishing common ends on the separate RNAs of the tripartite bromovirus genomes. Recombination has also contributed to the divergence of individual bromoviruses, as shown by several clearly bounded insertion/deletion differences between the BMV, CCMV and BBMV genomes.

Recombination can regenerate wild-type CCMV RNA3 from two co-inoculated mutants with inactivating mutations in the 3a and coat genes, respectively. The ability to assemble functional genomic RNAs from independently disabled mutants should help to offset the negative effects of the high mutation rates commonly found in RNA virus replication while allowing bromoviruses to realize the survival benefits of high variability.

Recombination events can be targeted between two bromovirus RNAs by engineering duplications that serve as hotspots for homologous recombination, or by engineering complementary regions that create the potential for forming heteroduplexes. These potential heteroduplexes become the sites of recombinational crossovers between the two RNAs. Mutations in the 1a or 2a RNA replication genes can influence the frequency and distribution of recombinational crossovers between bromovirus RNAs. Together with the engineered heteroduplex results, this suggests that template switching during RNA replication may be one mechanism for bromovirus RNA recombination.

Transmission

In the laboratory, bromoviruses are efficiently transmitted between plants by mechanical inoculation. In the field, bromoviruses can be mechanically transmitted by human activity, including walking, and presumably also by machinery. Seed transmission tests with bromoviruses have generally been negative, though seed transmission of BMV in one South African wheat cultivar has been reported.

Beetles reproducibly transmit bromoviruses, though inefficiently and for short periods of time. Nematodes have also been shown to transmit BMV. Aphid transmission tests with bromoviruses have been consistently negative except for one report using concentrated BMV as a feeding source. Despite experimental demonstrations, the natural significance of invertebrate vectors in bromovirus field epidemiology is uncertain. Multiyear field trials in the presence of potential beetle vectors found no spread of CCMV from infected source plants to surrounding cowpea or soybean plants. Lack of an efficient invertebrate vector may be one reason why bromovirus infections characteristically produce large yields of virus (1–5 mg g^{-1} infected tissue).

Infection Spread and Host Range for Systemic Infection

Although bromoviruses replicate and encapsidate their RNAs in directly inoculated cells from a wide variety of plants, they have fairly restricted host ranges for systemic infection of whole plants. The effective host range for bromovirus infection thus appears to be determined frequently at the level of initiating or sustaining infection spread from the sites of primary infection.

When CCMV is inoculated onto a leaf of a cowpea plant, a permissive host for CCMV systemic infection, infection initially spreads from primary inoculated cells only into directly contacting cells. Such direct cell-to-cell spread continues to expand the radius of the infection site at a rate of approximately one additional cell diameter per 5 h. When the infection reaches vascular elements, it advances along them more rapidly and secondary infections emerging from vascular elements become the dominant mode of infection spread.

Both RNA3 genes, *3a* and *coat*, are dispensable for RNA replication but required for systemic spread. Disruption of the *3a* gene blocks cell-to-cell infection spread, limiting infection to individual, directly inoculated cells. The 3a protein has a number of properties common to cell-to-cell movement proteins of other viruses, including the ability to bind cooperatively to single stranded RNA, to localize to the plasmodesmatal connections that are the presumed route of infection spread between cells, and to induce the formation of virion-containing tubules from the surface of BMV-infected protoplasts. For CCMV, disruption of the coat gene allows short-range cell-to-cell spread of infection at a reduced rate, but inhibits vascular spread, slowing spread within inoculated leaves and blocking virus spread to noninoculated leaves. For BMV, disruption of the coat gene similarly stops virus spread to noninoculated leaves. In at least some virus–host combinations, disruption of the BMV coat protein also appears to block cell-to-cell spread.

Exchanging genomic RNAs and individual genes shows that the host-specific adaptation of BMV to monocotyledonous plants and of CCMV to dicotyledonous plants depend on differences in multiple viral genes. These include the *3a* gene of RNA3 and incompletely determined features of RNA1 and RNA2. Some host-specificity determinants in bromovirus genes might alter the ability of virus-encoded functions to actively direct infection spread in particular plants, perhaps by adapting viral components to interact successfully with essential host factors that display some variation from host to host. Certain changes in BMV RNA1 that enhance systemic spread in cowpea plants, e.g. are correlated with a cowpea-specific increase in viral RNA accumulation. Alternatively or in addition, viral host specificity determinants might modulate induction of host defense responses, which appear to be a limiting factor for infection of some plants. Replacing the cowpea-adapted CCMV *3a* cell-to-cell movement gene with the monocot-adapted BMV *3a* gene, for example stops infection spread at an early stage, but only after multiple rounds of cell-to-cell spread. Thus, the misadapted *3a* gene is able to mediate cell-to-cell spread even in an inappropriate host, and further observations suggest that the eventual block to spread is associated with virus-induced host responses. Significantly for evolutionary considerations, only one or a small number of amino acid changes are needed to allow the monocot-adapted BMV *3a* gene to support systemic infection spread in dicot hosts.

Pathology

The external symptoms of bromovirus infections can vary markedly depending on the strain of virus, the host genotype, and the ambient physical conditions. BMV infections in cereals typically produce a mosaic or streaked pattern of chlorosis in infected leaves, often accompanied by stunting. The effects of CCMV and BBMV infections can range from a lack of visible symptoms to mild variegated chlorosis to a vivid yellow mottling. Particularly in directly inoculated primary leaves, localized reddish spots may also occur. Overall, there is not a simple correlation between symptom severity and bromovirus yield. Some changes in bromovirus symptoms, including dramatic exacerbation of chlorosis, have been associated with changes in the coat protein or in the subgenomic promoter sequences upstream of the coat protein gene.

Studies of bromovirus cytopathology have revealed a number of intracellular changes. Cytoplasmic vesicles, possibly derived by budding from the endoplasmic reticulum, occur and form part of cytoplasmic inclusion bodies that also include portions of the endoplasmic reticulum, virus particles, and electron-dense material. As infection proceeds, massive accumulation of virus particles occurs in the cytoplasm and sometimes in the central vacuole. RNA measurements indicate that up to several million progeny genomes accumulate per cell in bromovirus infection. Structurally altered chloroplasts have been seen in leaf cells from chlorotic tissue infected with CCMV. In later stages of bromovirus infection, reductions in the number and size of mitochondrial cristae have been reported.

See also: **Alfamovirus and Ilarviruses (*Bromoviridae*); Cucumoviruses (*Bromoviridae*): General features, Molecular biology; Replication of viruses.**

Further Reading

Ahlquist P (1992) Bromovirus RNA replication and transcription. *Curr. Opin. Genet. Dev.* 2: 71.

Bujarski JJ and Nagy P (1996) Different mechanisms of homologous and nonhomologous recombination in brome mosaic virus: role of RNA sequences and replicase proteins. *Semin. Virol.* 7: 363.

De Jong W and Ahlquist P (1991) Bromovirus host specificity and systemic infection. *Semin. Virol.* 2: 97.

Francki RIB (ed.) (1985) *The Plant Viruses*. Vol. I. *Polyhedral Virions with Tripartite Genomes*. New York: Plenum.

Speir JA, Munshi S, Wang G, Baker TS and Johnson JE (1995) Structures of the native and swollen forms of cowpea chlorotic mottle virus determined by X-ray crystallography and cryoelectron microscopy. *Structure* 3: 63.

Sullivan M and Ahlquist P (1997) *Cis*-acting signals in bromovirus RNA replication and gene expression: networking with viral proteins and host factors. *Semin. Virol.* 8: 221.

BUNYAVIRIDAE

Contents

General features

Neal Nathanson and **Francisco González-Scarano**, Departments of Microbiology and Neurology, University of Pennsylvania Medical Center, Philadelphia, Pennsylvania, USA

Introduction

The family *Bunyaviridae* (named after Bunyamwera, the type virus) is the largest single family of animal viruses, consisting of more than 300 individual virus species grouped into five genera: *Bunyavirus, Phlebovirus, Nairovirus, Hantavirus* and *Tospovirus*. The defining features of the family are both molecular and biological. They have a virion structure that consists of a lipid envelope about 100 nm in diameter surrounding an inner nucleocapsid, and possess a trisegmented genome of negative polarity with a roughly consistent coding pattern within each genome segment. However, the phleboviruses and tospoviruses use an ambisense coding strategy for one or two gene segments. Biologically, they are arboviruses, transmitted by arthropods and maintained in a vector–host cycle, although there are important exceptions: the hantaviruses appear to be transmitted from vertebrate-to-vertebrate without an intermediate vector, and the tospoviruses have a plant rather than an animal host.

The profusion of distinct viral species is probably explained by the arthropod-borne maintenance cycle, which limits the geographic distribution of individual viruses to the confined range of different vector species. Many different insect species, including mosquitoes, ticks, midges, flies and thrips can transmit bunyaviruses, but each individual virus is usually transmitted by a limited number of insect species. Since each vector species usually prefers to feed on a few preferred hosts, each virus is maintained in nature in a rather narrow cycle. Viruses cause nonlethal lifelong persistent infections in the insect vector but generally cause acute immunizing infections in the vertebrate host. Most bunyaviruses never infect humans or domestic animals, but a few infect and cause significant disease in humans and/or

Studies of bromovirus cytopathology have revealed a number of intracellular changes. Cytoplasmic vesicles, possibly derived by budding from the endoplasmic reticulum, occur and form part of cytoplasmic inclusion bodies that also include portions of the endoplasmic reticulum, virus particles, and electron-dense material. As infection proceeds, massive accumulation of virus particles occurs in the cytoplasm and sometimes in the central vacuole. RNA measurements indicate that up to several million progeny genomes accumulate per cell in bromovirus infection. Structurally altered chloroplasts have been seen in leaf cells from chlorotic tissue infected with CCMV. In later stages of bromovirus infection, reductions in the number and size of mitochondrial cristae have been reported.

See also: **Alfamovirus and Ilarviruses (*Bromoviridae*); Cucumoviruses (*Bromoviridae*): General features, Molecular biology; Replication of viruses.**

Further Reading

Ahlquist P (1992) Bromovirus RNA replication and transcription. *Curr. Opin. Genet. Dev.* 2: 71.

Bujarski JJ and Nagy P (1996) Different mechanisms of homologous and nonhomologous recombination in brome mosaic virus: role of RNA sequences and replicase proteins. *Semin. Virol.* 7: 363.

De Jong W and Ahlquist P (1991) Bromovirus host specificity and systemic infection. *Semin. Virol.* 2: 97.

Francki RIB (ed.) (1985) *The Plant Viruses*. Vol. I. *Polyhedral Virions with Tripartite Genomes*. New York: Plenum.

Speir JA, Munshi S, Wang G, Baker TS and Johnson JE (1995) Structures of the native and swollen forms of cowpea chlorotic mottle virus determined by X-ray crystallography and cryoelectron microscopy. *Structure* 3: 63.

Sullivan M and Ahlquist P (1997) *Cis*-acting signals in bromovirus RNA replication and gene expression: networking with viral proteins and host factors. *Semin. Virol.* 8: 221.

BUNYAVIRIDAE

Contents

General features

Neal Nathanson and **Francisco González-Scarano**, Departments of Microbiology and Neurology, University of Pennsylvania Medical Center, Philadelphia, Pennsylvania, USA

Introduction

The family *Bunyaviridae* (named after Bunyamwera, the type virus) is the largest single family of animal viruses, consisting of more than 300 individual virus species grouped into five genera: *Bunyavirus, Phlebovirus, Nairovirus, Hantavirus* and *Tospovirus*. The defining features of the family are both molecular and biological. They have a virion structure that consists of a lipid envelope about 100 nm in diameter surrounding an inner nucleocapsid, and possess a trisegmented genome of negative polarity with a roughly consistent coding pattern within each genome segment. However, the phleboviruses and tospoviruses use an ambisense coding strategy for one or two gene segments. Biologically, they are arboviruses, transmitted by arthropods and maintained in a vector–host cycle, although there are important exceptions: the hantaviruses appear to be transmitted from vertebrate-to-vertebrate without an intermediate vector, and the tospoviruses have a plant rather than an animal host.

The profusion of distinct viral species is probably explained by the arthropod-borne maintenance cycle, which limits the geographic distribution of individual viruses to the confined range of different vector species. Many different insect species, including mosquitoes, ticks, midges, flies and thrips can transmit bunyaviruses, but each individual virus is usually transmitted by a limited number of insect species. Since each vector species usually prefers to feed on a few preferred hosts, each virus is maintained in nature in a rather narrow cycle. Viruses cause nonlethal lifelong persistent infections in the insect vector but generally cause acute immunizing infections in the vertebrate host. Most bunyaviruses never infect humans or domestic animals, but a few infect and cause significant disease in humans and/or

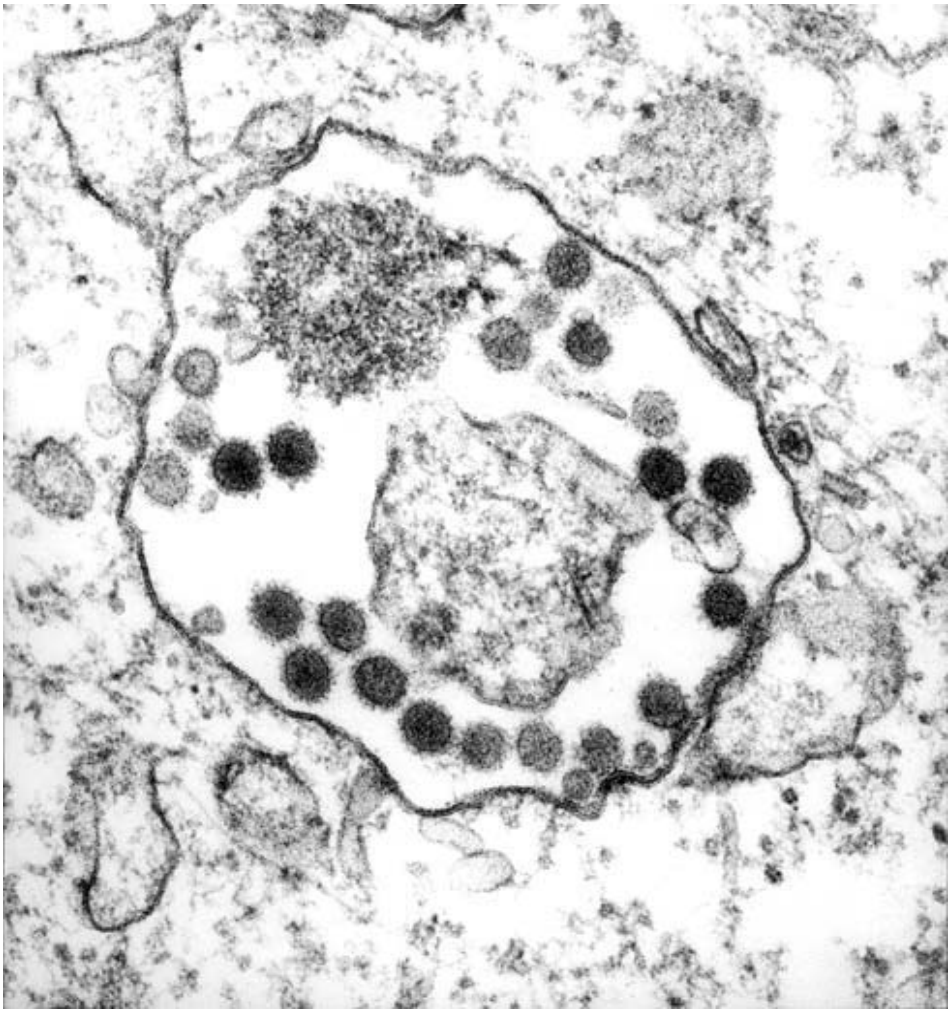


Figure 1 Cache Valley Virus, a member of the family *Bunyaviridae*. Electron Micrograph, 89 000 ×. Image kindly provided by C. Goldsmith and P. Rollin, Centers for Disease Control, Atlanta, GA, USA.

domestic animals. Humans are accidentally infected and almost always constitute deadend hosts without further transmission.

Taxonomy and Classification

The very large number of bunyaviruses in the family *Bunyaviridae* offer a taxonomic challenge. Molecular features are used to define genera, particularly the conserved nucleotide sequences at the termini of each RNA segment and the genome organization within each RNA segment. Classical serological methods are used to define serogroups within each genus. In general the complement fixation test defines antigenic determinants on the nucleocapsid protein, which tend to be conserved and will link related viruses, whereas the neutralization and hemagglutination inhibition define epitopes on the glycoproteins, and can be used to distinguish between different viruses. Currently, the individual bunyaviruses are grouped in over 30 serogroups within five genera, but there are more than 80 viruses that are not yet assigned to specific genera and/or serogroups (**Table 1**). Many bunyaviruses are poorly characterized as to their natural vectors and vertebrate hosts.

Structure and Organization of the Virion

Virions average about 100 nm in diameter (range 80–120 nm) and are spherical or pleomorphic depending on the method used for fixation and visualization (**Fig. 1**). There is an outer lipid envelope (about 4 nm thick) containing glycoprotein spikes (about 10 nm long) that surrounds a core containing the viral genome and its associated proteins (nucleoproteins and polymerase). During replication, the virions mature by budding through intracytoplasmic vesicles associated with the Golgi apparatus. Virions are

Table 1 *Bunyaviridae* genera, serogroups and selected viruses

Genus	*Serogroup or genetic complex*	*No. of viruses*	*Selected human (H) animal (A) or plant (P) pathogens*
Bunyavirus	Anopheles A	12	
	Anopheles B	2	
	Bakau	5	
	Bunyamwera	33	Cache Valley (A, H)
	Bwamba	2	
	Group C	14	
	California	13	La Crosse (H), Tahyna (H)
	Capim	10	
	Gamboa	8	
	Guama	12	
	Koongol	2	
	Minatitlan	2	
	Nyando	2	
	Olifantsvlei	5	
	Patois	7	
	Simbu	24	Akabane (A), Oropouche (H)
	Tete	6	
	Turlock	4	
	Ungrouped	4	
Phlebovirus	Phlebotomus fever	23	Sandfly fever (A)
	Group		Rift Valley fever (A, H), Toscana (H)
	Unkuniemi group	12	
	Ungrouped	16	
Nairovirus	Crimean–Congo hemorrhagic fever	3	Crimean–Congo hemorrhagic fever (H)
	Dera Ghazi Khan	6	
	Hughes	11	
	Nairobi sheep disease	2	Nairobi sheep disease (A)
	Qalyub	3	
	Sakhalin	7	
	Thiafora	2	
Hantavirus	Hantaan	>20	Hantaan (H), Dobrava (H), Seoul (H), Puumala (H), Sin Nombre (H), Andes (H)
Tospovirus	I	7	Tomato spotted wilt (P)
	II		Ground nut ringspot (P)
	III		*Impatiens* necrosis (P)
	IV		Watermelon silver mottle (P)
	V		Iris yellow spot (P)
Ungrouped	Bhanja	3	
	Kaisodi	3	
	Mapputta	4	
	Resistencia	3	
	Tanga	2	
	Upolu	2	
	Yogue	2	
	Ungrouped	23	
Total		>321	

After Calisher CH (1996) History, classification, and taxonomy of viruses in the family *Bunyaviridae*, p. 1. In: Elliott RM (ed.) *The Bunyaviridae*, New York: Plenum Press.

released by transport of vesicles to the cell surface followed by exocytosis or by lysis of the cell.

Bunyaviruses have a buoyant density of about 1.16 g per cm^3 in sucrose gradients and a sedimentation constant of about 450 S. The virion consists of nucleic acid (1–2% by weight), protein (>50% by weight), lipid (>30% by weight) and carbohydrate (2–7% by weight). When the virion is disrupted with nonionic detergents, the nucleocapsids can be isolated and resolved into three sizes by rate-zonal centrifugation. Bunyaviruses are readily inactivated by lipid solvents, detergents or betapropriolactone, and by exposure to 56°C or to low pH.

Bunyaviruses contain a single-stranded genome of negative polarity (see exceptions below) that is divided into three segments, large (L), middle-sized (M) and small (S). The L RNA segment codes for a single large (L) protein that is an RNA polymerase, the M RNA segment codes for two glycoproteins (G1 and G2) and a nonstructural protein (NSm), and the S RNA segment codes for the nucleocapsid (N) and a nonstructural (NSs) protein.

RNA segments and proteins differ in size in different bunyavirus genera. The L RNA segment is in the range 6.4–11.3 kb, the M RNA segment about 3.2–4.9 kb, and the S RNA segment 1–2.9 kb. The L protein is in the range 180–330 kDa, the G1 protein 55–125 kDa, the G2 protein 30–60 kDa, and the N protein 20–50 kDa for different genera. The L RNA segment encodes a single large open reading frame (ORF) in the virus-complementary sense. The M RNA segment encodes a single ORF in the virus-complementary sense and the polyprotein is processed into G1, G2 and NSm proteins. For the *Tospovirus* genus, the M RNA is ambisense since it has an additional virus-sense ORF, that encodes a 'movement' protein thought to be important for the cell-to-cell transmission of the viral nucleocapsids through the plasmodesmata of plant tissues. The S RNA segment encodes the N protein in the virus-complementary sense in all genera. The NSs protein is encoded in the same ORF used for the N protein in *Nairovirus* and *Hantavirus* genera, and in a different virus-complementary sense ORF for the *Bunyavirus* genus. In the *Phlebovirus* and *Tospovirus* genera, the NSs protein is encoded in a virus-sense ORF so that the S RNA segment utilizes an ambisense coding strategy for these genera.

In each virion there are about 600 copies of each of the glycoproteins, 2000 copies of the N protein, and 25 copies of the L protein. Each virion contains three nucleocapsids, each arranged in a noncovalently closed circle with a panhandle due to association of complementary sequences at the 3′ and 5′ ends of each RNA segment. The bases at both 3′ and 5′ ends of the genome are conserved for 9–11 nucleotides, and the two ends form a complementary palindrome. The terminal sequences are identical for all three RNA segments for all members of each genus, but differ between genera.

For viruses in the bunyavirus genus, the larger glycoprotein (G1) has been shown to carry several important biological properties, such as attachment to the putative receptor on animal cells, hemagglutination, neutralization and certain aspects of virulence. Fusion is probably dependent on both the G1 and G2 glycoproteins, since the G1 protein undergoes a conformational change at the pH of fusion while the G2 may carry the fusion domain. It has been postulated that the G2 protein is the ligand for a putative receptor on insect cells. The G1 and G2 proteins are sometimes referred to as GN and GC for their order on the ORF (GN = amino terminal; GC = carboxy terminal).

Cell Culture

Bunyaviruses replicate readily in many types of animal cell cultures although some, such as the hantaviruses, are very fastidious. Most bunyaviruses will replicate well in either BHK-21 or VERO E-6 cells, which are frequently used for virus isolation and the preparation of virus stocks. Bunyaviruses are cytolytic and usually plaque well in permissive animal cells, although some, such as the hantaviruses, are relative noncytolytic. Bunyaviruses will also replicate in insect cells such as mosquito cells, in which they typically cause persistent infections with relatively little or no cell death.

Genetics

Temperature sensitive (ts) mutants of bunyaviruses are easily obtained by standard methods. Within each bunyavirus serogroup, ts mutants readily complement each other if the mutations are in different gene segments. A complementation index can be obtained by initiating a dual infection at the restrictive temperature and then comparing the yield with that obtained when each of the mutants is grown separately at the restrictive temperature.

Reassortment of gene segments can occur between members of the same serogroup but apparently not between members of different serogroups even within the same genus. Reassortants can be constructed by co-infecting a permissive cell culture with two different viruses and then plaquing the progeny to obtain discrete genetic clones. To facilitate the selection of reassortants, ts mutants may be used as parental viruses and the progeny can be selected at the

restrictive temperature, assuming that the parental viruses have ts mutations in different RNA segments. The frequency of reassortment can be estimated by co-infecting cultures at the permissive temperature and assaying the progeny at both permissive and restrictive temperature. When two ts mutants in the California serogroup are crossed in this manner, the frequency of reassortants is 5–50%. Reassortment can also be obtained with clones that are not temperature sensitive, but requires use of genotyping to identify reassortants. Reassortment can occur in dually infected mosquitoes and analysis of field isolates suggests that some bunyaviruses may reassort in nature. Defective interfering virus can be generated by high multiplicity passage of bunyaviruses, and stocks containing high titers of defective virus can interfere with the replication of standard virus.

Infection of Vertebrate Hosts

Vertebrate hosts are usually infected by an insect bite, so that the virus is injected intradermally or subcutaneously. The early events after intracutaneous infection have not been studied in detail, but the virus probably moves via lymphatic channels into the blood whence it is distributed to peripheral tissues in which it can replicate, such as striated muscle in the case of California serogroup viruses. Peripheral replication leads to an active plasma viremia, which lasts for a relatively short interval, usually less than one week. Only during this limited period can the vertebrate host transmit to arthropod vectors. Viremia also delivers the virus to target organs, such as the brain, where replication leads to disease. Infection is acute and, if the host survives, virus is cleared between one and two weeks after exposure. Bunyaviruses elicit brisk immune responses, which lead to termination of viremia and sterilization of tissues. Likewise, a single infection probably produces lifelong immunity.

Host variables that influence susceptibility to infection include age and genetic determinants. Newborn animals are much more susceptible to peripheral infection than are older animals; they develop much higher titers of viremia and are readily killed, whereas older animals often undergo mild or inapparent immunizing infection. Individual inbred strains of mice or rats may exhibit dramatic differences in their susceptibility to infection and in the target organs affected, for instance, the brain or liver.

Laboratory strains of the California serogroup viruses can vary markedly in their ability to infect or cause disease, after either peripheral or intracerebral injection. Experiments with reassortant viruses have shown that attenuation of virulence can be associated with mutations in either the L RNA segment or the M RNA segment. Variant virus clones selected with monoclonal neutralizing antibodies against the G1 protein can be attenuated, indicating that single epitopes on the major glycoprotein can influence pathogenicity. Some variants are attenuated after peripheral injection only whereas others are attenuated after both peripheral and intracerebral injection. Reduced peripheral virulence is associated with reduced ability to replicate in striated muscle, the main tissue in which extraneural virus amplification occurs, whereas reduced intracerebral virulence is associated with reduced ability to replicate in neurons.

Infection of Invertebrate Hosts

Bunyavirus infection of arthropods has been studied most extensively in mosquitoes. Following the ingestion of virus, the cells lining the midgut are infected, with subsequent spread of virus to the hemocele from which it disseminates to many sites including neural and reproductive tissues, and the salivary gland. Large numbers of virions are discharged into the lumen of the salivary gland and these can be injected when the mosquito takes its next blood meal. The extrinsic incubation period (time between initial infection and development of infectivity) is 7–14 days for La Crosse virus. Infection produces little if any deleterious effects on the mosquito host and is lifelong.

Infection of ovaries, including oocytes, occurs as part of the systemic infection of mosquitoes with La Crosse virus. Transovarial transmission can then occur, with vertical transmission to both male and female mosquitoes. This permits further spread within the mosquito population, by venereal transmission and by further transovarial transmission.

Transmission, Ecology and Epidemiology

The patterns of transmission of different bunyaviruses are remarkably diverse between genera although they are quite consistent within each genus. Three of the five genera (*Bunyavirus, Phlebovirus, Nairovirus*) are arthropod-borne and are maintained in vector–vertebrate cycles, one (*Tospovirus*) is arthropod-borne but is maintained in a vector–plant cycle, and one (*Hantavirus*) is maintained in an exclusively animal-to-animal cycle. Each genus is transmitted by a limited range of insects, mainly mosquitoes (*Bunyavirus, Phlebovirus*), ticks (*Nairovirus*) or thrips (*Tospovirus*). Infection of insects is nonlethal and persistent, whereas infection of vertebrates is acute, with or without disease, and produces

a brief period of viremia during which transmission may occur. Infection of plants is also acute and often causes severe lesions and death. Many bunyaviruses have an alternative mode of virus perpetuation in which the virus is maintained exclusively in the insect host by transovarial and trans-stadial transmission (see above).

Hantaviruses differ from the other four genera in that there is no evidence for arthropod vectors. Each hantavirus is perpetuated in one or a few rodent species in which it causes persistent asymptomatic infection, which markedly enhances the probability of further transmission.

Human infections with most members of the family *Bunyaviridae* are incidental and occur only when humans intrude on the natural routes of transmission. In most instances, infected insect vectors mediate transmission to humans. However, hantavirus infections are caused by close contact with the natural rodent hosts. Nairoviruses and phleboviruses deserve special mention since Rift Valley fever and Crimean–Congo hemorrhagic fever viruses can be transmitted to humans by an alternate nonvectored route, i.e. exposure to infected animal tissue. Crimean–Congo hemorrhagic fever virus can also be transmitted by exposure to infected humans other body fluids as evidenced by numerous nosocomial outbreaks. Transmission not involving an insect is presumably mediated by aerosol or percutanoueous routes.

Epizootics and epidemics occur when the ecological variables lead to an amplification of the number of vectors and the proportion of vectors that are infected. For example, outbreaks of Rift Valley fever and Oropouche disease are often associated with conditions, such as heavy rainfall, which are conducive to intense vector breeding.

Bunyavirus Genus

The *Bunyavirus* genus is named after Bunyamwera virus and includes more than 150 individual viruses classified in 18 serogroups. Mosquitoes transmit almost all viruses in the genus (although *Culicoides* midges transmit a few) but they have a wide variety of vertebrate hosts. Some of the California serogroup viruses cause encephalitis in humans and a number of other viruses in the genus cause febrile illnesses in humans. A few of the Simbu complex viruses cause severe disease in livestock.

The California serogroup includes 14 viruses, each of which is transmitted by a few mosquito species, has a narrow range of vertebrate hosts, and a limited geographic distribution. Of these viruses, the most important pathogen is La Crosse virus, which is transmitted by *Aedes triseriatus* and infects woodland rodents, such as squirrels and chipmunks. La Crosse virus is also maintained by transovarial transmission in the absence of vertebrate infection. The virus occurs throughout the eastern United States, but most human infections and disease are limited to the midwestern United States. It is estimated that annually there are at least 100 cases of encephalitis, and well over 100 000 infections in humans. Most cases occur during the summer months in children and young adults who are exposed in wooded areas. Humans are dead-end hosts, and there is no human-to-human transmission. Encephalitis is relatively benign, with very few fatalities, and over 90% of patients recover without residual disease; the most common sequel is convulsions. The recent importation from Southeast Asia into the United States of *Aedes albopictus* could enhance the transmission of La Crosse virus to humans, since this mosquito is a major transmitter of other viruses of humans such as dengue virus and a competent vector for La Crosse virus.

La Crosse virus infects laboratory mice and produces encephalitis, with a spectrum of severity that depends on age, and route of injection. Strains of California serogroup viruses may vary in their virulence, either by subcutaneous or intracerebral routes of infection. The pathogenesis of infection involves stepwise replication of virus in striated muscle, viremia and invasion of the central nervous system, followed by cytolytic replication in neurons and encephalitis. Construction of reassortant viruses has been used to show that attenuation of virulence may be associated with mutations in either the large or the middle RNA segment, with reduced replication in striated muscle or in neurons, respectively.

The Simbu serogroup includes two pathogenic viruses, Oropouche and Akabane. Oropouche virus is transmitted by *Culicoides* midges, mainly *C. paraensis*, and causes large epidemics of febrile illness in humans in the Amazon delta of northern Brazil. It is postulated that the virus is maintained in a sylvatic cycle, but the vertebrate host(s) have not been identified. During epidemics, which are limited to the rainy season, the virus may be maintained in a midge–human cycle.

Akabane virus is transmitted by *C. brevitarsus* in Australia and probably by other *Culicoides* species in Japan, and is presumably maintained in a wildlife host, which has not been identified. Infection of pregnant cattle and sheep causes a variety of fetal anomalies (hydrancephaly, arthrogryposis, and poliomyelitis) depending on the gestational age at infection. Experimental infection of sheep or cattle reproduces the diseases seen under field conditions.

Cache Valley virus (**Fig. 1**) has recently been associated with a few cases of human encephalitis.

Hantavirus Genus

Hantaviruses are named after Hantaan virus, the cause of Korean hemorrhagic fever. With one exception, hantaviruses are rodent-borne viruses. Each hantavirus appears to be associated with a single (or a few) rodent species, with which it has coevolved, and the distribution of the virus is coextensive with the range of its rodent host(s). In its natural host, each virus causes lifelong infection with persistence in lungs, saliva, kidneys and urine, and is probably transmitted between rodents by contaminated urine or saliva. Transmission to humans is limited to persons who are in intimate contact with the rodent host, and is presumed to occur through aerosolization of contaminated excreta. In general, human-to-human transmission does not occur, although a recent outbreak of hantavirus pulmonary syndrome in South America appeared to involve person-to-person spread. Hantaviruses are notoriously fastidious but most of them replicate well in the Vero E-6 cell line.

The hantavirus genus is currently divided into three broad groups based on antigenic and genetic relationships and rodent reservoir host (the Murinae, Arvicolinae and Sigmodontinae subfamilies). Murinae-vectored viruses include the Hantaan (host *Apodemus agrarius*), Dobrava (*A. flavicollis*), and Seoul (*Rattus norvegicus*) viruses that cause hemorrhagic fever with renal syndrome (HFRS) of severe (Hantaan and Dobrava) to moderate (Seoul) severity in various areas of Asia and Europe. Hantaan virus, isolated near the Hantaan River in Korea, was discovered to be the cause of Korean hemorrhagic fever, after more than 25 years of searching for the etiologic agent. Hantaan virus occurs from Korea through northern Asia into Europe, and its most important host is the striped field mouse, *A. agrarius*. Seoul virus infects rats (*R. norvegicus*) and can be found in seaports throughout the world, as well as in colonies of laboratory rats.

The most significant Arvocolinae-vectored virus is Puumala virus, a cause of mild HFRS. Puumala virus occurs in Scandinavia, northern Europe, and western Russia, and its primary host is the bank vole, *Clethrionymus glareolus*.

The Sigmodontinae-vectored viruses were discovered with the identification of a newly recognized disease, hantavirus pulmonary syndrome (HPS). In 1993, a new disease was reported in the southwest United States involving pulmonary edema and a mortality of more than 50%. In a remarkable coordinated research effort, the agent was identified within one month, as a previously unknown hantavirus, that was subsequently named Sin Nombre (no name) virus. Sin Nombre virus occurs throughout the western and northeastern United States where the primary host is the deer mouse, *Peromyscus maniculatus* or the white-footed mouse, *P. leucopus*. Sin Nombre virus is the major cause of HPS in North America, although cases can also be associated with New York (*P. leucopus*), Black Creek Canal (*Sigmodon hispicus*), and Bayou viruses (*Oryzomys palustris*). Viruses related to Sin Nombre virus occur in South America. Andes (*Oligoryzomys longicaudatus*), Laguna Negra (*Calomys laucha*) and Juquitiba (host unknown) viruses have been associated with HPS outbreaks in Argentina, Paraguay and Brazil. Interestingly, a rare instance of hantavirus human-to-human transmission has been documented to have occurred during the Andes virus outbreak in Patagonia, Argentina.

Phlebovirus (and *Uukuvirus*) Genus

The genus *Phlebovirus* has been expanded to include the uukuviruses, which were originally considered to be a separate genus, in view of similarities in genome organization. The genus is named after phlebotomus fever virus. The genus *Phlebovirus* includes over 50 viruses. Of these, Rift Valley fever virus and the phlebotomus fever viruses cause significant illness in humans. Viruses in this genus are transmitted by mosquitoes or by phlebotomine flies (sandflies).

Rift Valley fever virus can be transmitted by a rather wide variety of mosquito species, including *Culex pipiens, Aedes* species and *Culex theileri*, and is probably maintained in several different wildlife cycles, although the vertebrate hosts have never been well defined. Epizootics characteristically occur in diverse ecological settings and are sporadic and unpredictable. During an epizootic, disease usually occurs first in domestic animals, particularly sheep, cattle and goats. Lambs experience a high mortality of about 90%, adult sheep about 25%, and pregnant ewes usually abort. The virus can be transmitted to humans both by mosquito bite and by contact with infected animals, the latter probably being the most important source of infection. Rift Valley fever virus has long been recognized to produce infection by aerosols, and those working with the virus must take extreme precautions.

Rift Valley fever virus has caused recurrent outbreaks throughout sub-Saharan Africa, and occasionally in West Africa and Egypt. In the Egyptian epidemic of 1977–79, there was an intense epizootic in sheep and cattle, and an estimated 200 000 human illnesses with 600 deaths. There are several types of human disease: an influenza-like syndrome, hepatitis with hemorrhagic manifestations, meningoencephalitis and retinitis. In sheep, hepatic necrosis and

hemorrhagic complications are the primary cause of death. In addition, the virus infects a wide variety of laboratory and domestic animals, and is often lethal. Inactivated vaccines have been made for Rift Valley fever and, although unlicensed, these have been used in laboratory personnel and probably protect against illness if not infection. Antiviral compounds, particularly ribavirin, have been shown to have some effect when used to treat experimental animal infections.

Phlebotomus fever viruses are transmitted by phlebotomus flies (sandflies), which are small flies, the size of midges, of the *Phlebotomus, Sergentomyia* and *Lutzomyia* genera. Sandfly fever viruses are enzootic in the Mediterranean basin, in the Middle East and across the Arabian peninsula into Pakistan and northwest India. Presumably, sandfly fever viruses are maintained in a vector–vertebrate cycle, but the vertebrate hosts have never been identified. During World War II, there were epidemics of sandfly fever in military troops, and these viruses continue to cause infections in North Africa and southwest Asia and are a potential risk for travelers. Toscana virus, a related virus, causes febrile illness and aseptic meningitis in Tuscany. In addition, similar viruses are found in South America, where they are probably maintained in sylvatic cycles, and cause sporadic infections in forest travelers. Phlebotomus fever is an acute febrile illness with associated malaise, which lasts 2–4 days, with complete recovery. Sandfly fever viruses replicate well in Vero cell cultures, are cytocidal, and produce plaques. However, these viruses replicate poorly in laboratory animals, even suckling mice, and there is no animal model of infection.

Nairovirus Genus

Nairoviruses are named after Nairobi sheep disease virus, the type virus. There are more than 30 viruses in the genus classified into seven serogroups. The most distinctive feature of nairoviruses is that they are tick-borne, although a few have been isolated from mosquitoes. Crimean–Congo hemorrhagic fever is the most medically important of these viruses.

Crimean–Congo hemorrhagic fever (CCHF) virus is transmitted by Hyalomma ticks, and is found over the wide geographic range of these ticks, extending from South Africa to the Middle East and into western China. CCHF virus is probably maintained in nature by two cycles, a transovarial and transstadial cycle in ticks, and a tick–vertebrate cycle. In South Africa the wildlife hosts include large herbivores (wild and domestic) and hares. Infection of humans is relatively infrequent even in enzootic areas, and occurs either by tick bite or by direct contact with infected animals or their tissues. Agricultural and abattoir workers are particularly at risk. In addition to tick and zoonotic transmission, person to person spread can also occur and has caused many small nosocomial outbreaks. In humans, CCHF is a serious disease with a mortality of 10–50%. After an initial febrile and influenza-like illness, hepatitis and hemorrhagic signs develop, that often progress to a fatal shock syndrome. Although CCHF virus will cause encephalitis in laboratory animals, there is no model for the hemorrhagic syndrome seen in humans.

Tospovirus Genus

The *Tospovirus* genus is named after its type species, tomato spotted wilt virus (TSWV). Tospoviruses are arthropod-borne viruses, that are transmitted by several species of thrips, minute insects that feed exclusively on plants. Thrips can be infected only during the larval stages and can transmit during the last larval stage and throughout adulthood. Thus, tospoviruses differ from other bunyavirus genera where many of the vectors can be infected only as adults. To date, five distinct groups, including seven viruses, have been defined based on serology and genetic sequence, and additional viruses continue to be identified. The most distinctive genetic feature of tospoviruses is the presence of an additional virus sense ORF in the M RNA segment. This ORF codes for a movement protein involved in the intercellular spread of nucleocapsids through plasmodesmata – channels in the rigid cellulose walls that separate plant cells.

TSWV was first described as a distinct disease of tomatoes in 1915, was shown to be a virus disease in 1930, and only identified as a bunyavirus in the 1980s. TSWV is known for its broad host range, including both monocots and dicots, and can infect over 500 plant species, many of which are members of the Solanaceae and Compositae families. TSWV is endemic worldwide in the tropics, subtropics and temperate zones. The virus has recently spread extensively in North America and Europe causing considerable economic losses in agriculture and horticulture. The pathological changes caused by TSWV vary widely, due both to variation in the virulence of different virus strains circulating in nature and to different responses of different host plants to the same virus strain. Symptoms range from severe widespread necrosis, to mosaic or mild discoloration of leaves, and the infection usually becomes systemic but may remain localized to the site of inoculation in a few hosts. Ultrastructural studies indicate that the morphogenesis of the virus particle is similar, but not identical, to that observed with other

species of bunyaviruses. TSWV produces cytoplasmic inclusion bodies, associated with accumulation of either N or NSs protein, which has not been reported for other bunyaviruses.

See also: ***Bunyaviridae*: Replication; Hantaviruses (*Bunyaviridae*); Tospoviruses (*Bunyaviridae*).**

Further Reading

Elliott R (ed.) (1996) *The Bunyaviridae*. New York: Plenum Press.

Gonzalez-Scarano F and Nathanson N (1996) Bunyaviridae. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 1473. Philadelphia: Lippincott-Raven.

Replication

Richard M Elliott, Institute of Virology, University of Glasgow, Glasgow, Scotland

Introduction

Currently more than 300 mainly arthropod-transmitted viruses are contained within the family *Bunyaviridae*. The majority of these viruses are classified into one of five genera: *Bunyavirus, Hantavirus, Nairovirus, Phlebovirus* and *Tospovirus* (the only plant-infecting members of the family). The basic features which unite these viruses are similar virion morphology, tripartite single-stranded RNA genome and cytoplasmic site of viral replication with intracellular maturation in the Golgi. However, considering the large number of viruses within this one taxonomic grouping, it is not surprising that considerable diversity exists in terms of genome coding and replication strategies. In this article, gross generalizations are made based on results from one virus in a particular genus; future research may invalidate such generalizations. Where possible, genus-specific traits are indicated with the terms bunyavirus, hantavirus, etc., with the term *Bunyaviridae* being reserved for familial traits.

Virion Proteins and RNA Segments

The spherical *Bunyaviridae* virion consists of four structural proteins: two internal proteins (N and L) and two (perhaps three in some nairoviruses) external glycoproteins which are inserted in the viral membrane (**Fig. 1**).

By convention, the glycoprotein of greater molecular weight (or slower electrophoretic mobility in sodium dodecyl sulfate–polyacrylamide gels) is termed G1. There is no equivalent of a matrix protein to stabilize the virion structure. The three genomic RNA segments, which are designated L (large), M (medium) and S (small), characteristically have complementary terminal sequences, which are similar for the three segments of viruses within a genus; the genus-specific consensus sequences are shown in **Table 1.** A consequence of the terminal complementarity is that the ends of the RNAs may base-pair, and circular or panhandle *Bunyaviridae* RNAs have been seen in the electron microscope. Each RNA segment is associated with the N (~2100 copies per particle) and L (~25 copies per particle) proteins to form a ribonucleic protein complex known as the nucleocapsid. *Bunyaviridae* nucleocapsids are also circular, and the ends of the RNA segments are base-paired within these structures. It is probable that the

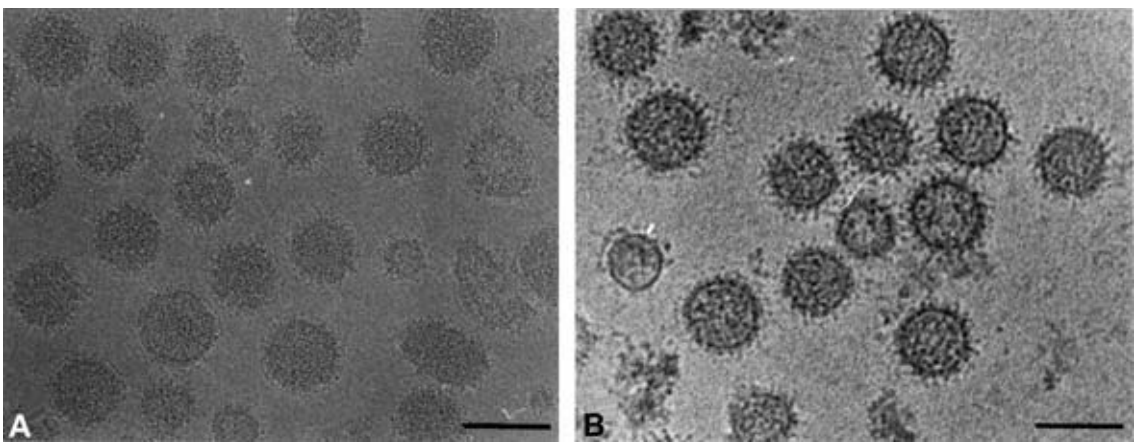


Figure 1 Electron micrographs of vitrified-hydrated La Crosse virions. (**A**) Small defocus value which demonstrates the membrane bilayer; (**B**) large defocus value which demonstrates the glycoprotein spikes (see Elliott (1990) for further details). Bar = 100 nm. The photographs were generously provided by Dr B. V. V. Prasad.

species of bunyaviruses. TSWV produces cytoplasmic inclusion bodies, associated with accumulation of either N or NSs protein, which has not been reported for other bunyaviruses.

See also: ***Bunyaviridae*: Replication; Hantaviruses (*Bunyaviridae*); Tospoviruses (*Bunyaviridae*).**

Further Reading

Elliott R (ed.) (1996) *The Bunyaviridae*. New York: Plenum Press.

Gonzalez-Scarano F and Nathanson N (1996) Bunyaviridae. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 1473. Philadelphia: Lippincott-Raven.

Replication

Richard M Elliott, Institute of Virology, University of Glasgow, Glasgow, Scotland

Introduction

Currently more than 300 mainly arthropod-transmitted viruses are contained within the family *Bunyaviridae*. The majority of these viruses are classified into one of five genera: *Bunyavirus, Hantavirus, Nairovirus, Phlebovirus* and *Tospovirus* (the only plant-infecting members of the family). The basic features which unite these viruses are similar virion morphology, tripartite single-stranded RNA genome and cytoplasmic site of viral replication with intracellular maturation in the Golgi. However, considering the large number of viruses within this one taxonomic grouping, it is not surprising that considerable diversity exists in terms of genome coding and replication strategies. In this article, gross generalizations are made based on results from one virus in a particular genus; future research may invalidate such generalizations. Where possible, genus-specific traits are indicated with the terms bunyavirus, hantavirus, etc., with the term *Bunyaviridae* being reserved for familial traits.

Virion Proteins and RNA Segments

The spherical *Bunyaviridae* virion consists of four structural proteins: two internal proteins (N and L) and two (perhaps three in some nairoviruses) external glycoproteins which are inserted in the viral membrane (**Fig. 1**).

By convention, the glycoprotein of greater molecular weight (or slower electrophoretic mobility in sodium dodecyl sulfate–polyacrylamide gels) is termed G1. There is no equivalent of a matrix protein to stabilize the virion structure. The three genomic RNA segments, which are designated L (large), M (medium) and S (small), characteristically have complementary terminal sequences, which are similar for the three segments of viruses within a genus; the genus-specific consensus sequences are shown in **Table 1.** A consequence of the terminal complementarity is that the ends of the RNAs may base-pair, and circular or panhandle *Bunyaviridae* RNAs have been seen in the electron microscope. Each RNA segment is associated with the N (~2100 copies per particle) and L (~25 copies per particle) proteins to form a ribonucleic protein complex known as the nucleocapsid. *Bunyaviridae* nucleocapsids are also circular, and the ends of the RNA segments are base-paired within these structures. It is probable that the

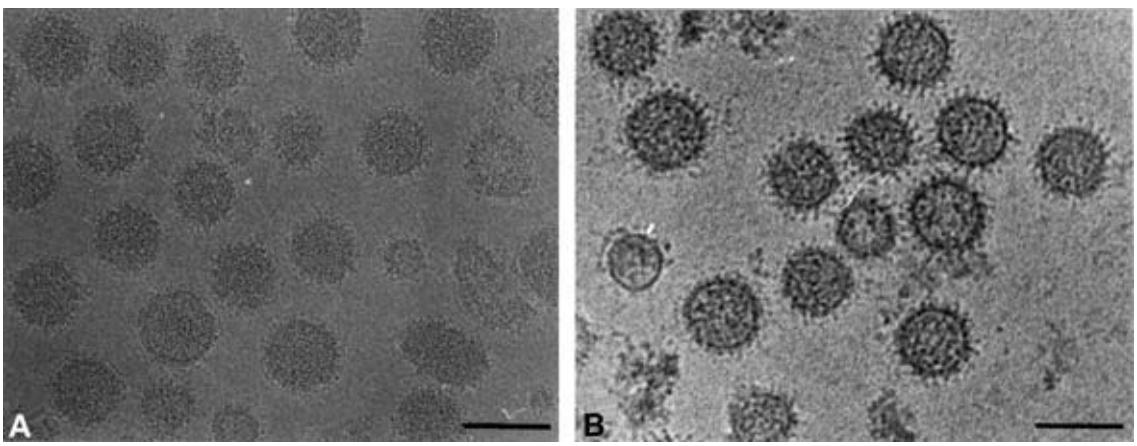


Figure 1 Electron micrographs of vitrified-hydrated La Crosse virions. (**A**) Small defocus value which demonstrates the membrane bilayer; (**B**) large defocus value which demonstrates the glycoprotein spikes (see Elliott (1990) for further details). Bar = 100 nm. The photographs were generously provided by Dr B. V. V. Prasad.

Table 1 Consensus 3′ and 5′ terminal nucleotide sequences of *Bunyaviridae* genome RNAs

Bunyavirus	3′UCAUCACAUGA....UCGUGUGAUGA 5′
Hantavirus	3′ AUCAUCAUCUG.....AUGAUGAU 5′
Nairovirus	3′ AGAGUUUCU.....AGAAACUCU 5′
Phlebovirus	3′ UGUGUUUC.....GAAACACA 5′
Tospovirus	3′ UCUCGUUAG.....CUAACGAGA 5′

encapsidation signal for the N protein is at the 5′ end of the RNA.

Coding Strategies of the Viral Genomes

Complete nucleotide sequences have been determined for at least one representative of each genus in the *Bunyaviridae* which has allowed the coding strategies of the individual genome segments to be elucidated. These are shown schematically in **Fig. 2.**

The L RNA encodes the L protein using a conventional negative-strand strategy, i.e. in a complementary positive-sense mRNA. The L protein contains motifs found in all RNA polymerases, and expression of the Bunyamwera bunyavirus and Rift Valley fever phlebovirus L proteins using recombinant vaccinia viruses demonstrated the L had RNA synthesis activity. The L protein is therefore a component, at least, of the virion-associated transcriptase.

The M segment encodes in the complementary sense mRNA the virion glycoproteins in the form of a precursor polyprotein; in the case of bunyaviruses, hantaviruses and phleboviruses this is cotranslationally cleaved to yield the mature proteins which are

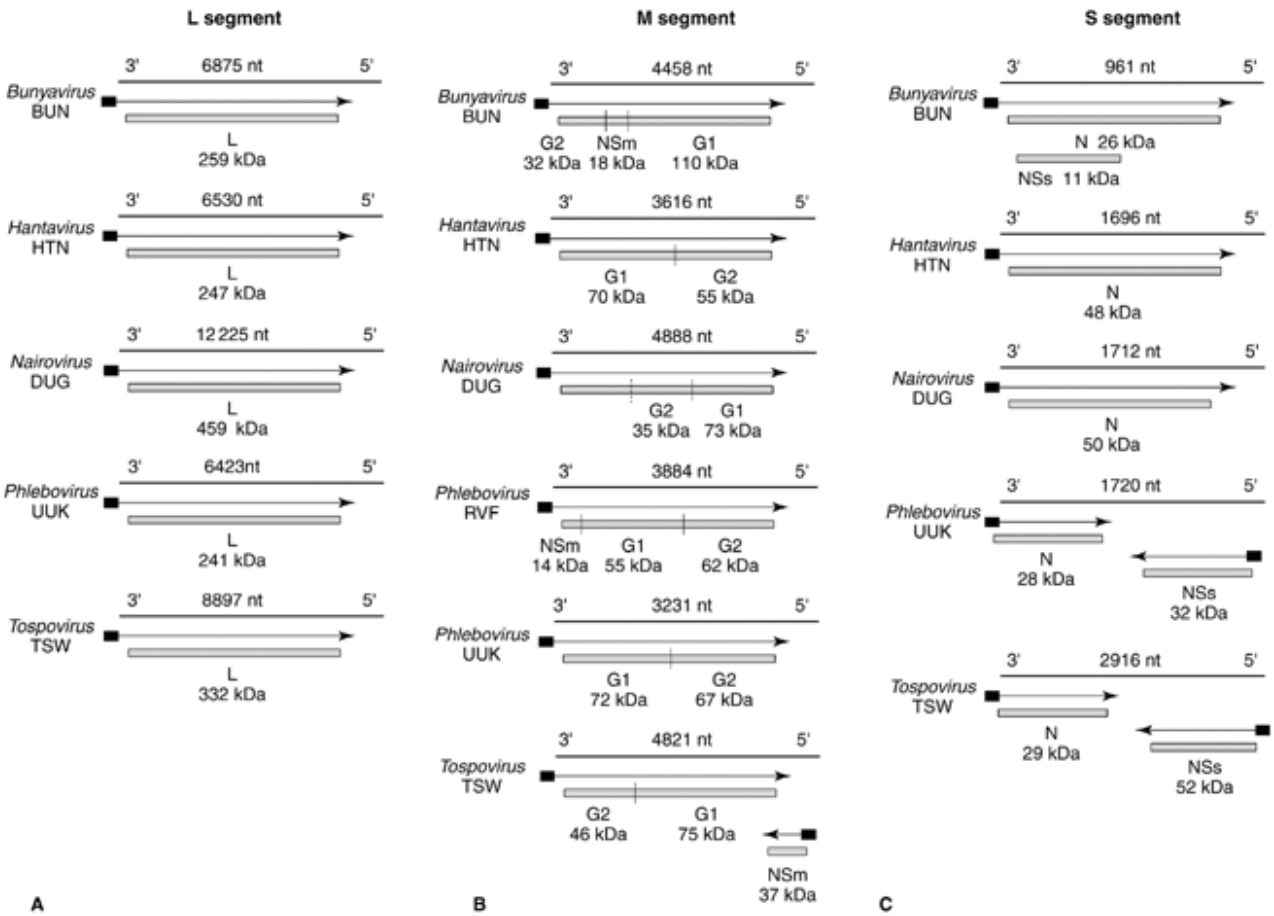


Figure 2 Coding strategies of *Bunyaviridae* genome segments. (**A**) L segment; (**B**) M segment; (**C**) S segment. Genomic RNAs are represented by thin lines (the length in nucleotides is given above each segment) and the mRNAs are shown as arrows (■ indicates host-derived sequences at 5′ end, ➤ indicates 3′ end). Gene products, with their apparent M_r, are represented by stippled boxes. Two examples of phlebovirus M segments are given which differ with respect to the presence or absence of NSm. Abbreviations: BUN, Bunyamwera virus; DUG, Dugbe virus; HTN, Hantaan virus; UUK, Uukuniemi virus; TSW, tomato spotted wilt virus; RVF, Rift Valley fever virus.

characteristically rich in cysteine residues. Processing of the nairovirus polyprotein is more complex, occurs over a period of several hours and is not yet understood. The M segment gene products have been implicated in many biological attributes of the virus including hemagglutination, virulence, tissue tropism, neutralization and cell fusion. For bunyaviruses and some phleboviruses a nonstructural protein, NSm, of unknown function, is also encoded as part of the precursor. Tospoviruses encode an NSm protein, which functions as a movement protein, using an ambisense strategy. The NSm coding region is contained in the 5′ terminal part of the viral genomic RNA, but is translated from a subgenomic mRNA. This mRNA is transcribed from the full-length complement of the genomic RNA.

The S RNA segments also show great diversity in their coding strategies. The bunyavirus S segment encodes two proteins, N, and a nonstructural protein, NSs, in different overlapping reading frames in the complementary-sense RNA. The two proteins are translated from the same mRNA species, the result of alternative initiation at different AUG codons. For hantaviruses and nairoviruses a single open reading frame, encoding the N protein, is found in the S segment complementary-sense RNA. The S segments of phleboviruses and tospoviruses employ an ambisense coding strategy: the N protein is encoded in the complementary-sense RNA corresponding to the 3′ half of the genomic S segment, whereas the coding sequence for the NSs protein is contained in the 5′ half of the genomic RNA. The proteins are translated from separate subgenomic mRNAs.

The function(s) of the NSs protein is unknown. It is noteworthy that viruses which apparently do not encode NSs (hanta- and nairoviruses) have N proteins about twice as large as those that do encode NSs (**Fig. 2**), so one might speculate that the function of NSs may have been transferred to the larger N protein.

Viral Replication Cycle

Attachment, entry and uncoating

Infection of the target cell is mediated by one or both of the virion glycoproteins interacting with the cellular receptor, though no receptor has yet been identified for any member of the *Bunyaviridae*. The relative importance of either of the glycoproteins in attachment has not been fully elucidated and may differ between the genera. For bunyaviruses, it has been suggested that G1 is the major attachment protein for vertebrate cells, whereas G2 may contain the major determinants for attachment to mosquito cells. Neutralization and hemagglutination-inhibition sites have been mapped to both glycoproteins encoded by hantaviruses and phleboviruses, suggesting that for these viruses both G1 and G2 may be involved in attachment. In common with many other enveloped viruses the *Bunyaviridae* can fuse cells at acidic pH; for bunyaviruses at least this is accompanied by a conformational change in G1. Based on electron microscopic studies of phleboviruses, entry into cells is by endocytosis. It is probable that uncoating occurs when endosomes become acidified, thus initiating fusion of the viral membrane and endosomal membrane, followed by release of the nucleocapsids into the cytoplasm.

Transcription

The classical scheme for replication of a negative-strand RNA virus is that the infecting genome is first transcribed into mRNAs by the virion-associated RNA polymerase or transcriptase (**Fig. 3**). This process, termed primary transcription, is independent of ongoing protein synthesis. Following translation of the primary transcripts into viral proteins, the genome is replicated via a complementary full-length positive-strand RNA, the antigenome, and then further mRNA synthesis (secondary transcription) ensues. Transcriptase activity has been detected in detergent-disrupted virion preparations of representatives of most *Bunyaviridae* genera. The enzymatic activity was weak compared to, for example, the transcriptase of vesicular stomatitis virus, which has hampered extensive biochemical characterization of the enzyme. However, the bunyavirus transcriptase was shown to be stimulated by oligonucleotides of the (A)nG series, cap analogues (e.g. mGpppAm) and natural mRNAs such as alfalfa mosaic virus RNA 4. These appeared to act as primers for transcription. Further support for this notion was provided by sequencing studies of the 5′ ends of both *in vivo* and *in vitro* synthesized mRNAs, which showed they contained an additional 10–18 nontemplated nucleotides; a cap structure was present at the 5′ terminus. *In vitro*, an endonuclease activity which specifically cleaved methylated capped mRNAs was detected. Taken together, these data indicate that bunyavirus transcription is markedly similar to that of influenza viruses in using a 'cap-snatch' mechanism to prime transcription. In contrast to influenza viruses, bunyavirus transcription is not sensitive to actinomycin D or α-amanitin, and occurs in the cytoplasm of infected cells. The apparent reiteration of viral terminal sequences at the junction between the primer and viral sequence itself suggests that the polymerase may slip during transcription; further analysis of hantavirus RNAs suggests that this may occur during

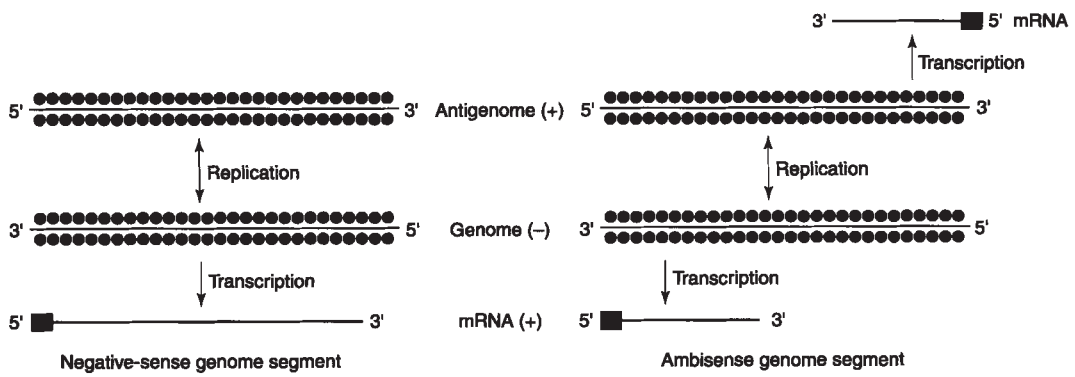


Figure 3 Transcription and replication scheme of *Bunyaviridae* genome segments. The left panel shows the scheme for a negative-strand segment and the right panel that for an ambisense segment. The genome RNA and the positive-sense complementary RNA known as the antigenome RNA are only found as ribonucleoprotein complexes and are encapsidated by N protein (•). The mRNA species contain host-derived primer sequences at their 5′ ends (■) and are truncated at the 3′ end relative to the vRNA template; the mRNAs are not polyadenylated. (Adapted from Elliot RM (1997) Emerging viruses: the *Bunyaviridae*. *Molecular Medicine* 3: 572–577, with permission, Picower Institute Press).

replication as well, and has been dubbed 'prime and realign'.

Analysis of bunyavirus primary transcription *in vivo*, however, appeared initially to produce results incompatible with the presence of a virion transcriptase, in that no mRNA synthesis could be detected in the presence of protein synthesis inhibitors in certain virus-cell systems. Further work showed that only short transcripts were produced in the absence of protein synthesis *in vivo;* subsequent gel electrophoresis analyses of the *in vitro* transcriptase products showed that these too were short transcripts. If the *in vitro* reaction was supplemented with rabbit reticulocyte lysate, however, full length RNAs were synthesized. The translational requirement was not at the level of mRNA initiation, but rather during elongation, or more precisely to prevent the transcriptase from terminating prematurely. A model to account for these observations proposes that in the absence of ribosome binding and protein translation the nascent mRNA chain and its template can base-pair, thereby preventing progression of the transcriptase enzyme. This translational requirement is not ubiquitous, however, since concurrent translation is not needed for efficient readthrough of premature termination sites in some strains of BHK cells or in the C6/36 mosquito cell line. Reconstitution and mixing experiments suggest that the translational requirement is mediated by a host cell factor, present in some BHK cell lines, which may promote interaction between the nascent mRNA and its template.

The 3′ ends of the *Bunyaviridae* mRNAs are not coterminal with their genome templates. For the nonambisense segments, mRNAs terminate 50–100 nucleotides before the end of the template, though there does not appear to be a universal termination signal in the *Bunyaviridae*: for instance 3′ GUUUUU 5′ and 3′ ACCCC 5′ are two sequences that have been mapped as termination sites. The subgenomic mRNAs transcribed from ambisense S segments terminate within the noncoding intergenic sequences in the RNA; for some, but not all, viruses the intergenic region has the potential to form a stable hairpin structure, though the role of secondary structure in transcription termination is unclear. *Bunyaviridae* mRNAs are probably not 3′ polyadenylated, but many have the potential to form stem–loop structures which may confer stability.

Genome replication

In order to replicate the negative-sense genome RNA a full-length complementary, positive-sense RNA, the antigenome, must be synthesized (**Fig. 3**). This molecule differs from the positive-sense mRNA in that it does not have the 5′ primer sequences and the 3′ end extends to the 5′ terminus of the genomic RNA template. It is not known what controls the switch from transcriptive to replicative mode of the polymerase. Two events differ between transcription and replication: initiation, which does not require a primer, and readthrough of the mRNA termination signal. The difference in initiation may be because the RNA polymerase is modified by another viral or cellular protein. In the infected cell antigenomes are only found as nucleocapsids; therefore encapsidation of the nascent antigenome RNA may prevent its interaction with the template, thereby overcoming the mRNA termination signal.

Assembly and release

Maturation of the *Bunyaviridae* characteristically occurs at the smooth membranes in the Golgi apparatus, and hence is inhibited by monensin, a monovalent ionophore. The viral glycoproteins accumulate in the Golgi complex and cause a progressive vacuolization. However, the morphologically altered Golgi complex remains functionally active in its ability to glycosylate and transport glycoproteins destined for the plasma membrane. Using vaccinia virus recombinants it has been shown that the targetting of the *Bunyaviridae* glycoproteins to the Golgi is a property of the glycoproteins alone, and does not require other viral proteins or virus assembly. Electron microscopic studies revealed that viral nucleocapsids condense on the cytoplasmic side of areas of the Golgi vesicles where viral glycoproteins are present on the luminal side. The absence of a matrix-like protein in the *Bunyaviridae,* which for other viruses may function as a bridge between the nucleocapsid and the glycoproteins, suggests that direct transmembrane interactions between the *Bunyaviridae* nucleocapsid and the glycoproteins may be a prerequisite for budding. After budding into the Golgi cisternae, vesicles containing viral particles are transported to the cell surface via the exocytic pathway, eventually releasing their contents to the exterior.

There are important exceptions, however, to the above maturation scheme; Rift Valley fever phlebovirus has been observed to bud at the surface of infected rat hepatocytes, and it appears that a characteristic of the newly described American hantaviruses which cause hantavirus pulmonary syndrome is that assembly and maturation occur at the plasma membrane.

Reverse Genetics

Reverse genetic approaches to the study of bunyavirus and phlebovirus RNA synthesis have been described, in which transiently expressed recombinant N and L proteins transcribe and replicate synthetic RNA transcripts containing a reporter gene. More recently a system to recover infectious bunyavirus entirely from cloned cDNA copies of the three genome segments has been developed. This is a significant accomplishment which promises to allow future in-depth studies on the functions of all the viral proteins as well as detailed investigation of biological properties such as virulence, tissue tropism, vector competence, etc. It is expected that similar systems will soon be developed for the other *Bunyaviridae* genera, and in the longer term the design of modified viruses having potential as vaccines may be feasible.

Persistent Infections

Arboviruses share a common biological property in their capacity to replicate in both vertebrate and invertebrate cells. The outcomes of these infections can be markedly different: whereas infection of vertebrate cells is often lytic, leading to cell death, infection of invertebrate cells is often asymptomatic, self-limiting and leads to a persistent infection. For the *Bunyaviridae* this has been demonstrated both at the organismic level and in cultured cells. Studies on bunyavirus persistent infections of mosquito cells showed no inhibition of host cell protein synthesis in sharp contrast to the situation in mammalian cells. A feature of the persistently infected cells was the excess amount of S segment RNA they contained, but although the level of S mRNA remained high the amount of N protein translated declined. The block to N protein synthesis was shown to be because N was able to encapsidate its own mRNA, thereby preventing its translation. Defective L segment RNAs were also found in the persistently infected cells, but these were not packaged into virions. In contrast, a novel type of defective interfering particle was produced which contained only S segment RNA. It is highly probable that host cell factors contribute to these events, but the identity of these factors awaits further investigation.

See also: ***Bunyaviridae*: General features; Hantaviruses (*Bunyaviridae*); Influenza viruses (*Orthomyxoviridae*): General features, Molecular biology, Structure of antigens; Persistent viral infection; Tospoviruses (*Bunyaviridae*).**

Further Reading

Bridgen A and Elliott RM (1996) Rescue of a segmented negative-strand RNA virus entirely from cloned complementary DNAs. *Proc. Natl. Acad. Sci. USA* 93: 15400.

Elliott RM (1990) Molecular biology of the *Bunyaviridae*. *J. Gen. Virol.* 71: 501.

Elliott RM (ed.) (1996) *The Bunyaviridae*. New York: Plenum Press.

Schmaljohn CS (1996) *Bunyaviridae*: the viruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology,* 3rd edn, p. 1447. Philadelphia: Lippincott-Raven.

C

CALICIVIRUSES (*CALICIVIRIDAE*)

Michael J Studdert, The University of Melbourne, School of Veterinary Science, Victoria, Australia

History

In 1932 there occurred in southern California in pigs fed uncooked garbage a vesicular disease that was provisionally diagnosed as foot and mouth disease. The disease was eradicated by slaughter and quarantine. However, in 1933 the disease recurred at which time it was realized that the disease was not foot and mouth disease but a new disease that was called vesicular exanthema of swine. Between 1932 and 1951, the disease continued to occur in southern California and more than 2.5 million pigs or 21% of the total pig population in southern California were involved in outbreaks. There was a clear linkage between the disease and the feeding of raw, i.e. uncooked, garbage containing, it was assumed, pork scraps and outbreaks of the disease continued to occur despite the introduction of laws requiring that all garbage fed to pigs be cooked. In late 1951, a train left San Francisco and off-loaded in Wyoming, to a pig rancher, garbage containing pork scraps that were fed to his pigs. When a few of the pigs in the herd developed vesicular lesions the owner shipped the pigs for sale following which, between 1952 and 1956, outbreaks of vesicular exanthema occurred in 40 of the 48 states. The last outbreak occurred in New Jersey in 1951. By 1956 rigid enforcement of laws that required all garbage fed to pigs be cooked or a total ban on the feeding of garbage resulted in the disappearance of the disease from the national swine herd in the United States, the only country in which it has ever occurred and no further outbreaks of the disease have been recorded.

In 1972 a calicivirus was isolated from sea lions during an investigation of an outbreak of abortion on San Miguel Island off the southern Californian coast. This virus closely resembles vesicular exanthema virus and is transmissible to pigs. It is believed that the origin of vesicular exanthema virus was a consequence of feeding uncooked sea lion carcasses that were washed up on the beaches of southern California, to pigs. Subsequently caliciviruses have been isolated from other pinniped species including Northern fur seals inhabiting the Pribilof Islands, Alaska.

Acute upper respiratory disease of cats is common and in 1957 the first feline calicivirus, originally identified as a picornavirus, was isolated and shown to be one of the two major viral causes of respiratory disease in cats, the other virus being feline herpesvirus.

In 1984 a new, highly infectious disease of the European rabbit, *Oryctolagus cuniculus*, was identified in China. It was characterized by hemorrhagic lesions particularly affecting the lungs and liver, that was eventually called rabbit hemorrhagic disease. It killed some 470 000 rabbits in the first six months and by 1985 had spread throughout China. By 1988 it had spread throughout eastern and western Europe and had reached North Africa. In December 1988 cases occurred in Mexico City. Both wild and domestic *O. cuniculus* were affected, but all other species of mammals except the European hare appear to be resistant to infection. The disease was unknown in Europe before 1984; however a very similar disease called European brown hare syndrome had been recognized in the early 1980s affecting *Lepus europaeus* and subsequently some other *Lepus* spp. Rabbit hemorrhagic disease is caused by a calicivirus that is different from the virus that causes European brown hare syndrome.

Rabbit hemorrhagic disease virus was imported into Australia in 1991 to a high security laboratory. Australian native animal species susceptibility studies were conducted prior to determining whether the virus would be a safe and effective biocontrol agent. In Australia, rabbits are in plague numbers perhaps as many as 100 million and are estimated to cause $600 million in annual losses some of which, including native species and habitat destruction, may be permanent. The virus was transferred from the high security laboratory to Wardang Island for further pen trials and during these trials it escaped to the mainland

possibly by insect vector transmission (mosquitoes, bush flies) or carrion-eating birds (crows, eagles) and subsequently spread to many areas prior to 'official' release as a biocontrol agent. In New Zealand in mid-1997 the virus was illegally introduced and spread probably by farmers, who were irate after a decision of the government not to allow legal importation of the virus until more was known about it particularly in relation to its known or potential host range.

Viruses with typical calicivirus morphology have been identified but not cultivated often from enteric infections from other species including humans, cattle, swine (new type), dog, rabbit, chicken, reptile, amphibian and insects. Human hepatitis E is also caused by a calicivirus-like although the final classification of this virus is debated.

Classification and Properties

Although superficially resembling picornaviruses, with which they were once classified, in some of their properties the distinctive properties led to the creation of the family *Caliciviridae*. The family *Caliciviridae* contains four genera: two of which contain members that are the subject of this entry one of which includes the related viruses of vesicular exanthema virus and San Miguel sea lion virus as well as feline calicivirus (*Vesiculovirus* genus) and the second the related viruses that cause rabbit hemorrhagic disease and European brown hare syndrome (*Lagovirus* genus). Two other genera include Norwalk ('Norwalk-like viruses') and Sapporo viruses ('Sapporo-like viruses') that cause diarrhea in humans. Hepatitis E virus ('hepatitis-E-like viruses' genus) and a related virus isolated from pigs in midwestern USA have not been assigned to a family. Probable caliciviruses have also been recovered from monkeys, cattle, mink, swine (in addition to vesicular exanthema virus), dog, rabbit, chicken, reptile, amphibian and insect, but have not been fully characterized; viruses from some of these animal species cause gastroenteritis, and a probable strain has been isolated from dogs with a vesicular genital disease.

The family name *Caliciviridae* derives from the cup-shaped (*calix* = cup) surface depressions that give the virion its unique appearance. By cryoelectron microscopy virions are 40.5 nm with 32 cup-shaped surface structures comprising 90 arch-like capsomers arranged in $T = 3$ icosahedral symmetry. The individual capsomers are dimers of the ~60 kDa capsid protein and have three main structural domains consisting of an upper bilobed structure, a central stem and a lower shell. The capsid protein has six regions (A–F) that probably form hypervariable, loop-like structures on the surface of the folded protein and are sites of antigenic variation. Some calicivirus isolates lack the characteristic morphology and have a fuzzy edge. The virion is relatively resistant to heat and intermediate in its pH stability (>99% inactivated at pH 3).

The genomes are ~7.5 kb, positive sense, polyadenylated single-stranded (ss) RNA. The sequence of the genomes of feline calicivirus, rabbit hemorrhagic disease virus and European brown hare syndrome virus have been determined. Two different arrangements for the coding region, exemplified by feline calicivirus and rabbit hemorrhagic disease virus, in which there is a different arrangement of open reading frame (ORF) usage at the polymerase–capsid boundary. In the case of rabbit hemorrhagic disease virus the nonstructural proteins and the structural protein are encoded as a single polyprotein whereas for feline calicivirus these are separately encoded. There are three ORFs. For feline calicivirus, and probably also vesicular exanthema virus, two polyadenylated RNA species, one corresponding to the genome of 7.6 kb and the second, a subgenomic species of 2.4 kb that is bicistronic encode ORFs 2 and 3. Similar polyadenylated RNA species (7.5 and 2.2 kb) are recognized for rabbit hemorrhagic disease virus and it has been shown that both species are probably packaged into either the same or separate virions. The initiation codon for ORF 1 is at nucleotide 20 and for feline calicivirus the polyprotein encoded is 1763 amino acids whereas for rabbit hemorrhagic disease virus the polyprotein is 2344 amino acids. The nucleotide sequence at the junction between the polyprotein termination codon and the ORF 2 initiation codon is absolutely conserved and ORF 2 has a −1 frame shift relative to ORF 1. The 3′ terminal ORF 3 encodes a basic protein of 116 amino acids of unidentified function that is translated from the 2.4 kb mRNA with a −1 frame shift relative to ORF 2; it is not necessary for capsid assembly but may itself be a capsid protein. The 10–15 kDa VPg protein is bound to the 5′ terminus of both RNA species. The nonstructural proteins, 2C helicase, 3C trypsin-like serine protease and 3D polymerase, are similar to those found in picornaviruses. The individual nonstructural proteins are released from the polyprotein by specific viral protease activity in a cascade similar to that of picornaviruses.

Geographic Distribution

As noted, vesicular exanthema between 1932 and 1951 occurred only in southern California and subsequently in 40 of the 48 states of the USA before it was eradicated in 1956. Caliciviruses, including San Miguel sea lion virus, remain endemic in pinniped

species particularly along the western seaboard of North America. Feline calicivirus is the cause of a common, everyday disease of cats throughout the world. Rabbit hemorrhagic disease has occurred in China, eastern and western Europe, North Africa, Korea, probably Mexico and is now endemic in Australia and New Zealand.

Host Range and Virus Propagation

Pigs are a significant alternative host for at least some of the pinniped caliciviruses. The extent to which pinniped and perhaps other marine species share as hosts their caliciviruses has not been fully defined. Feline calicivirus is restricted to members of the family *Felidae* although most commonly infections are recognized in domestic cats. Rabbit hemorrhagic disease virus is known to infect and cause disease in the European brown hare (*Lepus europaeus*) and some other *Lepus* spp.

The pig, pinniped and feline viruses grow readily and rapidly in monolayer cell cultures derived from pig and feline tissues, respectively; most strains of vesicular exanthema virus grow in Vero cells. Rabbit hemorrhagic disease virus has not been cultivated in cell culture.

Serologic Relationships and Variability

Based on serum neutralization assays and cross-protection studies in pigs vesicular exanthema virus has a very high number of antigenic types. It was not uncommon for more than one type to be isolated during a single outbreak of disease or indeed for more than one virus type to be isolated from a single pig. The exact number of types cannot be recorded with certainty since some of the early collections of viruses were lost although at least 13 distinct antigenic types exist in one collection of viruses.

A similar pattern of antigenic variation (types) was recognized among feline caliciviruses, i.e. when rabbit antisera were used in serum neutralization assays, a large number of different antigenic types was identified in collections examined by the few individual laboratories that attempted the studies; all feline caliciviruses have never been examined by a single set of typing criteria. Curiously, however, when antisera raised in specific pathogen-free cats were used in virus neutralization assays to examine reasonably large collections of feline caliciviruses, although considerable antigenic variation was recognized, extensive crossreactions were identified essentially between all viruses. It was concluded that feline caliciviruses were related as a single antigenic type and these findings paved the way for the development of monotypic vaccines. Antigenic variation among rabbit hemorrhagic disease virus isolates has not been much studied primarily because of the lack of a cell culture system; however monotypic vaccines appear to be fully effective.

Epidemiology

Vesicular exanthema was initially and on an ongoing basis transmitted to pigs by the feeding of uncooked pinniped carcass meat and additional transmission occurred by feeding uncooked garbage containing pork scraps. Within a herd pig-to-pig transmission occurred since ruptured vesicles shed large quantities of virus into the environment such that transmission would readily occur by contact or via fomites.

Feline caliciviruses are transmitted by contact and particularly by sneezing when cats are closely confined as in multiple cat households, breeding and boarding establishments, cat shelters and veterinary hospitals. Recovered cats remain persistently infected with virus for many months or years presumably as a consequence of low-grade infection in pharyngeal tonsillar tissues.

Rabbit hemorrhagic disease virus is readily transmitted between rabbits via a fecal–oral route and over longer distances apparently by fomites including contaminated vehicles. Insects were believed to have played a role as mechanical vectors in the escape and subsequent dissemination of rabbit hemorrhagic disease virus from an offshore island location to the mainland of Australia. Infected rabbit carcass meat could be carried over considerable distances by predatory and carrion-eating mammalian and avian species.

Pathogenesis

Vesicular exanthema virus gains entry via abrasions usually around the snout and mouth or on the feet. Secondary vesicles may occur as a result of direct local spread or following viremia. Abortion may occur and death of baby pigs from agalactia in their dams also occurs.

Feline calicivirus produces vesicular lesions on the muzzle, and within the oral cavity and the respiratory tract. These tend to rupture quickly.

Rabbit hemorrhagic disease is a generalized infection in which viremic spread results in lesions in a wide range of tissues, which at post mortem are particularly evident as hemorrhagic necrosis of the liver and lung. The key to the pathogenesis is massive liver necrosis leading to disseminated intravascular coagulation.

Clinical Signs

Vesicular exanthema of pigs is clinically indistinguishable from the other three, so called vesicular diseases that affect pigs, i.e. foot and mouth disease, vesicular stomatitis and swine vesicular disease. Following an incubation period of 12–48 h there is a marked febrile response, anorexia and listlessness. Primary vesicles are blanched, raised areas of epithelium up to 3 cm diameter and up to 1 cm high filled with a serous virus-rich fluid. They easily rupture leaving raw, bleeding, exceedingly painful ulcers that subsequently become covered with a fibrinous pseudomembrane. Secondary vesicles appear 48–72 h postinfection. Notably these appear on the soles of the feet and in the interdigital space and at the coronary band. There is severe four-footed lameness. Secondary bacterial infection of lesions particularly of the feet occurs and prolongs recovery where slaughter and eradication are not pursued.

Feline calicivirus infection may produce subclinical, or an acute or subacute disease usually characterized by conjunctivitis, rhinitis, tracheitis, pneumonia (usually in young kittens) and by vesiculation/ulceration of the epithelium of the oral cavity and muzzle. There is fever, anorexia, lethargy, stiff gait and usually a profuse ocular and nasal discharge. Morbidity is high and mortality in untreated cases may reach 30%.

Rabbit hemorrhagic disease affects rabbits over two months of age; curiously rabbits less than 2 months old do not develop clinical disease following infection. The disease is often peracute, characterized by sudden death following a 6–24 h period of depression and fever. Infection is via the fecal–oral route. Morbidity rates of 100% and mortality rates of 90% are observed in rabbits older than 2 months. At postmortem there is congestion and hemorrhage in the lungs, with accentuated lobular markings, necrosis of the liver and splenomegaly. Massive blood clots are present throughout the vasculature.

Immunity

Recovered pigs are immune to the particular antigenic type of vesicular exanthema virus with which they were infected but not to other types. Since slaughter and eradication policies are pursued the questions of long-term immunity and vaccine development are not at issue.

Cats recovered from feline calicivirus infection or immunized with feline calicivirus vaccine appear to remain relatively free of disease when exposed further. This appears to be the case despite the considerable degree of antigenic variability recognized among feline caliciviruses. It is practice to recommend annual boosting of immunity.

Vaccines have been developed against rabbit hemorrhagic disease and are widely used particularly in those countries such as China and Italy where rabbit farming for meat and pelt production is a major industry, and also for the protection of pet and laboratory rabbits.

The basic features of calicivirus immunity have been best studied for the feline calicivirus in which antibody and cell-mediated immunity including the generation of cytotoxic T lymphocyte responses able to lyse autologous cells have been demonstrated.

Prevention and Control

Vesicular exanthema is effectively controlled by slaughter and is now considered an extinct virus disease.

Feline calicivirus infection is most difficult to control in large open cat populations. Vaccination is an important means of control. Clinically ill cats should be isolated and incoming cats of uncertain status should be held in isolation for at least a week before being introduced into the general colony. Although recovered cats remain persistently infected the amount of virus spread is not usually large so they do not pose the same threat by contact with other cats as do cats with obvious clinical disease.

Preventing entry of rabbit hemorrhagic disease virus into commercial rabbitries, either via fomites or via infected wild rabbits, creates a major challenge in control. Where feed and other supplies are delivered to multiple farms special care is required. Feeding of pellets that are sterilized should minimize transmission. Vaccines for the control of the disease are prepared as inactivated, adjuvanted, homogenates of infected rabbit tissues. Virus-like particles produced by recombinant DNA technology in baculovirus expression systems are effective as a vaccine following parenteral or oral administration but are not available commercially.

Future

Vigilance will be required to avoid the re-emergence of vesicular exanthema of swine either from marine sources or from laboratory escape sources. The host range of the virus strains currently known requires constant monitoring as does the emergence of variant strains. A clearer understanding of the molecular basis for virulence remains a major challenge. The molecular basis for antigenic variation among feline caliciviruses could be a research objective although research budgets for this work are difficult to identify

with a sense that currently used vaccines appear effective. The nature of the carrier state requires further understanding.

Further details of the molecular events in the replication cycle of caliciviruses in general including a definition of the total number of messenger RNA transcripts and their regulation should continue to emerge in the coming few years. The sequencing of the genome of at least one vesicular exanthema of swine virus should be undertaken. For rabbit hemorrhagic disease, in some ways the future arrived early; although the most recently identified calicivirus, the entire nucleotide sequence, comprising 7437 nucleotides was reported in 1991 and was the first calicivirus genome to be completely sequenced. Comparative sequence analysis among the caliciviruses will be as interesting and fascinating as it has been for other viruses.

At a more prosaic level the need to cultivate rabbit hemorrhagic disease virus is a high priority as is the need to understand why rabbit kittens less than two months of age are not susceptible to disease, a phenomenon contrary to and seemingly without precedent in any other virus disease.

For those countries such as Australia and New Zealand where wild rabbits are a plague upon the nations, reducing profits from farming and degrading the land, continuing assessment and enhancement of the effectiveness of rabbit hemorrhagic disease virus to control rabbits will be ongoing. The emergence of a smooth possibly nonvirulent virion form of rabbit hemorrhagic disease virus in Europe presumably as a mutation rather than a phenotypic change due to enzymatic digestion of outer peptide residues of the virion surface may be one of many factors that may in the long term diminish the effectiveness of rabbit hemorrhagic disease virus as a biocontrol agent. In many areas of Australia, rabbit numbers have declined precipitously (>60%) and there is evidence for the restoration of original habitats and species in unfarmed areas. It is too early to assess the long-term effectiveness and benefits following the introduction of the virus into Australia and New Zealand but it is likely that it, together with other established methods of control including myxomatosis (in Australia), will bring long-term benefit. The use of rabbit hemorrhagic disease virus as a biocontrol agent has caused conflict among farming groups and animal welfare groups on the one hand and those other greens among us concerned with the preservation and return of the environment to its more natural state as was before the arrival of the rabbit in Australia and New Zealand. The paradox created by the use of the virus as a lethal biocontrol and the recovery of some environments to near pristine state with the reappearance of native plant and animal species that were either much depleted or considered extinct will continue to be discussed. As in much of the rest of the world it is now essential in Australia and New Zealand to have effective vaccination programs in place to protect farmed, pet and laboratory rabbits. In contrast to the situation in the antipodes the preservation of wild rabbit populations for gun/dog hunting purposes throughout all of Europe has been a contentious political issue and the mass immunization of these wild populations will be considered.

See also: **Foot and mouth disease viruses (*Picornaviridae*); Persistent viral infection; Polioviruses (*Picornaviridae*): General features, Molecular biology; Vesicular stomatitus viruses (*Rhabdoviridae*).**

Further Reading

Clarke IN and Lambden PR (1997) The molecular biology of caliciviruses. *J. Gen. Virol.* 78: 291.

Berke T, Golding B, Jiang X *et al* (1997) Phylogenetic analysis of the caliciviruses. *J. Med. Virol.* 52: 419.

Meyers G, Wirblich C and Thiel H-J (1991) Rabbit hemorrhagic disease virus – molecular cloning and nucleotide sequencing of a calicivirus genome. *Virology* 184: 664.

Morise J-P (1991) Viral hemorrhagic disease of rabbits and European brown hare syndrome. *Rev. Sci. Tech. Off. Int. Epiz.* 10: 263.

Schaffer FL (1979) Caliciviruses. In: Fraenkel-Conrat H and Wagner RR (eds), *Comprehensive Virology*, vol. 14, p. 249. New York: Plenum Press.

Studdert MJ (1978) Caliciviruses. *Arch. Virol.* 58: 157.

Canine Distemper Virus

see **Rinderpest and Canine Distemper Viruses**

Canine Parvoviruses

see **Parvoviruses**

CAPILLOVIRUSES

LF Salazar, Department of Pathology, International Potato Center (CIP), Lima, Perú

Taxonomy and Classification

Members in the genus *Capillovirus* (from Latin *capillus* = hair) have not been assigned to a family. Potato virus T (PVT), was previously included in the genus *Capillovirus*, is now a member of genus *Trichovirus*. Viruses in these genera have particles with the same typical flexuous morphology but differ in their coat protein sequences and in their genome organization and replication strategy. Members of the genus *Capillovirus* are apple stem grooving virus (ASGV), citrus tatter leaf virus (CTLV) and lilac chlorotic leafspot virus (LCLV).

Virus Structure and Physicochemical Properties

ASGV and CTLV have flexuous filament virions of 640 × 12 nm, whereas LCLV has particles with a modal length of 1536 nm. Particles are built with nine or ten protein subunits per turn of the primary helix, with a pitch 3.4 nm, although 3.69 nm was reported for LCLV and 3.9 nm for NSPV. Particles have an $s_{20,w}$ of 96–112S, and their isoelectric point is about pH 4.3 at ionic strength 0.1 mol l^{-1}.

ASGV and CTLV contain a linear positive-sense single-stranded RNA of 6.5 kb that constitutes 5% of particle weight. The RNA is polyadenylated at its 3′ end. The complete sequence of ASGV and CTLV genomic RNA has been determined. Virus particles are composed of a single protein with an M_r about 27×10^3.

Host Range and Symptoms

Host range is restricted for NSPV and wide (nine plant families) for ASGV, CTLV and LCLV. Symptoms in natural hosts are stem grooves, graft union abnormalities, malformed leaves, interveinal chlorosis and symptomless infections. Symptoms in inoculated hosts are mainly chlorotic or necrotic, local and/or systemic.

Transmission

No vectors are known for capilloviruses. Seed transmission of ASGV and CTLV has been reported in *Chenopodium quinoa* and CTLV in seeds of lily. All viruses are transmitted by grafting and, excepting NSPV, also by sap inoculation.

Geographic Distribution

ASGV is probably distributed worldwide in apples. CTLV has been reported from China, Japan, Korea, the USA, Australia, Taiwan and South Africa. LCLV has been found in England and The Netherlands. NSPV was reported from the USA.

Detection and Control

The use of clean planting materials and elimination of infected plants from the field is the most effective method of control; therefore, sensitive and accurate methods for virus detection are fundamental. Capilloviruses are moderately inmunogenic and can be detected by enzyme-linked inmunosorbent assay (ELISA). For viruses that can be transmitted by sap inoculation, *Chenopodium quinoa* is a good indicator host. Heat therapy has been used to eliminate ASGV from some apple scions.

Further Reading

Ahmed NA, Christie SR and Zettler FW (1983) Identification and partial characterization of a closterovirus infecting *Nandina domestica*. *Phytopathology* 73: 470.

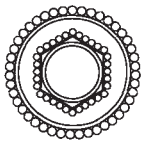
Murphy FA, Fauquet CM, Bishop DHL *et al* (eds) (1995) *Virus Taxonomy. Classification and Nomenclature of Viruses*. Sixth Report of the International Committee on Taxonomy of Viruses. Vienna: Springer.

Ohira K, Ito T, Kawai A (1994) Nucleotide sequence of the 3′-terminal region of citrus tatter leaf virus RNA. *Virus Genes* 8: 169.

Yoshikawa N and Takahashi T (1988) Properties of RNAs and proteins of apple stem grooving and apple chlorotic leaf spot viruses. *J. Gen. Virol.* 69: 241.

Yoshikawa N and Takahashi T (1992) Evidence for genomic translation of apple stem grooving capillovirus RNA. *J. Gen. Virol.* 73: 1313.

CAPRINE ARTHRITIS ENCEPHALITIS VIRUS (*RETROVIRIDAE*)



Gilles Quérat and **Robert Vigne**, INSERM U372, Campus de Luminy, Marseille, France

History

Caprine arthritis encephalitis (CAE) syndrome was first described in the USA by Cork, Hadlow, Crawford, Gorham and Piper, in 1974. This chronic disease in goats was primarily characterized by a progressive arthritis in adult animals, and by a demyelinating encephalomyelitis in goats younger than 6 months. It has been more recently recognized that arthritic adult ewes also suffer from mammitis and pneumonitis. The CAE syndrome is caused by a virus that was isolated from synovial membrane explants from an arthritic goat, in 1980, by Crawford, Adams, Cheevers and Cork. This virus was named caprine arthritis encephalitis virus (CAEV). Encephalomyelitis in CAE resembles visna, a demyelinating disease of sheep that reached epidemic proportions in Iceland in the 1940s. This disease was caused by visna virus, which was introduced into Iceland by a few Karakul sheep imported from Germany. The disease and the virus were eradicated from Iceland by the extensive slaughtering of affected flocks. Viruses similar to visna virus, however, are still in circulation in sheep and have been associated with pulmonary diseases (progressive pneumonia or maedi) in many countries. Similarly, infection and inflammatory diseases caused by CAEV in goats are almost worldwide in distribution.

Classification

CAEV belongs to the *Lentivirus* genus of the *Retroviridae* family. It can be further classified as an exogenous, nononcogenic retrovirus with a complex genome. In addition to CAEV and visna virus, the lentiviruses include human immunodeficiency viruses (HIV-1 and HIV-2), immunodeficiency viruses of numerous species of monkeys (SIV), equine infectious anemia virus (EIAV), feline immunodeficiency virus (FIV) and bovine immunodeficiency virus (BIV). Comparison of the nucleotide sequence of various isolates of lentiviruses infecting sheep and goats, i.e. visna virus and CAEV, reveal that these viruses are closely related and show less similarity to the other animal and human lentiviruses.

Lentiviruses cause lifelong persistent infections in spite of a vigorous immune response. They induce either degenerative and inflammatory diseases of specific connective tissue (CAEV and visna virus) or pathological changes to the immune system resulting in severe immune deficiency syndromes (immunodeficiency viruses).

Virion Particle and Genomic Structure of CAEV

CAE virions are enveloped particles 80–120 nm in diameter, with surface spikes of 8 nm, and an electron dense core resembling a truncated cone. The isopyknic density in a sucrose gradient is close to 1.155 g ml^{-1}. The genome is diploid, consisting of two usually identical, positive-sense, polyadenylated single-strand RNA molecules, 9.2 kb in size, which are noncovalently bound as a dimer with a strong secondary structure. The base composition of the genomic RNA is 38.2% A, 16% C, 25.1% G and 20.8% U.

The core shell (**Fig. 1**) is formed by the *gag*-derived capsid protein (CA, 25 kDa), bearing the group specific antigen. In the core, the nucleic acid binding protein (NC) is associated with the genomic RNA. It is a very basic protein with a calculated molecular weight of 9 kDa but which migrates in denaturing polyacrylamide gels as a 14 kDa protein. The core also encases, but in a minor proportion, the *pol*-derived enzymes, namely the heterodimer of reverse transcriptase (RT) and its associated RNase H (p66/p51), protease (PR, 12 kDa), dUTPase (DU, 17 kDa) and integrase (IN, 29 kDa). The matrix protein (MA, 17 kDa), unlike most of its counterparts in other mammalian retroviruses, is probably not myristoylated according to the predicted amino acid sequence and we still do not know how the *gag* and *gag-pol* polyproteins precursors migrate to the cellular membrane for virion assembly and budding. MA is believed to interact with the *env*-derived transmembrane protein (TM), although this has not been formally demonstrated for CAEV. The virion envelope originates from the cell membrane and bears the viral-specific 125 kDa surface glycoprotein (SU), the 38 kDa TM and probably numerous other cell-derived proteins.

In addition to the classical *gag*, *pol* and *env* genes common to all replication-competent retroviruses,

Figure 2

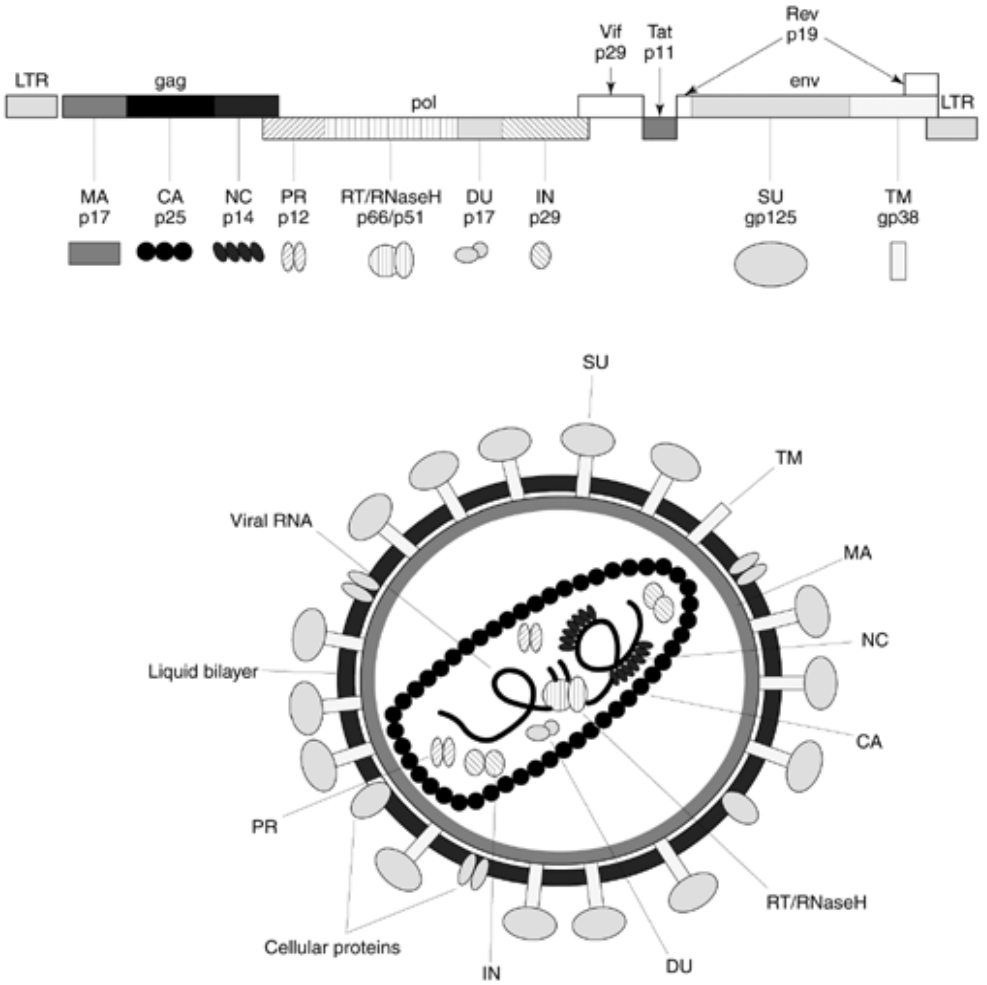


Figure 1 Genetic organization of the CAEV provirus and schematic representation of the viral particle.

CAEV and its close relative visna virus possess four other genes (**Fig. 1**).

Vif is believed to be analogous to the *vif* gene of HIV and SIV because of similar length (230 amino acids), location in the genome, overall composition of certain amino acids (rich in tryptophan) and its requirement for full replicative ability of the virus. Vif-mutants of CAEV have been engineered and were shown to replicate poorly in goat synovial membrane cells and very poorly in goat primary macrophages. Experimental infection of goats with *vif*-CAEV or *in vivo* transfection with the mutated proviral DNA led to seroconversion, but viral recovery from explanted tissues was unsucessful, further demonstrating the requirement of *vif* for efficient replication. Vif has been shown to act late in the viral replicative cycle at the stage of virion formation, release or maturation.

The *tat* gene (formally called ORF S) encodes a small protein (87 residues), which activates the viral transcription in *trans*. Unlike its HIV counterpart TAT_{CAEV} does not bind directly to viral RNA (nor DNA). The closely related Tat protein of visna virus is believed to interact with proteins of the fos/jun family and its target is believed to be an AP-1 recognition sequence upstream of the initiation site of transcription. The level of Tat-induced transactivation of expression for CAEV and visna virus seems to be considerably weaker than for HIV (at most 40 times, versus a thousand times), although this might reflect the strong basal activity of the visna and CAEV promotor in the cell type studied. There have been some conflicting data about the requirement of Tat for viral replication but it now appears that tat_{CAEV} is neither required for replication in goat synovial membrane cells in culture nor in goats experimentally infected with *tat*-mutant CAEV.

CAEV Rev is a protein which upregulates in *trans* the cytoplasmic level of viral RNA containing its

target, the Rev responsive element (RRE). Like its counterpart in HIV, RRE is a stable secondary structure of RNA, 200 nucleotides in length, present in the *env* gene region. In the absence of the *rev* gene product, all RNAs that contain RREs are sequestered in the nucleus, and only the multispliced *tat* and *tat-rev* mRNAs are accumulated in the cytoplasm. In the presence of Rev, the RRE-containing genomic and messenger RNAs which code for the structural Gag, Pol and Env proteins are efficiently transported to the cytoplasm. Rev_{CAEV} is encoded by two exons, the first one corresponds to the first 37 N-terminal residues of the env precursor, and the second one is located at the 3′ end of the genome, making a Rev protein of 133 amino acids. CAEV and visna Rev contain two distinct functional domains: a basic domain which is thought to be responsible for both nuclear localization and binding to the RRE; and a leucine-rich motif which corresponds to the activation domain and which may act as a nuclear export signal for the protein and its associated RRE-containing RNAs. Unlike *vif* and *tat* genes, *rev* is absolutely required for viral replication.

The dUTPase gene, which was formally called pseudoprotease, is located in the *pol* gene between the RNase H and the integrase domains. It encodes a virion-associated 17 kDa protein which breaks down dUTP into dUMP and PPi. This activity is superfluous for viral replication in dividing cells like goat synovial membrane cells but accelerates the replicative cycle in nondividing primary goat macrophages. Although the viral dUTPase is dispensable for replication *in vivo*, it definitely confers a replicative advantage in experimentally infected goats, as a revertant of a single point mutant appears during replication. DU was also found to be necessary for the timely development of bilateral arthritic lesions in goats. Lastly, DU prevents accumulation of G to A substitutions in the genome *in vivo*.

Replication of CAEV: Transcription, Translation and Virion Maturation

The virus binds to the target cell via the interaction of its surface envelope protein SU to an unknown receptor, which leads to fusion of the viral and the cell membranes. Reverse transcription of genomic RNA into proviral DNA begins in the cytoplasm and ends in the nucleus. The primer for minus-strand synthesis is a $tRNA^{Lys1,2}$, which is believed to be linked to the genomic RNA and the reverse transcriptase in the viral nucleocapsid. Once in the nucleus, some of the proviral DNA molecules integrate into the host chromosomal DNA. It has not been formally demonstrated that integration is a prerequisite for the transcription of mRNAs, although this seems likely owing to what is known in the HIV system.

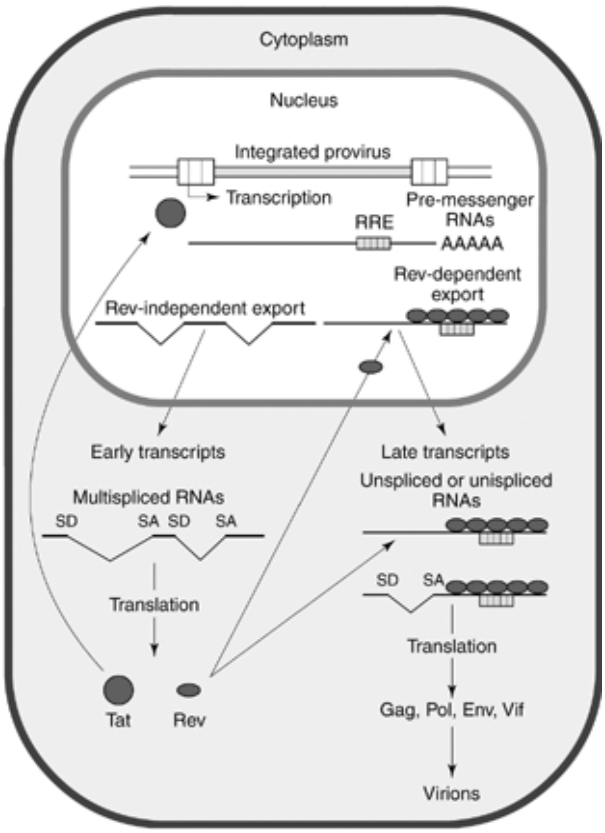


Figure 2 Regulation of the viral gene expression.

In permissive goat synovial membrane cell cultures, the transcription is a two-step process. Early after infection (24 h), the overall level of transcription is low and the major classes of cytoplasmic mRNAs are the multispliced *rev* and *tat-rev* RNAs of 1.2 and 1.5 kb. Later, i.e. 48 h after infection, there is a dramatic increase in the level of transcription and the appearance in the cytoplasm of a large amount of unspliced or singly spliced RRE-containing mRNAs coding for the structural proteins of the virion, i.e. Gag, Pol and Env, and the regulatory protein Vif (**Fig. 2**).

The genomic-sized RNA codes for the *gag* and *pol* gene products either as a $Pr55^{gag}$ polyprotein precursor or as a $Pr150^{gag\text{-}pol}$ precursor resulting from a (-1)-ribosomal frame shifting in the overlap between *gag* and *pol* reading frames. The precursors are then processed by the viral protease PR which is autocatalytically released from the Gag-Pol precursor, to yield (beginning from the N-terminus) MA-CA-NC for the *gag*-related products and PR-RT-DU-IN for

the *pol*-related products. Unlike most retroviruses, however, for which the processing of *gag*- and *pol*-related products takes place only at the budding stage of virion assembly, some of these precursors mature in the cytoplasm of the infected cells.

The Env precursor is translated from the 3.5 kb mRNA as a 107 kDa polyprotein with an unusually long N-terminal sequence preceding the hydrophobic signal peptide (about 80 residues), which is then cleaved after glycosylation of the precursor. The sequence RKKR/GVG defines the cleavage site of the precursor to yield the mature gp125 surface protein and the transmembrane gp38 protein.

Assembly of the viral particles takes place at the cell membrane during budding, as for a classical type C retrovirus, although there are some indications that it can also occur at the vacuole membrane of activated macrophages, therefore yielding a productively infected macrophage which releases a few viral particles into the extracellular space. Nevertheless, as previously mentioned, the mechanisms of targeting of the Gag and Gag-Pol precursors to the cell membrane are probably different from those of classical lentivirus like HIV, which exhibits myristoylated precursors.

In contrast to the high level of expression in acutely infected permissive cells *in vitro,* the expression of CAEV is highly restricted *in vivo,* particularly in circulating blood monocytes. There is no detectable free virus in the plasma and the cell-associated viral load, which is measured by the number of monocytes required to successfully explant the virus, is in the range of 1 in 10^5 monocytes. The differentiation of monocytes into macrophages in certain fluids or tissues, such as milk or lung, is accompanied by an activation of viral gene expression. Consequently the cell-associated viral load in either milk macrophages or alveolar macrophages is much higher and the proportion of macrophages expressing viral RNAs, as measured by *in situ* hybridization experiments, can amount to 1%. The mechanisms for the activation of expression of the viral genome *in vivo* from a restricted (possibly latent) state are presently largely unknown, although the differentiation-induced activation of *fos* and *jun* gene products, which are thought to act on the AP-1 sites of CAEV promoter, could well be implicated. The acute myeloid factor 1 (AML-1) which has been described as a binding factor in the long terminal repeat (LTR) of the ovine EV-1 lentivirus, may also enhance the CAEV transcription upon macrophage activation. Local secretions of interferon γ (IFN-γ) by activated lymphocytes could also lead to viral activation in bystander macrophages, owing to the presence of an IFN-γ-responsive element (GAS) in the LTR. It is worthy of note that *in situ* hybridization studies of synovia from infected goats have shown that viral transcription in tissue macrophages often colocalizes with IFN-γ-expressing T lymphocytes.

Host Range and Viral Propagation

Although CAEV is distributed worldwide and is associated with inflammatory diseases in goats, it is possible that it could propagate through the sheep species concomittantly with ovine lentiviruses (visna virus), as CAEV-like strains have been isolated from sheep with progressive pneumonia in the USA and Western Europe. These small ruminant viruses do not appear to have any hosts other than sheep and goats. The presence of related lentiviruses in wild ungulate animals is, however, not excluded.

Infected monocytes and macrophages are the cellular vectors that spread the virus into the specific target tissues within the same animal and probably from animal to animal. In the case of horizontal transmission to kids in the field, the colostrum is the major source of infectious virus. This secretory fluid contains infected macrophages which may convey the infection to the neonatal animals through the gastrointestinal tract. Prenatal transmission of the closely related visna virus has also been documented but its relevance for CAEV transmission and the epidemiology is not known. Horizontal transmission of CAEV is also possible between adult goats, either via the respiratory route by the dry cough containing free virions and/or infected macrophages or via the mammary glands from udder to udder by infected milk contaminating the milking machine.

Genetics and Evolution

Lentiviruses are notorious for their genetic variation. The molecular explanation for this variation is that the reverse transcriptase step occurs in the absence of a proofreading mechanism. Replication in nondividing cells like macrophages, where deoxynucleotide pools are low and highly imbalanced, may also lead to numerous nucleotide misincorporations. The additional guanine-to-adenosine hypermutation tendency that occurs in lentiviruses may also contribute to the high adenosine content of their genomes (*c.* 38% for CAEV) relative to other retroviruses (*c.* 24% for the oncovirus Rous sarcoma virus).

Comparison of the sequences from various field isolates of either ovine or caprine lentiviruses originating in Europe and the USA show that they form a group of the *Lentivirus* genus with nucleotide divergences in the range of 10–20% for the *gag* and

pol genes and 20–30% for the *env* gene. Phylogenetic studies suggest that the caprine lentiviruses originated from the ovine viruses clade. Previous attempts to date the divergences of the various lentivirus clades must be taken with caution as numerous studies have shown that there is no universal molecular clock.

Comparative sequence analysis of a hypervariable *env* gene domain during experimental infection suggests that the mutation rate of these viruses is approximately $5\ 10^{-3}$ nucleotide substitutions per site per year. Because misincorporation occurs only during replication, this rate reflects the number of replicative cycles per year in the animal. This rate is within the same range (but slightly lower) than those observed for HIV and SIV, suggesting that, although the viral replication in blood cells is considerably lower than observed for the primate lentiviruses, CAEV replicates quite actively in other organs of the body, such as synovia, lung or udder.

Epidemiology

Serological surveys show that the infection is common in goats, with a level of prevalence of around 70% in adult goats of US origin and of some countries of Europe (for example, France and Norway). Comparison of detections of the virus by either ELISA serology or polymerase chain reaction (PCR) show greater sensitivity but lower specificity for the PCR at the present time, although the PCR assay for CAEV and visna virus detection is still in its infancy.

The higher incidence of goats infected with CAEV, compared with visna virus in sheep, is probably due to the common practice in the dairy goat industry of feeding kids with pooled colostrum and milk from dairy mothers. In contrast, pooled milk is rarely fed to lambs. This enhanced transmission in dairy goats may therefore explain the high prevalence of CAEV infection in goats in Western Europe, Australia and North America, and the lack of such infections in African countries that have no dairy goat operations based on Western models. This marked difference in prevalence of infection between dairy and nondairy goats was also observed in Australia, where it was reported that Angora goats were much less infected than dairy goats.

Pathogenicity

The manifestation of the disease resulting from CAEV infection is a complicated process involving very complex interactions between the virus and host. Pathogenesis studies with CAEV mutants failed to pinpoint any 'pathogenic gene', suggesting that the overall level of viral replication in a particular tissue may be the best viral correlate to the level of tissue injuries. Any mutations affecting the fitness of the virus is therefore likely to affect its pathogenic potential. The host environment is also critical and a variety of host factors such as immune status, genetic background, age and nutritional status may be determinant factors. As an example of the relevance of the genetic background, it was reported that some class I major histocompatibility complex (MHC) haplotypes of the Saanen breed of Switzerland may be less sensitive to the CAE syndrome. Similarly, the European veterinary community pointed out the relative resistance of the ovine Merino breeds to visna and maedi syndromes. The relevance of the immune status and the age dependence is again questioned, as it is remarkable that the CAE syndrome has a high incidence in newborn goats and that the most severe clinical signs appear when the maternal antibodies disappear.

Clinical Features and Histopathology

CAEV is associated with several inflammatory diseases affecting the joints, brain, mammary glands and lungs of goats. Encephalomyelitis is the most frequent clinical symptom of infection in goat kids, occurring at between 2 and 6 months of age. The clinical feature of infection is posterior paresis, which sometimes progresses to paralysis of the hind legs and eventually to tetraplegia. When the affected kids recover from the neurological disease, they develop severe arthritis when they become adults. A slow progressive encephalitis rarely occurs in adult goats. This disease has clinical features that are very similar to those of visna in sheep. Arthritic disease is encountered most frequently in adult goats. It progresses over the years, with a gradual swelling in the carpal (knee) joints. In advanced cases, carpal swelling may be marked and hard, with extensive periarticular fibrosis and mineralization. Some animals are lame and lean. Chronic interstitial pneumonia with progressive dyspnea and weight loss, symptoms resembling maedi in sheep, are also observed in arthritic goats. Recently, mastitis was recognized as a prevalent ailment in arthritic dairy goats, with an acute phase at the kidding period. The consequence of this frequent manifestation is a decrease in the milk secretion.

Histopathological studies reveal that in all the target organs the lesions are of an inflammatory nature, with infiltration of a large number of plasmocytes (50–70% of all inflammatory cells in the synovium) and MHC class II-activated lympho-

cytes and macrophages. Arthritis is characterized by marked hyperplasia of the synovial membrane and villosities and by mineralization and fibrosis of the soft tissues. Lymphoid foci are numerous and often surround vessels. In the brain, inflammatory lesions are distributed in a multifocal pattern and are most prevalent in the periventricular white matter and/or the midbrain. In the spinal cord, focal necrosis is observed in the lateral white matter. The pulmonary lesions are similar to those described for progressive pneumonia in sheep, particularly with respect to the focal and diffuse infiltration of lymphocytes to the alveolar septa, mild septal fibrosis and hyperplasia of the cells lining the alveoli. Lastly, the interstitium of the mammary gland is diffusely infiltrated with mononuclear cells, although lymphoid foci may form around the mammary gland secretory ducts and/or within interlobular septa. In all the target tissues, the infected cells, detected by *in situ* hybridization, are predominantly macrophages, but other types of cell, such as epithelial, endothelial and fibroblast cells, are also infected.

The finding of many concomitant bacterial and parasitic infections, such as *Corynebacterium pseudotuberculosis* lymphadenitis, *Mycoplasma ovipneumoniae, Müllerius capillaris* pneumonia and intestinal coccidiosis, in the CAEV-infected goats suggests that CAEV, like HIV, may compromise the immune system.

Immune Response

A common characteristic among all lentiviral infections is that neither the cellular nor the humoral immune response is capable of eliminating the pathogenic agent from its natural host. Infected goats produce antibodies against all virus polypeptides, and specifically against envelope proteins, but these antibodies are poor at neutralizing the infectivity of the agent. Immunization of goats with attenuated live virus, killed virus or detergent-treated infected cells failed to induce protective, sterilizing immunity against homologous virus given as challenge. Moreover, for reasons that are still unclear, in some instances such a challenge was reported to result in a more severe pathology.

The immune system may even be responsible for some of the pathological changes in the affected tissues, as, for example, a high level of anti-SU antibodies is a good predictor of the development and severity of arthritic lesions. Immunodominant epitopes in the TM have also been described but their role in the pathogenesis is still unknown. Studies of cytokine expressions in the joints have suggested that either early after infection (6–12 days), or in older but clinically asymptomatic joints, the activated lymphocytes express predominantly IFN-γ, interleukin 2 (IL-2) and IL-10. This kind of pattern is consistent with a type 1 (Th1) response of T helper cells, leading to predominantly cellular immunity. In contrast, clinical arthritis is associated by predominant expression of tumor necrosis factor α (TNFα), IL-6 and MCP-1, a monocyte chemoattractant factor, a pattern which is consistent with a Th2 or predominantly humoral response. However, even in late clinical arthritis, foci of both kinds of cytokine expression patterns are present in a single synovia, suggesting that the evolution of the immunopathogenesis may be more complicated than a simple shift from Th1 to Th2 response.

Future Perspectives

CAEV infection of goats is an ideal model for studying the pathogenesis of macrophage-tropic lentiviruses in their natural host. Research efforts should be directed towards analyzing CAEV replication *in vivo* to determine the exact role of the different genes of CAEV in the rate of viral replication, the severity of the disease and the interaction of lentiviruses with different target cells. It is important to determine the role of *rev* and *tat* in maintaining the lentivirus as a latent form in undifferentiated blood monocytes. The receptor of CAEV on macrophages also needs to be identified in order to better characterize the cells that harbor and replicate the virus *in vivo*.

Research on the immune response is needed to improve our knowledge of the mechanisms implicated in the immunopathological changes. Very little is known about the T cell-mediated cytotoxic response, its target epitopes and its efficacy at killing the infected macrophages. The interplay between cytokine secretions, soluble viral regulatory proteins and the direct cytotoxicity of these molecules also needs to be investigated, as well as the possible presence of superantigens or of autoimmune responses. Such studies may permit us to find new therapeutic approaches to lentiviral diseases.

Vaccination research has many opportunities here, from the live attenuated virus to the naked DNA expressing a specific viral gene, in combination (or not) with specific cytokines.

See also: **Visna-Maedi viruses (*Retroviridae*); Human immunodeficiency viruses (*Retroviridae*): Molecular biology, Anti-retroviral agents, General features; Persistent viral infection; Latency.**

Further Reading

Coffin JM (1996) *Retroviridae:* the viruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields' Virology*, 3rd edn, p. 1767. Philadelphia: Lippincott-Raven.

Narayan O, Zink MC, Gorrel M *et al* (1993) The lentiviruses of sheep and goats. In: Levy JA (ed.) *Retroviridae (The Viruses)*, vol. 2, 2nd edn. New York: Plenum Press.

CARDIOVIRUSES (*PICORNAVIRIDAE*)

Douglas G Scraba (deceased, October 1997), University of Alberta, Edmonton, Alberta, Canada

Ann C Palmenberg, Institute for Molecular Virology, and Department of Biochemistry, University of Wisconsin, Madison, USA

History

The first cardioviruses were discovered inadvertently during attempts to adapt human polioviruses to laboratory mice. Following the protocol of Armstrong, who in 1939 successfully transferred the Lansing strain (type 2) of poliomyelitis from monkeys to cotton rats to mice, Jungeblut and Sanders, at Columbia University, reported the adaptation of the Yale-SK poliovirus to mice. Subsequent immunological studies, however, led to the conclusion that the agent, which caused a flaccid paralysis of the hind limbs of mice, followed by death, was a new and different neurotropic virus; it was designated Columbia-SK. In 1943, Jungeblut and Dalldorf made another attempt, starting with brainstem material from a fatal case (MM) of poliomyelitis. The result was the same: after intracerebral transfers in monkeys and hamsters a virus was isolated which induced encephalitic symptoms (tremor, uncertain movements), paralysis and death in mice, but which was not poliovirus. In retrospect, it seems most likely that the Columbia-SK and MM viruses were endemic in the laboratory mice, and that their virulence was enhanced by direct intracerebral transfer. However, it is possible that they came from the monkeys used in the experiments, because rhesus monkeys inoculated with brain tissue from dead mice developed a mild febrile illness.

In 1945, Helwig and Schmidt recovered pleural fluids from a gibbon and a chimpanzee which had died in captivity in Miami from pulmonary edema and myocarditis. When these edema fluids were inoculated into mice by the intracerebral (i.c.), intraperitoneal (i.p.) or intravenous (i.v.) route, the pathogen they contained produced hind limb paralysis, myocarditis and death within a week. The 'myocarditis-producing agent' was subsequently shown to be a virus, and was named encephalomyocarditis (EMC) virus. It probably originated from a group of wild rats that lived on the same farm as the primates; about half of the rats that were later trapped and examined had antibodies to EMC virus.

In 1948, Mengo virus was isolated by Dick, Smithburn and Haddow, at the Yellow Fever Research Institute at Entebbe in the Mengo district of Uganda, from a captive rhesus monkey that had developed lower limb paralysis. Blood or spinal fluid from this animal, when inoculated intracerebrally into mice, produced hind limb paralysis followed by prostration and death within a few days. The filterable Mengo virus was also isolated from mosquitoes and from a (healthy) captured mongoose. Crossneutralization studies done in 1949 showed that the Columbia-SK, MM, EMC and Mengo viruses were antigenically very closely related, and distinct from Theiler's murine encephalitis viruses.

A fifth member of this cardiovirus serological group was isolated by Gronnert, at the Bayer laboratories in West Germany in 1949, from the intestinal tissues of a sick mouse; this was called mouse-encephalomyelitis or maus-Elberfeld (ME) virus.

Taxonomy and Classification

The cardioviruses constitute a separate genus, *Cardiovirus*, in the family *Picornaviridae*. They are distinguished from other picornaviruses by special features of their genome organization, common pathological properties, and the dissociability of their virions at pHs between 5 and 7 (in 0.1 M NaCl). The intrinsic fragility of the cardiovirus capsid is reflected

Further Reading

Coffin JM (1996) *Retroviridae:* the viruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields' Virology*, 3rd edn, p. 1767. Philadelphia: Lippincott-Raven.

Narayan O, Zink MC, Gorrel M *et al* (1993) The lentiviruses of sheep and goats. In: Levy JA (ed.) *Retroviridae (The Viruses)*, vol. 2, 2nd edn. New York: Plenum Press.

CARDIOVIRUSES (*PICORNAVIRIDAE*)

Douglas G Scraba (deceased, October 1997), University of Alberta, Edmonton, Alberta, Canada

Ann C Palmenberg, Institute for Molecular Virology, and Department of Biochemistry, University of Wisconsin, Madison, USA

History

The first cardioviruses were discovered inadvertently during attempts to adapt human polioviruses to laboratory mice. Following the protocol of Armstrong, who in 1939 successfully transferred the Lansing strain (type 2) of poliomyelitis from monkeys to cotton rats to mice, Jungeblut and Sanders, at Columbia University, reported the adaptation of the Yale-SK poliovirus to mice. Subsequent immunological studies, however, led to the conclusion that the agent, which caused a flaccid paralysis of the hind limbs of mice, followed by death, was a new and different neurotropic virus; it was designated Columbia-SK. In 1943, Jungeblut and Dalldorf made another attempt, starting with brainstem material from a fatal case (MM) of poliomyelitis. The result was the same: after intracerebral transfers in monkeys and hamsters a virus was isolated which induced encephalitic symptoms (tremor, uncertain movements), paralysis and death in mice, but which was not poliovirus. In retrospect, it seems most likely that the Columbia-SK and MM viruses were endemic in the laboratory mice, and that their virulence was enhanced by direct intracerebral transfer. However, it is possible that they came from the monkeys used in the experiments, because rhesus monkeys inoculated with brain tissue from dead mice developed a mild febrile illness.

In 1945, Helwig and Schmidt recovered pleural fluids from a gibbon and a chimpanzee which had died in captivity in Miami from pulmonary edema and myocarditis. When these edema fluids were inoculated into mice by the intracerebral (i.c.), intraperitoneal (i.p.) or intravenous (i.v.) route, the pathogen they contained produced hind limb paralysis, myocarditis and death within a week. The 'myocarditis-producing agent' was subsequently shown to be a virus, and was named encephalomyocarditis (EMC) virus. It probably originated from a group of wild rats that lived on the same farm as the primates; about half of the rats that were later trapped and examined had antibodies to EMC virus.

In 1948, Mengo virus was isolated by Dick, Smithburn and Haddow, at the Yellow Fever Research Institute at Entebbe in the Mengo district of Uganda, from a captive rhesus monkey that had developed lower limb paralysis. Blood or spinal fluid from this animal, when inoculated intracerebrally into mice, produced hind limb paralysis followed by prostration and death within a few days. The filterable Mengo virus was also isolated from mosquitoes and from a (healthy) captured mongoose. Crossneutralization studies done in 1949 showed that the Columbia-SK, MM, EMC and Mengo viruses were antigenically very closely related, and distinct from Theiler's murine encephalitis viruses.

A fifth member of this cardiovirus serological group was isolated by Gronnert, at the Bayer laboratories in West Germany in 1949, from the intestinal tissues of a sick mouse; this was called mouse-encephalomyelitis or maus-Elberfeld (ME) virus.

Taxonomy and Classification

The cardioviruses constitute a separate genus, *Cardiovirus*, in the family *Picornaviridae*. They are distinguished from other picornaviruses by special features of their genome organization, common pathological properties, and the dissociability of their virions at pHs between 5 and 7 (in 0.1 M NaCl). The intrinsic fragility of the cardiovirus capsid is reflected

in the fact that empty capsids have never been isolated.

Comparative analyses of the RNA sequences from a variety of isolates have confirmed the homologous relationship (>45% nucleotide identity in both the coding and noncoding regions) among all cardiovirus genomes. Special features of the 5′ noncoding region (presence or absence of a poly(C) tract), the lengths of virion protein surface loops and serology allow subclassification into the EMC-like viruses (EMC, Mengo, MM, ME, Columbia-SK), the Theiler's-like viruses (BeAn, DA and GD VII strains). The Vilyuisk viruses, isolated in 1954–1957 by Farmanova and Chumachenko from clinical specimens of humans with acute and chronic encephalitis, share many genomic and sequence characteristics with other members of the genus, and a strain designated 'V1' has been proposed as a type species for a third subgroup of cardioviruses.

Properties of the Virion

The cardiovirion consists of a genomic single-stranded RNA molecule (molecular weight, K^+ salt $= 2.8 \times 10^6$) enclosed in an icosahedral capsid. The 20 amino acid residue protein, VPg, is covalently linked to the 5′-uridine residue of the RNA by a phosphotyrosine bond. There are four different polypeptide species (VP1, VP2, VP3 and VP4) in the capsid, and 60 copies of each are required for the complete structure. The molecular weights of the Mengo capsid polypeptides, in order, are 31700, 29000, 25100 and 7200. The viral RNA is tightly packed within the capsid, occupying about 75% of the available interior volume (**Table 1**). The remainder is filled with water, various polyamines and salts that neutralize the charge of the RNA. Minus-strand viral RNAs, cellular mRNAs and tRNAs are never detected in the particles. Nor do the particles contain other exogenously packaged viral or cellular proteins, although the viral protein $3D^{pol}$ sometimes appears to copurify with virions because of a strong hydrophylic interaction with the capsid surface.

Table 1 Physicochemical properties of the cardiovirion

Hydrated diameter	30 nm
Thickness of the capsid shell	5 nm
Diameter of the RNA-containing core	20 nm
Sedimentation coefficient ($s_{20,w}$)	155×10^{-13} s^{-1}
Diffusion coefficient ($D_{20,w}$)	1.47×10^{-7} cm^2 s^{-1}
Partial specific volume (ν)	0.67 ml g^{-1}
Particle weight	8.4×10^6
Percentage RNA by weight	33%
Virions/A_{260} unit	9.5×10^{12}

Virus Capsid: Three-dimensional Structure

X-ray crystallographic analysis of the Mengo virion at 0.3 nm resolution was reported in 1987 by Luo *et al.* The overall architecture of the capsid follows $T = 1$ icosahedral symmetry, with one asymmetric unit comprising one molecule each of VP1, VP2 and VP3. The 60 VP4 molecules occupy internal positions, extending from under the threefold axes to form part of an annular structure under the fivefold axes; there are no extensions of VP4, or the other capsid proteins into the RNA-containing core. The shell-forming domain of each of the VP1, 2 and 3 proteins is a wedge-shaped, eight-stranded antiparallel β barrel. This folding pattern has been called the RNA virus core (RVC) motif because it is shared by the capsid proteins of all picornaviruses and by the $T = 3$ icosahedral plant viruses. While Nature has adopted a common mode for capsid shell construction for both of these groups of viruses, the specific portions of the polypeptide chains which link the eight β strands (designated A to H from N- to C-terminal) are different for each virus and for each VP protein type within each virus. It is essentially the composite of these differences that distinguish the various species.

The arrangement of the RVC domains of the capsid polypeptides with respect to the surface of the Mengo virion is shown in **Fig. 1**. The asymmetric structure unit, or protomer, is outlined by a thick line, as is the pentameric subunit which serves as the assembly unit for the construction of progeny virions *in vivo*. The protomer is stabilized by extensive noncovalent interactions among the RVC domains of the three polypeptides and between the N-terminal extensions of VP1 and VP3, which are located on the inner surface of the capsid. Protomers can only be dissociated *in vitro* by boiling in sodium dodecyl sulfate (SDS). The association of protomers into pentamers, which is the first step in progeny virion assembly, is facilitated by hydrophobic contacts between the N-terminal extensions of VP3 and VP4 (actually, VP0 at the time of pentamer formation), which together form a β annulus in the interior portion of the shell directly under the fivefold symmetry axis. Pentamers can be dissociated into protomers by 2 M urea. The 12 pentamers that comprise the cardioviral capsid are held together by electrostatic interactions among the VP2 and VP3 polypeptides along the twofold and around the threefold axes; these can be broken in the presence of 0.14 M chloride or bromide ions at $5.6 < \text{pH} < 6.4$. Polio-, rhino- or foot-and-mouth disease (FMD)

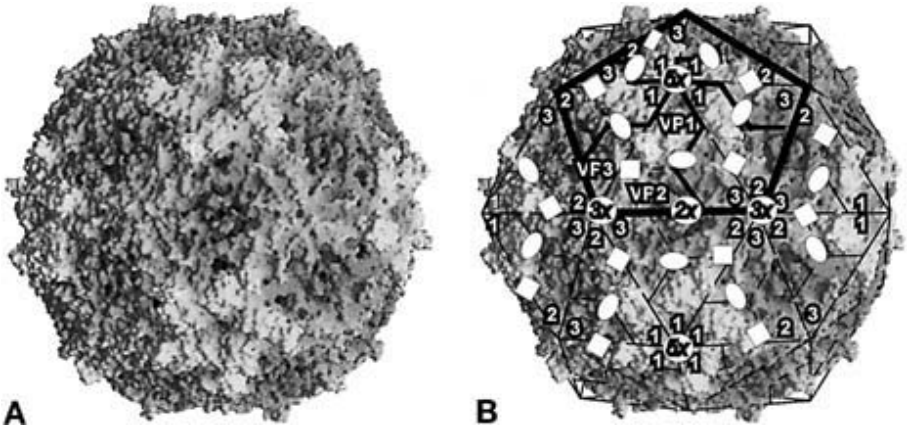


Figure 1 Exterior surface of the Mengo virus capsid. (**A**) Computer graphics representation of the Mengo virion surface at a resolution of 3.0 Å (Luo *et al.*, 1987). Light-colored regions define the higher 'massif' regions on the virion surface relative to the lower, dark-colored regions. (**B**) The surface figure is highlighted with the icosametric arrangement of polypeptides, protomers and pentamers (one pentamer, and the five protomers within it are outlined with thick lines). The icosahedral fivefold, threefold and twofold axes are marked, as are the locations of the 'pits', the putative receptor-binding sites (ovals) and the composite antigenic determinant (squares). The VP4 polypeptides occupy internal locations. Figures A and B, together, form a stereopair for three-dimensional perspective.

virions do not share this capsid fragility because their pentamer–pentamer contacts are more extensive: adjacent pentamers in polio- or rhinovirions actually interdigitate and those in FMD virions overlap, while the Mengo pentamers simply abut one another.

Among the noticeable surface features of the Mengo capsid is the regular disposition of 60 depressions, or 'pits', which are about 3 nm in diameter at the top and 2.2 nm deep. In accordance with Rossmann's 'canyon hypothesis', indirect experimental evidence implicates the 'pits' and the adjacent walls of the nearby fivefold 'massifs' as virion-attachment or receptor-binding sites. The outer diameter of the 'pit' is small enough to prohibit access by an antibody molecule (whose combining-site 'footprint' is about 3.5 nm in diameter), yet large enough to accommodate an elongated cell surface glycoprotein. Mutations changing amino acid residues around the 'pit' would be permitted, whereas the residues which line the 'pit' would have to be conserved in order for virus–cell interaction to occur. The immunoglobulin VCAM has been proposed as a cell surface receptor for the EMC-like cardioviruses; however, EMC and Mengo are also very efficient at agglutinating erythrocytes, and the specific receptor for this function on human cells is the sialoglycoprotein, glycophorin A. The sialic acid moiety of glycophorin A is specifically required for erythrocyte binding.

Figure 1 also shows the location of the composite antigenic determinant defined by a panel of neutralizing monoclonal antibodies. Although each antibody recognizes a specific epitope within the determinant (generally a conformational rather than a linear array of amino acid residues), crossneutralization experiments have shown that most epitopes are physically overlapping. The determinant involves residues in VP1 (T1100; in the conventional numbering system the first digit refers to the capsid polypeptide and the next three identify its position in the chain from the N-terminus), VP2 (K2075, N2144, R2145, S2147 and K1248) and VP3 (K3057 and S3068). These residues were identified by sequencing the capsid-coding regions of RNAs from neutralization-escape mutants. As expected, all of these residues are on the virion surface, generally near the outer wall of the 'massif' at the center of each asymmetric protomer, and spatially removed from the 'pit' areas.

Structure of the Genomic RNA

Cardioviral genomes are single-stranded, positive-sense RNA molecules of 7600 to 8000 nucleotides. The 3′ ends of cardioviral RNAs are polyadenylated. The 5′ ends are not capped as with typical eucaryotic mRNAs, but instead have a small, viral-coded, genome-linked protein (VPg) attached by a tyrosine-O^4-phosphodiester bond to the 5′-terminal uridylyl nucleotide. VPg sequences are rich in basic, hydrophilic amino acids and have only one tyrosine residue (the attachment site) at position 3 from the amino end of the peptide.

The genomic sequence begins with an unusually long 5′ untranslated region (5′ UTR), followed by an open reading frame for a large polyprotein, and terminates with a short 3′ UTR (**Fig. 2**). The EMC-

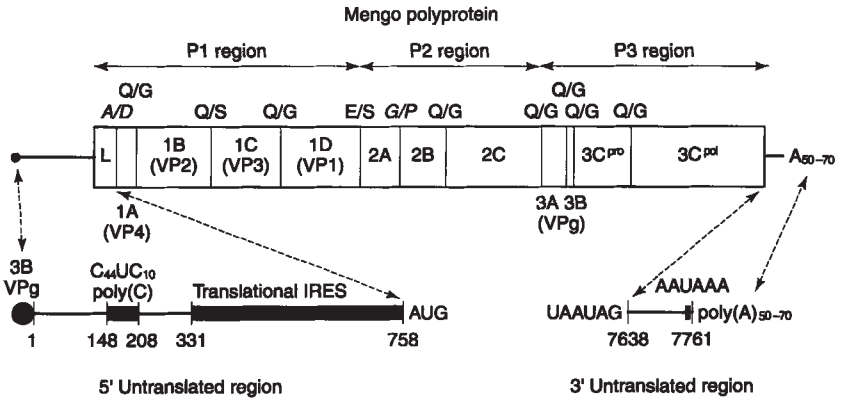


Figure 2 Mengo virus genomic RNA. The protein-coding region is indicated by the rectangle, and the nucleotide sequence coding for each gene product is to scale. There are 70 amino acid residues in VP4, 256 in VP2, 231 in VP3 and 277 in VP1. The total number of nucleotides (excluding the 3′ poly(A) tail) is 7761. The EMC RNA is 7835 nucleotides in length, the extra residues being accounted for by a longer poly(C) tract in the 5′ noncoding region.

like cardioviruses additionally contain an unusual polypyrimidine tract within the distal region of their 5′ UTRs. The tract can vary in length from 60 to 420 residues, depending upon the strain of virus; for Mengo(M) and EMC(R) the tracts have been sequenced as $C_{44}UC_{10}$ and $C_{125}UCUC_3UC_{10}$ respectively. Occasional U residues notwithstanding, this segment is referred to as the 'poly(C)'.

Computer modeling, biochemical data and phylogenetic comparisons suggest that, except for poly(C) region which is single-stranded, most cardiovirus RNAs have extensive, well-conserved base-paired structures within the remainder of their 5′ UTRs. Viral translation is directed by a 5′ IRES, or internal ribosomal entry site, that directs the viral RNA to act as an mRNA once it is free within the cellular cytoplasm. In EMC, the IRES is comprised of 430 bases arranged in multiple ordered structures, immediately 5′ to the AUG which begins the polyprotein (nucleotides 400–833). Translation actually begins at the sixth AUG from the 5′ end of Mengo RNA (nucleotides 759–761) and at the 12th AUG (834–836) in EMC RNA. Once a ribosome initiates protein synthesis, it continues along the RNA template through the entire coding region until it reaches the tandem termination signal (nucleotides 7638–7643 in Mengo RNA, 7710–7715 in EMC RNA) near the 3′ end.

The 3′ UTRs of cardiovirus, at 124–126 nucleotides in length, are the longest in any picornavirus. A poly(A) tail of 20–70 nucleotides is attached to the 3′ UTR. Approximately 20 of these poly(A) bases are template-encoded during viral RNA replication; the remainder are presumed to be added by cellular poly(A) elongation enzymes. In the EMC-like cardioviruses, the sequence AAUAAA precedes the poly(A) tract by seven and ten nucleotides. Although these are the only picornaviruses to display a polyadenylation initiation signal at the right place in their genomes, these signals are apparently not functional. Deletion of the complete poly(A) in genetically engineered Mengo or EMC is lethal, and viral RNA transcripts with fewer than 15 As are not recognized or repaired by cellular enzymes. The 3′ viral poly(A) is part of a recognition unit bound by recombinant $3D^{pol}$, and this sequence, plus nearby secondary and tertiary structures, is required for the initiation of minus-strand RNA synthesis.

Unencapsidated cardioviral RNAs are infectious to cultured cells and to animals. Whether extracted from virions, or synthesized as full-length transcripts from viral cDNAs, preparations of the intact RNAs, free of any protein, can initiate an infectious cycle when transfected into permissive cells (HeLa, L cells, BHK or others) or mice (with liposomes). The RNAs do not need to be capped or attached to 5′ VPgs to observe infectivities as high as 10^6 PFU (plaque forming units) per microgram of RNA.

Protein Expression and Processing

The cardioviruses have evolved robust and effective mechanisms to express their proteins. The IRESs allow these genomes to bypass normal translational requirements for 5′ cap structures, and efficiently lure the ribosomes into viral instead of cellular pathways. The captured ribosomes pass down the single, long open reading frame (2200 codons), creating polyproteins that are in reality tandem linkages of all structural and enzymatic units necessary for virulent infection. The individual protein fragments are liberated cotranslationally and post-translationally

in a proteolytic cascade that is a defining feature of the genus. No fewer that three viral encoded catalytic entities are required for complete processing, none of which has an exact cellular analogue. The special property of cardiovirus RNAs to initiate efficient translation in cell-free extracts and the remarkable completeness of the processing cascade during these reactions are hallmarks of these genomes.

The first, or primary cleavage within the processing cascade, occurs cotranslationally, as ribosomes traverse the middle (P2) region of the genome, and releases the N-terminal capsid precursor (L-P1-2A) from the growing polypeptide chain. With rhino- and enteroviruses, protein 2A, a product of P2 region translation, is responsible for this reaction: the *cis*-acting 2A protease cleaves its polyprotein at a Tyr-Gly junction between the P1 and P2 regions (1D/2A), and bears recognizable sequence and catalytic similarity to a second viral protease, $3C^{pro}$, encoded within the more distal region of the genome. The cardioviruses use a different mechanism for primary cleavage. The EMC polyprotein is cotranslationally cleaved at the 2A–2B junction by a short protein fragment, centering on an Asn-Pro-Gly-Pro (NPGP) sequence at this site, that has an inherent propensity for autocatalytic self-cleavage. The unusual 'suicide' activity and the sequence which causes it are unique among the cardio-, aphtho- and group-C rotaviruses.

After the primary break in the polyprotein, the secondary cardioviral processing cascade is carried out by viral protease $3C^{pro}$. This cysteine-reactive, chymotrypsin-like enzyme catalyzes a series of monomolecular and bimolecular reactions within the viral precursors, to the (near) exclusion of reactions with cellular proteins. Nearly all $3C^{pro}$ viral cleavage sites are at Gln-Gly or Gln-Ser sequences, but Glu-Ser, Glu-Asn and Gln-Ala substrates are found in some viruses. The EMC $3C^{pro}$ sequence is proteolytically active in each of its precursor forms (P3, 3ABC, 3CD). The use of each form, as well as the polyprotein sequence context and protein structure, have important regulatory roles that govern the orderly release of viral proteins throughout the infectious cycle; indeed, every aspect of the viral replication is strongly influenced, if not determined, by the rates at which individual polyprotein sites are selected and processed by the $3C^{pro}$ enzyme and its precursors' autocatalytic activities. Mutations that impinge on these pathways are invariably fatal, though sometimes for reasons not anticipated from study of mature protein products. For example, the proper binding of replicase precursor proteins (P2 + P3) to the 3′ UTR of the virion RNA probably helps display these substrates for efficient post-translational cleavage, and may even be an absolute requirement for $3C^{pro}$ cleavage at the 3A–3B site, a proteolytic reaction that is never observed in the absence of active RNA synthesis. Introduced mutations that prevent RNA synthesis also prevent cleavage of 3A–3B, even in the presence of $3C^{pro}$ or its precursors.

The final cleavage within picornaviral polyproteins, the maturation of the 1AB peptide (also called VP0), is not catalyzed by any identified viral or cellular proteases. Maturation reactions are normally observed *in vivo* only during the final stages of virion morphogenesis and are dependent upon RNA association into large capsid assembly structures. The cleavages occur at the interior surface of the protein capsid where it interfaces the RNA, leading to the assumption that the RNA itself may have a direct catalytic involvement. However, it is more likely that a transitional protein conformation, triggered by the unnaturally tight proximity of the packaged RNA, is responsible for VP0 cleavage. Mature cardiovirions contain 58–59 copies each (on average) of VP2 + VP4, the VP0 cleavage products, and an additional 1–2 copies of uncleaved VP0.

Polyprotein Organization

Cardioviral proteins and their precursors take their names (L, P1, P2, P3) from their locations within the polyprotein. The EMC and Mengo leader or 'L' proteins are about 7000 kDa in molecular weight and play an undefined role in host or tissue tropism. Genetically engineered Theiler's viruses with altered L sequences exhibit altered neurovirulence in mice. L may also be involved in translational regulation of the IRES. The four P1 peptides, 1A, 1B, 1C and 1D, are the capsid proteins. After incorporation into virions, they are called VP4, VP2, VP3 and VP1, respectively. VP0 is the uncleaved precursor of VP4 + VP2. The capsid proteins derived from a common P1 precursor stay together as a protomer unit throughout particle assembly. The P2 portion of the polyprotein contains peptides 2A, 2B and 2C (in Mengo: 16000, 17000, and 36000 kDa). Beside encoding the primary cleavage peptide, and possible roles in RNA synthesis, the functions of 2A and 2B are unknown. Protein 2C is the genetic locus of the guanidine resistance marker, a compound that affects the initiation of RNA synthesis. All cardiovirus 2C sequences contain RNA helicase and nucleotide-binding motifs. However, 2C is not a polymerase, and its contribution to the replication cycle remains unclear. The P3 peptides, 3A, 3B, 3C and 3D (in Mengo: 10000, 2000, 22000, and 51000 kDa) are more closely associated with RNA replication. Preparations of $3D^{pol}$ can catalyze elongation of nascent RNA chains in primer-dependent reactions, an activity that identifies this enzyme

as the central element of viral polymerase complexes. Protein 3B is VPg, the peptide attached to the 5′ end of the genome. Initiation of positive- and negative-strand RNA synthesis requires VPg, perhaps as free protein, or as part of a donor peptide, 3AB. Protein $3C^{pro}$ is the viral protease described above, and is the primary catalyst of the polyprotein cleavage cascade.

Initiation of Infection

The cardiovirion initiates infection by attaching to a specific cell surface glycoprotein receptor. Interactions between the receptor and amino acid residues in the 'pit' cause conformational changes which affect the pentamer–pentamer contacts and dispose the capsid to dissociation. Polio- and rhinovirions undergo a two-stage dissociation process: after attachment and conformational alteration, these virions are taken into the cell in endocytic vesicles, and subsequent acidification is required to release VPg-RNA from the capsid and into the cytoplasm. Such a two-stage process may not apply to the cardioviruses. The noncovalent pentamer–pentamer interactions which maintain the integrity of the cardiovirus capsid are much less extensive than those in other picornavirions: either interaction with a cellular receptor or decreasing the pH of a physiological salt solution will cause the cardiovirion to dissociate into pentamers, with the release of VPg-RNA and the VP4 polypeptides. Also, treatment of cells with chemicals which raise the pH of endosomes causes a significant reduction in the infectivity of polio- or rhinovirions, but not of cardiovirions. It has been proposed that a cardiovirion is uncoated at the cell plasma membrane as an immediate consequence of binding to the receptor, and that VPg-RNA enters the cytoplasm, perhaps with the assistance of myristoylated VP4s, directly from that site.

Synthesis of Components for Progeny Virions

Once in the cellular cytoplasm, the infecting viral RNA (with its VPg removed) acts as messenger RNA. The translation of this RNA into a polyprotein and the proteolytic processing of the polyprotein have been discussed. The infecting RNA must also be able, at some stage, to free itself from the translation machinery and act as a template for the synthesis of a complementary (minus-strand) RNA. How this is accomplished is not known. The minus-strand RNA acts as a template for the synthesis of progeny plus strands (virion RNAs), with several plus strands being synthesized simultaneously. These structures can be isolated from endoplasmic membrane fractions

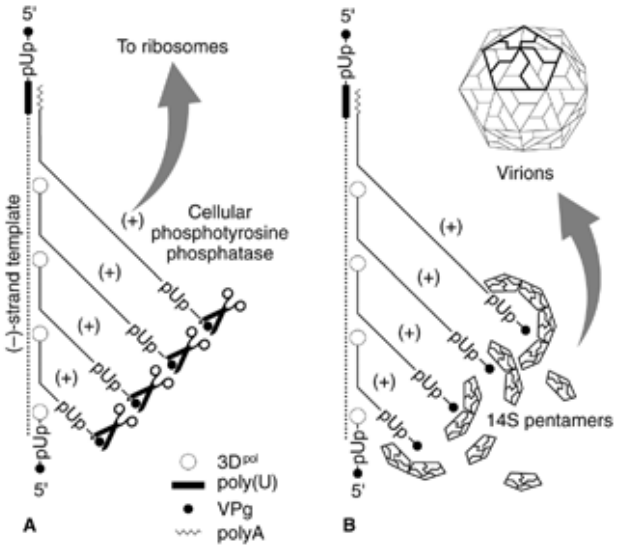


Figure 3 Regulation of cardiovirus replication. **(A)** At early times after infection (0–5 h), the cellular phosphotyrosine phosphatase has easy access to the nascent plus-strands of viral RNA in RI replicative intermediate structures. It removes the 5′-VPg proteins from these RNAs, making them templates for continuing viral protein biosynthesis. **(B)** At later times (5–10 h), 14S pentamers accumulate and compete with the phosphatase for the VPg RNAs. As time goes on, more progeny plus-strand RNAs from the RIs are packaged into progeny virions, and fewer become mRNAs.

of infected cells, and are called replicative intermediates (RIs; **Fig. 3**). The proteins encoded by the P2 and P3 region of the viral genome are responsible for RNA replication: VPg initiates each newly synthesized molecule, plus or minus strand, and $3D^{pol}$ is the elongation polymerase. There is a dramatic asymmetry in RNA synthesis, with a large excess of plus strands and no evidence for RIs composed of a plus-strand template and nascent minus strands. The mechanism(s) by which RNA synthesis is regulated is not understood.

Inhibition of Host Cell RNA and Protein Synthesis

Cellular RNA and protein biosynthesis begin to decline within the first few hours after cardiovirus infection, at about the time viral RNA synthesis enters its exponential phase. The decrease in cellular RNA synthesis, which can fall to ~10% of normal, depending upon the type of cell infected, may reflect a competition among polymerases for ribonucleoside triphosphates. The decrease in host cell protein synthesis is similar in magnitude, and is caused primarily by the ability of the IRES in the 5′-noncoding region of the viral RNA to outcompete capped cellular mRNAs for ribosomes and initiation

factors. There is no virus-induced cleavage of the p220 component of the cap-binding complex, eIF–4F, in cardiovirus-infected cells; this is in contrast to the situation in cells infected with poliovirus, rhinovirus or FMD viruses. Cardioviruses may gain their competitive advantage in part by causing modifications to ribosomes (an inhibitor of the translation of capped mRNAs *in vitro* can be washed off ribosomes from Mengo virus-infected cells) and/or to eIF2 (partial phosphorylation results in partial inactivation with respect to cellular, but not viral, mRNAs).

Assembly of Progeny Virions

One molecule each of VP0, VP3 and VP1 generated by $3C^{pro}$ cleavage of a P1 precursor remain together as a capsid protomer, which sediments at 6 S and can be isolated. The proteolytic release of L from P1 during the $3C^{pro}$ processing cascade, allows the N-terminal sequence of P1 (G-N-S-T-S-) to be recognized by a cellular enzyme and *N*-myristoylated. This covalent modification, and the $3C^{pro}$-mediated cleavages between VP0 and VP3, VP3 and VP1 and VP1 and 2A, must be completed before the protomer can build further. Five mature protomers associate by (largely) hydrophobic interactions to form the pentameric assembly unit, which sediments at 14 S. Cardiovirus pentamers will not form empty capsids in infected cells because such structures are intrinsically unstable; therefore, virion assembly may begin with a specific attachment of VPg RNA, extruded from active replication complexes, to a free pentamer. It is possible that protein–protein interaction between VPg and the inner surface of the pentamer (the *N*-myristoyl moieties may be involved) is the triggering event. Once initiated, the assembly process continues with the addition of another 11 pentamers, the condensation and enclosure of the RNA and the RNA-dependent cleavage of 58–59 VP0 molecules to VP4 + VP2.

Assuming that a pentamer–RNA interaction may initiate progeny virion assembly, overall regulation of the viral replicative process could be achieved as shown in **Fig. 3.**

Geographic Distribution and Host Range

Cardioviruses have a worldwide distribution and are associated with a wide variety of species including humans, other primates, domestic animals, rodents, birds and mosquitoes. A study done in Hawaii in 1978 found EMC virus-neutralizing antibodies in humans (6% of individuals tested), cats (3%), cows (20%), pigs (23%), mongooses (36%), rats (36%), boobies (9%) and shearwaters (20%). The natural reservoir of these viruses would appear to be rodents, with susceptibility to infection varying among species. Rats or mice, which can be infected orally, will contact a mild illness and recover (unless the virus dose is high). They can transmit the virus to other species; for example, an epizootic outbreak of EMC virus disease among pigs in New South Wales in 1986, which affected 37 separate piggeries, coincided with an infestation of mice.

In pigs, cardiovirus infection is associated with reproductive failure or abortions in sows, and systemic infections of newborn or young animals with resultant high mortality (15–20%). From 1986 to 1991, outbreaks were reported in Greece, Minnesota, Western Australia and Quebec. A survey done in Italy in 1989 estimated that approximately 70% of the total swine population in that country were seropositive for EMC virus.

Virus Propagation

Laboratory animals such as mice, hamsters, gerbils and cotton rats can undergo a rapid and fatal infection after the administration of virus by any route. Guinea pigs usually develop a fever, which may be followed by hind limb paralysis. Subcutaneous inoculation of baboons or African green monkeys with 10^6 PFU of EMC virus causes myocardial and central nervous system damage leading to death within a week, whereas rabbits or albino rats acquire an inapparent infection at the same dose level. Cardioviruses grow well in a wide variety of cultured cells, including: primary mouse embryo fibroblast, adrenal or myocardial cells; Ehrlich or Krebs ascites tumor cells; L-929 mouse fibroblast cells; human cell lines such as HeLa or HEp-2; BHK (hamster) cells; and guinea pig embryo fibroblasts or tumor cell lines. They will also grow in embryonated eggs, but not in chick embryo fibroblasts. An abortive infection of Maden–Darby bovine kidney cells by Mengo virus has been described; these cells exhibit the usual cytopathology and eventually die, but progeny virus production is minimal (1000-fold less than in L-929 cells).

Genetics, Evolution and Serotype Stability

The complete nucleotide sequences of the genomic RNAs of EMC (strains R, B and D) and Mengo (strain M) viruses have been determined and many of these sequences have been cloned into infectious cDNAs. A comparison of these genomes reveals the evolutionary constraints on the propagation of naturally occurring mutations. Mutations may arise

Table 2 Differences in genomic nucleotide sequences and in polyprotein amino acid sequences between EMC(R) and Mengo(M) viruses

	Nucleotides		*Amino acids*	
Genome segment[a]	*Changes*	*%*	*Changes*	*%*
Total genome[b]	1453	18.9	—	—
Polyprotein	1383	20.1	148	6.5
5′ NTR[b]	56	6.6	—	—
L region	35	17.4	5	7.5
P1 region	517	20.7	25	3.0
P2 region	368	19.9	65	8.6
P3 region	463	20.0	65	8.4
3′ NTR	14	11.2	—	—

[a] The subdivisions of the genome are illustrated in **Fig. 2.**
[b] The comparison does not include the length differences in the poly(C) tract in the 5′ NTR (nontranslated region).

during viral RNA replication because the polymerase ($3D^{pol}$) lacks a 3′–5′ proofreading exonuclease activity; the mutation rate has been estimated to be about one incorrect nucleotide per 10^4 incorporated, or about one per progeny genome. Nevertheless, the common laboratory strains of EMC and Mengo have changed very little in their genomic sequence, relative to their original isolates, during more than 40 years of replication in cultured cells.

As **Table 2** shows, there is an overall divergence of nearly 20% between the EMC and Mengo genomic RNAs at the nucleotide level. In the capsid protein-coding (P1) region, however, only 3% of the amino acid residues are different, i.e. in this region nucleotide changes have been almost exclusively restricted to third positions in codons. Thus, there is little flexibility in capsid or nonstructural protein design among the cardioviruses, and a great pressure to maintain a common serotype.

Transmission and Tissue Tropism

Natural infections are acquired by the ingestion of virus-contaminated material. Gastrointestinal disease of infected mice, rats, squirrels or voles remains limited and is quickly resolved. When swine are infected per os, the virus replicates in the gut and is excreted in the feces. Viremia is detectable within a few days, and an unchecked infection can result in acute myocarditis and sudden death. Cardiovirus infections in breeding sows can result in stillborn or mummified fetuses; surviving piglets will suffer (and usually die) from interstitial pneumonia, meningoencephalitis and/or myocarditis. Surviving animals develop high levels of neutralizing antibodies, and possess immunity to reinfection. There have been reports that humans are susceptible to cardiovirus-induced disease. Dick, when first isolating Mengo virus, suffered 'an acute febrile illness characterized by signs and symptoms referable to the central nervous system' and virus was recovered from his serum. From 1949 to 1953, EMC virus isolates were reported from individual patients with Guillain–Barré syndrome, asceptic meningitis or paralytic poliomyelitis. In 1945–1946, an epidemic of a relatively mild '3 day fever' afflicted American soldiers stationed in Manila. EMC virus-neutralizing antibodies were found in a significant number of convalescent patients. Whether cardioviruses were primary pathogens in any of these cases is debatable; previous subclinical infection with these viruses is a plausible alternative. Few specific human illnesses associated with cardioviruses have been noted in recent years, despite the fact that many laboratory workers (and probably farmers and veterinarians) in contact with EMC or related viruses have developed neutralizing antibodies.

Pathogenicity in Laboratory Mice

Notwithstanding differences in virulence among laboratory strains of cardiovirus, and diversities in response to these viruses by genetically distinct strains of mice, inoculation by any route (i.p., i.v., i.m. or s.c.) generally leads to a severe and usually fatal encephalitis. As few as 100 PFU can be lethal. High titers of virus are recoverable from brain tissues, and a common sequela of infection is an acute, moderate to severe myocarditis. If virions are inoculated directly into the brain, 10 PFU or less will cause neurological symptoms followed by hind limb paralysis and death within 7–10 days.

In 1968, Craighead discovered a strain of EMC virus which, following intraperitoneal inoculation, produced diabetes mellitus in 'adult male mice'. Subsequent investigations of the EMC(D) variant revealed that it causes diabetes by specifically destroying the pancreatic β cells. Neither the relatively low interferon-inducing ability of EMC(D) nor an autoimmune destruction of the β cells appear to be major factors in the development of diabetes in EMC(D)-infected mice. RNA sequence comparisons between a nondiabetogenic strain (B) of EMC virus and the diabetogenic strain have implicated a mutation causing an amino acid change at residue 152 of capsid protein VP1 (T in EMC(B), A in EMC(D)). This residue is located on the surface of the virion, along one edge of a depression, or 'pit', which is most likely to form the binding site for a cellular receptor. Thus, it appears to be the unique ability of the EMC(D)

virus to bind to, infect and destroy pancreatic β cells, which eventually leads to diabetes in laboratory mice.

Cytopathology of Infected Cells

Electron micrographs of picornavirus (including cardiovirus)-infected cells reveal a characteristic cytopathological picture, the most striking feature of which is the dramatic proliferation of endoplasmic smooth membranes and vacuoles. As the infectious process continues, these vacuoles accumulate in the central part of the cell, displacing and compressing the nucleus. Virus replication occurs in the cytoplasm, with the smooth membranes providing anchorages for viral RNA replication complexes. Quasi-crystalline arrays of progeny virions can be visualized in the peripheral cytoplasm before the cell membrane ruptures at multiple sites and the cellular integrity is lost (8–12 h after infection). The yield of progeny virions per infected cell is about 100 000.

Immune Response

In mice, recovery from cardiovirus infection involves the production of virus-neutralizing antibodies. These bivalent reagents may reduce the infectivity of virions by crosslinking several particles into one effective unit, by crosslinking two pentameric structure units in a single virion and inhibiting its uncoating after contact with a susceptible cell, or by causing a conformational change in the viral capsid such that the receptor-binding sites are altered or occluded. Neutralization-resistant mutants, selected *in vitro* in the presence of monoclonal antibodies, have been used to map antibody-attachment sites (via RNA sequence comparisons) in the capsid structure. Such sites are found on surface loops or ridges, and usually comprise amino acid residues from more than one polypeptide (discontinuous epitopes).

Only mice with fully developed and functional immune systems have any likelihood of surviving an intraperitoneal injection of cardioviruses. Short-term immunity can be transferred to naive animals by injection of neutralizing IgG, but cell-mediated responses are crucial to recovery and to the development of long-term immunity. Studies using synthetic peptides to stimulate the proliferation of lymph node leukocytes from mice immunized with UV-inactivated Mengo virus have identified segments in VP2 (residues 2118–2132) and VP3 (3051–3063) as containing T cell epitopes. The cells that were stimulated to proliferate were CD4+, and the response was not H-2 restricted. No T cell epitopes were found in the VP1 or VP4 proteins.

Prevention and Control of Cardiovirus Infections

Given that the introduction of cardioviruses to domestic swine, zoo populations or captive primate colonies can constitute a considerable veterinary and economic problem, it would be desirable to have efficacious cardiovirus vaccines available to prevent epidemics.

In 1989, Duke and Palmenberg prepared several Mengo cDNAs in order to determine the nucleotide sequence of the genome. Their enzymatic procedures accidentally truncated the poly(C) tracts in the 5′ UTRs of several cDNAs (e.g. $C_{44}UC_{10}$ became $C_{13}UC_{10}$). The RNA transcripts from these cDNAs were discovered to be infectious upon transfection into HeLa cells, and genetically stable progeny virions could be propagated in the same manner as the wild-type isolates. However, these short poly(C) tract viruses, as well as subsequent, deliberately engineered isolates with deleted poly(C)s, were found to be significantly attenuated for virulence for laboratory mice, and at the same time to stimulate the production of high titer, long-lived neutralizing antibodies in innoculated animals. The truncation of the poly(C) apparently renders the virus suceptible to the full range of antiviral activities by the host, and even an initial injection of 10^{11} PFU (e.g strains vMC_{24} or vMC_0) is not enough to overcome the animal's immune response. The attenuated strains have proven safe and efficacious live vaccines in a wide variety of domestic and zoo animals (11 species of primates, pigs, tapirs, babirusa, peccaries, guanacos and porcupines), and can probably protect any animal (or human) suceptible to cardiovirus infection.

Acknowledgement

In the fall of 1997, Douglas Scraba passed away after a heroic bout with cancer. Honest and enthusiastic, with a positive influence on everyone he encountered, Doug was passionate about his research, and his family. This entry contains many of his seminal contributions that helped define the field of cardiovirology. An irreplacable colleague who is deeply missed, Doug has the lasting respect of everyone who had the privilege to work with him.

Ann C. Palmenberg

See also: **Immune response: Cell mediated immune response, General features; Polioviruses (*Picornaviridae*): General features, Molecular biology; Theiler's viruses (*Picornaviridae*); Viral receptors; Virus structure: Atomic structure, Principles of virus structure.**

Further Reading

Luo M, Vriend G, Kaner G *et al* (1987) The atomic structure of Mengo virus at 3.0 Å resolution. *Science* 235: 182.

Palmenberg AC (1989) Sequence alignments of picornaviral capsid proteins. In: Semler B and Ehrenfeld E (eds) *Molecular Aspects of Picornavirus Infection and Detection*, p. 211. Washington: American Society for Microbiology.

Rueckert RR (1990) Picornaviridae and their replication. In: Fields BN *et al* (eds) *Virology*, 2nd edn, p. 507. New York: Raven Press.

Scraba DG (1990) Functional aspects of the capsid structure of Mengo virus. *J. Struct. Biol.* 104: 52.

Warren J (1965) Encephalomyocarditis viruses. In: Horsfall FL and Tamm I (eds) *Viral and Rickettsial Infections of Man*, p. 562. Philadelphia: Lippincott.

CARLAVIRUSES

Sergei K Zavriev, Institute of Agricultural Biotechnology, Moscow, Russia

History

The carlaviruses acquired their name from the then best studied type member of this group, *Carnation latent virus* (CLV). The carlavirus-associated diseases of the infected plants usually are mild and in many cases symptomless. The first report of a carlavirus-linked infection dates back to 1923 when Schultz and Folsom described a leafrolling mosaic of American potato variety Green Mountain. Later this disease was found to be associated with a carlavirus potato virus M (PVM). Until recently carlaviruses did not attract special attention of phytopathologists since, with rare exceptions, they do not cause serious plant diseases – a characteristic feature of carlaviruses is the latency of infection. The finding of potato virus S (PVS) in 1964 prompted active studies on this virus group, because it turned out that PVS is found worldwide and infects most of the commercial potato varieties.

Taxonomy and Classification

Definite as well as possible or poorly characterized members of the genus *Carlavirus*, *Closteroviridae* family, are listed in **Table 1.** Many viruses have been included in the genus *Carlavirus* based mainly on their serological relatedness with CLV and their host range. The taxonomic status of a number of them still has to be verified. For example, a detailed molecular-biological study of the narcissus latent virus, which has always been considered a definite member of the genus *Carlavirus*, has shown that it has properties distinct from those of any previously described virus genus.

Virus Structure and Composition

The virions of carlaviruses are flexible rods of modal length 610–690 nm and diameter 12–14 nm. The fine structure of carlaviruses is characterized by rows of subunits separated by about four longitudinal furrows. Crossbanding and an axial canal are usually not visible. The viral particles are composed of 1600–2000 coat protein subunits of M_r 3.2×10^3–3.8×10^3 and a linear single-stranded RNA of M_r 2.3×10^6–3.0×10^6, arranged as a helix (pitch 3.3–3.4 nm) and constituting 5–7% of the particle weight. Purified preparations of some carlaviruses (potato virus S (PVS), Helenium Virus S (HVS)) contain small amounts of encapsidated subgenomic RNAs (sgRNAs).

Physicochemical Properties

The physicochemical properties of carlaviruses are known for only a few members. The thermal inactivation point for most carlaviruses is between 55 and 70°C, and the infectivity in sap at room temperature remains from 2 to 5 days. The UV-absorbance spectra of carlaviruses have maxima at 258–260 nm, and minima at 243–248 nm, with A_{max} / A_{min} ratios of 1.1–1.3. Particles sediment at 147–176S, and have a buoyant density in CsCl of 1.31–1.33 g cm^{-3}.

The carlavirus virions can be broken down with denaturing agents such as sodium dodecyl sulfate (SDS), pyridine, pyrrolidine, guanidine hydrochloride, urea, acetic acid or alkali. In most viruses of this group the capsid protein is partly degraded during purification and storage of virus preparations, which may give rise to errors in determining the molecular mass of the coat protein.

Further Reading

Luo M, Vriend G, Kaner G *et al* (1987) The atomic structure of Mengo virus at 3.0 Å resolution. *Science* 235: 182.

Palmenberg AC (1989) Sequence alignments of picornaviral capsid proteins. In: Semler B and Ehrenfeld E (eds) *Molecular Aspects of Picornavirus Infection and Detection*, p. 211. Washington: American Society for Microbiology.

Rueckert RR (1990) Picornaviridae and their replication. In: Fields BN *et al* (eds) *Virology*, 2nd edn, p. 507. New York: Raven Press.

Scraba DG (1990) Functional aspects of the capsid structure of Mengo virus. *J. Struct. Biol.* 104: 52.

Warren J (1965) Encephalomyocarditis viruses. In: Horsfall FL and Tamm I (eds) *Viral and Rickettsial Infections of Man*, p. 562. Philadelphia: Lippincott.

CARLAVIRUSES

Sergei K Zavriev, Institute of Agricultural Biotechnology, Moscow, Russia

History

The carlaviruses acquired their name from the then best studied type member of this group, *Carnation latent virus* (CLV). The carlavirus-associated diseases of the infected plants usually are mild and in many cases symptomless. The first report of a carlavirus-linked infection dates back to 1923 when Schultz and Folsom described a leafrolling mosaic of American potato variety Green Mountain. Later this disease was found to be associated with a carlavirus potato virus M (PVM). Until recently carlaviruses did not attract special attention of phytopathologists since, with rare exceptions, they do not cause serious plant diseases – a characteristic feature of carlaviruses is the latency of infection. The finding of potato virus S (PVS) in 1964 prompted active studies on this virus group, because it turned out that PVS is found worldwide and infects most of the commercial potato varieties.

Taxonomy and Classification

Definite as well as possible or poorly characterized members of the genus *Carlavirus*, *Closteroviridae* family, are listed in **Table 1.** Many viruses have been included in the genus *Carlavirus* based mainly on their serological relatedness with CLV and their host range. The taxonomic status of a number of them still has to be verified. For example, a detailed molecular-biological study of the narcissus latent virus, which has always been considered a definite member of the genus *Carlavirus*, has shown that it has properties distinct from those of any previously described virus genus.

Virus Structure and Composition

The virions of carlaviruses are flexible rods of modal length 610–690 nm and diameter 12–14 nm. The fine structure of carlaviruses is characterized by rows of subunits separated by about four longitudinal furrows. Crossbanding and an axial canal are usually not visible. The viral particles are composed of 1600–2000 coat protein subunits of M_r 3.2×10^3–3.8×10^3 and a linear single-stranded RNA of M_r 2.3×10^6–3.0×10^6, arranged as a helix (pitch 3.3–3.4 nm) and constituting 5–7% of the particle weight. Purified preparations of some carlaviruses (potato virus S (PVS), Helenium Virus S (HVS)) contain small amounts of encapsidated subgenomic RNAs (sgRNAs).

Physicochemical Properties

The physicochemical properties of carlaviruses are known for only a few members. The thermal inactivation point for most carlaviruses is between 55 and 70°C, and the infectivity in sap at room temperature remains from 2 to 5 days. The UV-absorbance spectra of carlaviruses have maxima at 258–260 nm, and minima at 243–248 nm, with A_{max} / A_{min} ratios of 1.1–1.3. Particles sediment at 147–176S, and have a buoyant density in CsCl of 1.31–1.33 g cm^{-3}.

The carlavirus virions can be broken down with denaturing agents such as sodium dodecyl sulfate (SDS), pyridine, pyrrolidine, guanidine hydrochloride, urea, acetic acid or alkali. In most viruses of this group the capsid protein is partly degraded during purification and storage of virus preparations, which may give rise to errors in determining the molecular mass of the coat protein.

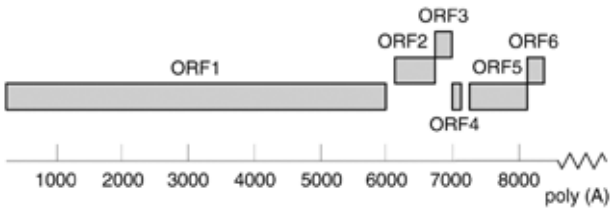


Figure 1 Schematic representation of the general structural organization of the carlavirus genome.

The concentration reached by carlaviruses in plant sap is moderate; dilution end point is usually 10^{-3}–10^{-4}. For PVS and red clover vein mosaic virus (RCVMV) in some hosts it reaches 10^{-6}. The purified viral RNA is far less infective than the intact virus containing an equimolar amount of RNA (with PVS the infectivity of the RNA is only 0.18%).

Genome Structure

The genome of carlaviruses consists of a monopartite plus-sense single-stranded (SS) RNA, which is encapsidated in the virus particles. To date, we know the complete genome structure of three carlaviruses, potato virus M (PVM) (8534 nucleotides (nt) long), blueberry scorch virus (BBSV) (8514 nt long), and garlic common latent virus (GaCLV) (8353 nt long) and extensive 3′ terminal regions of PVS (strains O and An), poplar mosaic virus (POPMV), lily symptomless virus (LSV), chrysanthemum virus B (CVB), HVS, carnation latent virus (CLV), shallot latent virus (SLV) and cowpea mild mottle virus (CPMMV). The RNA of carlaviruses is polyadenylated at the 3′ end, and there are several indirect evidences that it is capped at its 5′ end. The genome structures of the carlaviruses mentioned is very similar (with the only exception of SLV in some parameters), and can be schematically represented as in **Fig. 1.** The open reading frame (ORF) 1 codes for the putative virus-specific RNA replicase with a M_r 223 000 (for PVM and BBSV) and 219 000 (for GaLV).

Sequence analysis of the ORF 1 polypeptide has revealed that this product also contains putative methyltransferase and NTP-binding/helicase domains. In addition, it has been demonstrated that *in vitro* BBSV ORF 1 protein is autocatalytically processed by a papain-like proteinase. ORFs 2, 3 and 4 (the triple gene block) code respectively for proteins of M_r about 25 000, 12 000 and 7000 which are most probably involved in the virus transport and cell membrane modification. As with other triple gene block-containing viruses the ORF 2 protein contains a duplicate of the NTP-binding/helicase domain. All triple gene block proteins are possibly expressed *in vivo* in very small amounts – none of them could have been detected in extracts from carlavirus-infected plants. ORFs 2 and 3 proteins contain highly hydrophobic regions and may associate with membranes *in planta*. It has been shown that all of the triple block proteins of white clover mosaic potexvirus are required for transport. Since the design of the triple gene block of carla- and potexviruses is almost identical, it can be suggested that all of the triple gene block proteins of carlaviruses are involved in the virus transport.

ORF 5 encodes the virus coat protein of M_r 32 000–36 000, depending on the individual carlavirus. ORF 6 encodes a cysteine-rich protein of M_r 12 000–15 000 which contains a zinc finger motif and in the case of PVM has been shown to bind with single- and double-stranded nucleic acids *in vitro*. Genes encoding cysteine-rich proteins are also found in hordei-, furo- and tobravirus groups. The possible functions suggested for this protein are the ability of the virus to be vectored, the assistance in initiating viral assembly, and a regulatory role in the transcription or/and translation of the genome.

The viral RNA is flanked by noncoding regions; there are also two short internal noncoding regions between ORF 1 and ORF 2, and between ORF 4 and ORF 5. In CVB there is an additional 17 nt-long noncoding region between ORF 5 and ORF 6. Both the 3′ and 5′ noncoding RNA regions of carlaviruses sequenced to date are highly conserved. The 5′ terminal nontranslated leaders are less than 80 nt long, and the corresponding 3′ terminal non-coding regions vary in length from 51 to 107 nt, terminating in the poly(A) tail.

The only carlavirus for which a full-length infectious cDNA clone has been reported is BBSV.

The overall genome organization of carlaviruses is very close to that of potexviruses, with the exception that the latter lack ORF 6, and shallot virus X (ShVX) related viruses, which have an additional 40 000 M_r ORF between the triple gene block and the coat protein gene. There is a high level of homology in the amino acid sequences of the corresponding proteins within the carlavirus group, as well as between the carla-, potex- and ShVX-related viruses. It has been suggested that carlaviruses, potexviruses and ShVX-related viruses form a 'supergroup' of related plant viruses.

Viral Multiplication

Little is known of the carlavirus RNA replication mechanism. Carlavirus-infected plants contain double-stranded replicative forms of RNA (dsRNA) corresponding in molecular mass to genomic viral

RNA. Thus virus-specific dsRNAs of 5.5 MDa have been found in plants infected with RCVMV, pea streak virus (PeSV), dandelion latent virus (DLV), PVS, KLV, and CPMMV, and a dsRNA of 5 MDa in those infected with PVM. In all probability, besides the virus-encoded RNA replicase, the replication complex includes several host-cell factors.

Genome Expression

Only the virus-encoded RNA replicase (the ORF 1 protein) appears to be expressed from the genomic carlavirus RNA, whereas the other genes are expressed with the help of two major sgRNA about 3.0 and 1.5 kb long, which have been demonstrated in carlavirus-infected (PVS, PVM, HVS and BBSV) plants by Northern blot hybridization. Both sgRNA species identified are 3′ co-terminal to the genomic RNA and polyadenylated. It is believed that the ORF 2, ORF 3 and ORF 4 proteins are expressed from the long sgRNA, whereas the coat protein (the ORF 5 protein) and the ORF 6 protein from the short sgRNA. Possible promoters for the sgRNA synthesis have been identified for several carlaviruses – the sequence C/UUUAGGU, 19–43 residues upstream from both putative sgRNA initiation sites is suggested to be a part of the promoter region. No minor sgRNAs for expression of ORFs 3, 4 and 6 proteins were detected in carlavirus-infected plants. In case of PVS the 101 nucleotide-long sequence upstream from the AUG initiation codon of the coat protein gene was shown to act as a translational enchancer *in vitro* and *in vivo*.

Translation

The data on genomic RNA translation are available for PVS, PVM, CLV, BBSV, AHLV and HVS. Translation of PVM RNA *in vitro* (rabbit reticulocyte lysates) and *in vivo* (*Chenopodium quinoa* protoplasts) gives rise to a heterogeneous set of proteins, the largest of which has an M_r of 185 000–190 000. No viral coat protein is translated *in vitro* from the genomic RNA. The virion RNA of PVS (Andean strain) *in vivo* in the protoplasts of *Solanum tuberosum* directs the synthesis of three major products with M_r of 123 000 and 104 000 and the 34 000 M_r capsid protein. *In vitro* translation of the virion RNA of PVS (Peruvian strain) in rabbit reticulocyte lysates produces the coat protein and several high M_r polypeptides, the longest corresponding to M_r 130,000. These data confirm the suggestion that the ORF 1 polyprotein contains a proteinase domain, which provides for the autocatalytic processing step in the carlavirus translation strategy. Expression of the PVS coat protein from the virion RNA *in vitro* is most probably due to the presence in the virions of some sgRNA for the coat protein. Genomic HelVS RNA *in vitro* produces 190 000 and 160 000 M_r proteins whereas the coat protein is only translated from the appropriate 1.5 kb-long sgRNA.

For the 3′ terminal gene cluster (coat protein and the ORF 6 protein) of PVM, two *in vitro* translational strategies operating in the ORF 6 encoded gene have been identified. An internal initiation, resulting in the synthesis of an M_r 12 000 protein and a polypeptide as a coat protein and ORF 6 protein transframe product by ribosomal frameshifting. The products of about M_r 45 000–50 000 corresponding to the M_r of the coat protein and ORF 6 translational fusions, which can be immunoprecipitated with antisera against the virus particles, have been detected from the *in vitro* translational products of PVS, HVS and AHLV.

Viral Transmission

Carlaviruses are easily spread by vegetative propagation, transmitted mechanically and usually, nonpersistently, by aphids. The latter appears to be the most common way of transmission in nature. The efficiency of aphid transmission varies for different viruses and particular strains. Thus with PVM, PVS and PeSV the transmission efficiency for different strains varies greatly even with the same aphid species. No aphid or other vector has been found for Mulberry latent virus (MLV), Passiflora latent virus (PLV), PMV and CPMMV. For PMV, transmission by root grafts has been considered. As a rule, carlaviruses are not seed-transmitted, although for CPMMV it has been shown with three leguminous hosts that 2–90% of the seeds transmit the virus. Some carla-like viruses are transmitted by whiteflies. Particularly, transmission by whitefly *Bermisia tabaci* was shown for CPMMV.

Cytopathology

The distribution of carlaviruses in the infected plant is not tissue-specific; the particles are mostly found in the cytoplasm of various plant tissues, singly or more often in large aggregates which are sometimes banded. The virus particles have not been found in chloroplasts, nuclei or other organelles. They sometimes occur in bundles, or in groups with one end attached to membranes. Carlaviruses do not induce any pronounced cytopathological effects by which infection can be recognized. Infection with carlaviruses usually does not give rise to virus-specific inclusions in plant tissues. RCVMV is the only carlavirus that induces in infected plants crystalline cyto-

Table 1 Definite and possible members of the genus *Carlavirus*

Definite members	*Possible members*
American hop latent virus (AHLV)	Artichoke latent virus (ArLV)
Blueberry scorch virus (BBSV)	Caper vein binding virus (CapVBV)
Cactus virus 2 (CV-2)	Cardamine latent virus (CaLV)
Caper latent virus (CapLV)	Cassia mild mosaic virus (CasMMV)
Carnation latent virus (CLV)	Cassia brown streak virus (CasBSV)
Chrysanthemum virus B (CVB)	Chicory blotch virus (ChiBV)
Cowpea mild mottle virus (CPMMV) (tentative member)	Cole latent virus (CoLV)
Dandelion latent virus (DLV)	Cowpea mild mottle virus (CpMMV)
Elderberry carlavirus (ECV)	Cynodon mosaic virus (CynMV)
Garlic common latent virus (GaCLV)	Daphne virus S (DaVS)
Helenium virus S (HVS)	Dulcamara carlavirus A (DuCVA)
Honeysuckle latent virus (HnLV)	Dulcamara carlavirus B (DuCVB)
Hop latent virus (HpLV)	Eggplant mild mottle virus (EMMV)
Hop mosaic virus (HpMV)	Fig virus S (FVS)
Hydrangea latent virus (HdLV)	Fuchsia latent virus (FuLV)
Kalanchoe latent virus (KLV) (including strains named kalanchoe virus 1 and kalanchoe virus 2)	Gentiana carlavirus (GeCV)
Lilac mottle virus (LiMV)	Gladiolus carlavirus (GCV)
Lily symptomless virus (LSV)	Groundnut crinkle virus (GrCV)
Mulberry latent virus (MLV)	Gynura latent virus (GyLV)
Muskmelon vein necrosis virus (MuVNV)	Helleborus carlavirus (HeCV)
Nerine latent virus (NeLV)	Kalanchoe carlavirus (KaCV)
Passiflora latent virus (PLV)	Nasturtium mosaic virus (NasMV)
Pea streak virus (PeSV)	Plantain virus 8 (PlV8)
Pepino latent virus (PeLV)	Southern potato virus (SPV)
Poplar mosaic virus (PopMV)	Voandzeia mosaic virus (VoMV)
Potato virus M (PVM)	White bryony mosaic virus (WBMV)
Potato virus S (PVS)	
Red clover vein mosaic virus (RCVMV)	
Shallot latent virus (SLV)	
Sint-Jem's onion latent virus (SJOLV)	
Strawberry pseudo mild yellow edge virus (SPMYEV)	

plasmic inclusions consisting of protein and nucleic acid and containing spherical granules. CPMMV forms unusual brush-like intracellular inclusions.

Serology

In many cases the relatedness of a new virus to carlaviruses has been tested using an antiserum to CLV. Therefore, except for PMV, SLV and HLV all the viruses listed in **Table 1** are serologically close to CLV. Serological relationships among the intact particles of carlaviruses have been studied by different approaches which revealed similar groupings. A number of carlaviruses are more or less closely interrelated, with SDIs ranging from about 3.5 to 6.5, e.g. LV, CVB, HVS, PLV, PeSV, PeLV, PVM, PVS and CVMV. It is interesting that these viruses (PVS–VB and CLV–CVB) have different host ranges whereas two most remote pairs (RCVMV–PeSV and PVM–PVS) have similar host ranges.

Geographical Distribution

Most carlaviruses are found wherever their hosts are grown, some are restricted to certain parts of the world, and for others the distribution is somewhat nonuniform. Thus PeSV is found only in the USA, PVM is mostly encountered in the East European countries, and PVS is spread over practically all potato-growing regions worldwide.

Viral Epidemiology and Control

Carlaviruses usually produce symptomless infection in many plants. One of the main factors hindering virus detection is the lack of suit-

testing problem is especially important for carlaviruses infecting monocots. In this connection, carlavirus detection and testing mostly relies on serological and electron-microscopic methods. For industrial-scale virus control in the seed material, ELISA kits are commercially available for PVS and PVM. AGDIA, Inc. (IN, USA) also offers ELISA kits and reagents for BBSV, CLV, CVB, KLV, LSV and SLV. A polymerase chain reaction test based on degenerate carlavirus-specific primers can be used for the universal detection of carlaviruses in infected plants. This test is available now from AGDIA Inc. Most countries producing seed potatoes include obligatory tests for PVS. Meristem tip culture is known to be the most promising technique for the production of virus-free mother plants in vegetatively propagated crops. This technique is often used in combination with thermotherapy and the use of antiviral compounds which are simply incorporated in the medium during thermotherapy – in the case of carlaviruses this approach is found to be especially effective. PVS is the only carlavirus for which transgenic plants (potato and *Nicotiana debneyii*) resistant to virus infection have been reported.

Host Range

The host range of individual viruses is rather narrow, but taken together the group encompasses a wide range of monocotyledonous and dicotyledonous hosts. One plant group in which the carlaviruses are not detected are serial crops – no one definite carlavirus is detected in these plants.

Symptomatology

Most carlaviruses develop latently in infected plants, which is reflected in the names of many members of this group (see **Table 1**). In some cases stunting and mosaic symptoms are produced. Sometimes latency is found in certain hosts but not in others. A severe reduction of flower and pod formation numbers has been observed on RCVMV-infected chickpea plants.

Economic Significance and Ecology

Although a large portion of carlaviruses cause symptomless infection, some of them (PMV and legume carlaviruses) in their natural hosts cause serious diseases such as chlorosis, wilting, growth retardation, etc. Therefore, PMV and carlaviruses afflicting pea and red clover are a considerable economic problem. It should be noted that though the natural host range of carlaviruses is rather narrow, under experimental conditions they can heavily afflict plants of various families. The severity of the plant disease also depends on the virus strain (isolate) and on the plant variety. The main studies on this problem have been carried out with PVM. Thus, the leafrolling mosaic strain and the paracrinckle strain of PVM cause diseases of many potato varieties, which result in significant yield losses. In mixed infection carlaviruses appear to be capable of potentiating the pathogenicity of the partner virus: thus a complex of SLV with leek yellow stripe potyvirus induce a serious form of necrotic stripe and sometimes causes death of infected leek plants.

Evolution

Analysis of the RNA nucleotide sequences of a number of carlaviruses (see Genome Structure) allows them to be related to the supergroup of Sindbis-like phytoviruses. On the basis of the primary structure of the functionally important conserved domains in proteins, first of all those encoded in the triple gene block, carlaviruses can be united with four groups of other representatives of Sindbis-like phytoviruses: potex-, furo-, hordei- and ShVX-related viruses (the latter is not yet distinguished as a separate group). Taking into account the primary structure and the general genomic organization of carlaviruses, they can be considered most closely related to potexviruses and ShVX-related viruses.

See also: **Latency; Potexviruses; Sindbis and Semliki Forest viruses (*Togaviridae*).**

Further Reading

Foster GD (1992) The structure and expression of the genome of carlaviruses. *Rev. Virol.* 143: 103.

Hillman BI and Lawrence DM (1994) In: Singh RP, Singh US and Kohmoto K (eds) *Pathogenesis and Host Specificity in Plant Diseases*, vol. 3. *Carlaviruses*, p. 35. New York: Pergamon.

Milne RG (ed.) (1985) *The Plant Viruses*, vol. 4. *The Filamentous Plant Viruses*. New York: Plenum Press.

Zavriev SK, Kanyuka KV and Levay KE (1991) The genome organization of potato virus M RNA. *J. Gen. Virol.* 72: 9.

CARMOVIRUSES (*TOMBUSVIRIDAE*)

Feng Qu and **T Jack Morris**, School of Biological Sciences, University of Nebraska, Lincoln, Nebraska, USA

Taxonomy, Classification and Evolutionary Relationships

Carmovirus is one of two officially recognized genera of *Tombusviridae*. Members of *Tombusviridae* all have icosahedral virions of about 30 nm in diameter with $T = 3$ symmetry that consists of 180 coat protein (CP) subunits of about 38–43 kDa and a single-stranded (ss) RNA genome ranging in size from 4.0 to 4.7 kb. Carmoviruses share recognizable sequence similarity in both the polymerase and structural genes with members of the other genus, *Tombusvirus*. Sequence comparisons and phylogenetic analysis of these genes support the view that the genera are distinct but closely related. In addition, the genome organization of the carmoviruses is characteristically different from that of the tombusviruses.

Carmoviruses contain a single-component positive-sense genome of about 4.0 kb. The genome, as exemplified by turnip crinkle virus (TCV) in **Fig. 1**, consists of five open reading frames (ORFs) which encode proteins of about 28, 88, 8, 9 and 38 kDa from the 5′ to the 3′ end, respectively. The virions are icosahedral and consist of 180 CP subunits of approximately 38 kDa. The genus name is derived from the first member of the genus to be sequenced, carnation mottle virus (CarMV). Much more detailed knowledge about virus structure and genome function is, however, known for TCV because its crystal structure has been determined and it was the first carmovirus for which infectious transcripts were produced from a cDNA clone of the genome. To date, the nucleotide sequences of six definitive carmoviruses have been determined (**Table 1**). These sequenced members share similar morphological and physicochemical properties with about 20 other viruses listed in **Table 1** that are recognized as species or tentative species, depending on the characterized molecular detail of the viruses. Unlike the majority of tombusviruses, carmoviruses are sufficiently distant from each other to prevent them crossreacting in standard RNA hybridization or serological tests.

Carmoviruses and tombusviruses share properties with numerous other small spherical RNA viruses. Structural similarities with members of the *Dianthovirus* genus (e.g. red clover necrotic mosaic virus, RCNMV) are significant and include sequence similarity of CP as well as similarity in subunit topology and interactions within the virion. There also exists a marked sequence similarity in the RNA-dependent RNA polymerase (RDRP) genes of carmoviruses and viruses of the following genera: *Dianthovirus, Necrovirus* (e.g. tobacco necrosis virus, TNV), *Machlomovirus* (e.g. maize chlorotic mottle virus, MCMV) and *Luteovirus* (e.g. barley yellow dwarf viruses of the PAV type). Several newly sequenced viruses have been proposed for consideration as new genera of *Tombusviridae,* based primarily on similarities of nucleotide sequences and genome organization (e.g. pathos latent virus, oat chlorotic stunt virus and panicum mosaic virus). In a broader context, phylogenetic comparisons of viral RNA polymerase genes have identified the *Tombusviridae* as a representative plant virus cluster for one of three RNA virus supergroups with relatedness to animal viruses of *Flaviviridae* and the family of small RNA phage (*Leviviridae*).

Distribution, Host Range, Transmission and Economic Significance

Carmoviruses occur worldwide and are generally reported to cause mild asymptomatic infections on relatively restricted natural host ranges. Most accumulate to high concentrations in infected tissues and are mechanically transmitted with facility. Beetle transmission has been reported for a number of members, as has transmission in association with soil and/or irrigation water, and in some cases in association with fungal zoospores. Most of the viruses have been reported to cause diseases in a limited number of plants or plant types.

A number of carmoviruses have been identified in association with ornamental hosts and have been widely distributed in such hosts by vegetative propagation. CarMV is the most noteworthy, being widespread in cultivated carnations, and recognized as one of the more important components of viral disease complexes in this crop worldwide. It accumulates to high concentrations without producing severe symptoms and spreads primarily by contact transmission and vegetative propagation. It has a broad experimental host range that includes over 30 species in 15 plant families. PFBV is widespread in vegetatively propagated *Pelargonium* species, causing disease in association with other viruses. The incidence of NTNV in

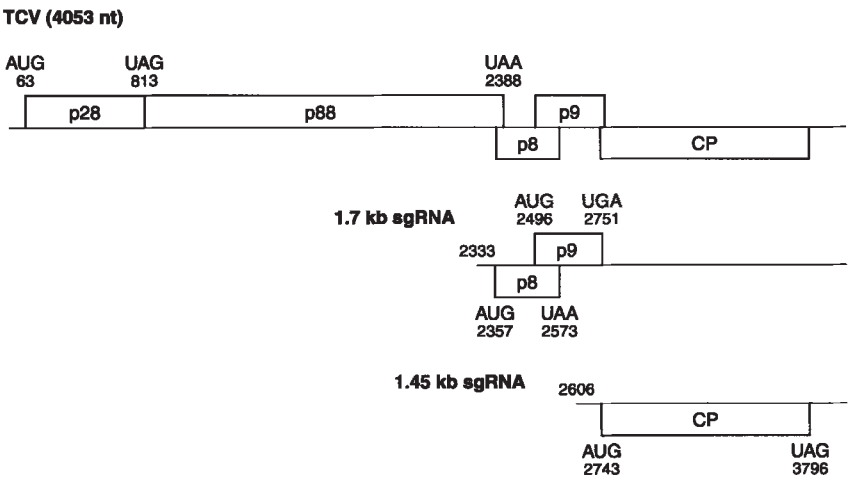


Figure 1 Genome organization of a typical carmovirus as represented by turnip crinkle virus. The genomic and subgenomic RNAs are depicted as solid lines and the sizes of the RNAs are noted. The ORFs are indicated by the boxes, with the numbers in the boxes identifying the polypeptide encoded by the ORF. The numbers above and below the diagrams give the transcriptional start sites for the sgRNAs and the nucleotide locations of the codons defining each ORF.

Narcissus cultivars and HCRSV in *Hibiscus* primarily reflects distribution of infected nursery stock.

Numerous small RNA viruses have been reported to naturally infect cucurbits, causing significant disease problems. Several of these viruses are recognized tombusviruses, while others such as MNSV, CLSV, CSBV have been identified as carmoviruses based on sequence and genome organization properties. MNSV occurs worldwide in greenhouse cucurbits and is both soil and seed transmitted, while CSBV has been primarily restricted to infrequent outbreaks around the Mediterranean. All three viruses have been reported to be transmitted in association with the fungus *Olpidium bornovanus*.

Several carmoviruses have been discovered in natural leguminous hosts, with GMoV being potentially the most important, causing serious disease losses in legumes in Africa. BMMV has been reported to be a latent virus widely distributed in bean cultivars in El Salvador, and BMoV has been found in *Vigna* species in Asia. Beetle vectors have been identified for these viruses, but seed transmission may also be an important factor in their distribution.

TCV is neither common nor widespread in nature in spite of the fact that it is reportedly beetle transmitted. It has a relatively wide experimental host range in some 20 plant families, including experimentally useful *Arabidopsis* and *Brassica* species, in which it accumulates to extremely high concentrations, often approaching a level equivalent to 0.5% of the fresh weight of the plant tissue. CCFV was first discovered in the Mount Kosiusko alpine region of Australia in *Cardamine lilacina*, a wild perennial *Brassica*. It has also been shown to infect *Arabidopsis* and other *Brassica* species.

Table 1 List of species in the genus *Carmovirus*

Sequenced species	
Carnation mottle	(CarMV)
Cardamine chlorotic fleck	(CCFV)
Cowpea mottle	(CPMoV)
Melon necrotic spot	(MNSV)
Saguaro cactus	(SCV)
Turnip crinkle	(TCV)
Classified species	
Ahlum water-borne	(AWBV)
Bean mild mosaic	(BMMV)
Cucumber leaf spot	(CLSV)
Cucumber soil-borne	(CSBV)
Galinsoga mosaic	(GaMV)
Hibiscus chlorotic ringspot	(HCRSV)
Pelargonium flower break	(PFBV)
Weddel water-borne	(WWBV)
Tentative species	
Blackgram mottle	(BMoV)
Elderberry latent	(ELV)
Glycine mottle	(GMoV)
Narcissus tip necrosis	(NTNV)
Plaintain 6	(PIV-6)
Squash necrosis	(SqNV)
Tephrosia symptomless	(TeSV)

Other carmoviruses have been isolated worldwide from natural hosts with little apparent disease and are presumably of little agricultural concern. These include TeSV from legumes in Kenya, GMoV from glycine in Australia, SCV from saguaro cactus in Arizona, GaMV from potato weed in Australia and PIV-6 from plantain weed in England. The infrequent isolation of these genetically similar viruses in remote locations around the world has prompted the speculation that ancestor carmoviruses may have been introduced into their natural hosts well before the last Ice Age and have since coevolved in isolation in their diverse host plants.

Virion Structure and Assembly

TCV is the only carmovirus for which refined structural studies, including high resolution X-ray crystallography, have been performed. The detailed information about CP structure and subunit–subunit interactions established that TCV and tomato bushy stunt tombusvirus (TBSV) share marked structural conservation. In this regard, the common structural features shared by other members of *Tombusviridae* have been primarily deduced from alignment of the amino acid sequence of the coat proteins of TBSV and TCV. TCV consists of a $T = 3$ icosahedral capsid of 180 subunits of the 38 kDa CP. The individual CP subunit folds into three distinct domains typical of the CP subunit of the tombusviruses. The relatively basic N-terminal R domain extends into the interior of the virus particle and presumably interacts with viral RNA. The R domain is connected by an arm to the S domain which constitutes the virion shell. The S domain is attached through a hinge to the P domain which projects outward from the virion surface. The protein subunits are believed to form dimers in solution and during assembly.

TCV is also the only carmovirus on which detailed *in vitro* assembly studies have been performed. The virion has been shown to dissociate at elevated pH and ionic strength to produce a stable RNA–CP complex (rp-complex) and free CP subunits. Reassembly under physiological conditions in solution could be demonstrated using the isolated rp-complex and the soluble CP subunits. This rp-complex, consisting of six CP subunits tightly attached to viral RNA, could be generated *in vitro* and was shown to be important in selective assembly of TCV RNA. A model for assembly was proposed in which three sets of dimeric CP interact with a unique site on the viral RNA to form an initiation complex to which additional subunit dimers could rapidly bind. Preliminary characterization of the 'origin of assembly' for this virus identified two possible sites based on the identification of RNA fragments in the rp-complex protected from RNase digestion by CP. Further *in vivo* studies narrowed the assembly origin site to a bulged hairpin loop of 28 nt within a 180 nt region at the 3′ end of the CP gene.

Genome Structure

Complete nucleotide sequences have been determined for six carmoviruses as listed in **Table 1**: CarMV (4003 nt), TCV (4053–4054 nt), MNSV (4262 nt), CCFV (4041–4072 nt), CPMoV (4029 nt), and SCV (3879 nt). Comparative studies of the deduced ORFs revealed that all these viruses encode a similar set of genes that are closely related and in the same gene order as illustrated for TCV in **Fig. 1.** The genome organization of the carmoviruses is quite compact, with most of the identified ORFs overlapping each other. Both the product of the most 5′ proximal ORF (26–28 kDa) and its readthrough product (86–89 kDa) have been shown to be essential for replication of the TCV genome. The 3′ proximal gene encodes the viral CP which varies from 37 to 42 kDa for the different viruses. All of the sequenced carmoviruses characteristically encode two small ORFs in the middle of the genome that have been shown in TCV to both be indispensable for cell-to-cell movement (movement proteins or MPs). Although the genome organizations of all sequenced carmoviruses are quite similar, there are some unique features evident in the individual carmoviruses. In CarMV, a second readthrough event could extend the translation of the polymerase gene into the p9 MP gene to produce a p98 product. This double readthrough event is not predicted for any other carmovirus. In another example, the two small central ORFs in MNSV (p7a and p7b) are connected by an in-frame amber codon that could result in the production of a 14 kDa fusion protein of the two ORFs. An unusual feature of the CPMoV sequence is the prediction of a sixth ORF (p28) nested within the 3′ proximal CP gene.

There is no direct evidence for the existence of a cap structure at the 5′ end of carmovirus genomes. Absence of a cap seems most likely given the recent identification of cap-independent translational enhancer sequences in the leaders of the genomic and subgenomic RNAs of TCV. The 5′ noncoding region varies from 34 nt in CPMoV to 88 nt in MNSV. No extensive sequence homology was observed within this region. The 3′ noncoding region of carmoviruses varies from about 200 to 300 nt in length and possesses neither a poly(A) tail nor a tRNA-like structure.

Replication and Gene Expression

Carmoviruses replicate to very high concentrations in protoplast infections, with the genomic RNA accumulating to levels approaching that of the ribosomal RNAs. Upon infection of susceptible plants, carmoviruses transcribe two 3′ coterminal subgenomic RNAs (sgRNAs) for expression of the MP and CP genes. The smaller sgRNA (*c.* 1.5 kb) is the mRNA for CP. The larger sgRNA (*c.* 1.7 kb) presumably functions as the mRNA for the two MP genes utilizing a leaky scanning mechanism. This is supported from *in vitro* translation experiments performed for TCV and SCV. Recent results involving transgenic expression of the p8 and p9 gene products of TCV in *Arabidopsis* plants has demonstrated that both of them are essential for viral cell-to-cell movement and that they function by *in trans* complementation in the same cell. It was also shown that cell-to-cell movement of TCV in *Arabidopsis* does not require the CP gene.

Viral specific double-stranded RNAs (dsRNAs), corresponding in size to the genomic RNA and sgRNAs, characteristically accumulate in infected plant tissue. The product of the 5′ proximal ORF (p28 in TCV) and its readthough product (p88 in TCV) are the only virus encoded components of the polymerase complex. Mutagenesis studies demonstrated that p28 and p88 are both essential for viral RNA replication in protoplasts. When expressed from two separate molecules, p28 and p88 complemented *in trans* to enable the genome replication. Putative host factors needed for viral RNA replication have not yet been well studied, although extracts from infected turnip plants have been shown to contain RDRP activity capable of synthesizing complementary full-length molecules from both (+)-strand and (−)-strand TCV associated RNA templates. Experiments utilizing these RDRP extracts have provided useful information about *cis*-elements important in replication and gene expression. For example, a stable stem–loop structure at the 3′ end of the TCV genome has been identified as the promoter for (−)-strand synthesis from the (+)-strand TCV genome. A similar stem–loop structure has also been identified in other carmoviruses, including CarMV and CCFV, by sequence comparisons. The essential promoter sequences for the two subgenomic RNAs have also been mapped in TCV. The promoter for (+)-strand genomic RNA synthesis has not yet been identified. Another potentially important *cis*-acting hairpin structure in TCV (the FaFf element, see **Fig. 1**) has been identified in the vicinity of the UAG stop codon that punctuates the polymerase gene. A regulatory function for this element is proposed because it binds CP with high affinity, and disruption of the RNA structure, but not the coding sequence, abolishes replication.

Satellites, Defective-Interfering RNAs and RNA Recombination

TCV is the only carmovirus in which replication of associated small subviral RNAs in infected plants has been characterized and the situation for this virus is curiously complex. For example, TCV infections have been shown to contain defective-interfering RNAs derived totally from the parent genome (e.g. RNA G of 342–346 nt), satellite RNAs of nonviral origin (e.g. RNAs D, 194 nt and F, 230 nt), and chimeric RNAs (e.g. RNA C of 356 nt) with a 5′ region derived from satellite RNA D and a 3′ region derived from the 3′ end of the TCV genome. All three types of small RNAs depend on the helper virus for their replication and encapsidation within the infected plant. The different satellite and defective-interfering RNAs have been shown to affect viral infections in different ways. Both RNA C and G intensify viral symptoms while interfering with the replication of the helper virus, whereas RNAs D and F seem to produce no detectable effects on either expression of symptoms or helper virus replication.

TCV has proved to be a good model for studies on RNA recombination. The frequency and diversity of the small RNA species that appear to have arisen spontaneously in plant infections suggests that RNA recombination occurs frequently and with facility. The *de novo* generation of defective-interfering RNAs in plants after inoculation with cDNA-derived transcripts of the genome is an obvious example. Detailed studies in Anne Simon's laboratory, utilizing the chimeric satellite RNA C as a model, have provided some detailed insight into the copy-choice mechanisms that are important in viral RNA recombination. They have shown, for example, that the majority of recombinants produced are not viable for further replication. These studies have also identified a novel 3′-end repair mechanism by which the RDRP complex synthesizes short oligoribonucleotides (up to 8 nt) complementary to the 3′ end of TCV RNA, which in turn serve as primer for RNA synthesis using a different template (e.g. satellite RNA C with 3′ end up to 6 nt deleted).

Virus–Host Interaction

Studies are beginning to emerge in the area of viral–host interactions utilizing *Arabidopsis* and TCV as a model system. In one study from L. A. Heaton's laboratory an *Arabidopsis* encoded protein, Apt8, was identified as interacting specifically with the TCV

p8 protein in a yeast two-hybrid screen. Apt8 was shown to be localized in plasma membrane and possibly linked to the cytoskeleton. Transgenic *Arabidopsis* and tobacco plants expressing antisense Apt8 mRNA were resistant to TCV infection.

Some studies suggest that the CP of carmoviruses may also interact with host plants in other ways besides assembly. For example, minor amino acid changes within TCV CP have been shown to modify symptoms of infection. In addition, the CP has been implicated as a possible viral determinant in the observed resistance of *Arabidopsis thaliana* ecotype Di-0 to TCV infection. In these studies, systemic invasion by chimeric virus occurred in the Di-0 ecotype when the TCV CP ORF was replaced by the CCFV CP ORF. It was also shown that TCV replicated normally in Di-0 protoplasts and inoculated leaves, suggesting the genetic resistance was due to restricted systemic movement.

CP has also been found to be important in satellite RNA C interactions in the host plant. Normally, the presence of RNA C results in symptom intensification in TCV infections. However, when the TCV CP ORF is either deleted or replaced by the CCFV CP ORF (see above), RNA C will then attenuate symptoms caused by the helper virus. Additional experiments in protoplasts suggested that the CP either downregulated the replication of RNA C or enhanced its own competitiveness.

Finally, the replicase gene has also been implicated in the symptom modification by satellite RNA C. The 3′ end of the TCV genome, common in TCV RNA, RNA C and defective-interfering RNA G, was also suggested to be a symptom determinant. Environmental conditions were also found to affect the extent of resistance of *Arabidopsis* plants to TCV.

See also: **Defective interfering viruses; Dianthoviruses (*Tombusviridae*); Luteovirus; Necroviruses (*Tombusviridae*); Tombusviruses; Virus structure: Atomic structure, Principles of virus structure.**

Further Reading

Hacker DL, Petty ITD, Wei N and Morris TJ (1992) Turnip crinkle virus genes required for RNA replication and virus movement. *Virology* 186: 1.

Morris TJ and Carrington JC (1988) Carnation mottle virus and viruses with similar properties. In: Koenig R (ed.) *The Plant Viruses*. Vol. 3: *Polyhedral Virions with Monopartite RNA Genomes*. New York: Plenum Press.

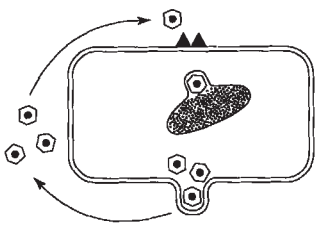
Nagy P, Carpenter CD and Simon AE (1997) A novel 3′ end repair mechanism in an RNA virus. *Proc. Natl Acad. Sci. USA* 94: 1113.

Qu F and Morris TJ (1997) Encapsidation of turnip crinkle virus is defined by a specific packaging signal and RNA size. *J. Virol.* 71: 1428.

Russo M, Burgyan J and Martelli GP (1994) Molecular biology of *Tombusviridae*. *Adv. Virus Res.* 44: 381–428.

Song C and Simon AE (1994) RNA-dependent RNA polymerase from plants infected with turnip crinkle virus can transcribe (+)- and (−)-strands of virus-associated RNAs. *Proc. Natl Acad. Sci. USA* 91: 8792.

CELL STRUCTURE AND FUNCTION IN VIRUS INFECTIONS



Samuel Dales, Rockefeller University, New York, USA

Introduction

The subject matter of this article concerns the structure and function of a prototypical animal cell in its role as a host for viruses (**Fig. 1**). It is now clearly evident from the information acquired during the recent past, particularly from data developed by molecular biology techniques, that viruses in the form of quasi-autonomous macromolecular complexes are propagated by expression of genomes which originated from their hosts. The viral genes have undergone alteration to a varying degree during evolutionary selection so as to give the virus parasite an advantage in metabolic efficiency. Detailed knowledge about cell organization reveals the existence of a great variety of relationships connecting cellular components of direct relevance to every stage of the infectious process with individual virus types. Owing to the brevity of this treatise it has been necessary to omit information about cell organelles and structures that have only a minor or peripheral relationship to the virus life cycle.

p8 protein in a yeast two-hybrid screen. Apt8 was shown to be localized in plasma membrane and possibly linked to the cytoskeleton. Transgenic *Arabidopsis* and tobacco plants expressing antisense Apt8 mRNA were resistant to TCV infection.

Some studies suggest that the CP of carmoviruses may also interact with host plants in other ways besides assembly. For example, minor amino acid changes within TCV CP have been shown to modify symptoms of infection. In addition, the CP has been implicated as a possible viral determinant in the observed resistance of *Arabidopsis thaliana* ecotype Di-0 to TCV infection. In these studies, systemic invasion by chimeric virus occurred in the Di-0 ecotype when the TCV CP ORF was replaced by the CCFV CP ORF. It was also shown that TCV replicated normally in Di-0 protoplasts and inoculated leaves, suggesting the genetic resistance was due to restricted systemic movement.

CP has also been found to be important in satellite RNA C interactions in the host plant. Normally, the presence of RNA C results in symptom intensification in TCV infections. However, when the TCV CP ORF is either deleted or replaced by the CCFV CP ORF (see above), RNA C will then attenuate symptoms caused by the helper virus. Additional experiments in protoplasts suggested that the CP either downregulated the replication of RNA C or enhanced its own competitiveness.

Finally, the replicase gene has also been implicated in the symptom modification by satellite RNA C. The 3′ end of the TCV genome, common in TCV RNA, RNA C and defective-interfering RNA G, was also suggested to be a symptom determinant. Environmental conditions were also found to affect the extent of resistance of *Arabidopsis* plants to TCV.

See also: **Defective interfering viruses; Dianthoviruses (*Tombusviridae*); Luteovirus; Necroviruses (*Tombusviridae*); Tombusviruses; Virus structure: Atomic structure, Principles of virus structure.**

Further Reading

Hacker DL, Petty ITD, Wei N and Morris TJ (1992) Turnip crinkle virus genes required for RNA replication and virus movement. *Virology* 186: 1.

Morris TJ and Carrington JC (1988) Carnation mottle virus and viruses with similar properties. In: Koenig R (ed.) *The Plant Viruses*. Vol. 3: *Polyhedral Virions with Monopartite RNA Genomes*. New York: Plenum Press.

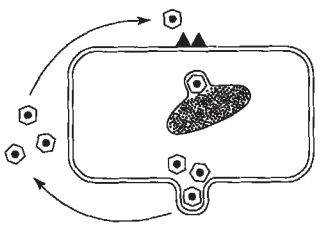
Nagy P, Carpenter CD and Simon AE (1997) A novel 3′ end repair mechanism in an RNA virus. *Proc. Natl Acad. Sci. USA* 94: 1113.

Qu F and Morris TJ (1997) Encapsidation of turnip crinkle virus is defined by a specific packaging signal and RNA size. *J. Virol.* 71: 1428.

Russo M, Burgyan J and Martelli GP (1994) Molecular biology of *Tombusviridae*. *Adv. Virus Res.* 44: 381–428.

Song C and Simon AE (1994) RNA-dependent RNA polymerase from plants infected with turnip crinkle virus can transcribe (+)- and (−)-strands of virus-associated RNAs. *Proc. Natl Acad. Sci. USA* 91: 8792.

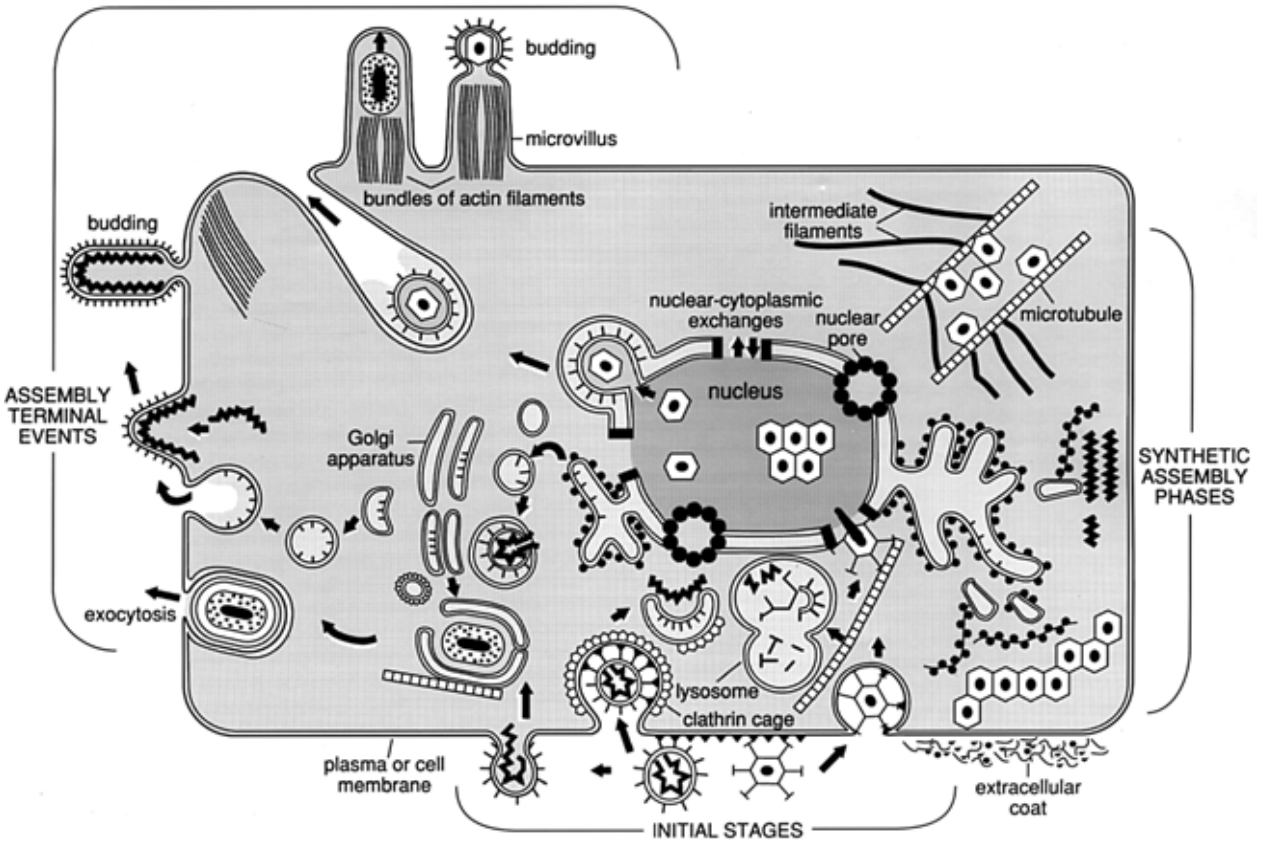
CELL STRUCTURE AND FUNCTION IN VIRUS INFECTIONS



Samuel Dales, Rockefeller University, New York, USA

Introduction

The subject matter of this article concerns the structure and function of a prototypical animal cell in its role as a host for viruses (**Fig. 1**). It is now clearly evident from the information acquired during the recent past, particularly from data developed by molecular biology techniques, that viruses in the form of quasi-autonomous macromolecular complexes are propagated by expression of genomes which originated from their hosts. The viral genes have undergone alteration to a varying degree during evolutionary selection so as to give the virus parasite an advantage in metabolic efficiency. Detailed knowledge about cell organization reveals the existence of a great variety of relationships connecting cellular components of direct relevance to every stage of the infectious process with individual virus types. Owing to the brevity of this treatise it has been necessary to omit information about cell organelles and structures that have only a minor or peripheral relationship to the virus life cycle.



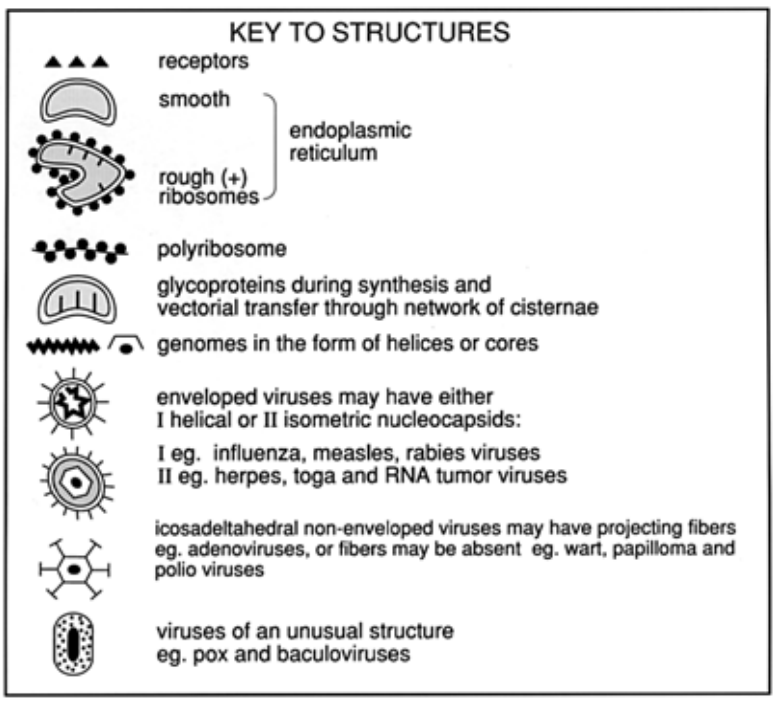


Figure 1 Schematic representation of a prototypical animal cell. The illustration emphasizes cellular organization as it relates to various phases of the virus replication cycle.

The Surface

Tissue organization and cell polarity

When discussing cellular structure and function with respect to virology, one must realize that within the animal host, initiation and spread of virus infections frequently follows encounters at epithelial and endothelial surfaces such as those in skin, lung, gut and vascular lining. These surface sheets are assemblies of polarized cells that possess apical and basolateral membranes exposed to the fluid milieu. The free movement of particulates from one side of these sheets to the other is, however restricted by lateral cell-to-cell contacts termed tight junctions. This manner of organization at cellular surfaces determines the routes of virus ingress, egress and dissemination within the animal. Data derived from infected animals and explants of cultured epithelia demonstrate that specific viral receptors may be confined to either the apical or basolateral surfaces, depending on the virus type, although dual receptors at opposite sides of polarized cells have been uncovered. Cell polarization also influences the membrane sites at which envelopment and assembly of viruses can occur. This may be due primarily to mechanisms for sorting and vectorial transfer of nascent glycoproteins destined for incorporation into the virus progeny within specialized membrane components. The interrelationship between molecular sorting and vectorial transfer is clearly exemplified by differences between influenza virus, which is assembled on the apical side, vesicular stomatitis virus, which buds preferentially on the basolateral face, and the coronaviruses, which use for assembly membranes of intracellular vesicles neighboring the Golgi apparatus. The direction of vectorial movement through the cellular membrane network is actually inherent in specific amino acid sequences of polypeptides and glycoproteins, whether they are encoded by cells or viruses.

Extracellular coats

Ubiquitous glycoprotein and glycolipid molecules, which are integral to the organization of the plasma membrane bilayer, are usually decorated by extracellular branched polysaccharide chains. A surface meshwork of such chains forms the glycocalyx. Carbohydrate residues of the glycocalyx provide initial attachment sites for ligands on the virus inoculum, exemplified by adsorption of influenza hemagglutinin to *N*-acetylneuraminic (or sialic) acid of the cell receptor. At the terminal formative stages of enveloped viruses, regions of cell surface contiguous to those of the emerging particles become desialylated by viral neuraminidases, thereby ensuring that individual virions are released efficiently from the glycocalyx. Other types of carbohydrates such as the elongated mucopolysaccharides that coat cells of the respiratory epithelium represent another type of barrier which must be dispersed by enzyme on the virus before the inoculum can reach the cell receptor to establish a stable link. Other types of extensive coats also exist, termed extracellular matrix, with various properties adapted to specialized functions of the tissue where they occur. The substances present in the extracellular matrix include hydrated gel-like glycosaminoglycans linked to proteins forming proteoglycans. Proteoglycans can be permeated by a meshwork of fibrous proteins, exemplified by collagen and materials such as fibronectin and laminin, which promote cell adhesion to supporting layers. The extracellular matrix is involved in kidney diseases such as certain forms of glomerulonephritis which may develop during chronic persistent infections when massive quantities of virus–antibody immune complexes filtrated out of the circulation are deposited in the matrix. This disease process occurs among human carriers chronically infected with hepatitis B virus and other agents.

The cell or plasma membrane

The cell membrane, interchangeably termed plasma membrane, forms a semipermeable barrier enclosing the contents of the cell. Like all cellular membranes it is organized into a bilayer of amphipathic phospholipids and glycolipids, transversed by a great variety of transmembrane polypeptides and glycoprotein molecules. The carbohydrates of the glycoproteins, present as multiple branches of oligosaccharides, are frequently attached to the externally protruding N-termini of the polypeptide. Organization into bilayers favors a high degree of membrane flexibility during a constant state of flux and the lateral mobility of molecules inserted within the bilayer. The dynamic state of membranes allows for their rapid deformability into invaginations from the surface leading to formation of vesicles. Evaginations may result in the formation of transient elongations and more permanent processes such as microvilli. Membrane dynamics also provide mechanisms for membrane-to-membrane fusion or, conversely, separation between neighboring membrane segments, phenomena that occur frequently throughout the cell and do not compromise the bilayer continuity. These properties of membranes make the plasma membrane a site for the formation of autonomous endocytic vesicles, and coalescence with shuttle vesicles, which carry materials for exocytosis. Sealing between membranes can be promoted by the influence of fusogenic proteins. The

constant traffic away from and towards the surface facilitates continuous circulation and turnover of membrane components not only at the surface but throughout the cell. Sideways mobility of molecules within the plane of the lipid bilayer provides a mechanism for establishing intermolecular contacts and conformational changes related to metabolic activities. Specificity of interactions within the membrane bilayer between proteins and glycoproteins can create heteromeric or multimeric molecular assemblies, functioning themselves as receptors or via connections to the receptors. Such groupings of molecules are commonly involved with immune response phenomena, with formation of toxins or transmitter-gated channels of the type present in interneural and neuromuscular junctions. Rapid physiological responses can occur at the surface initiated via receptors recognizing extracellular signals, whether they are of the autocrine variety like lectins and growth factors, of an endocrine type such as insulin or in the category of second messengers represented by the cyclic nucleotides. The rapid transfer of externally initiated signals towards intracellular response foci can be mediated through connections provided by elements of the cytoskeleton, or by membrane-to-membrane coupling or through vesicular shuttles trafficking between membrane-enclosed compartments and the cell exterior.

Homeostasis of the cell with respect to cell volume, ion concentration and pH is maintained by means of proteinaceous ion pumps present in the plasma membrane. Such pumps facilitate internalization of Na^+ and Cl^- and removal of excess H^+ or $HCO3^-$ derived from cell metabolism. Other membrane proteins catalyze transmembrane transport of metabolites or, inadvertently, the uptake of chemotherapeutic drugs. Overall the cell membrane can be conceptualized as a mosaic of domains, with each type of domain fulfilling a specialized function in the life of a cell. Regions of dense coat along the inner cytoplasmic face of the plasma membrane are due to formation of cages or basket-like scaffolds organized out of triskeletal macromolecular assemblies of the protein clathrin. Clathrin cages also enclose endocytic and other specialized vesicles shuttling to and from the surface. Other types of specialized coated vesicles, surrounded by proteins other than clathrin, can also be formed and participate in trafficking with the plasma membrane.

As one of its primary functions, which also has a bearing on viruses, the plasma membrane selectively concentrates and internalizes extracellular material. Sequestration of particulates inside endocytic vesicles is termed endocytosis, and uptake of fluid-phase components is pinocytosis. Endocytosis becomes highly efficient and selective due to the sequential binding via a zippering-like process termed receptor-mediated endocytosis of ligands. The ligands present on the particulate destined for engulfment become bound to multiple receptors mobilized by lateral diffusion along the plasma membrane. Receptor-mediated endocytosis decrees the precise configuration of envelopment in endosomes. The internal milieu of newly formed endosomes is close to neutrality, but during their inward movement away from the surface the fluid contents of endosomes become acidified by accumulation of H^+ pumped out of the cytosol. The presence of a repertoire of acidic hydrolases, acquired via transfer vesicles from the Golgi apparatus, converts the late endosomes into prelysosomes, the latter predestined to coalesce with mature lysosomes. The lysosomes containing acidic enzymes able to hydrolyze lipids, nucleic acids and proteins function as the primary sites for intracellular digestion to dispose of damaged organelles or any undesirable materials acquired accidentally by engulfment. The strategies that viruses employ to utilize the properties of the plasma membrane during initial and terminal stages of their life cycle clearly manifest coevolutionary origin with that of the host. A stable attachment is established when a virus ligand makes contact with host cell receptor during the initial collisions at the surface. The presence of multiple ligands on the virus surface and an abundance of receptors, perhaps 10^4–10^6 on each host cell, increases the probability that a productive encounter will occur. The display of certain receptors on specific differentiated cell types and/or along specialized cell surfaces can be the determinant regulating tissue or organ tropisms for different virus types. Among notable examples are CD4 molecules on human T cells which function as receptors for the human immunodeficiency virus (HIV), β-adrenergic receptors on neurons in mouse brain which can bind reovirus type 3, receptors for the neurotransmitter acetylcholine at neuromuscular junctions where attachment of rabies virus is evident and the asialoglycosphingolipids in gut epithelium which function as receptors for rotaviruses. Receptors for polio-, rhino- and cardiovirus groups are macromolecules that are functional in uninfected cells related to a super family of glycoproteins with the basic configuration of immunoglobulin. As already mentioned, dual receptors for viruses such as herpes simplex can occur on distinctive surfaces on polarized epithelial cells, or alternative receptors may be present on different host cells, as is evident with attachment of HIV to either its principal T cell CD4 receptors or to galactocerebroside receptors on certain glial cells in the nervous system.

The entry of virus inoculum particles to effect an intracellular release or uncoating of their genomes depends on fusogenic activity carried by the virus which is used to disrupt temporarily membrane integrity. Viral fusogens are rapidly activated at the surface by specific host proteases. Fusion of this type, exemplified by paramyxoviruses, can be activated at neutral pH, bringing about a comingling between viral and cell membranes at the surface so as to create an entrance for the virus nucleocapsid into the cytosol. Access of encapsidated virus genomes into the cytosol, facilitated by fusion of viral envelopes with the cell membrane, can be regulated by interactions with coreceptors. With HIV they are chemokine receptors CCR5 in the case of macrophage tropic and CXCR for T lymphocyte tropic virus strains.

Other fusogens are activated under acidic conditions inside endosomes where virus membrane fusion creates an escape passage, observed during penetration of influenza, rhabdo-, toga-, adeno- and other virus groups. Sequestration inside endosomes rapidly destabilizes the icosahedral picornavirus coats effecting release of the RNA genome, which then passes across the membrane into the cytosol. Other types of icosahedral agents such as the adenoviruses can rupture endosome membranes, escaping thereby into the cytosol without apparent loss of integrity (**Fig. 2**). From the above examples it should be evident that viruses are able to initiate infections successfully because they circumvent the usual transfer and hydrolysis of engulfed particulates in lysosomes. Under circumstances that abolish or block the activity of viral fusogens, as happens on denaturation at elevated temperature or as a result of steric hindrance after attachment of specific antibodies, the engulfed inoculum virus is, in fact, transferred into lysosomes where it is destroyed.

Membrane network

The intracellular network of membrane-enclosed cisternae, vesicles and membranous organelles occupies about one-half of the volume of the cytoplasmic or cytosol compartment. Numerous membranous organelles that reside in the cytosol, among them the mitochondria, which are involved in energy metabolism, and microbodies, which participate in detoxification reactions, are omitted from **Fig. 1** and are not further discussed because they have only a peripheral role in virus infections. The predominant structures enclosed by membranes are the endoplasmic reticulum (ER) and Golgi apparatus, which are transiently interconnected with each other and the plasma membrane by transport vesicles. A specialized continuum of the ER are the cisternae enclosed by the nuclear envelopes surrounding the nucleus. This network of lacunae functions as a conduit for an orderly flow of materials between intracellular compartments and the exterior.

Some regions of the ER which are devoid of ribosomes are termed smooth ER (SER) whereas other regions encrusted with ribosomes on the membrane facing the cytosol are designated as the rough ER (RER). Synthesis of proteins destined for cisternae or membrane-enclosed organelles or for transfer to the cell surface is initiated on RER when ribosomes already engaged with messenger or mRNA in a translation complex become bound to receptors on the SER. The growing polypeptide chain is orientated from the ribosome across the membrane into the lumen of ER cisternae. The vectorial insertion of the nascent polypeptide is due to recognition of signal sequences of hydrophobic amino acids. The destination to which polypeptides synthesized in RER are dispatched is also determined by specific amino acid sequences. Some nascent molecules enter into organelles such as the lysosomes and mitochondria, others are destined to become plasma membrane components, and yet others are scheduled for removal from the cell by exocytosis. Many proteins moving through the ER are modified by addition of carbohydrate chains in a complex sequence of reactions involving enzymes bound to the luminal side of the membrane, termed glycosyltransferases. Following the formation of intermediate mannose-rich oligosaccharide chains linked to a dolichol lipid carrier, the carbohydrate becomes attached to asparagine residues at specific signal sites on the polypeptide forming an N-linkage. In addition to a role in producing proteins and glycoproteins, the other functions of the ER include synthesis of phospholipids and glycolipids required for membrane addition and turnover throughout the cell.

The ER provides a surface for enzymes such as those that catalyze the detoxification of drugs prominent in the liver, and as an attachment surface for ion pumps such as those for sequestering Ca^{2+} in the sarcoplasmic reticulum of muscle. Carrier vesicles engaged in transfer of materials between various cisternae are also generated from the ER. The Golgi apparatus, which is organized into aggregates or stacks of flat interconnected cisternae, is the compartment in which completion of synthesis, sorting and distribution of glycoproteins towards various membrane compartments takes place. The groups of Golgi cisternae facing towards the cell interior, termed *cis*, are involved in dispatching molecules to centrally positioned organelles, whereas *trans* cisternae, oriented towards the surface, are associated with products transferred to the lysosomes or to the

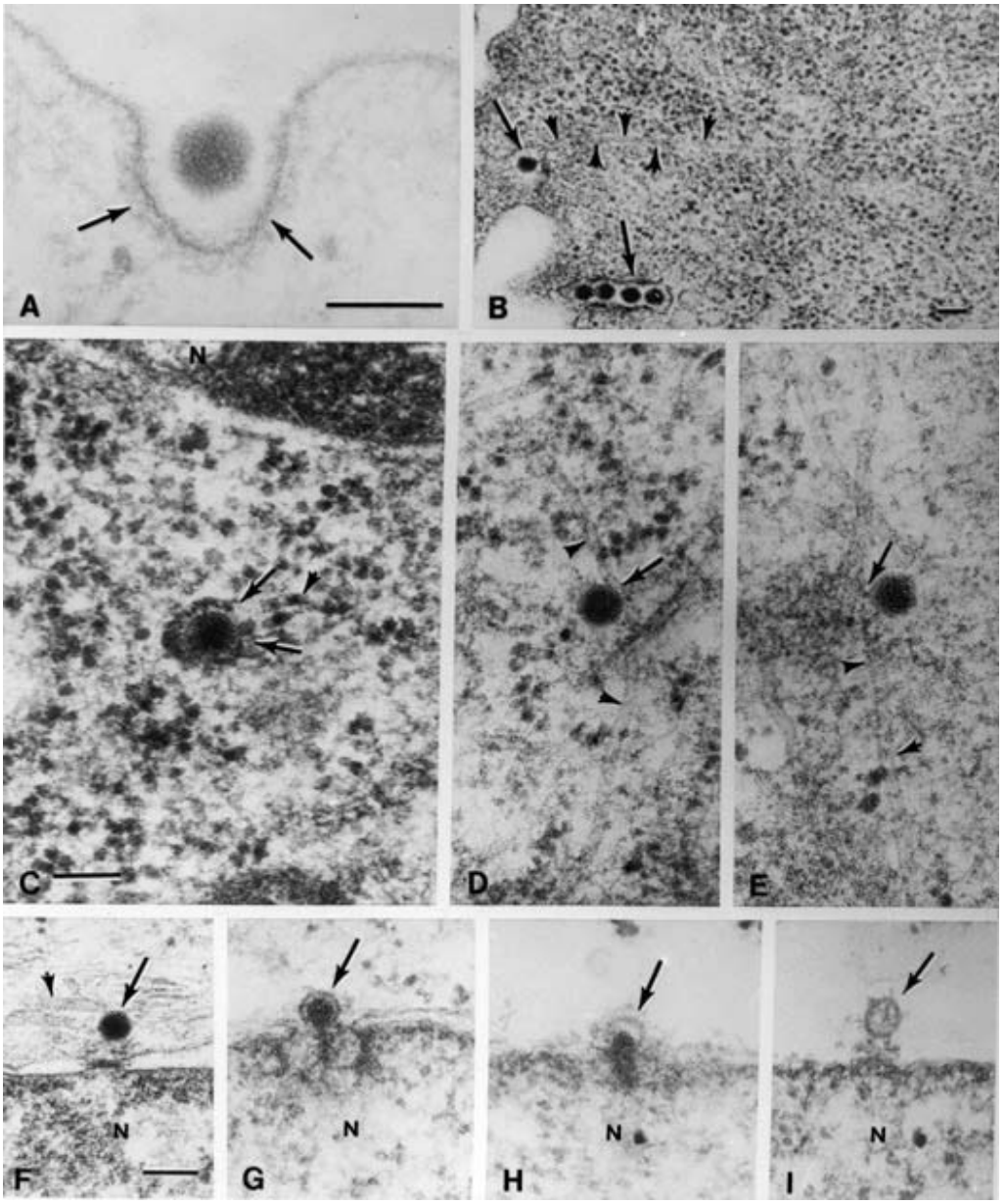


Figure 2 Intracellular events during the penetration and uncoating of inoculum adenoviruses. (**A, B**) Engulfment of virions at the plasma membrane is indicated by arrows. In (**B**) the microtubule, delineated by arrowheads, appears to be in contact with the plasma membrane. In (**C**) arrows indicate where the endosome membrane has been ruptured. (**D, E**) Arrows point to contacts between virions free in the cytosol and microtubules. (**F–I**) Progressive stages of uncoating at the nuclear pore complexes indicated by arrows. Following injection of dense, fibrous DNA into the nuclear compartment (N), illustrated in (**G**) and (**H**) the empty capsids in (**I**) remain behind. Magnifications (**A**) ×140 000; (**B**) ×40 000; (**C–E**) ×80 000; (**F–I**) ×65 000. (Reproduced from (**A**) Chardonnet Y and Dales S (1970) *Virology* 40: 462; (**B–I**) Dales S and Chardonnet Y (1973) *Virology* 56: 465.

plasma membrane by carrier vesicles, some enclosed within clathrin cages. Additional glycosyltransferase enzymes in the Golgi are used to modify the N-linked oligosaccharides, produced in the ER, by deletion and terminal addition of carbohydrate. These enzymes also synthesize exclusively in the Golgi oligosaccharides attached by the -O- linkage to serine residues of the polypeptide. Certain virus groups such as the coronaviruses, which possess the O-linked carbohydrate of an envelope glycoprotein, are assembled not at the cell surface but on membranes of Golgi vesicles. The majority of enveloped virus types is organized into particles by budding from the plasma membrane (**Fig. 3**). Therefore, the cell surface becomes a region

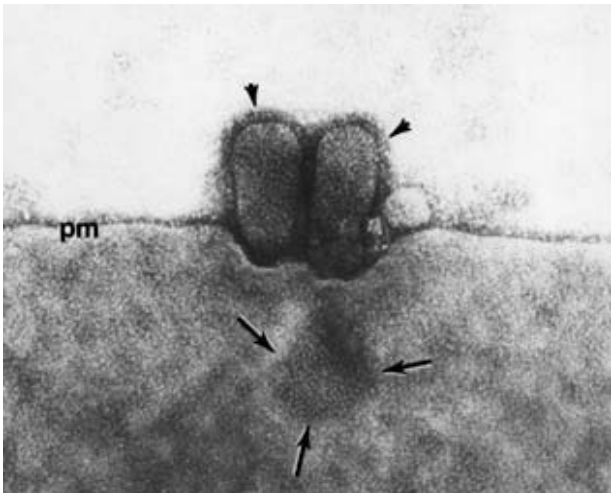


Figure 3 Emergence of influenza virus by budding from the plasma membrane (pm). In the electron micrograph of an infected primate cell arrowheads indicate presence of peplomers on the virions' surface. Arrows point towards a grouping of peplomers where a presumptive virion is assembling; magnification ×165 000. (Reproduced from Choppin PW (1963) *Virology* 21: 278.)

for organizing the glycoprotein molecules that constitute the peplomers or spikes on the virus envelope after they have been delivered to the plasma membrane in transfer vesicles from the Golgi cisternae. Once they are in place, peplomers become foci for mobilizing the nucleocapsid and inner membrane protein(s), then budding takes place. Some virus-induced glycoproteins, which are not a constituent *per se* of virus particles, are nevertheless inserted into cell membranes, thus altering their properties. For example, the cisternae may be formed into rigid channels through which particles of herpes simplex virus can pass to the cell surface, or, as in the case of poxviruses, membrane modifications promote recognition between surfaces on the virus and Golgi vesicles which then surround the virus particle and fuse with each other to form a continuous double-membrane cisterna. The wrapped virus is moved towards the surface where exocytosis occurs as a result of fusion between the outer wrapping and plasma membranes (**Fig. 4**).

The Cytosol

The cytosolic compartment occupies the cytoplasm exclusive of the membrane network and membrane-enclosed organelles. Proteins formed in the cytosol, termed 'free', are translated from mRNA on polyribosomal complexes that do not become attached to the ER. The 'free' proteins, whether they remain in the cytosol or enter into the nuclear compartment, are components of enzyme assemblies, which may be attached to membranes on the cytosolic side. They also form organelles such as the ribosomes, fibrous filaments of the cytoskeleton and nucleosome proteins of the chromatin. It is notable that the polyribosomes engaged in synthesis of large precursor polyproteins of viruses such as polio are attached to SER vesicles at one end. Virus-specified proteins synthesized in the cytosol accumulate into pools of material out of which individual icosahedral virions can be assembled. The proteins are sometimes sufficiently abundant for formation of higher order crystalline aggregates out of assembled virus particles (**Fig. 5**).

The cytoskeleton represents an array of three principal types of fibrous materials, actin microfilaments arranged in bundles, intermediate filaments of several types and cylindrical microtubules. These three elements are interconnected with each other, and can make contacts via a large repertoire of associated proteins with membranes around cell organelles, the nucleus and at the cell surface. Actin filament bundles, prominent in the peripheral cortex region of the cytosol and inside microvilli, are involved in surface mobility during formation of pseudopodia and participate in other changes in cell form. The filaments which have a directional polarity are continually disassembled and reassembled from actin protomers. Circumstantial evidence connects actin filaments with virus infections during initial stages of penetration, migration of the progeny through microvilli and assembly by budding at the cell surface. The intermediate filaments contain four chemically distinguishable but related fibrous proteins. In all cells, they form a net of nuclear lamins situated next to the nuclear envelopes. The organ or tissue in which a cell occurs determines the composition of the intermediate filament(s). Intermediate filaments in cells of epithelia, connective tissue, muscle and the nervous system contain respectively cytokeratins, vimentin, desmin, glial fibrillary acidic protein and neurofilament protein. Contact between intermediate filaments and other components most probably plays a role in mechanical support, as, for example, in neuronal and astrocytic processes and intercellular contacts where adhesion foci, such as the desmosomes, are present. Intermediate filaments have been implicated in the assembly of some picorna- and reoviruses by their presence among the progeny aggregates of virus. Microtubules, the thickest of the cytoskeletal elements, contain α and/or β tubulins organized into cylindrical walls of helical protofilaments. The presence of microtubules imparts the shape and polarity to cells and provides a mechanism for orderly segregation of daughters during cell division. The microtubules provide surfaces along

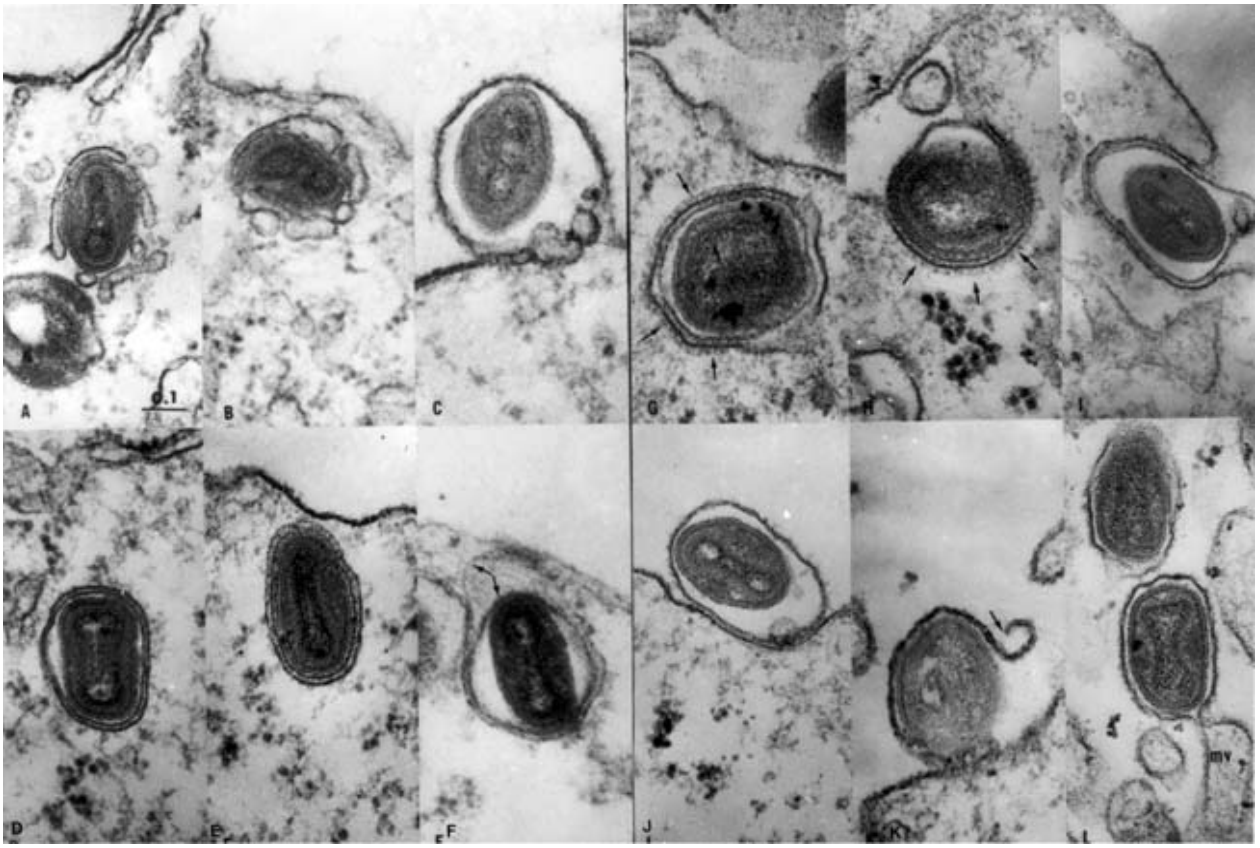


Figure 4 Electron micrographs illustrating a reconstructed sequence of the envelopment, migration to the surface and release through the plasma membrane of progeny vaccinia virus. (**A, B**) Envelopment in vesicles that failed to fuse into a continuous cisterna. (**C**) Two vesicles interposed between the plasma membrane and the inner cisterna membrane surrounding a discharged virion. (**D, E**) Migrating virions enclosed by double membrane cisternae. In (**F**) arrows indicate a separation of the two cisterna membranes. (**G, H**) Arrows indicate the presence of dense coats on some wrapping membranes. (**I, J**) Release of virions wrapped by the inner cisterna membrane, which has been ruptured in (**K**). (**L**) Virion in the process of discharge through a microvillus (mv), the alternative method of egress. Magnification ×61 000. (Reproduced from Ichihashi Y *et al* (1971) *Virology* 46: 507.)

which movement of vesicles and other membrane-enclosed organelles can be directed. Among the notable functions are: (1) fast axonal transport along groups of microtubules which connect the region of synthesis in the cell body with synaptic termini, (2) involvement in the beating of cilia or flagella and (3) as pathways for the rapid and reversible movement of granules in pigment epithelial cells. The energy for effecting translocations along the microtubules is obtained from the hydrolysis of ATP.

Microtubules are utilized by viruses during infection in various ways. They provide attachment sites for inoculum adeno- and herpes simplex viruses and for conveying them vectorially towards the nuclear pore complexes where uncoating and injection of the DNA occur (**Fig. 2**). The virions may be propelled along the microtubules by the action of a molecular motor, kinesin. Microtubules and connected intermediate filaments become loci for assembly of reovirus type 3 progeny, as is evident in fibroblasts and axons of neurons.

With another virus type there exists an interaction between neuronal microtubules and the N nucleocapsid protein of coronavirus JHM (**Fig. 6**). This relationship occurs perhaps due to molecular mimicry, as revealed by sequence relatedness of N with cellular microtubule-associated proteins *tau* and MAP-4 (**Fig. 7**). This mimicry draws attention to evolution of viruses which, as intracellular parasites, exploit the cell's structure and function properties for self propagation.

The Nucleus

Maintained as an autonomous compartment during the interphase of the cell cycle, the nucleus is where

Figure 5 An area of the cytosol in a human cell infected 7 h previously with poliovirus type 1. A prominent crystalline aggregate of virions occupies the center of the electron micrograph. Magnification ×83 000. (Reproduced from Dales S *et al* (1965) *Virology* 26: 379.)

synthesis occurs relevant to duplication of the DNA and gene expression in the form of transcription into messenger, ribosomal, transfer and other RNA species. The genomic DNA of the cell and associated proteins, including such basic proteins as the histones, form the chromatin, which consists of a multitude of primary units, the nucleosomes. Nucleosomes are organized into hierarchical orders to form the ultimate units of structure, the chromosomes. At interphase, during the most active synthetic phase, the chromatin is dispersed maximally. Chromatin compaction into cytologically identifiable chromosomes commences upon initiation of cell division. In the chromatin, individual genes can be expressed transcriptionally as units. DNA synthesis and transcription each require specific polymerase enzymes and numerous additional cofactors. These factors, some acting cooperatively and others competing with the polymerases, can bring about relaxation of the double-stranded DNA template, promote the copying of the forward and retrograde DNA strands and act in transcription of individual genes. Since the DNA is organized into interrupted stretches of informational sequences (exons) separated by intervening noncoding sequences (introns), a functional RNA template for translation into protein is formed from the primary RNA transcript by removal or splicing out of the introns, usually catalyzed by specific protein complexes termed spliceosomes. Further enzymatic modifications of the RNA template bring about an addition at the 5′ end of specific nucleotide sequences termed 'caps' and at the 3′ end stretches of polyadenylic acid. The above metabolic activities within the cell nucleus are diverted for their own use by viruses with a nuclear phase of their life cycles. Members of the papovavirus group, which possess circular DNA genomes organized as nucleosomes, can subvert DNA and RNA polymerases as well as other cellular factors to promote viral gene expression by splicing and terminal modifications of the mRNA in the manner of the host. To initiate their replication, papovaviruses require the host DNA to be in a synthetic phase of the cell cycle. The viral genome expression is favored over that of the host because a sequence at the origin of viral DNA replication can bind avidly the virus-induced T antigen protein which, in turn, has a high binding affinity for host polymerase. Other DNA viruses, including those of the herpes- and adenovirus groups, also initiate their replication by utilizing, to a varying degree, host polymerases. Replication of influenza viruses exemplifies another highly specific, albeit limited, requirement for the cell's metabolism. These agents possess fragmented RNA genomes of the minus sense which are transcribed into mRNAs by an RNA polymerase carried within the inoculum particles. However, to become functional, the influenza mRNAs must acquire their 5′ terminal caps from among those synthesized inside the nucleus under the aegis of host enzymes. Thus, association of influenza viruses with the nucleus is best explained in terms of transcapping of the viral mRNAs.

Nuclear–Cytoplasmic Exchanges

The mRNA, transfer RNA and ribosomal RNAs of large and small subunits are products of the nucleus which become components of a cytoplasmic translation complex. The individual ribosomal subunits are assembled in the nucleolus, a specialized site of the nucleus, out of ribosomal RNA and numerous types of ribosomal polypeptides, the latter synthesized in the cytoplasm. Compartmentalization of the above synthetic events highlights the necessity for a continual and efficient transfer of transcription products into the cytoplasm and the reverse movement of proteins made in the cytoplasm into the nucleus. Passive transfer of smaller proteins of less than

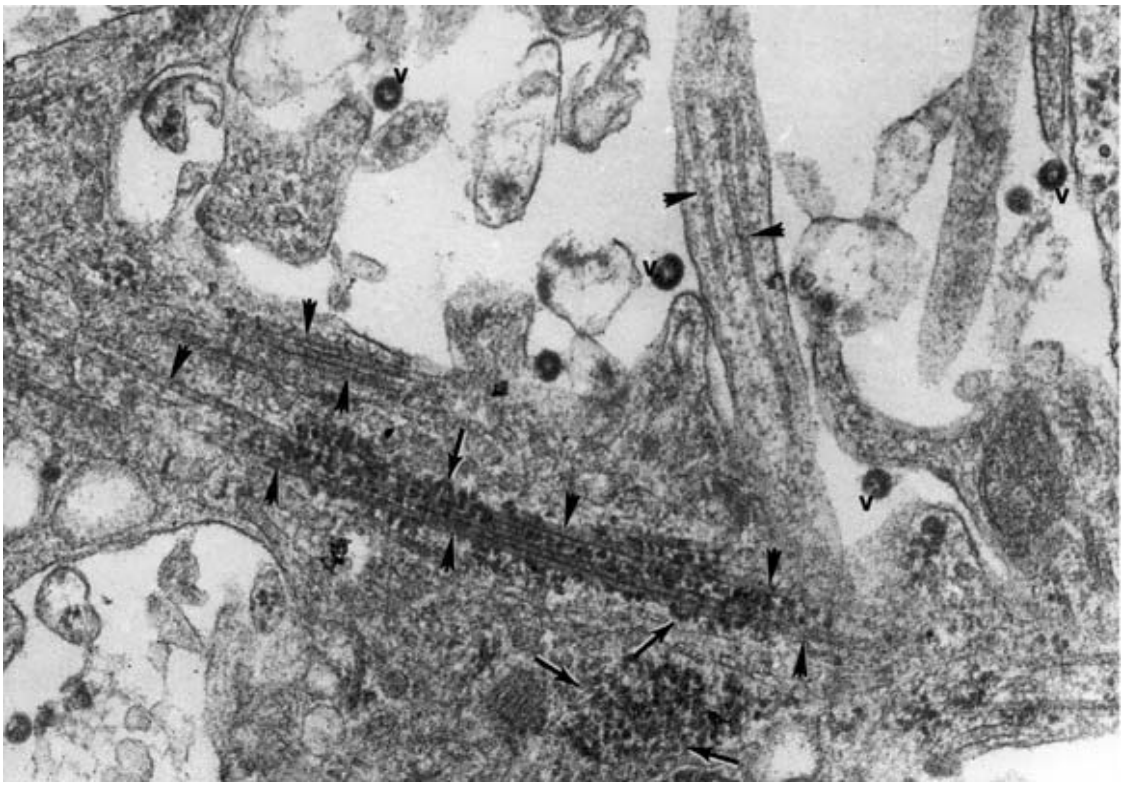


Figure 6 Association between the nucleocapsids of coronavirus JHM (arrows) and bundles of microtubules (arrowheads) within neurites of an infected hippocampal neuron of a mouse. Extracellular virions are marked 'v'. Magnification ×115 000. (Reproduced from Kalicharran K and Dales S (1996) *Trends Microbiol.* 4: 264.)

20 kDa occurs rapidly, within 2 min of synthesis, and that of larger proteins of about 40 kDa takes place more slowly, after perhaps 30 min. Proteins of even greater molecular mass can enter the nucleus only by means of active transport. Such proteins are first bound to specific receptors on the cytoplasmic side of nuclear pores, and are then able to enter into the nucleus because they possess specific signal sequences for import through the pores, as is evident in the case of 90 kDa T antigen of papovavirus. Another function of nuclear–cytoplasmic exchanges in virus infections includes uncoating by injection of adenovirus and baculovirus DNA through nuclear pores (**Fig. 2**). Cytological evidence for the presence inside nuclei of large numbers of assembled adeno- and herpesvirus particles, sometimes grouped into crystalline arrays, demonstrates that large quantities of viral proteins can enter the nucleus. This type of evidence is supported by biochemical studies which reveal very rapid and efficient intranuclear sequestration of nascent viral polypeptides.

```
N..........GSKLELVKKNSGG 339
           |||  :.:. :||
tau........GSKDNIKHVPGGG 207
           |||||||||||||
MAP-4.......GSKDNIKHVPGGG 1021
```

A

```
tau............SGEPPKSGER 126
               |:...:||.|
N..............SAPASRSGSR 208
               |||||||||:
MAP-4..........SAPASRSGSK 803
```

B

Figure 7 Sequence relatedness between the nucleocapsid protein N of coronavirus JHM and the microtubule-associated proteins tau and MAP-4. (**A**) The tandemly repeated sequence involved in binding to microtubules is matched with a sequence of N. (**B**) Shows homologies at the serine- and proline-enriched segments. In the optimal alignment amino acid identity is shown by connecting lines and relatedness, depending on closeness, by one or two dots. (Reproduced from Kalicharran K and Dales S (1996) *Trends Microbiol.* 4: 264.)

Concluding Remarks

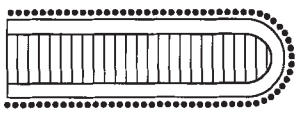
This very brief account of cellular structure and function emphasizes throughout the contribution of the host towards the infectious process. The depen-

dence and close intimacy between viruses and their host cells demonstrates adaptation through evolution by the parasite to take every advantage for self-propagation. The examples used to describe cell organization and metabolism in the context of virology should provide the reader with hints about the use that has been made of viruses as incisive probes of the living cell itself.

See also: **Pathogenesis: Animal viruses, Plant viruses; Replication of viruses; Viral receptors; Virus–host cell interactions.**

Central European Encephalitis Virus *see* **Encephalitis Viruses**

CHANDIPURA, PIRY AND ISFAHAN VIRUSES (*RHABDOVIRIDAE*)



P. De Bishnu and **Amiya K. Banerjee**, Department of Molecular Biology,
The Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, Ohio, USA

History and Classification

Chandipura, Piry and Isfahan viruses are members of the genus *Vesiculovirus* of the family *Rhabdoviridae*. The prototype of this genus is the vesicular stomatitis virus (VSV) of two serotypes, VSV-New Jersey (VSV_{NJ}) and VSV-Indiana (VSV_{IND}), which is known for its association with sporadic epizootics of vesicular disease among horses, cattle, and swine. Chandipura, Piry and Isfahan viruses along with a number of other viruses such as Alagoas, Calchaqui, Cocal, Jurona, La Joya, Maraba, Perinet and Yug Bugdanovac are the least studied members of this genus.

Chandipura (CHP) virus was first isolated by P. N. Bhatt and F. M. Rodrigues in 1965 from human patients at Nagpur county, India. Thus it is quite distinct from VSV with respect to geographic origin and host species origin, because the latter has been isolated almost exclusively from horses and cattles in the western hemispere. Piry virus was isolated by R. E. Shope in 1960 from spleen and liver samples of an opossum (Philander opossum) at Belem, Brazil. Isfahan (ISF) virus was isolated by R. B. Tesh and coworkers in 1975 from humans as well as gerbils and sand flies at Isfahan province, Iran.

These viruses have been classified as *Rhabdoviridae* based on their typical bullet-shaped morphology, approximately 180 nm in length and 65 nm in width, as well as nucleic acid and protein compositions which are similar to those of VSV (**Fig. 1A**). A low level but distinct crossneutralization of CHP and Piry viruses with VSV-IND was previously reported. However, subsequent studies using hyperimmune guinea pig as well as rabbit serum failed to detect any crossreactivity between these viruses, even when the infective ribonucleoprotein (RNP) particles were used, indicating that these viruses possess distinct antigenic determinants. The Piry virus constituent proteins were also found to be antigenically distinct from several other viruses such as lymphocytic choriomeningitis virus, herpes simplex virus, and 62 arboviruses.

Molecular Aspects: Properties of Genomes and Proteins

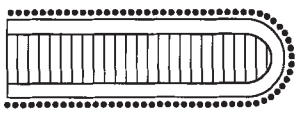
The molecular biology of these viruses remains less studied as compared to that of VSV. Complementation studies and analysis of transcription initiation, termination and intergenic sequences of CHP and Piry virus indicate that the organization of the genome is similar to that of VSV (**Fig. 1B**). The CHP virus has a single-stranded negative-sense RNA genome of approximately 11 kb long which encodes five structural proteins: the nucleocapsid protein N, the phosphoprotein P, the matrix protein M, the glycoprotein G and the large protein L. The genome RNA is tightly encapsidated by the N protein and is associated with the RNA polymerase (a complex of L and P protein) to form an RNP complex. Transcription of the genome RNA by RNA polymerase gives rise to five

dence and close intimacy between viruses and their host cells demonstrates adaptation through evolution by the parasite to take every advantage for self-propagation. The examples used to describe cell organization and metabolism in the context of virology should provide the reader with hints about the use that has been made of viruses as incisive probes of the living cell itself.

See also: **Pathogenesis: Animal viruses, Plant viruses; Replication of viruses; Viral receptors; Virus–host cell interactions.**

Central European Encephalitis Virus *see* **Encephalitis Viruses**

CHANDIPURA, PIRY AND ISFAHAN VIRUSES (*RHABDOVIRIDAE*)



P. De Bishnu and **Amiya K. Banerjee**, Department of Molecular Biology,
The Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, Ohio, USA

History and Classification

Chandipura, Piry and Isfahan viruses are members of the genus *Vesiculovirus* of the family *Rhabdoviridae*. The prototype of this genus is the vesicular stomatitis virus (VSV) of two serotypes, VSV-New Jersey (VSV_{NJ}) and VSV-Indiana (VSV_{IND}), which is known for its association with sporadic epizootics of vesicular disease among horses, cattle, and swine. Chandipura, Piry and Isfahan viruses along with a number of other viruses such as Alagoas, Calchaqui, Cocal, Jurona, La Joya, Maraba, Perinet and Yug Bugdanovac are the least studied members of this genus.

Chandipura (CHP) virus was first isolated by P. N. Bhatt and F. M. Rodrigues in 1965 from human patients at Nagpur county, India. Thus it is quite distinct from VSV with respect to geographic origin and host species origin, because the latter has been isolated almost exclusively from horses and cattles in the western hemispere. Piry virus was isolated by R. E. Shope in 1960 from spleen and liver samples of an opossum (Philander opossum) at Belem, Brazil. Isfahan (ISF) virus was isolated by R. B. Tesh and coworkers in 1975 from humans as well as gerbils and sand flies at Isfahan province, Iran.

These viruses have been classified as *Rhabdoviridae* based on their typical bullet-shaped morphology, approximately 180 nm in length and 65 nm in width, as well as nucleic acid and protein compositions which are similar to those of VSV (**Fig. 1A**). A low level but distinct crossneutralization of CHP and Piry viruses with VSV-IND was previously reported. However, subsequent studies using hyperimmune guinea pig as well as rabbit serum failed to detect any crossreactivity between these viruses, even when the infective ribonucleoprotein (RNP) particles were used, indicating that these viruses possess distinct antigenic determinants. The Piry virus constituent proteins were also found to be antigenically distinct from several other viruses such as lymphocytic choriomeningitis virus, herpes simplex virus, and 62 arboviruses.

Molecular Aspects: Properties of Genomes and Proteins

The molecular biology of these viruses remains less studied as compared to that of VSV. Complementation studies and analysis of transcription initiation, termination and intergenic sequences of CHP and Piry virus indicate that the organization of the genome is similar to that of VSV (**Fig. 1B**). The CHP virus has a single-stranded negative-sense RNA genome of approximately 11 kb long which encodes five structural proteins: the nucleocapsid protein N, the phosphoprotein P, the matrix protein M, the glycoprotein G and the large protein L. The genome RNA is tightly encapsidated by the N protein and is associated with the RNA polymerase (a complex of L and P protein) to form an RNP complex. Transcription of the genome RNA by RNA polymerase gives rise to five

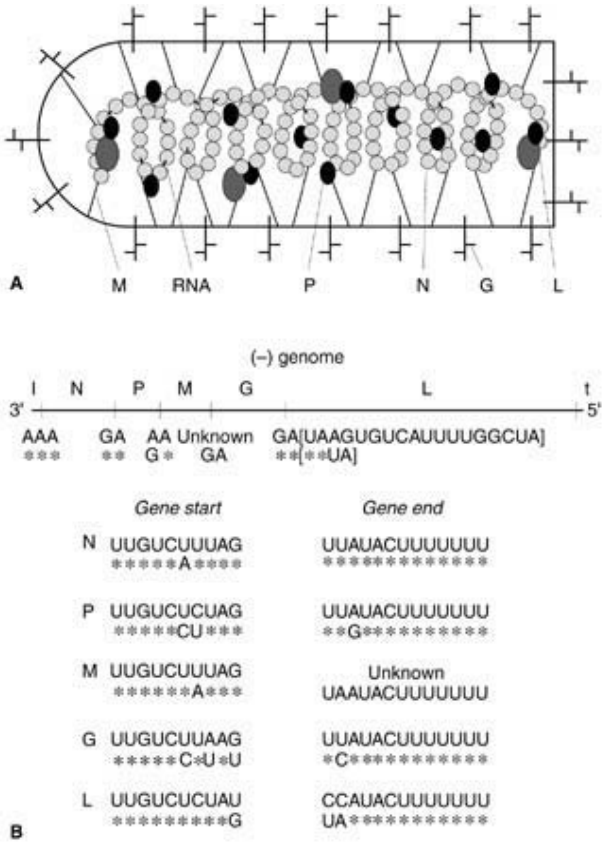


Figure 1 (**A**) Schematic representation of the morphology of vesiculoviruses. The envelope, inner nucleocapsid, and associated proteins are shown. (**B**) The gene order and intergenic regulatory sequences of CHP (upper) and Piry (lower). Asterisks indicate identical sequence in Piry virus. The sequence in brackets represents the insert in the G-L junction.

mRNAs for the five structural proteins. In agreement with serological studies, very little nucleotide sequence homology is found between CHP, Piry and VSV in genomic RNA hybridization analysis.

Nucleotide sequences of the *N*, *P* and *G* genes, G-L junction, part of the *L* gene and the 3′ end of the genome of CHP and Piry virus are known (**Table 1**). Comparison of the deduced amino acid sequences of these proteins with the corresponding sequences of various serotypes of VSV and other rhabdoviruses indicate that these viruses maintain a close evolutionary relationship. In the case of CHP virus, the N mRNA is 1291 nucleotides (nt) long and encodes a protein of 422 amino acids. The N protein is at least 50% homologous to the N proteins of each of the VSV serotypes, and a region near the center of the protein has been identified which is conserved among members of both the rhabdovirus and paramyxovirus families. The P mRNA is 908 nt long and encodes a protein of 293 amino acids. Comparison of P protein sequence with those of the two serotypes of VSV revealed homologies of only about 22%. Although no consecutive stretches of more than four amino acids were identical among these P proteins, a predominantly basic 20 amino acid-long region near the C-termini appears to be highly conserved. Interestingly, overall structure of these P proteins, as assessed by amino acid composition and by the relative hydropathicity distribution, is very similar. Recently, by using bacterially expressed P protein, the phosphorylation of P has been shown to be mediated by the cellular casein kinase II (CK II). By site-directed mutagenesis, Ser62 was identified as the site of phosphorylation. Substitution of Ser62 by Ala resulted in the total loss of P protein function in transcription indicating that the CK II-mediated phosphorylation at Ser62 is required for CHP virus transcription. Interestingly, CK II phosphorylates the VSV_{NJ} P protein in Ser59 and Ser61 and the VSV_{IND} P protein in Ser60, Thr62 and Ser64 residues whereas CHP virus P protein is phosphorylated at a single site, Ser62, in the corresponding region. Importantly, in all these cases CK II-mediated phosphorylation is required for P protein function in transcription. Furthermore, like VSV_{NJ} P protein, the CHP virus P protein also formed dimers after phosphorylation with a concomitant increase in alpha-helicity which is believed to favor transcription activation. The G mRNA is 1681 nt long and encodes a protein of 524 amino acids. The amino acid sequence alignment revealed that the CHP virus G glycoproteins is 39% homologous to each of the VSV G proteins. Analyses of the primary sequence of CHP glycoprotein also revealed the presence of the following consensus motifs: a cytoplasmic face at the C-terminus marked by an abundance of charged amino acid residues (this region contains four cysteines which are potential sites for palmitic acid attachment via thioester linkage); a highly hydrophobic sequence (VVLILVVVLIYGVL) just upstream of the cytoplasmic domain which may serve as a membrane anchor domain. In the case of piry virus, by sequencing the 3′ half of the genome, it has been established that the piry virus, like other vesiculoviruses, contains the genes for N, P, M, G and L in that order. The N mRNA is 1338 nt long and encodes a protein of 427 amino acids. Comparison of amino acid sequences of the N protein of piry virus with that of CHP and VSV, showed about 50% identity, indicating that piry virus is as distantly related to Chandipura virus as it is to the VSV. Analysis of the G protein sequence suggests that piry and CHP are related to each other as closely as the two serotypes of VSV to each other. The P mRNA is 999 nt long and encodes a protein of 328 amino acids. The deduced amino acid sequences of P proteins of piry and VSV

Table 1 CHP and Piry virus mRNAs and proteins characterized by nucleotide sequence analysis

	mRNA (protein)					
Virus	*N*	*P*	*M*	*G*	*L*	*Comments*
CHP	1291 (422)	908 (293)	–	1681 (524)	–	Phosphorylation of P protein by CKII is critical for RNA synthesis by the RNA polymerase complex (L-P). The region (aa 55–70) of P containing the site(s) of phosphorylation of CHP, Piry (?), VSV_{NJ} and VSV_{Ind} are shown below.
Piry	1338 (427)	999 (328)	745 (230)	1683 (529)	–	55 70 CHP: DECEEED*SEEDDDNLP Piry: GSMLKESDESESVDDD VSV_{NJ}: EEES*SD*SDTDYNAEHL VSV_{Ind}: QAADD*SD*TE*SEPEIED

Nucleotide lengths of mRNAs excluding poly(A) tail are shown, with the protein size in amino acids in parentheses. The asterisks indicate CKII phosphorylation sites. In the case of Piry virus, the phosphorylation site(s) remains unknown. However, three Ser residues, Ser 72, Ser 89 and Ser 94 in an acidic region (not shown) conform to the consensus CKII recognition motif.

are highly dissimilar. However, the hydropathicity patterns of these proteins are similar, indicating that the overall secondary structure of P proteins may be conserved. Gene sequences of ISF virus are not known to date.

Host Range, Transmission, Geographic Distribution and Pathogenicity

All three viruses, CHP, piry and ISF, display a wide host range encompassing a large number of species including vertebrates and insects. In nature, these viruses are found in mammals as well as in blood-sucking arthropods such as sand flies, ticks, and mosquitoes, hence their classification as arboviruses. As in the case of other vesiculoviruses, the reservoir cycles for these viruses are poorly understood. Although originally isolated from a human patient, CHP virus was later also recovered from infected hedgehogs (*Atelerix albiventris*) in Nigeria, Africa, and phlebotomine sand flies in West Africa. Antibody to CHP virus has been detected in a variety of mammals, such as horse, donkey, cattle, sheep and goat from various parts of India. Available evidence indicates that insects can act both as host and as vector for transmission between mammals in nature. Transmission of CHP virus by experimentally infected mosquitoes of various species and phlebotomine sand flies to laboratory animals has been documented. Transovarial (vertical) transmission of CHP virus in sand flies has been demonstrated, and the virus has been shown to grow up to 4 logs within 24 h of intrathoracic inoculation in sand flies. Antibody to Piry virus is found in many marsupials as well as in monkeys, edentates, rodents, pigs and water buffaloes. In the laboratory, all three viruses grow well when injected intracerebrally into newborn (suckling) mice, causing viremia and eventual death. Frozen brains of such mice are excellent media for long-term storage of virus specimens. These viruses grow in most mammalian and avian cell lines producing characteristic cytopathic effect (CPE) in liquid cultures and viral plaques under agar.

Mosquitoes infected with rhabdoviruses including CHP, Piry, and ISF develop a lethal sensitivity to CO_2 (cf. Sigma rhabdoviruses of *Drosophila*). Although *Culex* (*Culex quinquefasciatus*) and *Toxorhynchites* (*Toxorhynchites amboinensis*) mosquitoes are CO_2-sensitized by all three viruses, *Aedes* (*Aedes albopictus*) mosquitoes are CO_2-sensitized by CHP and Piry viruses only. In addition, Piry and ISF viruses also produce enhanced CO_2-sensitivity in the fruit fly, *Drosophila melanogaster* (see below), although it has no detectable CPE in *Drosophila* cells *in vitro*.

Serological evidence of natural infection with these viruses has been demonstrated in a wide variety of wild and domestic mammals. The available data suggest that mammals may represent a dead-end host rather than a vector because horizontal transmission is rare and viremia is transient. CHP and Piry viruses have been reported to produce disease in humans. The disease is usually characterized by an influenza-like illness with fever, myalgia, headache, and malaise of 3–6 days duration. Human laboratory infection with Piry virus has been reported due to aerosol inhalation, splashing virus in the eye and contamination of a cut or scratch with infected material. Antibodies to ISF virus have also been reported in humans, but it is unknown whether this infection resulted in clinical illness. No epizootic or epidemic is known for these viruses. Pathogenicity and immunogenicity of CHP and ISF viruses for laboratory animals have been studied. Both viruses were highly pathogenic for mice by the intracerebral route.

Genetics

Spontaneous and mutagen-induced temperature-sensitive (ts) mutants of CHP virus have been classified into complementation groups. Analysis of 50 ts mutants resulted in the identification of six groups designated ChI to ChVI containing 44, 2, 1, 1, 1 and 1 mutant, respectively. These results are in agreement with the presence of five protein-coding genes in the virions. The sixth complementation group probably represents a protein encoded by an alternative open reading frame in the P protein gene. The group ChI, which contains the majority of the mutants, presumably represents the largest gene *L* (see below). Group ChII may represent the gene encoding the glycoprotein G, whereas group ChV may represent the matrix protein M gene. In addition to having a viral degradation phenotype *in vivo*, a mutant of group V overproduced viral mRNAs, suggesting a negative regulatory role of M protein in transcription, as observed for the M protein of VSV *in vitro*. Complementation between ts mutants derived from CHP and different serotypes of VSV is not observed.

Conditional ts mutants of CHP that are unable to multiply in chick-embryo cells at 39°C, the tdCE phenotype, exhibit thermosensitive *in vitro* RNA polymerase activity. Viral protein synthesis was reduced or absent in chick-embryo cells at 39°C indicating that transcription was also defective *in vivo* under these conditions. Although the exact mechanism of inhibition is unknown, it does not appear to involve a defect in methylation of the mRNA 5′ cap structure. Host-induced inactivation of the viral polymerase at the nonpermissive temperature remains a possibility.

Host range mutants of Piry virus designated agD (affected growth in *Drosophila*) that have reduced ability to multiply and induce CO_2 sensitivity in adult *D. melanogaster* (Paris strain) have been isolated. The majority of them were also ts in chicken embryo cells, and some were classified into complementation groups. In the case of one of these mutants, it was shown that the restriction was under the control of a gene on chromosome III. The host gene that affects the multiplication of Piry virus was not one of the five genes known to be involved in the control of sigma virus. However, another class of ts mutant of Piry virus may be restricted by a *Drosophila* gene (*ref*) which also restricts sigma virus. Most agD mutants are generally restricted in all strains of *Drosophila*, whereas other mutants designated rgD (rapid growth in *Drosophila*) have lost general restriction in *Drosophila* but retained specific restriction in the Paris strain. Both agD and rgD mutants have been classified into complementation groups by a UV-inactivation–rescue procedure in which cells are infected with one mutant at low multiplicity in the presence of UV-inactivated wild-type virus or another mutant. The complementation analyses suggest that both kinds of mutants were L protein mutants resulting in a loss of the RNA transcriptase function under nonpermissive conditions. These observations are consistent with the belief that host range properties of vesiculoviruses are determined primarily by specific interactions between cellular factors and the viral polymerase.

See also: **Rhabdoviruses (*Rhabdoviridae*): Ungrouped mammalian, bird and fish rhabdoviruses; Vesicular stomatitus viruses (*Rhabdoviridae*).**

Further Reading

Barik S (1992) The phosphoprotein (P) gene of the rhabdovirus Piry; its cloning, sequencing, and expression in *Escherichia coli*. *Nucleic Acids Res.* 20: 5843.

Chattopadhyay D and Chattopadhyay D (1994) Cloning of the chandipura virus phosphoprotein encoding gene and its expression in *Escherichia coli*. *Cell. Mol. Biol. Res.* 40: 693.

Chattopadhyay D, Raha T and Chattopadhyay D (1997) Single serine phosphorylation within the acidic domain of chandipura virus P protein regulates the transcription in vitro. *Virology* 239: 11.

Masters PS and Banerjee AK (1987) Sequences of chandipura virus N and NS genes: evidence for high mutability of the NS gene within vesiculoviruses. *Virology* 157: 298.

Masters PS, Bhella RS, Butcher M *et al.* (1989) Structure and expression of the glycoprotein gene of chandipura virus. *Virology* 171: 285. Errata: *Virology* 174: 630 (1990).

Pringle CR (1987) Rhabdovirus genetics. In: Wagner R (ed.), *The Rhabdoviruses* p. 167. New York: Plenum.

Shope RE and Tesh RB (1987) The ecology of rhabdoviruses that infect vertebrates. In: Wagner RR (ed.) *The Rhabdoviruses*, p. 509. New York: Plenum.

Channel Catfish Virus *see* **Fish Herpesviruses**

Chicken Herpesvirus *see* **Marek's Disease Virus**

Chickenpox Virus *see* **Varicella-Zoster Virus**

CHIKUNGUNYA, O'NYONG NYONG AND MAYARO VIRUSES (*TOGAVIRIDAE*)

Charles H Calisher, Arthropod Borne and Infections Diseases Laboratory, Colorado State University, Foothills Campus, Fort Collins, Colorado, USA

History

Chikungunya (CHIK) means 'that which bends up', in the language of the people of the Makonde plateau, Newala Province, Tanzania (formerly Tanganyika). It is also the name given to a virus first isolated from human serum collected during an outbreak of febrile illness in that country in 1953. A second outbreak of CHIK fever occurred in South Africa in 1956, and CHIK virus continues to cause epidemics in Africa and elsewhere (see Geographic Distribution, below). O'nyong nyong (ONN) virus was first isolated in 1959 from the serum of a febrile human during an epidemic in Acholi, Uganda; ONN means 'weakening of the joints' in the Acholi dialect. This epidemic of ONN subsequently spread through Uganda to Kenya, Tanzania, Zambia, Malawi and Mozambique, involving several millions of humans. Both the CHIK and ONN epidemics were originally thought to be dengue. Although ONN virus was first isolated during an epidemic in 1959, it was not isolated again until 1978. In 1996 ONN fever reappeared in Uganda after a 35-year absence, although serologic studies of febrile patients in Kenya in 1994 and 1995 suggested that this virus may have been circulating there at that time.

Mayaro (MAY) virus was first isolated from the serum of a human in Mayaro County, southeastern Trinidad, in 1954; although not febrile at the time the serum was collected, the person became febrile the next day; MAY virus was isolated from other patients in Trinidad that year. In 1955, MAY virus was isolated from quarry and forest workers during an epidemic near Belém, Brazil and, also during an epidemic, from recent settlers from Okinawa who were living in a rain forest in eastern Bolivia. MAY virus continues to cause periodic outbreaks of disease, including Brazilian outbreaks in 1991–1993. MAY virus was isolated for the first time in Peru in 1994; as is common with CHIK and ONN, these cases were first diagnosed as dengue fever. Additional cases occurred in Peru in 1997, further suggesting the endemic nature of this virus in Amazonia.

Classification

CHIK, ONN and MAY viruses have been placed in the genus *Alphavirus* of the family *Togaviridae;* the other genus in the family (*Rubivirus*) includes rubella virus. MAY virion diameter is 60–65 nm (cores are 40 nm), and the nucleocapsid is surrounded by a lipid envelope, which is apparently derived from host cell membrane during budding. Originally classified by antigenic means, the togaviruses were designated 'Group A' arboviruses (*ar*thropod-*b*orne *viruses*), along with eastern equine encephalitis, western equine encephalitis, Venezuelan equine encephalitis, Getah, Ross River, Sagiyama, Semliki Forest, Sindbis and others. The two envelope glycoproteins, E1 and E2, compose the external surface of the virion and take part in attachment of virions to cell surfaces, hemagglutination and neutralization. Antigenic subtypes and varieties are numerous for most viruses of this genus.

Geographic and Seasonal Distributions

CHIK virus has been isolated from humans and mosquitoes in Tanzania, South Africa, and most areas of tropical Africa (Angola, Central African Republic, Nigeria, Senegal, Uganda, Zaïre, Zambia, Zimbabwe), and tropical Asia (Campuchea, India, Malaysia, Thailand), including Indonesia. CHIK virus caused large epidemics, outbreaks or isolated cases in Burma, India, Philippines, Sri Lanka, Thailand and Vietnam. Because of the prominent articular manifestations and other clinical criteria, it has been suggested that CHIK caused epidemics in Indonesia (1779), East Africa (1823, 1870), India (1824, 1871, 1901, 1923), the Far East (1901–1902), West Africa (1925), and the southern United States (1827–1828).

The epidemic of ONN spread from Uganda to Kenya, Tanzania, Zambia, Malawi and Mozambique, as shown by virus isolation or antibody detection. In addition, ONN virus has been isolated in Senegal.

MAY virus has been isolated from humans in Trinidad, Brazil, Bolivia and Suriname; a single isolation of MAY virus was made from a northbound migrant bird in Louisiana in April 1967.

Human infections with CHIK, ONN and MAY viruses occur primarily in the rainy season, when mosquito populations are highest. Antibody surveys have shown that 20–90% of a given population may

be immune to CHIK virus. In addition to virus isolation data, such surveys provide evidence for the widespread presence of the virus on the African and Asian continents.

Host Range and Virus Propagation

Humans, laboratory mice and other rodents (*Mystromys albicaudatus*), African vervet (*Cercopithecus aethiops*) and Asian monkeys and other primates (rhesus monkey *Macaca radiata,* baboon *Papio ursinus*), prosimians (*Galago senegalensis*) and bats (*Tadarida, Pipistrellus* and *Scotophilus*) are susceptible to CHIK virus and develop high titer viremias. ONN virus has been isolated only from humans, mosquitoes and sentinel mice. MAY virus has been isolated from a rodent (*Tropidurus torquatus hispidus*) and a lizard (*Ameiva ameiva ameiva*) in Brazil and from a migrating bird entering Louisiana. Development of viremia does not necessarily correlate with production of disease in experimentally infected animals. Antibody to CHIK, ONN and MAY has been detected in various primates and domestic animals and in other vertebrates. Field strains of CHIK, ONN and MAY viruses are nonpathogenic to adult mice but are pathogenic for suckling mice inoculated intracranially; suckling mice are less susceptible to ONN than to CHIK and MAY viruses. Birds appear to be refractory to infection with CHIK virus.

Various cell cultures from vertebrates (Vero, LLCMK2, BHK-21, hamster kidney, chicken embryo, duck embryo) and invertebrates (C6/36 cells from *Aedes albopictus* mosquitoes) are susceptible to these viruses and useful for characterizing them. These cells can be used as substrates for enzyme-linked immunosorbent assays (ELISA) or for tests to detect neutralizing antibodies to CHIK, ONN and MAY viruses, in which these viruses form plaques and cause cytopathic effects.

Genetics

Increasingly, genetic information is becoming available for CHIK, MAY and ONN viruses. An abundance of information is available about eastern equine encephalitis, Ross River, Sindbis and Semliki Forest viruses. Alphavirus genomes contain about 12 000 nucleotides arranged as single, nonsegmented positive sense strands of RNA. The genomes are polyadenylated at the 3′ end and capped with 7-methylguanosine at the 5′ end. Preliminary phylogenetic studies indicate that CHIK and ONN viruses occupy distinct evolutionary lineages and provide no support for the hypothesis that ONN outbreaks result from the emergence and epidemiological alteration of African CHIK virus strains. Phylogenetic relationships among isolates of MAY virus are being done by reverse transcription–polymerase chain reaction but results are not yet available.

Genome transfer into the cytoplasm of the infected cell precedes three sequential stages of viral amplification, i.e. ranslation, transcription and replication of genomic RNA. Subgenomic mRNA (referred to as 26S RNA) is formed during replication and contains about 4100 nucleotides, the sequence of which is identical to the 3′ terminal third of the genomic RNA. This RNA is also polyadenylated and capped and is the template for the four viral structural proteins; the 49S RNA nonstructural region serves as the template for four nonstructural proteins and the capsid. Alphavirus genomes can accept mutations in the conserved regions without destroying biologic activity. Only sequences at the 3′ and 5′ termini are indispensable for the genome to be amplified and packaged. In the region of the junction of nonstructural and structural genes, 19 nucleotides upstream from the beginning of the 26S RNA sequence and five nucleotides downstream from it are needed for production of 26S RNA.

Evolution

Although both structural and nonstructural proteins of alphaviruses are conserved to some extent, the latter are more conserved than the former. Because amino acid sequences of various alphaviruses are encoded by different codons, alphavirus replication may allow the use of different paths to the finished product but retention of similar requirements for accomplishment of fundamentals. Indeed, the original antigenic classification of the alphaviruses depended on the phenotypic expression (antigens) of the gene sequences (genotype). Because the protein structure of Sindbis alphavirus corresponds to (is conserved among) protein structures of certain plant viruses that have genomic organizations dissimilar from that of the alphaviruses, because all alphaviruses and many plant viruses are arthropod-borne at some stage in their natural cycle and because mosquitoes belonging to some of the species that transmit alphaviruses typically feed on plants as well as on vertebrates, alphaviruses and plant viruses may have had common ancestors. Highly conserved regions of alphavirus genomes may provide insights into more recent phylogeny. Relatively minor differences between viruses and virus variants may indicate relatively recent evolutionary drifts, and relatively major differences between viruses may indicate more remote genetic shifts.

Serologic Relationships and Variability

Alphaviruses have common antigenic determinants, as shown by many serologic tests, including hemagglutination–inhibition, complement-fixation, ELISA and immunofluorescence. These viruses have been classified as belonging to one of six antigenic complexes. Viruses in such complexes are more closely related to each other than they are to other viruses within the serogroup (Group A arboviruses). The six complexes are: (1) eastern equine encephalitis; (2) Middelburg; (3) Ndumu; (4) Semliki Forest; (5) Venezuelan equine encephalitis; and (6) western equine encephalitis. CHIK, ONN and MAY viruses belong to the Semliki Forest complex, which includes four viruses: Semliki Forest, CHIK [subtypes CHIK (several varieties) and ONN], Getah (subtypes (Getah, Sagiyama, Bebaru and Ross River) and MAY (subtypes MAY and Una). These viruses can be distinguished from one another by the use of virus- or strain-specific monoclonal antibodies employed in hemagglutination–inhibition, neutralization, ELISA and other tests. Because no two virus isolates are exactly the same, the definition and identification of an alphavirus depends on generalities as to placement in the serogroup, specific identification as to its nearest antigenic neighbor, and final placement with regard to type, subtype or variety. When type-specific antigens (polypeptides) and antibodies (against glycoproteins E1 and E2 and against nucleocapsids) become available, they can be used as reagents for rapid determination of serogroup, complex and virus type and for serodiagnosis of alphavirus infections. Distinguishing CHIK and ONN viruses from each other (MAY virus occurs only in the Americas, where neither CHIK nor ONN are known to occur; ONN has been recognized only in Africa) and from other alphaviruses can be done using neutralization and other tests.

Both antigenic and biologic variation have been detected between strains of CHIK virus from widely different geographic areas. Although antigens participating in hemagglutination and neutralization appear to be conserved, plaque-reduction neutralization tests to distinguish CHIK and ONN viruses are successful when single-dose (infection-immune) antisera are used. African and Asian strains of CHIK virus have been distinguished by using kinetic hemagglutination–inhibition tests or by using monoclonal antibodies in any of various tests, including ELISA.

Epidemiology

CHIK, ONN and MAY viruses are transmitted between vertebrate hosts by hematophagous mosquitoes; aerosol transmission of CHIK virus has been recorded and poses a threat to laboratory workers. In the savannahs and forests of tropical Africa, CHIK has been isolated from mosquitoes of the genus *Aedes*, including *Ae. aegypti, Ae. africanus, Ae. cordillieri, Ae. furcifer, Ae. luteocephalus, Ae. otok* and *Ae. taylori* and also from *Mansonia africana* and from mosquitoes of other genera and species. The principal epidemic vectors in Africa are thought to be those belonging to the *Ae. furcifer-taylori* group, with secondary transmission by *M. africana*. No evidence of transovarial transmission in arthropods has been found.

Humans develop high titer viremias and the peridomestic *Ae. aegypti* transmits CHIK virus to humans in urban and village settings. Nonhuman primates are the primary vertebrate hosts, with human infections occurring secondarily under epidemic conditions (virus, climatic factors that regulate populations of competent mosquito vectors, and nonhuman primate and human populations with low herd immunity).

CHIK virus is maintained in Asia in a human-to-human cycle, with *Ae. aegypti* mosquitoes transmitting the virus. Although Asian nonhuman primates develop high titer viremia, they do not appear to serve as the principal vector or reservoir of CHIK virus, as they do in Africa. Devastating epidemics, some lasting 4–6 years, others sweeping through a city and infecting essentially all the human populace within a few months, have been recorded.

The mosquito vectors of ONN virus are *Anopheles funestus* and *An. gambiae. An. funestus* rests in huts and feeds in the evenings, presumably the reason that human cases of ONN cluster by home, compound or village. Primate and other vertebrate hosts of ONN virus are unknown.

MAY virus has been isolated principally from *Haemagogus janthinomys* mosquitoes. Experimentally infected marmosets and other primates develop high titer viremia. Little else is known about the epidemiology of this virus.

Transmission and Tissue Tropism

These viruses are transmitted to humans by the bite of transmission-competent, infected mosquitoes. When human viremic titers reach such levels that subsequently feeding mosquitoes become infected by feeding on them, these mosquitoes can then transmit virus to people on whom they next feed. If an infected human does not develop a high viremia titer, that person cannot serve as a virus reservoir and becomes a dead-end host. The target tissues for these viruses is

unknown but may include circulatory, synovial, and epidermal cells.

Pathogenicity

These three viruses are pathogenic for humans and other animals, although only some infected persons develop signs and symptoms of illness. Different strains of these viruses do not appear to differ in pathogenic potential.

Clinical Features of Infection

CHIK, ONN and MAY viruses cause illnesses characterized by sudden onset, fever, severe chills, arthralgias, leukopenia and rash. After an incubation period of 2–3 days (range, 1–12 days), otherwise healthy humans infected with CHIK virus typically fall suddenly and seriously into a state of illness. Fever and shaking chills lasting 2–3 days herald the onset; biphasic fevers occur. Severe polyarthralgia commences at or soon after onset of other symptoms. These predominantly affect the small joints of the hands, wrists, ankles and feet but migrate among joints and may affect more than one joint at a time. Joint pain is intense, aggravated by exercise, and relieved but not abolished by rest; generalized myalgias are common. Arthralgias usually resolve within a week after onset but may persist for weeks, months or, rarely, years. More than 12% of patients with CHIK disease develop chronic joint symptoms. Maculopapular rash has been seen from as early as the day of onset to as late as the tenth day after onset. However, rash usually appears at the time the fever abates and lasts 1–5 days. Mild headache, photophobia, retroorbital pain, anorexia, nausea, vomiting, abdominal pains and other generalized signs and symptoms occur but are not usually severe or pathognomonic. Exanthema range from mild to extensive and are often confused with rashes caused by dengue viruses, with which CHIK virus is known to circulate. Petechiae may be present, but hemorrhagic manifestations are unusual. Severe arthralgia is the symptom most indicative of illness caused by CHIK virus.

The clinical syndrome caused by ONN virus is similar to that caused by CHIK virus. After at least an 8-day incubation period the patient experiences sudden onset of joint pain. Rash, often accompanied by resolution of illness, occurs about 4 days later in most patients and lasts 4–7 days. Fever is lower than in CHIK infections but, in contrast to CHIK virus infection, lymph nodes are enlarged. As determined by coordinated serologic and clinical surveys, most infections produce typical symptoms.

As with illnesses due to CHIK and ONN viruses, MAY virus infections are characterized by sudden onset, fever, chills, headache, myalgias and arthralgias of the small joints of the hands and feet. Between 2 and 5 days after onset, fever begins to resolve and a maculopapular rash appears. Arthralgias may persist for many months.

Virus isolation is the test of choice for laboratory diagnosis of these infections. CHIK, ONN or MAY virus can be isolated in either cell cultures or in suckling mice, although considerably more isolates of CHIK and MAY than ONN virus are pathogenic for suckling mice.

Pathology and Histopathology

Patients infected with CHIK, ONN or MAY virus usually do not die; therefore, tissues from these patients are not readily available for histologic examination. Biopsy of a skin lesion showed lymphocytic perivascular cuffing and extravasation of erythrocytes from superficial capillaries. A small proportion of children and an even smaller proportion of adults develop severe illness. In infants and young children infected with CHIK virus, mild hemorrhagic manifestations have been observed and the rare fatality recorded. Whether these were due to an underlying convulsive disorder or to some other preexisting abnormality is not known. Nevertheless, although rare convulsions in infants might be due to sudden febrile episodes, the occurrence of focal seizures, repeated seizures and convulsions in adults suggests that CHIK virus may directly involve the brain. Treatment of CHIK, ONN and MAY infections consists primarily of supportive therapy. Symptoms respond partially to nonsteroidal anti-inflammatory drugs and chloroquine phosphate has been evaluated for treatment of CHIK disease.

Immune Response

Infection with CHIK, ONN or MAY virus leads to production of immunoglobulin (Ig) M, IgG and, probably, IgA antibodies. As detected by IgM-capture ELISA, antibody to these viruses crossreacts with heterologous alphaviruses but is most reactive with other viruses of the same antigenic complex. That is, IgM antibody from patients with CHIK have highest titers to CHIK but higher titers to ONN and MAY viruses than to, for example, eastern equine encephalitis virus. IgM antibody peaks 2–3 weeks (3–5 weeks in CHIK virus infections) after onset and persists for about 2 months. IgG antibody also appears relatively soon after onset but, unlike IgM, persists for many months or years after the illness. Hemagglutination–

inhibition, complement-fixation, immunofluorescence and neutralization tests simply detect IgM and IgG antibodies in other configurations. IgM antibody is hemagglutinin-inhibiting, immunofluorescing and neutralizing; IgG antibody reacts in these tests and also in complement-fixation tests. Thus, IgM ELISA is the diagnostic test of choice for determining recent infections (if confirmed by neutralization tests) and may be applied to single serum samples in some instances. The presence of IgG antibody to any of these viruses is simply an indication of past infection and, without demonstration of a significant (fourfold or greater) increase or decrease between paired acute-phase and convalescent-phase serum samples, cannot be used to implicate the virus in the etiology of the illness; usefulness of IgG assays is limited because of crossreactivity.

Prevention and Control

Because CHIK, ONN and MAY viruses are transmitted by mosquitoes, prevention of human infection requires reduction of contact between the susceptible human and the vector. This can be effected by mosquito control operations, using larvicide or adulticide techniques, either on a large (spraying of insecticides from airplanes) or a small (spraying of insecticides from back-pack units) scale. Public education projects (with on-going publicity) and legal means are often used to direct intervention. On-going clinical, virologic, serologic and arthropod surveillance techniques should be used to ascertain the presence and prevalence of these viruses. In certain countries, this often requires considerable political will.

For preventing contact of mosquito vectors with humans, the use of netting and window and door screening is recommended. Removing and subsequently destroying mosquito breeding sites (source reduction), providing piped water, maintaining liquid waste systems and managing irrigation systems have been successful ways of reducing populations of both maintenance and amplification vectors.

Future Perspectives

If measures taken to control arthropod vectors of CHIK, ONN and MAY viruses are not effective or are not possible because of economic, political or geographic considerations, vaccines hold some potential for prevention of disease in humans at risk of infection. Although there may not be a need for a vaccine to prevent ONN virus infection, the prevalence of CHIK in both Africa and in Southeast Asia, the presence of competent vectors for this virus (*Ae. albopictus, Ae. aegypti*) in the United States and focally through a substantial portion of the Americas, and a human population completely susceptible to these viruses are sufficient justifications for developing such a vaccine. With rapid air transport, an increasing number of tourists are exposed to potential infection (one human infection with MAY virus was recently diagnosed in the US in a patient who had recently traveled to the Amazon region). Whether a return home in the incubation (viremic) periods of CHIK or MAY virus infections can infect local mosquito populations is unknown. An experimental vaccine against CHIK virus has recently been developed and is being tested in humans. Because of the close antigenic relationships between these viruses, a CHIK vaccine may prove to confer protection not only against CHIK virus but against ONN, MAY and other closely related pathogenic alphaviruses as well.

See also: **Equine encephalitis viruses (*Togaviridae*); Sindbis and Semliki Forest viruses (*Togaviridae*).**

Further Reading

American Committee on Arthropod-Borne Viruses (1985) In: Karabatsos N (ed.) *International Catalogue of Arboviruses Including Certain Other Viruses of Vertebrates*, American Society of Tropical Medicine and Hygiene.

Calisher CH, Shope RE, Brandt W *et al* (1980) Proposed antigenic classification of registered arboviruses I. *Togaviridae, Alphavirus*. *Intervirology* 14: 229.

Johnston RE and Peters CJ (1996) Alphaviruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, pp. 843–898. New York: Lippincott-Raven Press.

Jupp PG and McIntosh BM (1988) Chikungunya virus disease. In: Monath TP (ed.) *The Arboviruses: Epidemiology and Ecology*, vol. II, p. 137. Boca Raton, FL: CRC Press.

Rwaguma EB, Lutwama JJ, Sempala SDK *et al* (1997) Emergence of epidemic O'nyong-nyong fever in southwestern Uganda, after an absence of 35 years. *Emerg. Infect. Dis.* 3: 77.

Schlesinger A and Schlesinger MJ (1996) *Togaviridae*: The viruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, pp. 825–841. New York: Lippincott-Raven Press.

Theiler M and Downs WG (1973) *The Arthropod-borne Viruses of Vertebrates*. New Haven/London: Yale University Press.

Chilo Iridescent Virus *see* **Iridoviridae – invertebrate**

Chimpanzee Herpesvirus *see* **Herpesvirus – Baboon and Chimpanzee**

Chlorella Virus *see* **Algal Viruses**

CLOSTEROVIRUSES (*CLOSTEROVIRIDAE*)

Sylvie German-Retana and **Thierry Candresse**, Station de Pathologie Végétale, Institut National de la Recherche Agronomique, Villenave d'Ornon, France

Giovanni Martelli, Dipartimento di Protezione delle Piante dalle Malatie, Universita degli Studi and Centro di Studio del CNR sui Virus e Virosi delle Colture Mediteranee, Bari, Italy

History

The name closterovirus was first coined in the early 1970s for a taxonomic group of plant viruses characterized by an elongated, very flexuous particle morphology and an open particle structure. The name is derived from the Greek 'kloster' meaning thread or filament. The group was approved by the International Committee on Taxonomy of Viruses in 1976.

Very early, it was observed that closteroviruses showed some variations in a number of their properties, such as genome size, cytopathology, tissue tropism and relation with insect vectors. This initially led to several attempts to divide the group. In the early 1990s, the availability of genomic sequences led to the splitting of the group to separate the trichoviruses from the true closteroviruses. Due to their localization in the phloem and the difficulties in particle purification, closteroviruses have traditionally been a genus which, with few exceptions, has attracted relatively little attention. However, recent years have seen a remarkable increase in the number of publications on closteroviruses. This can probably be explained by two factors: the significant improvement brought to these studies by the introduction of molecular biology techniques, and the emergence of a number of 'new' closteroviruses, apparently linked to the explosion of insect vector populations. As a result, characterization and sequence data are now accumulating at a rapid rate. This has already led to a further splitting of the *Closterovirus* genus, in order to accommodate in a separate taxon the species with a divided genome (*Crinivirus*). The two genera (*Closterovirus* and *Crinivirus*) are now included in the recently created *Closteroviridae* family.

Regardless of whether monopartite or bipartite, closterovirus genomes have a similar structure and contain unique genes coding for a homologue of the cellular heat shock protein (HSP) 70 proteins and a divergent copy of the coat protein. These genes are present in all species sequenced to date. Closteroviruses are phloem-associated and induce the formation of characteristic cytopathic structures in infected cells. Transmission is semipersistent by a variety of vectors, i.e. aphids, whiteflies, and pseudococcid or coccid mealybugs. Some can be transmitted (with difficulty) by mechanical inoculation. These properties are common to all species and should be regarded as characterizing traits of the family.

Taxonomy and Classification

The major problem in dealing with the taxonomy of members of the *Closteroviridae* is that, for many of these viruses, only limited information is available. This is mostly due to the fact that particles are difficult to purify because of low virus titer in the plants (linked with their phloem localization). The particles are also labile and they tend to break or aggregate during purification.

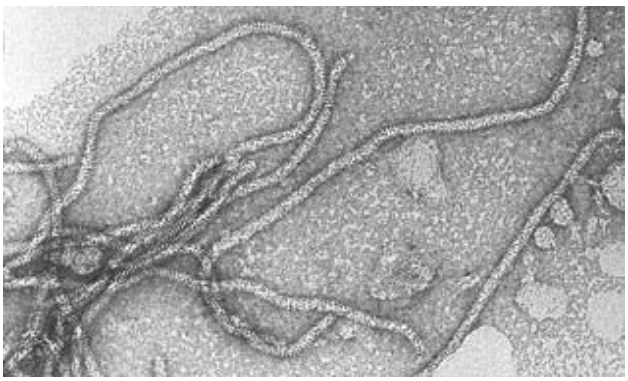


Figure 1 Particles of grapevine leafroll associated virus 2 (GLRaV-2) observed by electron microscopy. Original magnification approximately ×250 000.

Closterovirus taxonomy has rapidly evolved during the last few years as nucleotide sequence data began to accumulate. Major differences in genome size and organization, biological properties (type of vector, host range) and the results of phylogenetic analysis, suggested that the family *Closteroviridae* be divided into at least two genera (**Table 1**):

- Genus *Closterovirus*, typified by beet yellows virus (BYV), comprised of species with monopartite genomes, transmitted by aphids, mealybugs or whiteflies.
- Genus *rinivirus*, typified by lettuce infectious yellows virus (LIYV), comprised of whitefly-transmitted species with bipartite genomes.

Particle Structure and Composition

Although resembling that of some other flexuous filamentous plant viruses, such as tricho-, capillo- and vitiviruses, the very flexuous and open particle structure is still the most common and conspicuous trait of members of the *Closteroviridae* family (**Fig. 1**). In fact, in preliminary studies, this is often the only indication that a given virus might be a member of this family. Particles have, in general, a consistent diameter of about 12 nm, with a length varying between 650–900 and 2000 nm, depending on the virus. The RNA content is usually 5–6%. For some viruses (BYV, citrus tristeza virus (CTV), LIYV, beet yellows stunt virus (BYSV), little cherry virus (LChV)), two types of coat protein (CP) subunits have been identified: the genuine CP and a CP homologue that probably arose by gene duplication. With BYV and CTV, the duplicate protein coats an extremity of the virions, forming a distinct structure, for which the terms 'rattlesnake' or 'heterodimeric' have been coined. Given that the presence of a CP duplicate is found in all closteroviruses so far sequenced, such an unusual particle structure may well be a general characteristic of the family.

In addition to the end-to-end aggregation and breakage problems encountered during purification, it should be noted that closterovirus particles may assume slightly different dimensions depending on the negative stain used for electron microscopy, further complicating the determination of their size. In the case of CTV, smaller than full-length particles have been detected, which could encapsidate subgenomic or defective interfering RNAs.

The pitch of the primary helix, which can usually be measured from the obvious crossbanding of the particle, is in the 3–3.8 nm range, giving estimated ratios of four nucleotides per protein subunit and of about ten protein monomers per turn of the helix. The CPs of most members of the family fall in a restricted size range, usually 22–30 kDa. Notable exceptions are the CPs of mealybug-transmitted viruses, such as LChV or the grapevine leafroll associated viruses (GLRaVs), that have molecular masses of up to 46 kDa. An unusual property of the CP of some closteroviruses, such as BYV and carnation necrotic fleck virus (CNFV), is that they are devoid of tryptophan, which explains their high (1.4–1.8) A_{260}:A_{280} absorbance ratios. However, the CP of CTV does contain a significant amount of tryptophan (A_{260}:A_{280} of 1.21–1.22). The particles of several virus species such as CTV are not stable in CsCl solutions but are stable in Cs_2SO_4, with a buoyant density of 1.24–1.27 g ml^{-1}.

Genome Structure

Members of the *Closteroviridae* family have either monopartite or bipartite single-stranded RNA genomes of positive polarity. The genomic RNAs of BYV, CTV and LIYV are infectious, and have messenger activity *in vitro*. The genomic RNAs of

Table 1 Tentative grouping, particle length and coat protein molecular weight of members of the *Closteroviridae* family

Virus	*Particle length (nm)*	*Mol. wt (× 10^3)*
Genus *Closterovirus*		
A. Definitive species		
Aphid-transmitted		
Beet yellows virus (BYV)	1250–1450	22
Beet yellow stunt virus (BYSV)	1250–1400	24
Carnation necrotic fleck virus (CNFV)	1400–1500	23.5
Wheat yellow leaf virus (WYLV)	1600–1850	?
Burdock yellows virus (BuYV)	1600–1750	?
Citrus tristeza virus (CTV)	1900–2000	22
Carrot yellow leaf virus (CYLV)	1600	?
Mealybug-transmitted		
Grapevine leafroll-associated virus 3 (GLRaV-3)	1800–2100	43
Little cherry virus (LChV)		46
Vector unknown		
Grapevine leafroll-associated virus 2 (GLRaV-2)	1800	24
B. Tentative species		
Aphid-transmitted		
Clover yellows virus (CYV)	1700–1800	?
Dendrobium vein necrosis virus (DVNV)	1850	?
Heracleum virus 6 (HV6)	1400–1660	?
Mealybug-transmitted		
Pineapple mealybug wilt-associated virus 1 (PMWaV-1)	?	?
Pineapple mealybug wilt-associated virus 2 (PMWaV-2)	?	?
Sugarcane mild mosaic virus (SMMV)	1500–1600	?
Whitefly-transmitted		
Beet pseudo-yellows virus (BPYD)	1500–1800	?
Diodea vein chlorosis virus (DVCV)	?	?
Cucurbit chlorotic spot virus (CCSV)	1250–1450	?
Vector unknowm		
Grapevine leafroll associated virus 1 (GLRaV-1)	2200	39
Grapevine leafroll associated virus 4 (GLRaV-4)	1800	36
Grapevine leafroll associated virus 5 (GLRaV-5)	1400–1700	35
Grapevine leafroll associated virus 6 (GLRaV-6)	1800	36
Grapevine leafroll associated virus 7 (GLRaV-7)	1500–1700	37
Festuca necrosis virus (FNV)	1700	?
Alligator weed stunting virus (AWSV)	1700	?
Megakepasma mosaic virus (MeMV)	?	?
Genus *Crinivirus*		
A. Definitive species		
Abutilon yellows virus (AYV)	800–850	?
Cucurbit yellow stunting disorder virus (CYSDV)	825–900	?
Lettuce chlorosis virus (LCV)	800–850	33
Lettuce infectious yellows virus (LIYV)	650–700	28
Sweet potato sunken vein virus (SPSVV)	850	29
Tomato chlorosis virus (ToCV)	800–850	32
Tomato infectious chlorosis virus (TICV)	850–900	32

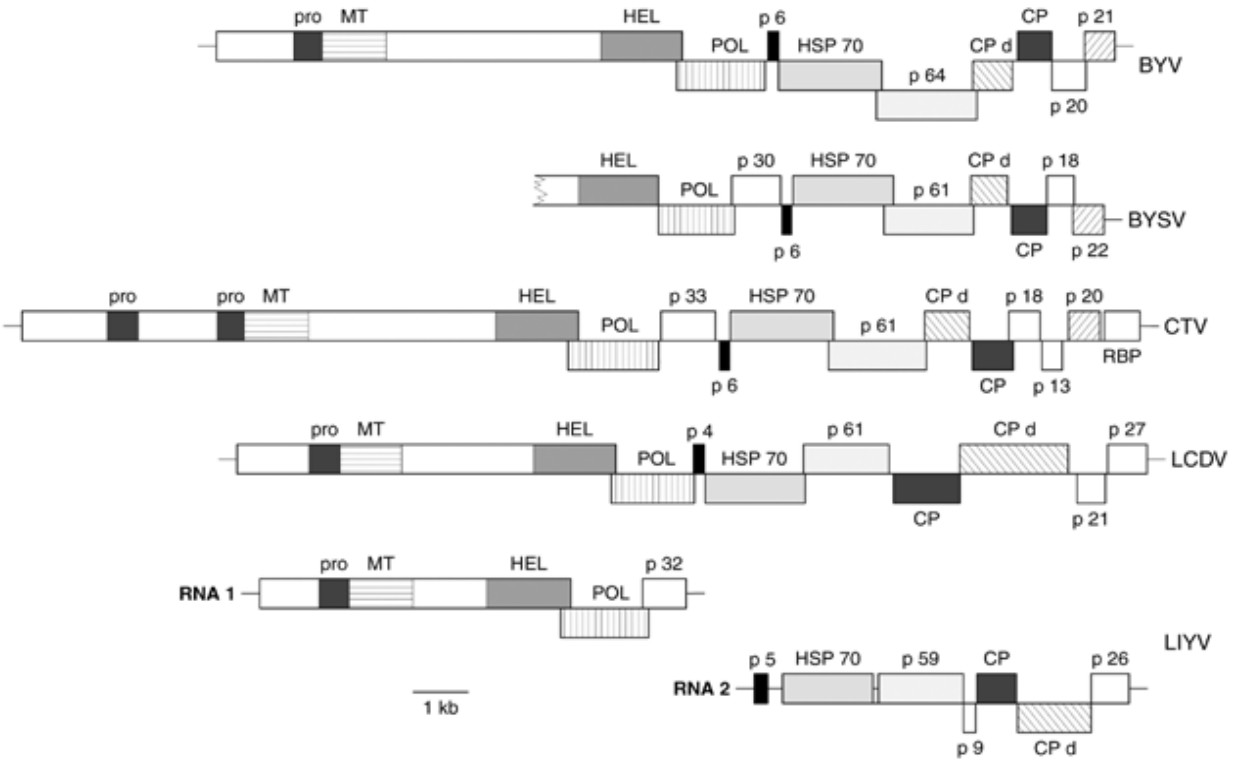


Figure 2 Genomic organization of some members of the *Closteroviridae* family. BYV, beet yellows virus; BYSV, beet yellow stunt virus; CTV, citrus tristeza virus; LChV, little cherry virus; LIYV, lettuce infectious yellows virus; pro, domain characteristic of papain-like proteinases; MT, methyltransferase domain; HEL, helicase domain; POL, RNA-dependent RNA polymerase domain; HSP 70, protein homologue of the cellular HSP 70 heat shock protein; CP, coat protein; CPd, diverged duplicate of coat protein; RBP, RNA-binding protein.

BYV, CTV, LChV and LIYV do not appear to include a poly(A) tract, but terminate with a heteropolymeric sequence devoid of conspicuous features other than the frequent presence of potential hairpins near the 3′ end. Indirect evidence suggests that the 5′ end of the genome of family members is capped.

Genome Organization and Expression: Affinities with Other Virus Taxa

To date, complete genomic sequences have been determined for only five viruses in the family: BYV, LIYV, LChV and two isolates of CTV (CTV-T36, a decline isolate from Florida, and CTV-VT, a yellows isolate from Israel), while the 3′-terminal sequence is available for beet yellows stunt virus (BYSV), grapevine leafroll-associated virus 2 (GLRaV-2), grapevine leafroll-associated virus 3 (GLRaV-3), and tomato infectious chlorosis virus (TICV). Relevant accession numbers are: (X73476: BYV), (RNA1 U15440, RNA2 U15441: LIYV), (Y10237: LChV), (U16304: CTV-T36), (U56902: CTV-VT), (U51931: BYSV), (Y14131: GLRaV-2).

The 15 500 nt genome of BYV shows an arrangement of nine open reading frames (ORFs) (**Fig. 2**). The largest 5′-proximal ORF, 1a, encodes a large polyprotein with calculated molecular mass of 295 kDa, containing domains characteristic of papain-like proteinase (P-PRO), methyltransferase (MT) and helicase (HEL). The downstream ORF 1b encodes a 48 kDa putative RNA-dependent RNA polymerase (POL), which is probably expressed via a +1 ribosomal frameshifting, as a 348 kDa ORF 1a/1b fusion protein. ORF 2 encodes a 6.4 kDa protein with membrane-binding properties, homologous to the small hydrophobic proteins of potex- and carlaviruses. ORF 3 codes for a 65 kDa homologue of the cellular HSP 70 heat shock proteins. The proteins of this family are molecular chaperones found in all cell types, and are implicated in protein–protein interactions. The 65 kDa protein of BYV has ATPase activity associated with the conserved N-terminal domain. However, it apparently lacks the ability to interact with unfolded protein chains, another property typical of cellular HSP 70 proteins. Recent experimental work indicates that this protein is essential for cell-to-cell movement of the virus. ORF

4 overlaps ORF 3 and encodes a 64 kDa protein with sequence similarity to proteins of the HSP 90 family of heat shock proteins. ORFs 6 and 5 code for the capsid protein (22 kDa) and its diverged duplicate (24 kDa), respectively. ORFs 7 and 8 code for proteins that do not have sequence similarity to any proteins in the database, with the exception of the homologous proteins of CTV. ORF 2, 3, 4, 5 and 6, coding for the small hydrophobic protein, the HSP 70 homologue, the HSP 90 homologue, the diverged copy of the CP and the CP itself, constitute a five-gene module present in all species sequenced so far.

The 19296 nt genome of CTV-T36 (the largest known plant virus genome) contains 12 ORFs potentially encoding at least 17 protein products. The general gene arrangement is similar to that of BYV, with two gene blocks or modules easily identified. The first block is the replication-associated complex (including the MT, HEL and POL domains, with the POL expressed via a +1 ribosomal frameshifting) preceded by a similar papain-like protease (apparently duplicated in the case of CTV). The second block is the five-gene module. Outside of these modules, other genes in CTV and BYV differ. In addition to a supplementary copy of the leader protease, CTV has four unique genes that do not have counterparts in the BYV genome, including a 3′-terminal ORF encoding a 23 kDa protein which probably has RNA-binding activity.

The genome of LIYV consists of two RNA components. The 8118 nt RNA1 contains three ORFs that include the domains for a papain-like protease, methyltransferase, helicase and RNA polymerase (expressed via a +1 ribosomal frameshift). The 7193 nt RNA2 includes six ORFs, which include the five-gene module. However, LIYV is distinct from monopartite closteroviruses in several ways: its genome consists of two RNAs; the order of the capsid protein gene and the gene for its diverged copy is reversed as compared to BYV and CTV (the same is also observed in the mealybug-transmitted LChV, GLRaV-3 and in the whitefly-transmitted sweet potato sunken vein virus (SPSVV)). The diverged copy (52 kDa) is also much larger than the CP (28 kDa) and could be the result of a triplication of the CP gene, with the CP gene followed by two fused, highly diverged duplicates. In addition, LIYV includes ORFs that are not related to ORFs found in the other viruses.

Members of the *Closteroviridae* share common and original expression mechanisms. In all members of the family for which sufficient information is available, the POL domain is encoded by a separate ORF overlapping with the 3′-terminal portion of the large upstream ORF, including the MT and HEL domains. It has been suggested that both ORFs are expressed as a large fusion protein resulting from a +1 ribosomal frameshifting. Such a +1 ribosomal frameshifting is a unique feature among positive-strand plant viruses. *In vitro* translation experiments have demonstrated the autocatalytic cleavage of the N-terminal portion of the ORF 1a product of BYV that contains the papain-like proteinase domain. Furthermore, site-directed mutagenesis of the predicted catalytic amino acid residues abolished the proteolysis, thus corroborating the identity of the protease. With BYV, CTV, BYSV and LIYV, the products of the ORFs downstream, ORFs 1a and 1b, are expressed via the formation of a nested set of 3′-coterminal subgenomic RNAs (sgRNA). Therefore, the genome expression of these viruses (and likely of all members of the family) is based on polyprotein processing, translational frameshifting and multiple sgRNA generation, thus resembling that of coronaviruses. However, unlike coronaviruses, the closterovirus polymerase belongs to the Sindbis virus-like lineage of polymerases.

Another common feature of members of the family is the presence of the gene coding for the HSP 70-related protein. This gene, first reported for BYV, has now been identified in 12 additional closteroviruses (CTV, LChV, BPYV, BYSV, CCSV, CNFV, GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-7, PMWaV-1, PMWaV-2) and five criniviruses (CYSDV, TICV, LIYV, LCV, ToCV). In the cell, the molecular chaperones of the HSP 70 family perform several functions, including protein folding, protein trafficking and secretion. Like some HSP 70 proteins, the 65 kDa protein of BYV binds to microtubules *in vitro*, this binding being stimulated by ATP hydrolysis. Several functions have been postulated for this HSP 70 homologue: mediation of cell-to-cell movement through plasmodesmata via interactions with the cellular translocation machinery (such as cytoskeletal or secretional proteins); involvement in the assembly of multisubunit complexes for genome replication and/or subgenomic RNA synthesis, or assembly of viral particles. Recent evidence, showing that BYV mutants in which the expression of the HSP 70 protein is abolished are restricted to inoculated cells, demonstrates the role of this protein in cell-to-cell movement of the virus.

A further peculiarity of members of the *Closteroviridae* is the duplication of the capsid protein gene. To date, this is the only known case of such a duplication in viruses with elongated particles. The capsid proteins of BYV and CTV, and their homologues, show a high degree of sequence conservation and, according to the pattern of conserved amino acid residues, the duplicate copies probably retain the general spatial folding and some crucial properties of

the CPs. Interestingly, in LIYV, LChV, GLRaV-2 and GLRaV-3, the CP gene and its tandem copy have the opposite order as compared to BYV and CTV, and the similarity is much lower.

The large size of the *Closteroviridae* genomes has been proposed to be due to the acquisition of foreign coding sequences via RNA recombination (protease, HSP 70 protein) and gene duplication, which may also explain the significant variability in genome organization observed between genera and among members of the same genus. Phylogenetic reconstructions based on POL, HEL and MT amino acid sequence comparisons indicate that the *Closteroviridae* should be regarded as members of the 'alpha-like' supergroup of viruses, their closest affinities being with the family *Bromoviridae*. Sequence comparisons show that the CPs of BYV and CTV are related (24% homology). These proteins belong to a lineage that includes the CPs of many other filamentous plant viruses (carla-, potex-, capillo-, tricho-, viti- and potyviruses) but they are not closely related to the CPs of members of any of these groups.

Host Range and Geographic Distribution

Host ranges of individual members of the *Closteroviridae* vary considerably but are usually rather restricted. While BYV was reported to infect over 120 species in 15 families, many other viral species have host ranges limited to a single botanical family. This is the case of CNFV, CTV, wheat yellow leaf virus (WYLV), LChV, and the GLRaVs. Very different geographic distribution situations are reported, depending on the individual virus considered: while some viruses cover essentially the totality of their host's geographic range (e.g. CTV, BYV, CNFV, GLRaVs), others have rather restricted distributions.

Virus–Host Relations: Cytopathic Effects

Some closterovirus, but not crinivirus species, are transmitted by sap inoculation, though with great difficulty and low efficiency, most likely because of their phloem-restricted condition. The cells of infected plants show two types of cytopathic effects: nonspecific subcellular changes accompanying chlorotic or necrotic reactions (e.g. membrane proliferation, vesiculation of chloroplasts, degeneration and vesiculation of mitochondria, accumulation of osmiophilic granules) and specific reactions, in the form of inclusion bodies. Two types of inclusions are observed: aggregates of virions and membranous vesicles, or combinations of the two. Virions occur either in crossbanded aggregates made up of stacked tiers of particles packed side by side, or in loose wavy paracrystalline aggregates, or, more typically, in irregular bundles intermingled with single or clustered membranous vesicles containing a network of fine fibrils. Inclusion of the latter kind are one of the hallmarks of members of the *Closteroviridae*, and are often referred to as BYV-type inclusion bodies. They are limited to the phloem of infected cells, i.e. sieve tubes, companion cells and phloem parenchyma, but occasionally, as with BYV, can also be found in the mesophyll and epidermis near local lesions. The fibrillar material contained within the membranous vesicles has been interpreted as possible double-stranded RNA, thus suggesting a role for these structures in viral replication, a contention that needs experimental verification.

Virus Transmission

BYV, the type species of the *Closterovirus* genus, has aphid vectors. However, other types of vectors (mealybugs, whiteflies) have been reported for members of the same genus. In many cases, the mode of transmission or the vector has not been determined (**Table 1**). Studies have mostly dealt with aphid transmission of BYV and CTV. Information on the transmission of other viruses is still very limited and, in many instances, transmission has only been demonstrated under laboratory conditions. In the case of BYV, CTV and other closteroviruses, aphid transmission is semipersistent, i.e. long acquisition and transmission periods (minimum feeding time for acquisition and transmission in the range of 15 and 30 min, respectively), relatively long retention of the virus (up to 2–3 days) and absence of a latency period. The virus does not appear to be retained through molts or to be transmitted to the vector progeny. In general, relatively short acquisition or transmission feeding periods result in lower efficiency of transmission, this being correlated with the length of these periods (up to 24 h for acquisition, up to 6 h for transmission). The molecular bases of the semipersistent mode of aphid transmission are currently not understood. In particular, the localization of virions in viruliferous aphids is not known, nor are the reasons for the long retention period. On the other hand, the close association of the viruses with phloem tissues accounts for some of the transmission characteristics, such as the length of the acquisition and transmission feeding periods and the lack of transmission during brief probings. One hypothesis, which remains to be verified, is that the CP duplicate may facilitate the interaction between virions and the insect's foregut.

Vector ranges vary from rather wide to restricted, depending on the virus: BYV has been shown to be transmitted by 23 aphid species (*Myzus persicae* and *Aphis fabae* being the major natural vectors), CTV by seven species (*Toxoptera citricida* and *Aphis gossypii* being the most efficient vectors) and a number of other viruses, such as CNFV and WYLV, by a single species.

Some of the closterovirus species and all those of the *Crinivirus* genus are transmitted semipersistently by whiteflies: namely, BPYV, TICV and CCSV, transmitted by *Trialeurodes vaporariorum*; LIYV, CYSDV and SPSVV, transmitted by *Bemisia tabaci*; LCV, transmitted by *Bemisia argentifolii*; diodia vein chlorosis virus (DVCV) and abutilon yellow virus (AYV), transmitted by *Trialeurodes abutilonea*. Persistence and specificity of transmission of whitefly-transmitted viruses in their respective vectors has been used as a biological character for virus species differentiation. For example, LIYV is retained by viruliferous vectors for a maximum of 3 days, while LCV and CYSDV can be retained for 4 and 9 days, respectively.

Pseudococcid (*Planococcus, Pseudococcus, Phenacoccus, Saccharicoccus* and *Dysmicoccus*) and coccid (*Pulvinaria*) mealybugs have been reported to transmit five different closteroviruses (**Table 1**). The mode of transmission, determined so far only for GLRaV-3, is semipersistent, and may not be vector-specific, as this virus can be transmitted by both pseudococcid (*Planococcus* and *Pseudococcus*) and coccid (*Pulvinaria*) mealybug genera.

Diseases and Their Economic Significance

The most frequent type of symptoms induced by members of the *Closteroviridae* family are of the yellowing type, frequently accompanied by phloem necrosis. Phloem localization of these viruses probably accounts for these types of symptoms, which are also caused by luteoviruses, another group of phloem-restricted viruses. The economic impact varies widely with the virus: while some are known to affect weeds only, many others have a significant detrimental impact on crop plants. These include BYV, CTV, CNFV, whitefly-transmitted viruses like BPYV, LIYV, and the GLRaVs.

BYV is responsible for serious losses of beet and spinach. Together with beet mild yellowing and beet western yellows luteoviruses, this virus is responsible for the so-called yellowing diseases of beet. In some European countries, losses of sugar beet yield have been estimated to be as high as 15–30% in some years. This reduction is mostly due to decreased root yields, with only marginal effects on the sugar content. Severity of losses correlates with the time of infection and the susceptibility of the affected cultivar. In artificial infection trials, yield losses as high as 40–60% have been recorded, but late season infection has only marginal effects on the yield.

CTV is undoubtedly one of the major citrus pathogens, recognized as one of the top ten viruses with the highest economic impact in eight out of ten wide geographic areas of the world. It is estimated that over the past 50 years or so, CTV has caused the loss of over 80 million trees worldwide. The name 'tristeza' is derived from a condition leading to a quick decline of sweet orange and numerous other commercial citrus species grafted on sour orange rootstocks, a symptom due to a scion–rootstock combination problem, with no decline appearing on plants grafted on tolerant rootstock. The most devastating disease induced by CTV is probably stem pitting of a number of citrus species, such as grapefruits, some sweet orange varieties, and sour and sweet limes. Stem pitting is usually accompanied by loss of plant vigour, decline and reduced fruit size. Other major types of diseases caused by CTV include vein clearing of sour lime and seedling yellows, a severe stunting disorder developed by some varieties when infected at an early stage. The pattern of symptoms induced by CTV is complicated by large differences in species and varietal susceptibility and by the existence of an extremely large biological variability between CTV strains.

Whitefly-transmitted viruses are the widespread agents of emerging diseases causing major losses to a number of crops, such as lettuce, sugar beet and cucurbits. A clear-cut estimate of yield losses caused by viral infections is complicated by the difficulty of correctly identifying the agent, because field symptoms (stunting, yellowing and reduced vigour) can also be attributed to the whitefly vectors. In documented cases of severe LIYV infection, losses of up to 80% have been reported from southwestern USA. Epidemic virus outbreaks have been estimated to induce, in a single season, losses of lettuce, sugarbeet, melon and squash worth $US8 million. The recently observed increase in the impact of these viruses appears to be directly linked to the explosion of whitefly vector populations during the last few years, which may have been favoured by a number of factors, i.e. the widespread use of synthetic organic insecticides, resistance to pesticides, changing climatic conditions, intensified agricultural practices and international distribution of plant material contaminated by whitefly populations.

Leafroll disease is one of the most economically relevant diseases of the grapevine and occurs world-

wide. It causes yield losses of 10–70%, lowers the sugar content, soluble solids and phenolic compounds of the berries, and reduces graft take and rooting ability. The etiology of the disease is being unravelled, for at least three (GLRaV-1, GLRaV-3, and GLRaV-7) of the seven serologically distinct closteroviruses associated with it have been experimentally proven to be genuine leafroll agents. GLRaV-2 is the cause of both leafroll and a graft incompatibility condition.

Virus Epidemiology and Control

Transmission by vectors may not be the major means of long-distance closterovirus dispersal, which is primarily due to distribution of contaminated propagating material through trading. This is obviously the case for CTV, CNFV and GLRAVs, and the cause of the transfer of BYV from Europe to the USA. In regions where a given virus is absent, quarantine is the best protective measure. In infected areas, infections can be controlled through the production and use of virus-free propagative material, along with control of insect vector populations and destruction of virus reservoirs. When possible, these measures can be coupled with the use of resistant or tolerant varieties or rootstocks. A search for natural resistance has been successful for CTV, but not for GLRaVs, and is currently underway for criniviruses.

The most popular detection techniques for certification of propagation and planting material include bioassays on susceptible hosts (indexing) and enzyme-linked immunosorbent assays (ELISA). Detection of double-stranded RNAs associated with viral infection has also been used, though often inconclusively. The potential of molecular techniques (hybridization, polymerase chain reaction (PCR)) is increasingly being exploited. PCR appears of particular interest when targeting the conserved HSP70 coding region.

In most cases, it has been possible to obtain virus-free plants through the use of meristem tip culture, thermotherapy or a combination of both. A special mention should be made of the use of crossprotection to control tristeza. This strategy is based on the pre-inoculation of trees with mild CTV isolates to protect them from the expression of symptoms caused by more severe superinfecting isolates. Crossprotection is not without risk, but with CTV it has been used commercially and, in general, with great success in a number of countries, to protect several million trees.

Future Perspectives

Members of the *Closteroviridae* family are important pathogens of plants but, despite the recent remarkable advances, information on their genomic organization and interactions with hosts and vectors is still limited. Stumbling blocks are the large genome size of these viruses and the difficulties encountered with their characterization and purification. As molecular data has accumulated, the taxonomy of this formerly heterogeneous group of viruses, previously based on morphological criteria, has evolved towards more phylogenetically based groupings. In some crops, such as grapevine, new viruses continue to be discovered. At the same time, in the western USA, an explosion of the whitefly populations and establishment of the new species *B. argentifolii,* has led to the discovery of several new diseases and viruses. Comprising both very old diseases of high incidence and emerging viruses, the field of closterovirology is rapidly becoming a very active and dynamic research area. The recent development of synthetic inocula using cDNA clones from several of these viruses offers new opportunities for the understanding of the relationships with their hosts and vectors. At the same time, molecular biology techniques have greatly improved our ability to detect and characterize these viruses, which should rapidly translate into increased capability to fight the diseases they cause.

See also: **Capilloviruses; Trichoviruses; Plant virus disease – economic aspects.**

Further Reading

Agranosky VV (1996) Principles of molecular organization, expression, and evolution of closteroviruses: over the barriers. *Adv. Virus Res.* 47: 119.

Bar-Joseph M and Murant AF (1982) Closterovirus group. *CMI/AAB Description of Plant Viruses,* no. 260.

Bar-Joseph M, Garnsey SM and Gonsalves D (1979) The closteroviruses: a distinct group of elongated plant viruses. *Adv. Virus Res.* 25: 93.

Bar-Joseph M, Lee RF and Pappu HR (1994) The closteroviruses. In: Singh RP, Singh US and Kohmoto K (eds) *Pathogenesis and Host Specificity in Plant Diseases.* Vol. III: *Viruses and Viroids,* p. 65. Oxford: Pergamon Press

Coffin RS and Coutts RHA (1993) The closteroviruses, capilloviruses and other similar viruses: a short review. *J. Gen. Virol.* 74: 1475.

Dolja VV, Karasev AV and Koonin EV (1994) Molecular biology and evolution of closteroviruses: sophisticated build-up of large RNA genomes. *Annu. Rev. Phytopathol.* 32: 261.

Francki RIB, Milne RG and Hatta T (1985) Closteroviruses. *Atlas of Plant Viruses* 2: 219. CRC Press, Boca Raton.

Lister RM and Bar-Joseph M (1981) Closteroviruses. In: Kurstak E (ed.) *Handbook of Plant Virus Infections and Comparative Diagnosis,* p. 809. Amsterdam: Elsevier/North Holland.

the *in vivo* process requires that all aspects of the *in vivo* process be mimicked *in vitro*.

Further Reading

Baas PD and Jansz HS (1988) Single-stranded DNA phage origins. *Curr. Top. Microbiol. Immunol.* 136: 31.

Denhardt DT, Dressler DH and Ray DS (eds) (1978) *The Single Stranded DNA Phages*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Dokland T, McKenna R, Ilag LL *et al* (1997) Structure of a viral procapsid with molecular scaffolding. *Nature* 389: 308.

Hayashi M, Aoyama A, Richardson DL and Hayashi MN (1988) Biology of the bacteriophage ϕX174. In: Calendar R (ed.) *The Bacteriophages*, vol. 2. New York: Plenum Publishing Corp.

Jennings B and Fane BA (1997) Genetic analysis of the ϕX174 DNA binding protein. *Virology* 227: 370.

Ng JY and Marians KJ (1996) The ordered assembly of the ϕX174-type primosome. *J. Biol. Chem.* 271: 15649.

Roof WD and Young R (1995) ϕX174 lysis requires *sly*D, a host gene which is related to the FKBP family of peptidyl-prolyl *cis–trans* isomerases. *FEMS Microbiol. Rev.* 17: 213.

COLIPHAGE LAMBDA (*SIPHOVIRIDAE*)

Allan M Campbell, Stanford University, Stanford, California, USA

Introduction

The double-stranded (ds) DNA phage lambda belongs to a group of related temperate phages (the lambdoids) whose natural hosts are *Escherichia coli* and other members of the *Enterobacteriaceae* and whose distribution is worldwide. Most lambdoid phages share a common genetic map but have undergone substantial divergence in sequence and specificity of traits such as repression or prophage location; collectively they present a combinatorial array of such specificities almost certainly generated by natural recombination. The major collections of lambdoid phages used in comparative studies were isolated in France (phages 21, 82, 381, 424, 434, 466), Japan (ϕ80), Hong Kong ('HK' phages) and the area formerly known as the Soviet Union.

Lambda-related sequences are present in the genomes of many enterobacterial isolates, even where active phage is not produced. These appear to result from lysogenization followed by progressive mutation to loss of function, including deletion of large segments of the prophage. Lambda was found in the K-12 strain of *E. coli*, which harbors at least four incomplete lambdoid prophages. In research, a derivative phage selected for larger plaques and better lytic growth than shown by natural lambda is standardly used.

The K-12 strain was isolated from a Stanford hospital patient in 1926. The presence of lambda was first reported in 1951. Because the genetics of the host was already developed at that time, lambda was studied more intensively than other temperate phages. Largely because of this happenstance, lambda became the model temperate phage, in which repression, regulation and integration were studied in depth. When methods for DNA cloning were developed in the 1970s, the accumulated information on lambda facilitated manipulation of its genome to make it the most commonly used double-stranded phage cloning vector.

Taxonomy and Classification

Coliphage lambda is in the genus 'Lambda-like phages' of the *Siphoviridae* family.

Virion Structure

Each lambda virion contains one linear dsDNA molecule 48 514 bp in length, with complementary 12-base single-stranded 5′ overhangs at the ends. The DNA is packaged within a regular icosahedral head 54 nm in diameter, attached to a flexible tail 173 nm in length. The major capsid proteins (gp*D*, gp*E*) are present in 420 monomer units each, in a $T = 7$ configuration with gp*E* grouped as trimers and gp*D* as hexamers. Heads lacking gp*D* can package and inject DNA but have a smaller diameter than normal heads

and cannot package a full-length lambda genome. The major tail protein gp*V* forms a hollow tube 9 nm in diameter through which the DNA is injected on infection. The tail terminates in a single fiber 23 nm long composed of a few molecules of gp*J*, which binds to the maltose-uptake protein LamB of the bacterial envelope in the first step of attachment to the host cell.

Genome Structure

The complete DNA sequence of a laboratory strain of lambda has been determined. For natural lambdoid phages, some sequences of some complete or nearly complete genomes (as well as many shorter segments) are available. Genes are arranged in functional clusters, the main groups being (in order along the DNA) head genes, tail genes, integration genes, regulatory genes, replication genes and lysis genes. The recognition sites of proteins for regulation, integration and replication are generally close to or within the structural genes for these proteins, e.g. the origin of replication lies within the *O* gene, the product of which determines specificity of initiation.

When any two lambdoid phages are compared, the sequences are very similar in some parts of the genome and highly divergent in others, the transitions from similarity to dissimilarity generally being abrupt. Each phage pair has its unique pattern of similarity and dissimilarity indicating that the phages are related by frequent recombination in nature.

The base composition of lambda DNA is 50% AT:50% GC. Within each strand, the ratios of the four bases are close to 1:1:1:1. However, the base ratios are not statistically uniform throughout the genome. The left 44% of the genome is 57% GC, the right 56% is 44% GC and within each half smaller AT-rich and GC-rich segments occur. The same pattern of base sequence heterogeneity is observed in lambdoid phages, even within segments that have diverged extensively from lambda. Also, within a strand, the G:C ratio is not uniform throughout but shifts between regions of leftward and rightward transcription. As in *E. coli*, the G:C ratio within genes is slightly higher in the coding versus the noncoding strand, because of the pattern of codon usage.

Growth Cycle (Lytic)

In infection, DNA is injected through the phage tail and the cell envelope. Soon after injection the complementary 12 bp terminal overhangs pair, and the nicks are sealed by the host-encoded DNA ligase, converting the linear DNA into a ring. This ring subsequently replicates bidirectionally from a unique origin. At late times, the major mode of replication is rolling circle, where multigenomic double-stranded tails are spun off by replicating circles. These multigenomic tails are the major natural substrate for DNA packaging into preformed heads. Head assembly requires several viral proteins as well as the host-encoded chaperonin GroE. In packaging, DNA is cut at specific sites (*cos*) in a manner that generates the 12-base single-stranded overhangs ('cohesive sites') found in virion DNA. Packaging is processive and proceeds from left to right on the conventional map. Lambdoid phages frequently differ in their recognition sites for *cos* cutting and in their cognate recognition proteins even where the actual cutting sites are identical.

Replication proteins are preferentially synthesized at early times, whereas virion components and other assembly proteins and lysis proteins are made later. Temporal coordination of the various syntheses is achieved through a balance of positive regulation by antitermination and negative regulation by repression.

Following injection, early transcription by host RNA polymerase is initiated divergently from two promoter sites, *pL* and *pR*. The first gene transcribed leftward is gene *N*, whose product gp*N* renders transcripts from *pL* and *pR* resistant to termination at transcription stop signals, by a mechanism dependent on N-utilization (*nut*) sites downstream from and close to *pL* and *pR* respectively. Rightward transcription from *pR* produces Cro protein, which binds to sites overlapping *pL* and *pR* and represses transcription from them. When antiterminated by gp*N*, the rightward transcript proceeds through the replication genes *O* and *P* and the late regulator gene *Q*. Antitermination of the leftward transcript results in expression of genes (*red*) that affect recombination and enhance replication.

The accumulation of Cro protein with time gradually reduces early gene transcription. As transcription of the *cro* gene itself is repressed by Cro, the level never reaches the point where shutoff is complete. Concurrently, the number of phage genomes available for transcription is amplified by replication, and the late genes for lysis and virion components are turned on through antitermination effected by gp*Q*. The time of lysis is determined by the accumulation of gp*S*, which destroys the cell membrane, providing access of the other major lytic protein gp*R* to its substrate, the rigid murein layer of the cell envelope. At lysis, about 100 progeny virions per cell are liberated. The time from infection to lysis is about 35 min at 37°C.

Lysogeny

Only a fraction of infected cells undergo a lytic cycle. The others survive infection and thereafter harbor the phage genome as a quiescent prophage. The genetic switch determining which pathway is chosen has been studied intensively. The decision to survive and become lysogenic requires expression of gene *c*I (which encodes a repressor that shuts off lytic functions) and of gene *int* [which encodes the integrase protein which mediates reciprocal recombination between specific sites (*att*) on phage and host DNA, resulting in insertion of the circular phage DNA into the bacterial chromosome]. Synthesis of repressor and integrase is coordinated because both of their structural genes are transcribed from promoters that are activated by the same macromolecular effector, gp*c*II. The gp*c*II protein is subjected to rapid *in vitro* proteolysis unless stabilized by a second viral protein gp*c*II. Transcriptional activation by gp*c*II is highly concentration dependent. Because gp*c*II is unstable (even with gp*c*III), achievement of a concentration high enough to initiate the lysogenic pathway requires a rapid rate of gp*c*II synthesis.

The *c*II and *c*III genes are located in the early right and left transcript respectively downstream from the first site for transcription termination. Therefore, a high rate of *c*II and *c*III transcription requires antitermination by gp*N* and is prevented by repressing concentrations of Cro. Being fairly stable, Cro accumulates with time, eventually reducing the rate of early transcription so that an effective concentration of gp*c*II can never be achieved and the cell is committed to the lytic pathway. Thus the decision of an individual cell to lyse or to become lysogenic ultimately turns on a race between *c*II and *N* on the one hand and *cro* on the other. The system is so poised that, under a wide range of ambient conditions, some cells go in one direction and some in another. The proportion of cells choosing the lysogenic pathway increases with multiplicity of infection, with intracellular cyclic AMP concentration and with concentration of divalent cations.

Once lysogeny is established, repressor turns off the transcription of *c*II and *c*III as well as genes for repression and late gene activation. Repressor synthesis continues in the absence of gp*c*II because *c*I transcription is now initiated at a *c*II-independent promoter. The maintenance promoter is adjacent to the operator sites that control early rightward transcription and is activated by repressor binding, so that repressor synthesis, once established, is self-sustaining.

In a lysogenic culture, repression breaks down in occasional cells, which then switch to the lytic cycle. The prophage is excised from the chromosome, and phage development follows. The extent of the rare spontaneous derepression depends strongly on the host RecA protein.

Mass derepression of a lysogenic culture is induced by treatment with ultraviolet light or other DNA-damaging agents that turn on a battery of repair genes (SOS response). SOS induction results from cleavage of the LexA protein, which represses the repair genes. The initiating event is activation (by products of DNA damage) of the RecA protein's ability to accelerate LexA cleavage. The lambda repressor, like LexA, is cleaved in the presence of activated RecA. Mutations in the *c*I gene that alter the cleavage site render the repressor resistant to RecA and make the phage noninducible by SOS-inducing treatments.

More synchronous mass derepression is conveniently achieved experimentally by heating a lysogen, the prophage of which makes a mutant, thermolabile repressor.

Prophage insertion and excision require a phage protein, integrase. The site-specific recombination takes place within a 15 bp segment that is identical in phage and host. Initial cleavage at a precise position within the 15 bp on DNA strands of identical polarity in phage and host, with transient covalent binding of enzyme to DNA, is followed by crossjoining of the broken strands to give a Holliday structure. Branch migration by 7 bp occurs next, and crossjoining of the other two strands completes the recombination. All this proceeds without loss of phosphodiester bond energy or complete relaxing of supercoiling. The specific sequence required on the bacterial partner is 21 bp, including the 15 bp identical segment within which branch migration occurs. The specific sequence required on the phage DNA is more than 200 nucleotides and includes several integrase-binding sites in the arm regions flanking the crossover point. Several integrase molecules bind concurrently to these sites, bending the DNA around a complex termed an intasome. A host protein, integration host factor (IHF), required for insertion and excision, binds to DNA in the arm regions and promotes DNA binding. IHF is also a positive regulator of gp*c*II synthesis. Excision of prophage from the chromosome requires, in addition to integrase and IHF, a small protein, excisionase, encoded by a gene just upstream from *int* and overlapping it by 20 bp.

When an infected cell becomes committed to lysogeny, integrase is transcribed by a *c*II-activated promoter from a start site within the excisionase gene. Thus integrase but not excisionase is formed. In a derepressed prophage, excisionase and integrase are transcribed coordinately from *pL* on a transcript antiterminated by gp*N*. In infected cells committed to

lysis, leftward transcription begins in the same way; however, downstream from the *int* gene, the transcript consists of phage rather than host DNA and includes an RNase III processing site (*sib*) which allows 3′ → 5′ degradation of the transcript. This regulatory effect of a downstream site (retroregulation) serves to reduce the amount of integrase in cells committed to lysis. Finally, in a repressed prophage, integrase is expressed at a low basal level from the gp*c*II-activatable promoter, while excisionase expression is undetectable. Thus at every stage of the life cycle, the *int* and *xis* genes are regulated in a manner consistent with cell fate.

Genetic Recombination

Comparison of lambdoid phage genomes indicates that recombination in nature is frequent. The most common confrontation generating natural recombinants is probably between an infecting phage and a prophage or defective prophage of the host.

In the laboratory, recombination is generally studied by mixed infection. Three recombination pathways are known: the host pathway (dependent on the *recABCD* genes), the phage general recombination pathway (dependent on phage *redX* and *redB*) and the integrase pathway, causing recombination only at the *att* site. Phages mutant in *red* genes produce less DNA and fewer progeny than wild-type phage, indicating that the most direct role of the pathway in phage biology is probably resolution of replication intermediates rather than generation of diversity in natural phage populations. Individually and jointly, the *rec* and *red* pathways generate linear recombination maps congruent with the disposition of genes on linear virion DNA.

In Vitro Replication

The steps in replication initiation have been studied in detail and resemble those for *E. coli*. Initiation requires formation and subsequent dissociation of a specialized nucleoprotein complex. First, four dimers of lambda protein gpO bind to DNA sites at the replication origin. Protein–protein interactions bring the gp*O* dimers together, with a stretch of DNA wound around them. The lambda protein gp*P* forms a heterodimer with the host helicase DnaB, and two such heterodimers bind to gpO. Interaction with the host heat-shock protein complex DnaJ–DnaK–GrpE causes, in several steps (one of which is ATP-dependent), expulsion of gp*P* together with DnaJ–DnaK–GrpE. The two copies of DnaB then unwind the DNA in opposite directions initiating bidirectional replication.

The phage-coded proteins gpO and gp*P* thus function only in initiation, serving to position DnaB so that elongation can begin. Subsequent replication is carried out by host enzymes.

Restriction/Modification

Lambda is subject to the restriction/modification systems of the hosts on which it grows. The first Type I restriction system known (that distinguishing the K-12 and B strains of *E. coli*) was discovered with lambda. A phage gene, *ral*, reduces the severity of restriction, probably by promoting modification of infecting DNA before restriction enzymes reach it. Lambda does not encode any restriction/modification system of its own. However, lambda prophages interfere with the development of some unrelated phages (such as *r*II mutants of phage T4) by virtue of two genes *rexAB* cotranscribed with the repressor gene *c*I. Neither *rex* nor *ral* is required for the lytic or lysogenic cycle, and these genes are not present in all lambdoid phages.

Plasmid Formation

Wild-type lambda is not perpetuated as a plasmid. However, lambda mutants defective in the early regulatory gene *N* as well as certain deletions removing most of the lambda genome (λ *dv*) can persist as highly unstable plasmids. The minimal requirements for plasmid formation are the rightward promoter *p*R, the replication genes *O* and *P*, and the *cro* gene to prevent their overexpression.

Cloning Vehicles

The unique sequence of virion DNA, the ability to package DNA *in vitro* into phage particles, and the formation of stable lysogens have made lambda a convenient vector for DNA cloning. Many derivatives of lambda have been constructed with improved utility as cloning vectors.

In the early 1970s, lambda derivatives were engineered to contain single cleavage sites for some common restriction enzymes. A lambda-size DNA molecule is expected to contain about 10 cleavage sites for a six-cutter like *Eco*RI. Lambda contains five. Four were removed by deletion of nonessential DNA or by recombination with lambdoid phages to substitute segments that lacked *Eco*RI sites of lambda.

Lambda can package and inject DNA molecules about 75–110% of the normal 48 kb genome length. The genes necessary for lytic development comprise only about 60% of the 48 kb. Deletions removing most of the dispensable 40% increase the possible insert length. Even longer inserts can be cloned into

vectors containing the *cos* sites but lacking much of the functional phage DNA, which can be perpetuated in the presence of a helper lambda phage.

Early vector construction was influenced by safety regulations dictating minimization of the probability that lambda carrying foreign DNA might multiply outside the laboratory. Lytic replication was forestalled by the presence of multiple amber mutations. Survival as prophage was made unlikely by deletion of the insertion site and integrase gene and by mutation (or disruption during cloning) of the *cI* gene. The possibility of plasmid formation was reduced by deleting a terminator site for rightward transcription (*nin5* deletion), allowing lytic replication even in absence of gp*N*. Legal restrictions have now been relaxed, but some current vectors still bear traces of these early constructions.

See also: **Host-controlled modification and restriction; Lysogeny and prophage; Phage Homologous Recombination; Phages as cloning vehicles.**

Further Reading

Campbell A (1994) Comparative molecular biology of lambdoid phages. *Annu. Rev. Microbiol.* 48: 193.

Ptashne M (1992) *A Genetic Switch*, 2nd edn. Oxford: Blackwell.

COMOVIRUSES (*COMOVIRIDAE*)

George P Lomonossoff and **Michael Shanks**, Department of Virus Research, John Innes Centre, Norwich, UK

Taxonomy and Classification

There are 15 different viruses in the genus *Comovirus* of the family *Comoviridae*. These are cowpea mosaic virus (CPMV; type member of the group), Andean potato mottle virus (APMV), bean pod mottle virus (BPMV), bean rugose mosaic virus (BRMV), broad bean stain virus (BBSV), broad bean true mosaic virus (BBTMV), cowpea severe mosaic virus (CPSMV), glycine mosaic virus (GMV), pea green mottle virus (PGMV), pea mild mosaic virus* (PMiMV), quail pea mosaic virus (QPMV), radish mosaic virus (RaMV), red clover mottle virus (RCMV), squash mosaic virus (SqMV) and ullucus C virus (UCV). Two additional genera, *Fabavirus* and *Nepovirus*, are in the *Comoviridae* family.

Biological Properties of Viral Particles

Viral particles generally reach a yield of between 0.1 and 2 g per kg of fresh plant tissue. The thermal inactivation point of viral particles in plant sap is in the range 60–75°C and the particles have a longevity in sap of between 2 and 112 days at room temperature.

Serology

The viral particles of all comoviruses are good antigens in rabbits and antiserum titres of 1/1000 are easily obtainable. Occasionally titres as high as 1/8000 have been reported. Every member of the group has been shown to be serologically related to at least one other member, although sometimes the relationship is distant.

Physical Properties of Viral Particles

Comovirus preparations consist of nonenveloped isometric particles, 28 nm in diameter, which can be separated into three components designated top (T), middle (M) and bottom (B) by centrifugation on sucrose density gradients. The exact sedimentation coefficients of the components vary slightly with the virus being analysed but are typically in the range 50–60 S for T, 90–100 S for M and 110–120 S for B components. The three components have identical protein compositions, containing 60 copies each of a large (L) and small (S) coat protein. The sizes of the two proteins vary, depending on the virus, but lie in the ranges 37–49 kDa for the L protein and 18–26 kDa for the S protein. The difference between the three centrifugal components is in their RNA contents. Top components are devoid of RNA, while middle and bottom components each contain single molecules of RNA of approximately 3.5 and 6.0 kb, respectively. The two RNA molecules were originally termed middle (M) and bottom (B) component RNA after the component from which they were isolated; however, more recently they have been referred to as RNA2 and RNA1, respectively. The three-compo-

* Commonly regarded as a strain of BBSV.

Coltiviruses *see* Orbiviruses and Coltiviruses

COLIPHAGE φX174 AND RELATED PHAGES (*MICROVIRIDAE*)

David T Denhardt, Department of Cell Biology and Neuroscience, Nelson Laboratories, Rutgers University, Piscataway, New Jersey, USA

Taxonomy, Classification and Ecology

φX174 and its close relative S13 are members of the family *Microviridae*, genus *Microvirus*. They were found to be among the smallest DNA bacteriophages in the early days of phage research (1920–1950) but they were not particularly popular as subjects of study because they were less easy to work with than the T phages and not as intriguing as temperate phages such as lambda. However, the physicochemical studies of Robert Sinsheimer and the genetic studies of Irwin Tessman convinced many members of a generation of phage workers to concentrate their efforts on these organisms (see Denhardt *et al* in the Further Reading list for a review of this period). A major defining characteristic of these phages is their possession of a single molecule of single-stranded circular DNA as their sole genetic material. Other members of this group, less closely related to φX, include α3, St-1 and G4. They all grow on various strains/species of Enterobacteriaceae, typically *Escherichia coli, Salmonella* and *Shigella* species, and are found wherever these bacteria are found. *E. coli* C is the best host for many isolates, though phage able to grow on *E. coli* B (e.g. α3) and *E. coli* K12 (e.g. St-1) are also known.

The reviews by Hayashi *et al* and Baas and Jansz in the Further Reading List contain details and references that of necessity are omitted here. Most of the specific information presented below concerns φX174 ('phi-ex'), and is assumed to apply to all members of this group unless indicated otherwise.

Virion Structure and Proteins

Several views of the virion are depicted in **Fig. 1**. There are 12 spikes projecting about 3 nm from an icosahedral core roughly 26 nm in diameter. The core capsid consists of 60 molecules each of F and J proteins enclosing a circular single-stranded DNA of 5386 nucleotides (almost 6000 for St-1). Interactions between the DNA and amino acids in the F and J proteins neutralize much of the negative charge on the DNA (polyamines and other cations neutralize the rest) and substantially restrict both base stacking and

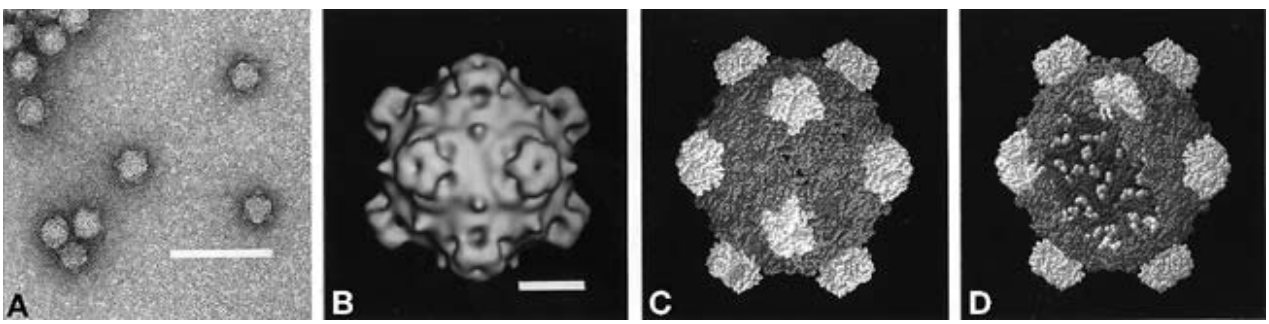


Figure 1 The isometric φX174 Virion. (**A**) Electron micrograph of a negatively stained preparation of phage (bar = 100 nm). (**B**) Reconstructed image from electron micrographs of unstained, frozen-hydrated preparations (bar = 8 nm). (**C**) Computer model based on x-ray diffraction data with each amino acid residue represented as a sphere centered at each Cα atom in the peptide chains (white, G protein; grey, F protein). (**D**) Same model as in (**C**) with several F and G molecules removed to show the inside surface of the protein shell where ordered nucleotides of the single-stranded DNA are bound (larger white spheres centered at the phosphorus atoms of the polydeoxynucleotide chain). ((**A**) and (**B**) are courtesy of Norman H. Olson and Timothy S. Baker, Purdue University. (**C**) and (**D**) are courtesy of Robert J. Hill and Nino L. Incardona, University of Tennessee–Memphis.)

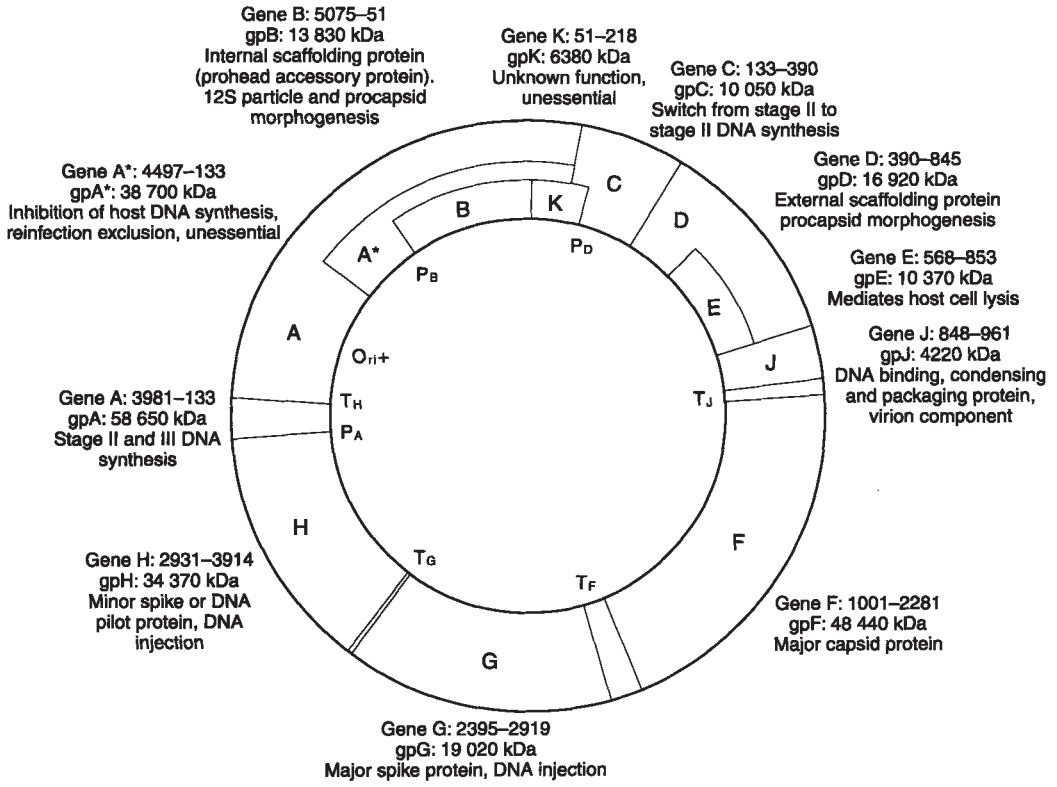


Figure 2 Genetic and functional map of ϕX174. The nucleotides in the 5386-nucleotide circle are numbered starting at the unique *PstI* site in gene A, which is near the 3′ end of the gene. Ori$^+$ indicates the site of gene A cleavage, the origin of RF replication. Genes A through H, K and J are shown along with the extent of gene overlap (B and E are completely within A and D respectively; K overlaps both A and C). The molecular weights indicated for each protein are those predicted by the base sequence. P_x and T_x indicate the major sites of transcription initiation and termination. Intergenic regions are unlabeled. Transcription of all the genes is in the clockwise direction. (Courtesy of Ben Fane.)

hairpin formation. Embedded in the capsid at the 12 vertices of the icosahedron with fivefold symmetry are the 12 spikes, each of which is composed of five molecules of protein G and one molecule of protein H. Mutations affecting host specificity have been found in genes F, G and H. The major motif in the F and G proteins is an eight-stranded antiparallel β barrel. About 180 water molecules stabilize the spike–capsid interactions. A hydrophilic channel that extends through each spike is plugged by a molecule of H protein.

Genome Structure

A genetic map of the circular genome with the approximate sizes and functions of the encoded proteins is shown in **Fig. 2**. The single-stranded DNA of each phage particle has the same polarity, defined as 'plus' since it is the same as the viral mRNA. ϕX was the first DNA-containing organism to have its genome completely sequenced, and a major surprise resulting from this work of Fred Sanger was the revelation that genes could overlap – that the same DNA sequence could encode information for more than one protein using different reading frames. This strategy to maximize genome utilization is of particular advantage to these phages because of the stringent limitation on the amount of DNA the capsid can contain. Some of the complications that arise as the consequence of gene overlap have been discussed (see Hayashi *et al* in the Further Reading list). Together, 11 genes have been defined by virtue of their protein products; eight of these (A, B, C, D, F, G, H and J) are essential for virus reproduction. Gene A* protein acts to suppress host DNA replication, gene E is responsible for cell lysis, and gene K appears somehow to enhance phage yields.

Replication

The virion attaches first to the core oligosaccharide of the lipopolysaccharide (LPS) of sensitive bacteria. Cooperative interactions of several spikes with several LPS molecules accompanied by host-induced

alterations in the coat structure of the virus make the adsorption process irreversible. Genetic analysis of host cell adsorption mutants suggests that uncoating and effective infection (eclipse) require the interaction of the virion proteins with several host cell proteins in the cell wall/membrane. Release of the circular single-stranded DNA through an opening in one of the spikes into the cell occurs at points of adhesion between the cell wall and the inner membrane.

As it enters the cell the DNA is rapidly complexed with single-stranded DNA binding protein (SSB) and becomes a template for the synthesis of a complementary minus strand (stage I synthesis). Some experiments suggest that *in vivo* this process is facilitated by the gene H protein (carried into the cell with the DNA) and host membrane proteins. A DNA sequence in the H/A intergenic region, called the 'reduction' or 'incompatibility' sequence, can, when already present in the cell in other DNA molecules, inhibit stage I DNA synthesis. This is presumably a sequence in the DNA that interacts with a cellular component, possibly a membrane site, present in very limited amounts in the cell. The result is to exclude superinfecting phages from establishing a successful infection. Initial effects of virus infection on host cell metabolic processes and macromolecular syntheses are minimal. Later in infection however the A* protein, which has the same amino acid sequence as the C-terminal two-thirds of the A protein, interrupts host DNA replication.

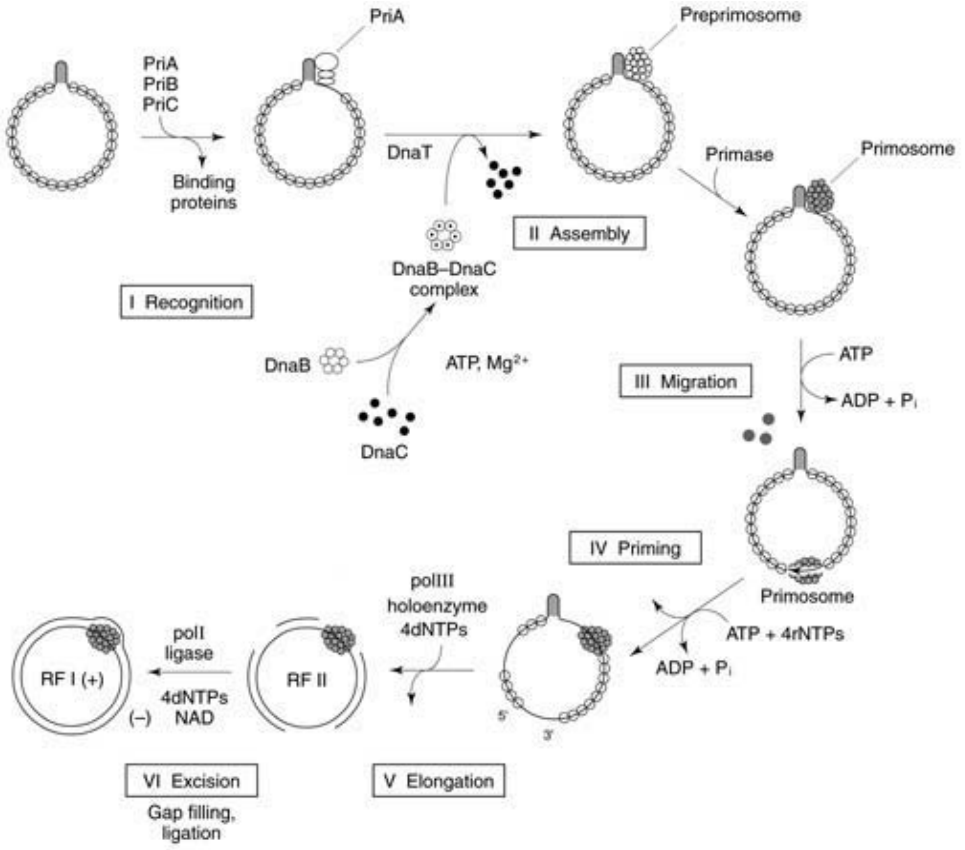
The replication of the viral genome occurs in three stages, models of which are shown in **Fig. 3**: stage I, synthesis of a complement (the 'minus' strand) to the viral strand; stage II, replication of the duplex viral DNA; stage III, generation of progeny single-stranded DNA. Interestingly, different members of the isometric and filamentous phages exploit different host enzyme systems to synthesize the complementary strand when the single-stranded DNA is complexed with SSB.

In stage I, the first event is the assembly of a preprimosome, composed of the proteins PriA, PriB, PriC, DnaT and DnaB, on single-stranded ϕX174 DNA coated with SSB. PriA recognizes a unique sequence (variously called n′ or *pas*, for primosome assembly site, in the F/G intergenic region) that is capable of forming stem-loop structures. PriB and PriC act as stability and specificity factors, while DnaT and DnaC promote the loading of DnaB. The hexameric DnaB is a bidirectional processive helicase that encircles ('clamps') the DNA. This preprimosome complex then associates with the DnaG primase to produce the primosome. As illustrated in **Fig. 3A**, the primosome migrates 5′ → 3′ (clockwise in **Fig. 2**) along the SSB-coated template strand, occasionally enabling the DnaG primase to synthesize a short RNA molecule that can serve to prime DNA synthesis by DNA polymerase III holoenzyme. After the RNA primers are replaced with DNA by DNA polymerase I, the adjacent DNA strands are joined by DNA ligase.

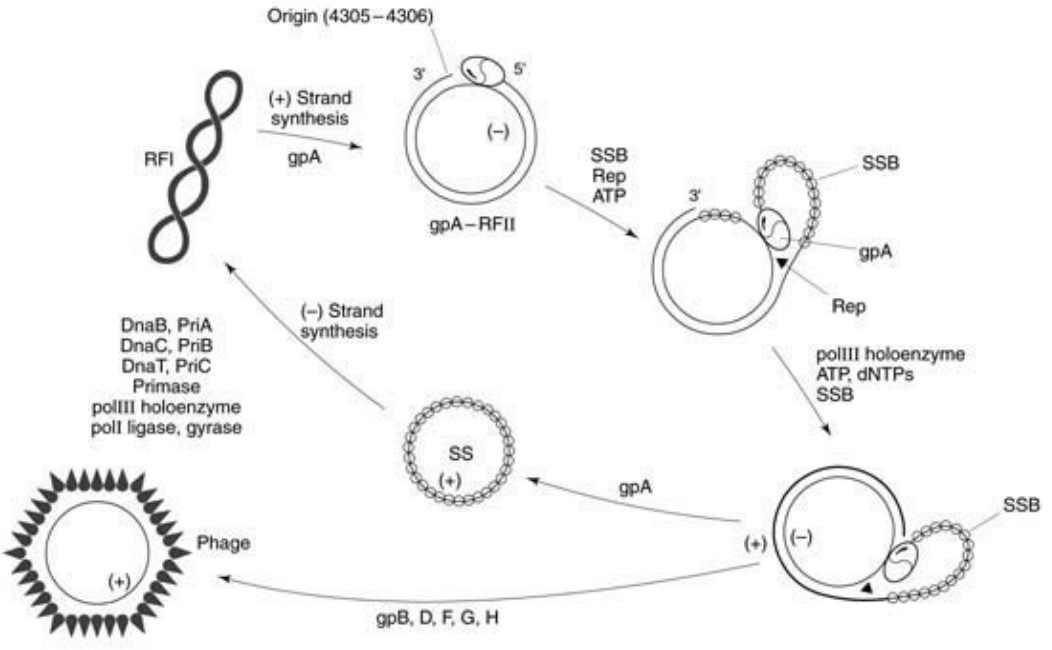
Unlike ϕX174, G4, α3 and St-1 all require only the DnaG primase to synthesize primers for complementary strand synthesis. This is because of the presence in the F/G (for G4) or G/H (for α3 and St-1) intergenic regions of a sequence forming several stem-loop structures that can interact with the DnaG protein in a manner that permits an RNA primer to be generated. As discussed elsewhere in this volume, the filamentous phages make use of RNA polymerase to synthesize a primer for complementary strand synthesis. None of the isometric phages appear to have a hairpin loop structure of the sort found in the filamentous phages that allows priming by RNA polymerase on SB-coated single-stranded DNA.

Completion of the complementary minus strand, including removal of RNA primers and ligation of the intermediate fragments, results in a closed circular duplex molecule called replicative form one (RFI). This molecule has a new topological property called

Figure 3 Replication of ϕX174 DNA. (**A**) Stage I synthesis starts in the upper left with the circular single-stranded DNA template (5′ → 3′ polarity is clockwise) coated everywhere with SSB except for the intergenic region between genes F and G which is believed to possess a stem-loop structure that renders it accessible to PriA protein (note that the orientation of the molecule differs from that shown in **Fig. 2**). Interactions with various bacterial proteins, described in the text, give rise to a protein complex (the primosome) that migrates around the circular DNA, leaving behind an occasional RNA primer synthesized in a retrograde fashion by primase. Successive actions by DNA polymerase III holoenzyme and DNA polymerase I generate a complementary minus-strand; polynucleotide ligase closes the nicks and generates a covalently closed circular RF structure, which becomes superhelical in part as the result of the action of DNA gyrase. The primosome may remain associated, as shown, with the RF molecule that contains the infecting viral strand, the 'parental' RF. (**B**) Both stage II and stage III synthesis begin with the cleavage of an RFI molecule by gpA at a unique location. The REP helicase interacts with gpA and begins to unwind the duplex. DNA polymerase III elongates the viral plus-strand to generate a new RF molecule shown largely complete at the lower right. The 'displaced' plus strand has two potential fates: in stage III synthesis, dependent upon gpC, it becomes incorporated into a virion as illustrated in **Fig. 4**. In stage II (RF replication) it is a substrate for minus-strand synthesis, which may occur by essentially the same process as described for stage I synthesis. (These illustrations are redrawn from Kornberg A and Baker T *DNA Replication*, 2nd edn, 1991, with permission from W. H. Freeman, New York.)


PriA
PriB
PriC
Binding proteins
PriA
Preprimosome
DnaT
Primase
Primosome
II Assembly
DnaB–DnaC complex
I Recognition
DnaB
ATP, Mg2+
DnaC
III Migration
ATP
ADP + Pi
IV Priming
Primosome
polIII holoenzyme 4dNTPs
ATP + 4rNTPs
ADP + Pi
polI ligase
4dNTPs NAD
RF I (+)
(–)
RF II
5'
3'
VI Excision
Gap filling, ligation
V Elongation

A


Origin (4305–4306)
3'
5'
(+) Strand synthesis
gpA
RFI
(–)
gpA–RFII
SSB
Rep
ATP
SSB
3'
gpA
Rep
DnaB, PriA
DnaC, PriB
DnaT, PriC
Primase
polIII holoenzyme
polI ligase, gyrase
(–) Strand synthesis
polIII holoenzyme
ATP, dNTPs
SSB
SS
(+)
gpA
SSB
(+)
(–)
Phage
(+)
gpB, D, F, G, H

B

the winding number – the number of times each polydeoxynucleotide strand winds about the other. This number is a topological constant for any given closed circular duplex; it can be broken down into the sum of two other numbers, the duplex winding number and the superhelical winding number. The latter can be positive or negative, and in either case (when not zero) causes the RF molecule to be in a more compact, superhelical (or supercoil) structure. A break in a phosphodiester bond of either strand permits free rotation about the bond opposite the break, allowing the winding number to change. The resulting molecule, RFII, is still circular but no longer topologically constrained. It can be distinguished from RFI in various ways, particularly its response to intercalating agents. The linear duplex is known as RFIII.

In stage II, the duplex RF serves first as a substrate for the host RNA polymerase and second, when the ϕX174 gene A protein (gpA) appears, as a substrate for replication by host DNA replication enzymes. Gene A protein cleaves a phosphodiester bond in the RF molecule at a unique sequence within gene A (the 'origin' region consisting of some 30 bp) and becomes covalently bound to the 5′ phosphate of the viral plus strand via a tyrosine residue. The *E. coli* Rep helicase interacts with the gpA/RFII molecule and in an ATP-dependent process, likely facilitated by SSB, unwinds the two strands of the duplex. In a coordinated process DNA polymerase III holoenzyme, using the 3′-OH of the cleaved plus-strand as a primer, synthesizes a new plus-strand on the minus-strand template. This is called 'rolling circle' replication, the hallmark of which is a replication intermediate containing a longer-than-unit-length viral DNA molecule. When the primosome assembly site on the displaced SSB-coated single-stranded plus strand becomes available, the process of synthesizing a new minus-strand may commence in a manner considered similar to that used in stage I synthesis.

When the REP/gpA–5′DNA end complex completes its circuit around the circular minus-strand template, displacing the original plus-strand from its minus-strand partner, the bond joining the new and old viral strands (the regenerated origin) is cleaved, and the protein-bound 5′ end of the old viral strand is joined to its newly created 3′ end with transfer of gpA to the newly created 5′ end, thereby setting the stage for a new round of replication. There appear to be two nearby tyrosine residues in gpA that participate in this exchange reaction, taking turns during each round of replication in serving to join gpA to the 5′ terminus. Completion of synthesis of the new minus-strand on the displaced plus-strand, presumably by a process like that described for stage I synthesis, produces the second of the two products of RF replication. The obvious asymmetry in the two products of replication likely explains why the RF molecule with the original infecting viral strand is preferentially replicated in repeated rounds of replication (the 'parental' RF, possibly in association with a special region of the host cell inner membrane).

Stage II replication generates 10–50 RF molecules, all of which serve as templates for RNA synthesis; some of them also become substrates for the synthesis of progeny plus-strand circular DNA molecules that occurs during the second half of the ϕX life cycle. The switch from stage II to stage III is precipitated by gpC (not shown in **Fig. 3B**), which binds to the gpA/REP/RFII molecule and somehow blocks them from serving as substrates for RF replication and redirects them to participate in the exclusive synthesis of single-stranded viral DNA. This latter process requires additional virus-encoded proteins, and is intimately tied into the morphogenesis of the virus particle as shown in **Fig. 4**.

The first step in the morphogenesis of the virion entails the assembly of the F and G proteins into 9S and 6S pentamers, which then come together under the influence of the B protein to form the 12S subunit. Further interactions with monomeric H and tetrameric D proteins yield the 108S procapsid structure, which is poised to interact with an RF molecule made ready by interactions with the A, C, J and REP proteins to generate, with the help of host cell enzymes, a single-stranded circular DNA molecule. Studies by Incardona, Rossmann and their colleagues have established that proteins B and D serve as internal and external scaffolding proteins, stabilizing intermediate structures and catalyzing steps in the assembly of the virion. B protein is displaced from the virion by the J protein that enters with the DNA, consistent with the fact that B and J both interact with the same interaction site on F. Formation of the mature 114S phage particle and loss of the external scaffolding protein gpD is stimulated by the increase in Ca^{2+} that occurs upon cell lysis.

Shortly after the appearance of mature virus-particles the bacterial cells begin to lyse as the result of the expression of gene E. E protein is produced in small amounts (several hundred molecules per cell) because of a weak ribosome-binding site, the presence of many rare codons in the message and possibly competition from ribosomes translating the overlapping D gene. Frameshifting of ribosomes initially translating the gene D message may also contribute to gene E expression. Gene E protein is not a lysozyme, and no enzymatic activities that might cause cell wall degradation have been detected in ϕX-infected cells. Only growing cells with a fluid membrane, an active

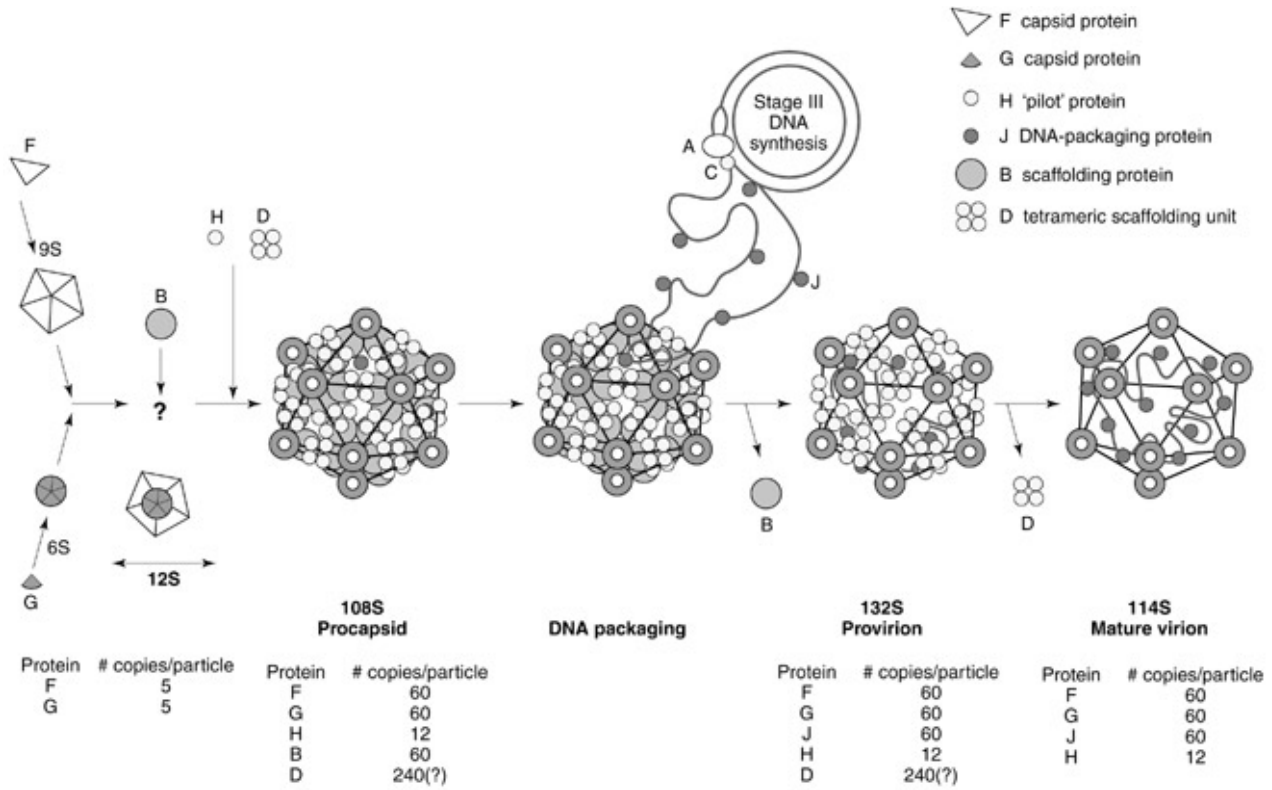


Figure 4 Morphogenesis of the φX174 virion. Please see the text for details. (From Ilag LL, Olson NH, Dokland T *et al* (1995) DNA packaging intermediates of bacteriophage φX174. *Structure* 3: 353–363. Figure courtesy of Michael Rossmann, Purdue University, and redrawn by permission of Current Biology Ltd.)

proton motive force, and appropriate genotype (*sly*D$^+$, *lyt*A$^+$) are lysed. SlyD is a peptidyl-prolyl *cis-trans* isomerase, or rotamase, that potentiates E function. Lysis occurs as the result of a defect induced by E protein in the cell pole (site of cell elongation) or the incipient septum (site of cell division). This defect results in a tunnel through the cell wall through which the membrane-encased cytoplasm begins to extrude in the form of a bleb (**Fig. 5**). The bleb bursts (unless osmotically stabilized) and releases the cytoplasmic contents of the cell into the medium. Murein degradation is minimal; the lysed cell retains most of its structural integrity and most of the periplasmic proteins remain sequestered. Under favorable conditions a burst size of several hundred phages can be obtained; with certain mutant phages even larger burst sizes are observed.

Gene Expression/Transcription/ Translation/*Cis*-acting Proteins

Compared to other phages, these phages have one of the least elaborate sets of controls over gene expression. Most RNA transcripts originate (see **Fig. 2**) at one of three promoters (P_A, P_B and P_D) in the RF. The degree of superhelicity has a minor effect on the relative efficiency with which the different promoters are used. There are four major transcription terminators (T_J, T_F, T_G and T_H) acting with moderate efficiencies independently of *rho* for the most part and giving rise (with the three promoters) to some 10 species of RNA. With exceptions for the overlapping genes, particularly E, proteins needed in larger amounts are encoded in the more abundant classes of message. Most of the mRNA molecules are polycistronic, and each gene has its own translational start and stop signals. mRNA molecules terminating at T_J have a half-life a few minutes longer than the others because of the presence of a stabilizing sequence in the J/F intercistronic region. Also contributing to the differential synthesis of the viral proteins is the efficiency with which the viral mRNAs are translated.

The gene A protein is an example of what is known as a '*cis*-acting' protein. These are proteins that phenomenologically (in genetic complementation studies) appear to act only on the specific DNA molecule from which the mRNA encoding them is

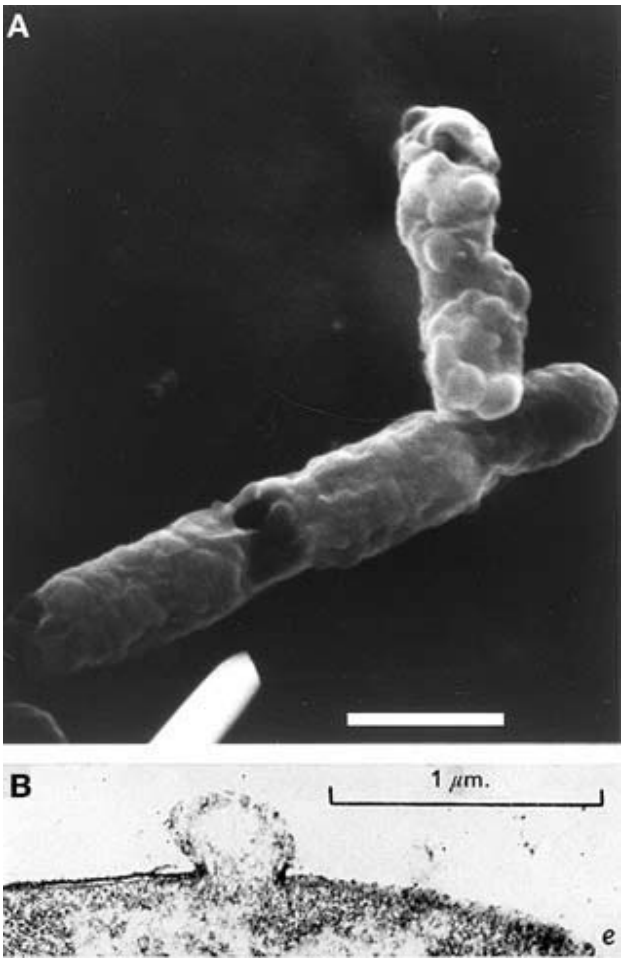


Figure 5 Gene E-mediated lysis. (**A**) is a high-resolution field emission scanning electron microscope image of an empty bacterial ghost after E-induced lysis. Cytoplasm apparently forming a bleb extruding from a hole in the division zone can be seen towards one end, whereas towards the other end there appears to be a hole in the division zone. Bar = 1 μm. (From Witte *et al* (1990) *J. Bacteriol.* 172: 4109. Reproduced with permission of the American Society for Microbiology.) (**B**) Electron micrograph of a incipient lysis bulge in *E. coli* 14 min after α3 infection. (Reproduced with permission from Bradley *et al* (1969) J. Gen. Virol. 5:113. For further interpretation of these figures see Young (1992) *Microbiol. Revs.* 56: 430.)

transcribed. Although other explanations are possible, a reasonable hypothesis is that such *cis*-acting proteins are proteins that bind to a DNA sequence in or near the gene encoding it; in this case gpA binds to the origin sequence within the gene. Most functionally inactive (mutant) versions of gpA likely retain the ability to bind to the origin sequence, and in these cases they would then physically prevent functional (nonmutant) gene A protein, made by another viral RF molecule at a geographically distant location, from accessing the substrate.

Recombination: Use as a Cloning Vehicle

The replicative forms of these phages appear to undergo normal recombination using host cell enzymes; however, because of the small size of the genome and the short life cycle, which limits the time available for recombination to occur, recombination is relatively rare and usually limited to a single event in any one infected cell.

Because of the constraints on the size of the DNA that can be incorporated into the mature virion, the isometric phages are not very useful as cloning vehicles; however, recent efforts to increase the amount of DNA that can be packaged by mutating the J protein have permitted larger amounts of DNA to be packaged.

In vitro Replication

The small size of the DNA of these phages made them extremely attractive experimental systems for the development of *in vitro* DNA-replicating systems. Because even such a simple step as the synthesis of a complement to the ϕX174 single-stranded DNA requires many of the same enzymes that *E. coli* requires for replicating its more complex duplex DNA, studies of this process have contributed enormously to our understanding of the enzymology of bacterial DNA replication. Pioneering efforts in the laboratories of Arthur Kornberg and Jerald Hurwitz uncovered many aspects of the process of synthesis of DNA complementary to the viral strand, and this work was later extended to studies on replication of the double-stranded RF molecule. Research in these laboratories and others using highly purified proteins and DNA templates complexed with the *E. coli* single-stranded DNA-binding protein has clarified many details of the *in vivo* replication process.

The conversion of the single-stranded circle to a double-stranded molecule is a model for the synthesis of Okazaki fragments on the so-called lagging strand of duplex DNA – that DNA strand whose polarity is such that it must be synthesized in the opposite direction from the direction the replicating fork is moving. The synthesis of the viral plus-strand has provided one model for how the 'leading strand' could be synthesized, at least at the origin of replication. Stage III replication and its dependence on phage morphogenesis was largely worked out in Masaki Hayashi's laboratory. Although biochemical studies in the test tube are essential for a complete understanding of events in the intact cell, they are only indicative of what *can* happen; proof that a mechanism elucidated *in vitro* accurately describes

the *in vivo* process requires that all aspects of the *in vivo* process be mimicked *in vitro*.

Further Reading

Baas PD and Jansz HS (1988) Single-stranded DNA phage origins. *Curr. Top. Microbiol. Immunol.* 136: 31.

Denhardt DT, Dressler DH and Ray DS (eds) (1978) *The Single Stranded DNA Phages*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Dokland T, McKenna R, Ilag LL *et al* (1997) Structure of a viral procapsid with molecular scaffolding. *Nature* 389: 308.

Hayashi M, Aoyama A, Richardson DL and Hayashi MN (1988) Biology of the bacteriophage ϕX174. In: Calendar R (ed.) *The Bacteriophages*, vol. 2. New York: Plenum Publishing Corp.

Jennings B and Fane BA (1997) Genetic analysis of the ϕX174 DNA binding protein. *Virology* 227: 370.

Ng JY and Marians KJ (1996) The ordered assembly of the ϕX174-type primosome. *J. Biol. Chem.* 271: 15649.

Roof WD and Young R (1995) ϕX174 lysis requires *sly*D, a host gene which is related to the FKBP family of peptidyl-prolyl *cis–trans* isomerases. *FEMS Microbiol. Rev.* 17: 213.

COLIPHAGE LAMBDA (*SIPHOVIRIDAE*)

Allan M Campbell, Stanford University, Stanford, California, USA

Introduction

The double-stranded (ds) DNA phage lambda belongs to a group of related temperate phages (the lambdoids) whose natural hosts are *Escherichia coli* and other members of the *Enterobacteriaceae* and whose distribution is worldwide. Most lambdoid phages share a common genetic map but have undergone substantial divergence in sequence and specificity of traits such as repression or prophage location; collectively they present a combinatorial array of such specificities almost certainly generated by natural recombination. The major collections of lambdoid phages used in comparative studies were isolated in France (phages 21, 82, 381, 424, 434, 466), Japan (ϕ80), Hong Kong ('HK' phages) and the area formerly known as the Soviet Union.

Lambda-related sequences are present in the genomes of many enterobacterial isolates, even where active phage is not produced. These appear to result from lysogenization followed by progressive mutation to loss of function, including deletion of large segments of the prophage. Lambda was found in the K-12 strain of *E. coli*, which harbors at least four incomplete lambdoid prophages. In research, a derivative phage selected for larger plaques and better lytic growth than shown by natural lambda is standardly used.

The K-12 strain was isolated from a Stanford hospital patient in 1926. The presence of lambda was first reported in 1951. Because the genetics of the host was already developed at that time, lambda was studied more intensively than other temperate phages. Largely because of this happenstance, lambda became the model temperate phage, in which repression, regulation and integration were studied in depth. When methods for DNA cloning were developed in the 1970s, the accumulated information on lambda facilitated manipulation of its genome to make it the most commonly used double-stranded phage cloning vector.

Taxonomy and Classification

Coliphage lambda is in the genus 'Lambda-like phages' of the *Siphoviridae* family.

Virion Structure

Each lambda virion contains one linear dsDNA molecule 48 514 bp in length, with complementary 12-base single-stranded 5′ overhangs at the ends. The DNA is packaged within a regular icosahedral head 54 nm in diameter, attached to a flexible tail 173 nm in length. The major capsid proteins (gp*D*, gp*E*) are present in 420 monomer units each, in a $T = 7$ configuration with gp*E* grouped as trimers and gp*D* as hexamers. Heads lacking gp*D* can package and inject DNA but have a smaller diameter than normal heads

vectors containing the *cos* sites but lacking much of the functional phage DNA, which can be perpetuated in the presence of a helper lambda phage.

Early vector construction was influenced by safety regulations dictating minimization of the probability that lambda carrying foreign DNA might multiply outside the laboratory. Lytic replication was forestalled by the presence of multiple amber mutations. Survival as prophage was made unlikely by deletion of the insertion site and integrase gene and by mutation (or disruption during cloning) of the *cI* gene. The possibility of plasmid formation was reduced by deleting a terminator site for rightward transcription (*nin5* deletion), allowing lytic replication even in absence of gp*N*. Legal restrictions have now been relaxed, but some current vectors still bear traces of these early constructions.

See also: **Host-controlled modification and restriction; Lysogeny and prophage; Phage Homologous Recombination; Phages as cloning vehicles.**

Further Reading

Campbell A (1994) Comparative molecular biology of lambdoid phages. *Annu. Rev. Microbiol.* 48: 193.

Ptashne M (1992) *A Genetic Switch*, 2nd edn. Oxford: Blackwell.

COMOVIRUSES (*COMOVIRIDAE*)

George P Lomonossoff and **Michael Shanks**, Department of Virus Research, John Innes Centre, Norwich, UK

Taxonomy and Classification

There are 15 different viruses in the genus *Comovirus* of the family *Comoviridae*. These are cowpea mosaic virus (CPMV; type member of the group), Andean potato mottle virus (APMV), bean pod mottle virus (BPMV), bean rugose mosaic virus (BRMV), broad bean stain virus (BBSV), broad bean true mosaic virus (BBTMV), cowpea severe mosaic virus (CPSMV), glycine mosaic virus (GMV), pea green mottle virus (PGMV), pea mild mosaic virus* (PMiMV), quail pea mosaic virus (QPMV), radish mosaic virus (RaMV), red clover mottle virus (RCMV), squash mosaic virus (SqMV) and ullucus C virus (UCV). Two additional genera, *Fabavirus* and *Nepovirus*, are in the *Comoviridae* family.

Biological Properties of Viral Particles

Viral particles generally reach a yield of between 0.1 and 2 g per kg of fresh plant tissue. The thermal inactivation point of viral particles in plant sap is in the range 60–75°C and the particles have a longevity in sap of between 2 and 112 days at room temperature.

Serology

The viral particles of all comoviruses are good antigens in rabbits and antiserum titres of 1/1000 are easily obtainable. Occasionally titres as high as 1/8000 have been reported. Every member of the group has been shown to be serologically related to at least one other member, although sometimes the relationship is distant.

Physical Properties of Viral Particles

Comovirus preparations consist of nonenveloped isometric particles, 28 nm in diameter, which can be separated into three components designated top (T), middle (M) and bottom (B) by centrifugation on sucrose density gradients. The exact sedimentation coefficients of the components vary slightly with the virus being analysed but are typically in the range 50–60 S for T, 90–100 S for M and 110–120 S for B components. The three components have identical protein compositions, containing 60 copies each of a large (L) and small (S) coat protein. The sizes of the two proteins vary, depending on the virus, but lie in the ranges 37–49 kDa for the L protein and 18–26 kDa for the S protein. The difference between the three centrifugal components is in their RNA contents. Top components are devoid of RNA, while middle and bottom components each contain single molecules of RNA of approximately 3.5 and 6.0 kb, respectively. The two RNA molecules were originally termed middle (M) and bottom (B) component RNA after the component from which they were isolated; however, more recently they have been referred to as RNA2 and RNA1, respectively. The three-compo-

* Commonly regarded as a strain of BBSV.

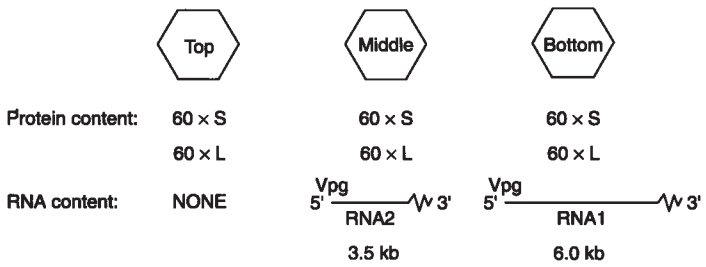


Figure 1 The three-component nature of comoviruses indicating the protein and RNA content of each component.

nent nature of comovirus preparations is summarized in **Fig. 1**.

Because of their differing RNA contents, the three components of comoviruses also differ in density and can hence be separated by isopyknic centrifugation. Generally, T, M and B components have densities of approximately 1.29, 1.40 and 1.41 g ml^{-1}, respectively. However, the pattern obtained is often more complex than that seen with sucrose gradients and depends on the precise conditions used and virus being examined. For example, while BPMV gives the expected three components when centrifuged on cesium chloride gradients under a wide range of conditions, the bottom component of CPMV can be resolved into two forms of differing density under alkaline conditions.

Preparations of comoviruses are often not only centrifugally heterogeneous but can also be separated into two forms, fast and slow, electrophoretically. Both electrophoretic forms contain all three centrifugal components. The proportion of the two electrophoretic forms in a given viral preparation varies, both with the time after infection at which the virus was isolated and the age of the preparation itself. Conversion of one form to the other has been shown to be caused by loss of amino acids from the C terminus of the S protein.

In a number of instances, comovirus particles have been shown to contain polyamines, particularly spermidine. Such polyamines are believed to have a role in the neutralization of the negative charges of the RNA within the particles. It is the exchange of such polyamines for cesium ions that leads to the two forms of bottom component of CPMV seen under alkaline conditions.

Viral Structure

X-ray crystallographic studies on CPMV and BPMV have provided a detailed picture of the arrangement of the two viral coat proteins in the three-dimensional structure of comoviruses. Overall, the virions are icosahedrally symmetrical, with 12 axes of fivefold and 20 axes of threefold symmetry, and resemble a classic $T = 3$ particle. The two coat proteins taken together consist of three distinct β-barrel domains, two being derived from the L and one from the S protein. Thus, in common with the $T = 3$ viruses, each CPMV particle is made up of 180 β-barrel structures. The S protein, with its single domain, is found at the fivefold symmetry axes and therefore occupies a position analogous to that of the A type subunits in $T = 3$ particles (**Fig. 2**). The N- and C-terminal domains of the L protein occur at the threefold axes and occupy the positions equivalent to those of the C and B type subunits of a $T = 3$ particle respectively (**Fig. 2**). Comovirus capsids are also structurally homologous to those of picornaviruses, with the N- and C-terminal domains of the L protein being equivalent to viral protein VP2 and viral protein VP3, respectively, and the S protein being equivalent to viral protein VP1 (**Fig. 2**). However, comoviral particles are structurally less sophisticated than those of picornaviruses. The L and S subunits lack the extended N- and C-termini found in VP2, VP3 and VP1 of picornaviruses and there is no equivalent of VP4. Moreover, comoviral subunits lack the relatively large insertions between the strands of β sheet, sequences which form the major antigenic determinants of picornaviruses. Knowledge of the three-dimensional structure of the capsids of CPMV has enabled the particles to be developed as carriers of foreign antigenic sites.

In the case of BPMV, crystals of purified middle component were found to contain strong electron density in the middle of the particle due to ordered RNA. Seven well-ordered ribonucleotides lie in a pocket formed by the covalently linked N- and C-terminal domains of each of the 60 copies of the L protein. The overall structure of the ordered RNA is that of one strand of an A-type RNA helix. The interactions of the RNA with the protein are mainly van der Waals. No equivalently ordered RNA is visible in the bottom component of BPMV or in either of the nucleoprotein components of CPMV. There are no obvious repeats in the sequence of BPMV RNA2

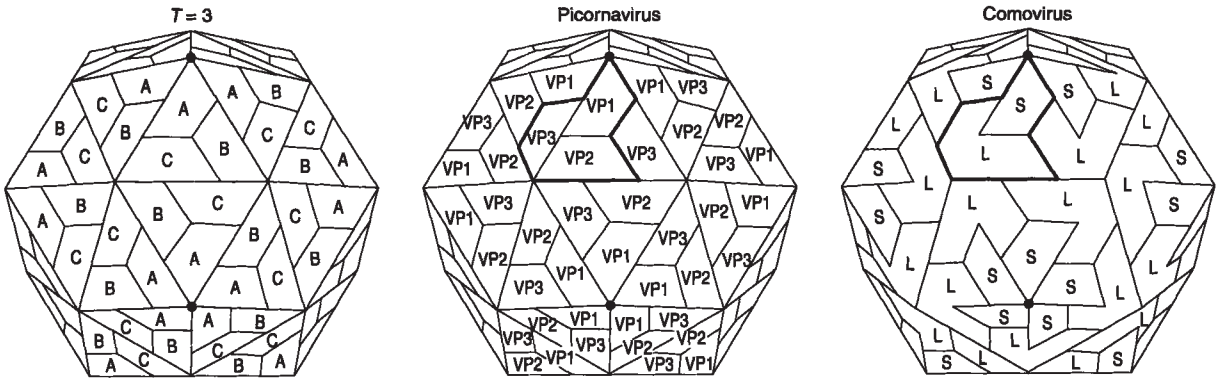


Figure 2 Arrangement of the coat protein subunits of comoviruses compared with those of simple $T = 3$ viruses and picornaviruses. The asymmetric unit of $T = 3$ viruses contains three β barrels contributed by three coat protein subunits with identical amino acid sequences (labelled A, B and C). The asymmetric unit of a picornavirus also contains three β barrels, but in this case each is contributed by a different coat protein (labelled viral protein VP1, viral protein VP2 and viral protein VP3). The comovirus capsid is similar to that of a picornavirus except that two of the β barrels (corresponding to viral protein VP2 and viral protein VP3) are fused to give the L protein. (Reproduced with permission from Lomonossoff *et al* (1991) *Proc. Phytochem. Soc. Eur.* 32: 76.)

which could account for this particular RNA being visible within particles.

Genome Structure

The genomes of comoviruses consist of the two RNAs (RNA1 and RNA2) encapsidated in bottom and middle components. Both RNA molecules are positive-sense and both are required for an infection of whole plants. However, in the case of CPMV, RNA1 has been shown to be capable of independent replication in isolated protoplasts. Both genomic RNAs have a small basic protein (VPg) covalently linked to their 5′ termini and are polyadenylated. The VPg is linked to the viral RNA via the β-hydroxyl group of its N-terminal serine residue. The VPg is not required for the viral RNAs to be infectious.

By the end of 1997, the complete nucleotide sequences of both RNAs of four comoviruses, CPMV, RCMV, CPSMV and BPMV, had been determined. An almost complete sequence of RNA2 of APMV is also available. Where both RNAs of a given comovirus have been examined it has been found that the two viral RNAs have little sequence homology apart from at the 5′ and 3′ termini. All comovirus RNAs examined to date share the common 5′-terminal sequence, VPg-pUAUUAAAAU-. Full-length infectious cDNA clones of CPMV and CPSMV have been constructed, allowing the genomes of these viruses to be manipulated.

Expression of the Viral Genome

All comovirus RNAs sequenced so far contain a single long open reading frame that occupies over 80% of the length of the RNA. A combination of *in vitro* translation and protoplast studies has unravelled the basic mechanism of expression of the genomic RNAs of the type member of the group, CPMV. More limited studies on other members of the group such as CPSMV, BPMV, RCMV and SqMV suggest that the mode of gene expression deduced for CPMV is applicable to all members of the group.

Both RNAs of CPMV are expressed through the synthesis and subsequent cleavage of large precursor polyproteins. Translation occurs directly off the genomic RNAs, with no subgenomic RNAs being produced in infected cells. On RNA1, initiation of translation occurs at the first AUG encountered on the sequence (at position 207) and results in the synthesis of a protein of approximately 200 kDa (the 200K protein). This initial product undergoes rapid co-translational autoproteolysis to give proteins with apparent sizes of 32 and 170 kDa (the 32 and 170K proteins). The 170K protein undergoes further cleavages to give the range of virus-specific proteins shown in **Fig. 3**. *In vitro* translation studies using mutant RNA1 molecules have shown that all the cleavages occur most efficiently in *cis*. The 170K product can initially be cleaved at three different sites to give three different combinations of secondary cleavage products, 58K + 112K, 60K + 110K and 84K + 87K. *In vitro*, and probably also *in vivo*, the 60K and 110K products are stable and do not undergo further cleavage reactions, at least not at an appreciable rate. This is particularly curious in the case of the 110K protein as it contains both the 24K proteinase domain and a cleavage site. By contrast the 112K and 84K proteins do undergo further cleavages. The endproducts of the cleavage pathway of the 170K

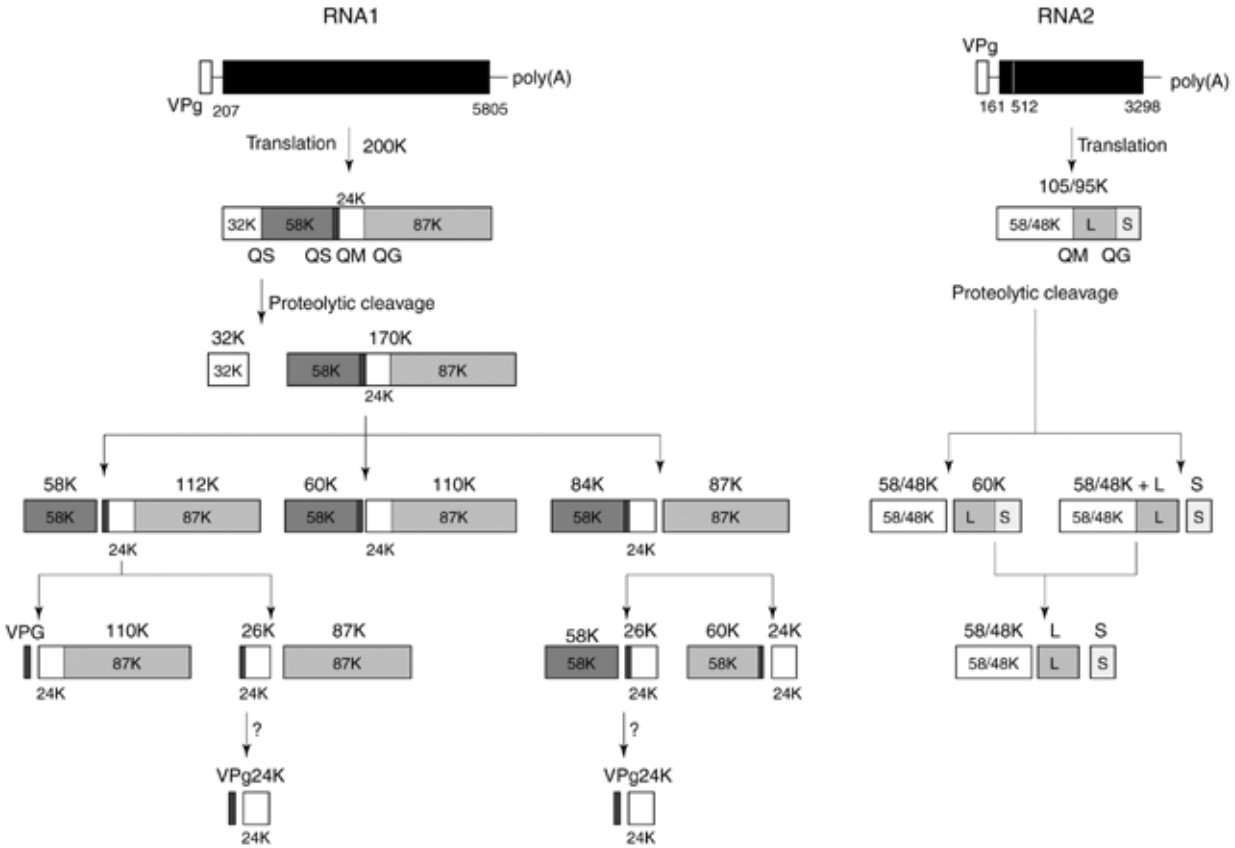


Figure 3 Expression of comoviral RNA1 and RNA2. Both RNAs contain a single long open reading frame. The details, such as the positions of the initiation and termination codons and the dipeptides at the proteolytic processing sites, are correct for CPMV; however, the overall processing scheme is probably applicable to all comoviruses.

protein are, from N- to C-terminus, the 58K protein, the VPg, the 24K proteinase and the 87K protein.

Initiation of translation of RNA2 occurs at two different positions on the RNA and results in the synthesis of two carboxy-coterminal proteins, the 105 and 95K proteins (**Fig. 3**). This double initiation phenomenon is found with the RNA2 molecules of all comoviruses. In the case of CPMV, synthesis of the 105K protein is initiated from an AUG at position 161, while initiation from an AUG at position 512 directs the synthesis of the 95K protein. CPMV RNA2 has an additional AUG (position 115) upstream of both these initiation sites but this feature is not conserved in the RNA2 molecules of other comoviruses. There has been some dispute as to whether initiation to give the 95K protein is a result of internal initiation or leaky scanning, and this has not, as yet, been fully resolved. Both RNA2-encoded primary translation products are cleaved by the RNA1-encoded proteolytic activity to give either the 58 or the 48K protein (depending on whether it is the 105 or 95K protein being processed) and the two viral coat proteins. Processing of the RNA2-encoded polyproteins, at least at the site between the 48K and L coat protein, has been shown to require the presence of the 32K protein as well as the 24K proteinase. For many years it was believed that the 60K protein (**Fig. 3**), containing the sequences of both the L and S coat proteins, was the direct precursor of the two mature capsid proteins; however, recent experiments involving the transient expression of the separate L and S proteins in protoplasts have cast doubt on this interpretation, and the role, if any, of the 60K protein in the virus replication cycle is unclear.

Functions of the Viral Proteins

Functions have been ascribed to most of the regions of the polyproteins encoded by both RNA1 and RNA2 of CPMV. In most cases, however, it is not certain at what stage(s) in the cleavage pathway they manifest their activity.

In the case of RNA1, the 32K protein, which is rapidly cleaved from the N-terminus of the 200K primary translation product, appears to be a cofactor which modulates the activity of the virus-encoded

protease. As described earlier, the presence of the 32K protein appears to be required for the cleavage of the RNA2-encoded 105 and 95K proteins but is not essential for the cleavage of the RNA1-encoded 170K protein. It does, however, seem to play a role in determining the rate at which cleavage of the 170K protein occurs. When mutant RNA1 molecules carrying deletions in the region encoding the 32K protein are translated *in vitro*, the rate of processing of the 170K protein is greatly increased, indicating that the 32K protein acts as an inhibitor of processing. This inhibition may be achieved through the interaction of the 32K with the 58K domain of the 170K protein. The mechanism by which the 32K protein enables the 24K proteinase to cleave *in trans* is unclear. It could be due to a direct association between the 32K protein and the 24K proteinase (or an intermediate containing the proteinase domain) or it could be an indirect result of the 32K protein modulating the cleavage of the 170K protein, thereby allowing a 24K-containing intermediate with *in trans* cleavage activity to accumulate.

The RNA1-encoded 58K protein is associated with cell membranes and contains a nucleotide-binding motif. For a long time it was believed that the 60K protein (**Fig. 3**), containing the amino acid sequence of the 58K protein linked to VPg, was the immediate precursor of the VPg *in vivo*. However, more recent studies have indicated that processing of the 60K protein *in trans* is extremely inefficient, casting doubt on its role as a precursor. It now seems more likely that it is the 112K protein, containing the sequence of the VPg linked to 24K and 87K proteins, which is the immediate precursor of the VPg. The 24K protein is the virus-encoded proteinase which carries out all the cleavages on both the RNA1- and RNA2-encoded polyproteins. Its proteolytic activity has been shown to be expressed in a number of the processing intermediates that contain its sequence. Indeed, it is not known whether the free form of the protein has any biological significance. Although the proteinase contains a cysteine at its active site, it is structurally related to serine proteases, such as trypsin, rather than cellular thiol proteases, such as papain. In this regard it is similar to the 3C proteinases of picornaviruses. All comoviral cleavage sites identified so far have a glutamine (Q) residue at the −1 position. The enzyme encoded by a given comovirus is specific for the polyproteins encoded by that virus and cannot cleave the polyproteins from other comoviruses either in *cis* or in *trans*.

The 87K protein is believed to contain the virus-encoded RNA-dependent RNA polymerase (RDRP) activity as it contains the G-D-D sequence motif found in all such enzymes. It also has amino acid sequence homology to the 3D polymerases encoded by picornaviruses. However, when replication complexes capable of elongating nascent RNA chains were isolated from CPMV-infected cowpea plants they were found to contain the 110K protein (**Fig. 3**), consisting of the sequence of 87K protein linked to the 24K proteinase, rather than the free 87K protein.

In the case of RNA2, the 48K protein, derived from processing of the 95K protein, is involved in potentiating the spread of the virus from cell to cell. This protein has been found in tubular structures that are formed in the plasmodesmata of cells infected with either CPMV or RCMV. Tubules extending into the culture medium can also be seen in protoplasts either infected with CPMV or transiently expressing the 48K protein. Virus particles can be seen within these tubules when protoplasts are infected with CPMV but not when only the 48K protein is expressed. At present, no definite role has been assigned to the 58K protein, which is produced by processing of the 105K protein. Mutants in which translation of the 105K protein is disrupted replicate poorly, if at all. In the light of these observations it has been suggested that the 105K protein may play a role in the replication of RNA2. Apart from containing many hydrophobic and aromatic amino acids, the approximately 10 kDa of protein present in the 58K but not the 48K protein is not conserved between comoviruses. The viral coat proteins are required to enable capsids to be formed. As well as protecting the genomic RNAs, capsid formation is essential for the virus to be able to spread from cell to cell through modified plasmodesmata. Long-distance movement, probably through the xylem, also requires capsid formation.

Replication

Comoviruses generally replicate to high level in infected cells. Replication is believed to involve the initial transcription of the incoming positive-sense RNA into minus-strands, followed by initiation and synthesis of new plus-strands from the recently formed minus-strands. For CPMV, it has been shown that the 5′ ends of both the plus- and minus-strands are covalently linked to the VPg, suggesting that this protein has an essential role in the initiation of RNA synthesis. There also appears to be a tight linkage between the translation of the viral RNAs and their replication.

In the case of CPMV, replication of the viral RNAs has been shown to occur in the membranous cytopathological structures which are formed in the cytoplasm of cells during infection. Both CPMV-specific double-stranded replicative form (RF) RNA and an enzyme activity capable of completing nascent

RNA strands can be isolated from such structures. Purified preparations of the enzyme activity contain the RNA1-encoded 110K protein and two host-encoded proteins of 68 and 57 kDa. However, at present no enzymatic activity capable of initiating RNA synthesis *in vitro* has been described.

Relationships with Other Virus Groups

There is a high degree of similarity between the comoviruses and the fabavirus, broad bean wilt virus (BBWV). For example, the L and S proteins of BBWV are the same size as those of CPMV, as are the respective RNA1 and RNA2. Indeed, some authors have considered BBWV to be a member of the comovirus group. However, BBWV shows no serological relatedness to any comovirus, reaches only moderate concentrations in plant sap, has a very wide host range and is transmitted by aphids rather than by beetles. For these reasons, BBWV is generally considered to be a member of a distinct group of plant viruses.

Consideration of genome structure and organization, translational strategy and amino acid homologies between the virus-encoded proteins has led to comoviruses being grouped with the nepo-, poty- and picornaviruses as members of the picorna-like supergroup of viruses. Members of this group are all nonenveloped positive-strand RNA viruses with 3′-polyadenylated genomic RNAs which have a protein (VPg) covalently linked to their 5′ ends. All members of the supergroup have a similar mode of gene expression which involves the synthesis of large precursor polyproteins and their subsequent cleavage by a virus-encoded proteinase. The members of the supergroup all contain the similar gene order, membrane-bound protein–VPg–proteinase–polymerase (**Fig. 3**), there being significant amino acid sequence homology between the membrane-bound proteins, the proteinases and polymerases. Comovirus capsids are also clearly structurally related to those of picornaviruses (**Fig. 2**) and probably also to those of nepoviruses.

Geographic Distribution

Individual comoviruses tend to have a somewhat restricted distribution. Clearly, the apparent distribution of a virus is governed, at least in part, by whether or not infection is diagnosed and reported. The following list, therefore, contains only those geographical areas where the presence of the virus has, to date, been confirmed.

- CPMV: Nigeria, Kenya, Tanzania, Japan, Surinam and Cuba.
- APMV: Central and South America.
- BPMV: USA.
- BRMV: Central America.
- BBSV: Europe and Africa.
- BBTMV: Europe and Northwest Africa.
- CPSMV: North, Central and South America.
- GMV: Australia.
- PMiMV: New Zealand.
- QPMV: North and Central America.
- RaMV: USA, Japan and Europe.
- RCMV: Europe.
- SqMV: North Africa, Israel, China, Japan, Australia and North, Central and South America.
- UCV: Peru and Bolivia.

Host Range

The host ranges of individual comoviruses tend to be rather narrow. Of the 14 members of the group, 10 (CPMV, BPMV, BRMV, BBSV, BBTMV, CPSMV, GMV, PMiMV, QPMV and RCMV) infect predominantly, if not exclusively, legumes. The other four members of the group, APMV, RaMV, SqMV and UCV, infect predominantly members of the *Solanaceae, Cruciferae, Cucurbitaceae* and *Basellaceae*, respectively.

Transmission

In nature, comoviruses are usually transmitted by leaf-feeding beetles, especially by members of the Chrysomelidae. However, CPMV has been shown also to be transmitted by thrips and grasshoppers. The beetle vectors can acquire the virus by feeding for as little as 1 min and can retain and transmit the virus for a period of days or weeks. The virus does not, however, multiply in the insect vector. Experimentally, all comoviruses are mechanically transmissible. In the case of BBSV, BBTV, CPMV, CPSMV and virus SqMV, transmission through seed has been reported.

Epidemiology

The primary sources for infection of crop plants with comoviruses are transmission through seed and infection from wild hosts which act as reservoirs of the viruses. It is also possible that virus which has overwintered in the beetle vector can act as a primary source of infection. Within-field spread is by the beetle vectors.

Symptomatology and Cytopathology

Generally, each member of the group has certain hosts in which the virus can spread systemically, causing mosaic or mottling symptoms, and other hosts in which the infection is confined to local lesions. Stunting of the host plant is sometimes observed, as is occasional systemic wilting and seed discoloration.

Infection of plant cells with comoviruses results in a number of characteristic cytological changes. These include the appearance of viral particles, either individually or as crystalline arrays, in the cytoplasm, a proliferation of cell membranes and vesicles in the cytoplasm, the appearance of amorphous inclusion bodies near or surrounding the nucleus and a variety of modifications to plasmodesmata. In the case of CPMV, the ability to cause membrane proliferation has been shown to be associated with RNA1.

Economic Importance

At present, BPMV, CPMV, CPSMV and RCMV are considered to be significant pathogens of legumes. Infection of soybeans with BPMV alone can cause yields to be reduced by 10–17%; however, this figure can rise to 60% in plants doubly infected with BPMV and the potyvirus, soybean mosaic virus. In Nigeria, infection of cowpeas with CPMV causes a great reduction in leaf area, flower production and yield. Infection of cowpeas with CPSMV has been shown to cause a 50% reduction in plant fresh weight and in the number and weight of pods. RCMV is of economic importance in forage production as it sometimes heavily invests clover. SqMV is a significant pathogen of cucurbits, problems associated with it being exacerbated by its high rate of seed transmission.

See also: **Fabaviruses (*Comoviridae*); Plant virus disease – economic aspects; Polioviruses (*Picornaviridae*): General features, Molecular biology; Virus structure: Atomic structure, Principles of virus structure; Nepoviruses (*Comoviridae*).**

Further Reading

Gergerich RC and Scott HA (1996) Comoviruses: transmission, epidemiology, and control. In: Harrison BD and Murant AF (eds) *The Plant Viruses 5: Polyhedral Virions and Bipartite RNA Genomes*, p. 77. New York: Plenum Press.

Goldbach RW and Wellink J (1996) Comoviruses: molecular biology and replication. In: Harrison BD and Murant AF (eds) *The Plant Viruses 5: Polyhedral Virions and Bipartite RNA Genomes*, p. 35. New York: Plenum Press.

King AMQ, Lomonossoff GP and Ryan MD (1991) Picornaviruses and their relatives in the plant kingdom. *Semin. Virol.* 2: 11.

Lomonossoff GP and Johnson JE (1991) The synthesis and structure of comovirus capsids. *Prog. Biophys. Mol. Biol.* 55: 107.

Stace-Smith R (1981) Comoviruses. In: Kurstak E (ed.) *Handbook of Plant Virus Infections and Comparative Diagnosis*, p. 171. Amsterdam: Elsevier/North Holland.

CORONAVIRUSES (*CORONAVIRIDAE*)

Kathryn V. Holmes, Department of Microbiology, University of Colorado HSC, Denver, Colorado, USA

Classification

The *Coronaviridae* were recognized as a new virus family in 1968 because their virion morphology and intracellular budding site distinguished them from other RNA viruses. Characteristic features of their genome, replication strategy, structural proteins and polymerase later supported this classification. The toroviruses and coronaviruses were recognized as separate genera within the *Coronaviridae* family in 1993. The *Coronaviridae* and *Arteriviridae* are now classified as members of the *Nidovirales* order, viruses with monopartite plus-strand RNA genomes that are transcribed to yield a nested set of overlapping subgenomic mRNAs that have a common 3′ end.

Virion Structure and Proteins

A model of coronavirus virions is shown in **Fig. 1.** The enveloped virions are approximately 100 nm in diameter, and are characterized by large, petal-shaped spikes. The spikes are oligomers of the 180–200 kDa S

Symptomatology and Cytopathology

Generally, each member of the group has certain hosts in which the virus can spread systemically, causing mosaic or mottling symptoms, and other hosts in which the infection is confined to local lesions. Stunting of the host plant is sometimes observed, as is occasional systemic wilting and seed discoloration.

Infection of plant cells with comoviruses results in a number of characteristic cytological changes. These include the appearance of viral particles, either individually or as crystalline arrays, in the cytoplasm, a proliferation of cell membranes and vesicles in the cytoplasm, the appearance of amorphous inclusion bodies near or surrounding the nucleus and a variety of modifications to plasmodesmata. In the case of CPMV, the ability to cause membrane proliferation has been shown to be associated with RNA1.

Economic Importance

At present, BPMV, CPMV, CPSMV and RCMV are considered to be significant pathogens of legumes. Infection of soybeans with BPMV alone can cause yields to be reduced by 10–17%; however, this figure can rise to 60% in plants doubly infected with BPMV and the potyvirus, soybean mosaic virus. In Nigeria, infection of cowpeas with CPMV causes a great reduction in leaf area, flower production and yield. Infection of cowpeas with CPSMV has been shown to cause a 50% reduction in plant fresh weight and in the number and weight of pods. RCMV is of economic importance in forage production as it sometimes heavily invests clover. SqMV is a significant pathogen of cucurbits, problems associated with it being exacerbated by its high rate of seed transmission.

See also: **Fabaviruses (*Comoviridae*); Plant virus disease – economic aspects; Polioviruses (*Picornaviridae*): General features, Molecular biology; Virus structure: Atomic structure, Principles of virus structure; Nepoviruses (*Comoviridae*).**

Further Reading

Gergerich RC and Scott HA (1996) Comoviruses: transmission, epidemiology, and control. In: Harrison BD and Murant AF (eds) *The Plant Viruses 5: Polyhedral Virions and Bipartite RNA Genomes*, p. 77. New York: Plenum Press.

Goldbach RW and Wellink J (1996) Comoviruses: molecular biology and replication. In: Harrison BD and Murant AF (eds) *The Plant Viruses 5: Polyhedral Virions and Bipartite RNA Genomes*, p. 35. New York: Plenum Press.

King AMQ, Lomonossoff GP and Ryan MD (1991) Picornaviruses and their relatives in the plant kingdom. *Semin. Virol.* 2: 11.

Lomonossoff GP and Johnson JE (1991) The synthesis and structure of comovirus capsids. *Prog. Biophys. Mol. Biol.* 55: 107.

Stace-Smith R (1981) Comoviruses. In: Kurstak E (ed.) *Handbook of Plant Virus Infections and Comparative Diagnosis*, p. 171. Amsterdam: Elsevier/North Holland.

CORONAVIRUSES (*CORONAVIRIDAE*)

Kathryn V. Holmes, Department of Microbiology, University of Colorado HSC, Denver, Colorado, USA

Classification

The *Coronaviridae* were recognized as a new virus family in 1968 because their virion morphology and intracellular budding site distinguished them from other RNA viruses. Characteristic features of their genome, replication strategy, structural proteins and polymerase later supported this classification. The toroviruses and coronaviruses were recognized as separate genera within the *Coronaviridae* family in 1993. The *Coronaviridae* and *Arteriviridae* are now classified as members of the *Nidovirales* order, viruses with monopartite plus-strand RNA genomes that are transcribed to yield a nested set of overlapping subgenomic mRNAs that have a common 3′ end.

Virion Structure and Proteins

A model of coronavirus virions is shown in **Fig. 1.** The enveloped virions are approximately 100 nm in diameter, and are characterized by large, petal-shaped spikes. The spikes are oligomers of the 180–200 kDa S

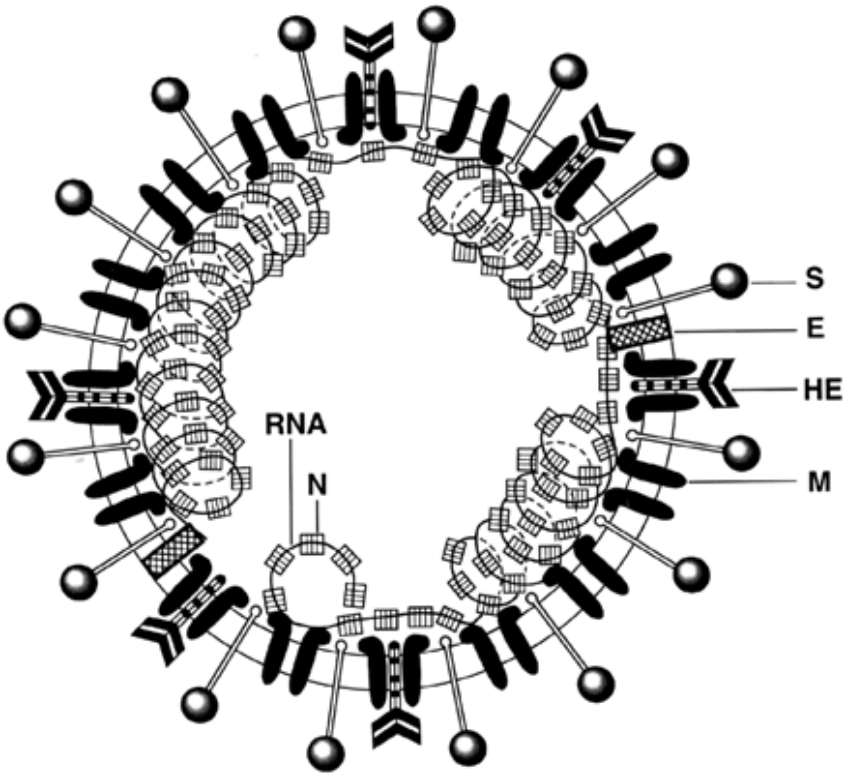


Figure 1 Model of coronavirus virion. The helical nucleocapsid containing the 30 kb, plus-sense RNA genome is coiled within an envelope that contains the S, M and E glycoproteins. Some coronaviruses related to MHV also express the HE glycoprotein. (Modified and published with permission from Fields BN, Knipe DM, Howley PM *et al* (eds) (1996) *Fields Virology*, 3rd edn. Philadelphia: Lippincott-Raven).

glycoprotein that binds to receptor glycoproteins and induces fusion of the viral envelope with cell membranes and, sometimes, cell–cell fusion. S proteins of several coronaviruses also bind to 9-*O*-acetylated sialic acid. A small envelope glycoprotein, M, traverses the lipid bilayer three times and interacts with the nucleocapsid in the virion. Intracellular transport of the M glycoprotein is arrested in the Golgi, which may determine the intracellular budding site of coronaviruses. The small E glycoprotein, originally believed to be a nonstructural protein, is present in small amounts in virions, and is essential for virus budding. Envelopes of some coronaviruses also contain a hemagglutinin-esterase glycoprotein, HE, which forms short spikes that bind *N*-acetyl-9-*O*-acetylneuraminic acid or *N*-glycolylneuraminic acid and have esterase activity. The nucleocapsid protein, N, encapsidates the monopartite, linear, single-stranded genomic RNA. The internal structure of coronaviruses was originally believed to be a helical nucleocapsid as shown in **Fig. 1**. Recently, however, cores that appear to have cubic symmetry were observed in detergent-treated virus preparations. Thus, the internal structure of coronaviruses is not yet understood.

Genome Structure

The 27–32 kb, plus-strand RNA genomes of coronaviruses are capped and polyadenylated. Transfection of genomic RNA into cells leads to production of infectious virions. The genomes of several coronaviruses have been sequenced, although no infectious coronavirus cDNA has yet been obtained. A map of the genome of murine coronavirus MHV (**Fig. 2**) illustrates the characteristic features of coronavirus genomic RNA. At the 5′ end of the genome is a cap with a leader RNA of approximately 70 bp. The order of the genes encoding the polymerase and structural proteins is the same in genomes of all coronaviruses (Pol, S, E, M, N), but several additional open reading frames (ORFs) are interspersed among these genes. These ORFs encode nonstructural (NS) proteins of unknown functions, and they differ in number, position and sequence among different coronaviruses.

For both *Coronaviridae* and *Arteriviridae*, the RNA-dependent RNA polymerase gene at the 5′ end of the genome consists of two ORFs in different reading frames, joined by a pseudoknot at a ribosomal frame shift site. The 5′ and 3′ ends of the genome contain predicted complex stem loop structures, and

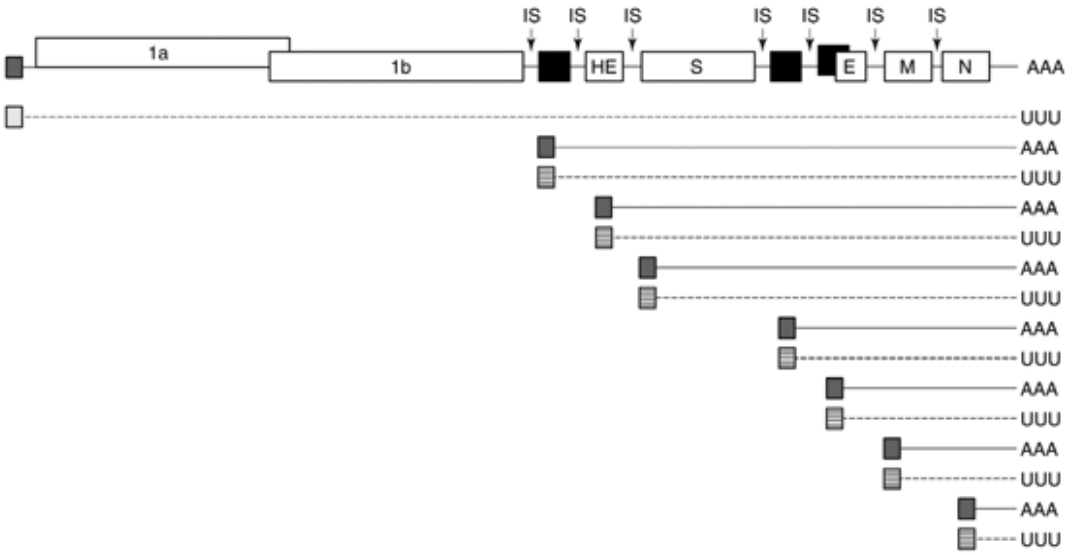


Figure 2 Map of the genome of murine coronavirus MHV and intracellular coronavirus RNAs. The polyadenylated plus-strand RNA genome is approximately 32 kb long. ORFs that encode structural proteins are shown in open boxes with the protein encoded by the gene inside. This gene order is the same in all coronaviruses. The RNA polymerase gene at the 5′ end of all coronavirus genomes consists of two ORFs, 1a and 1b, with a pseudoknot and a slippery sequence in the overlapping region. ORFs encoding putative nonstructural proteins (shown in filled boxes) vary in number and position among different coronaviruses. The conserved intergenic sequence on all plus-strand RNAs is shown by IS. The cap and approximately 70 bp leader RNA at the 5′ ends of each plus-strand RNA are indicated by a shaded box. Minus-strand templates are shown by dashed lines with a sequence complementary to the leader at the 3′ end indicated by a barred box, and a poly(U) tract at the 5′ end. Each subgenomic mRNA is translated to yield only the protein encoded by the 5′ end of the plus-strand RNA.

additional stem loops are predicted within some ORFs. Preceding every ORF in the genome is an intergenic sequence (IS) consisting of one or more UCUAAAC sequences for MHV or related sequences for other coronaviruses. The IS is included in a promoter that regulates the transcription of different viral genes. The location on the genome of packaging signals for the initiation of encapsidation of genomic RNA by the N protein appears to differ among coronaviruses.

Transcription, Replication, Translation and Genetics

Upon infection, the two large ORFs in the 20 kb polymerase gene of the genomic RNA are translated, via ribosomal frameshift, as a single, very large polyprotein that is cleaved during translation by 3-C-like and papain-like proteases within the nascent protein to yield a variety of peptides. The processing of the large polyprotein and the functions of the resulting peptides in RNA-dependent RNA polymerase activity are being analyzed in many laboratories.

Using the genomic RNA as template, the polymerase generates a full-length, minus-strand RNA with a 5′ poly(U) sequence. Infected cells also contain a 3′ coterminal nested set of polyadenylated subgenomic plus-strand mRNAs that have a cap and the approximately 70 bp leader sequence at their 5′ ends (**Fig. 2**). A negative-strand RNA template corresponding to each subgenomic mRNA is also found in infected cells (**Fig. 2**). A nonprocessive, leader-primed polymerase activity was postulated to account for the presence of the leader on each mRNA. A complex of polymerase and leader transcribed from the 3′ end of the full-length negative-strand template would bind to an IS on the template and act as a primer for synthesis of the subgenomic mRNA. This hypothesis does not explain the presence of the negative-strand templates for each subgenomic RNA. An alternative hypothesis suggests that polymerase jumping occurs during negative-strand synthesis, generating the subgenomic negative-strand templates for subsequent mRNA synthesis. Mutations in the IS preceding an ORF can prevent transcription of the mRNA with that ORF at its 5′ end. Although virus strains differ in the relative amounts of various mRNAs in infected cells, there is little temporal regulation of transcription.

Coronavirus replication is associated with a high frequency of mutations that arise by two mechanisms. First, during the replication of each approximately 30 kb RNA genome, several point mutations would

be expected to occur, based upon the known error frequency of RNA polymerases. Second, coronavirus genomes undergo a very high frequency of RNA recombination. Small or large deletions at the sites of recombination can produce mutant viruses, or defective interfering (DI) RNAs and subgenomic replicons. Large deletions in certain sites of the gene encoding the S glycoprotein and mutations in the N gene yield viruses with altered virulence, tissue tropism, or thermal stability. Thus, even after sequential single plaque isolations, coronavirus stocks always contain a mixture of quasispecies. Different virus variants may be selected during passage in different cell lines or under a variety of culture conditions. Therefore it is important to document the passage history of coronavirus strains or mutants. Rarely, recombination may occur between coronavirus genomic RNA and mRNAs of cells or unrelated viruses. Such an illegitimate RNA recombination event may have resulted in the incorporation of the mRNA that encodes the HE glycoprotein of influenza C into the genome of a coronavirus ancestral to the MHV group. Similarly, some of the NS ORFs may have been acquired through recombination with foreign mRNAs.

Genetic analysis of coronaviruses is somewhat limited because no full-length, infectious cDNA clones of coronavirus genomes are yet available. DI RNAs transfected into cells infected with wild-type virus can be replicated and have been used to study encapsidation and transcription initiation signals. Site-specific mutations have been introduced into several coronavirus genomes by targeted RNA recombination in infected cells transfected with a mutagenized subgenomic cDNA.

Coronavirus replication occurs in the cytoplasm and does not require the nucleus. Only the ORF at the 5′ end of each of the coronavirus mRNAs is translated within the infected cell or *in vitro*. The phosphorylated N protein, which is translated from one of the smallest mRNAs, is the most abundant viral protein in infected cells. It assembles with genomic plus-strand RNA in the cytoplasm to form helical nucleocapsids. Viral glycoproteins are translated on the rough endoplasmic reticulum, where S oligomerizes, then they are transported to the Golgi, where S proteins of some coronaviruses are cleaved by a trypsin-like host cell protease to yield S1 and S2 peptides. The S and HE glycoproteins are expressed on the plasma membrane. The viral S, HE, M and E glycoproteins and the nucleocapsid assemble by budding at a special pre-Golgi compartment, and the virions are apparently transported to the cell surface in large vesicles that fuse with the plasma membrane to release virions from the intact cell by exocytosis. Infected cells are characteristically coated with a thick layer of adsorbed virions.

Host Range, Tissue Tropism and Virus Propagation

Most coronaviruses cause epidemic disease in only one species, although limited replication, usually without disease, may result from experimental inoculation of other species. Coronaviruses typically cause respiratory or enteric diseases, although several can also cause hepatitis, infectious peritonitis, nephritis, myocarditis, sialodacryadenitis, or neurological, reproductive or immunological disorders. The viruses were named for their natural host and sometimes for the associated disease: for example, avian infectious bronchitis virus (IBV); mouse hepatitis virus (MHV); sialodacryadenitis virus of rats (SDAV); bovine coronavirus (BCoV); porcine hemagglutinating encephalomyelitis virus (HEV); turkey bluecomb coronavirus (TCoV); human respiratory coronaviruses (HCoV); transmissible gastroenteritis virus of swine (TGEV); porcine respiratory coronavirus (PRCV); canine coronavirus (CCoV); feline infectious peritonitis virus (FIPV) and feline enteric coronavirus (FeCoV); and rabbit coronavirus (RbCoV).

In vivo, coronaviruses bind to receptors expressed on the apical membranes of enteric and respiratory epithelial cells, and are released either from the apical or basolateral borders or both, depending upon the virus. Although strains of many coronaviruses have been adapted to growth in continuous cell lines, isolation of human coronaviruses from infected patients may require human fetal tracheal organ cultures. Some coronaviruses, such as rabbit coronavirus or enterotropic strains of MHV, cannot be propagated in cell culture, but require animal passage. Coronavirus-like particles seen in electron micrographs of human feces (human enteric coronaviruses, HECV) have not been adapted to serial passage in cell culture. IBV and TCoV can be propagated in embryonated eggs, and some strains grow in avian cell lines. Although cells of the natural host species are generally required for infection by virions, purified coronavirus genomic RNA can infect cells across species barriers. Receptors have been identified for several coronaviruses: MHV uses murine biliary glycoproteins in the immunoglobulin superfamily; HCoV-229E and TGEV use human and porcine aminopeptidase N (APN), respectively; FIPV and FeCoV use feline APN, which can also be utilized by HCoV-229E and TGEV; and BCoV and HCoV-OC43 use *N*-acetyl-9-*O*-acetyl neuraminic acid moieties. Expression of the cloned receptor glycoproteins

in cells of a foreign species can render them susceptible to infection with coronavirus virions. Thus, coronavirus–receptor interactions are an important determinant of the species specificity of coronavirus infection.

Coronavirus infection of cells may be inapparent or cause cell fusion, vacuolization, rounding and/or cell death. Cytopathic effects are minimized, and virus yield and stability are increased at acid pH. Some strains of MHV infect cells by receptor-dependent fusion of the viral envelope with the plasma membrane, but other strains of MHV and other coronaviruses appear to enter via fusion with endosomal membranes. For some coronaviruses, cleavage near the middle of the S glycoprotein at a sequence of basic amino acids yields the noncovalently linked S_1 and S_2 peptides and enhances viral infectivity and/or cell fusion. S glycoproteins of many other coronaviruses, such as FIPV, lack the protease target sequence and do not require protease activation for infectivity or cell fusion.

Serologic and Evolutionary Relationships

There are three coronavirus serogroups. One group includes HCoV-229E, TGEV, PRCoV, CCoV, FCoV, and others. A second group includes MHV, BCoV, SDAV, HEV, HCoV-OC43 and others. Avian IBV strains make up the third serogroup. Phylogenetic analysis of coronavirus N and S genes correlate well with the division of coronaviruses into these three groups. Nucleic acid sequence analysis also shows that certain pairs of coronaviruses that cause different syndromes in the same host should probably be considered strains of a single virus. Thus, PRCoV and TGEV of swine, which cause epizootic respiratory and enteric disease, respectively, are highly homologous, except for a deletion of more than 675 nucleotides in the S gene of PRCoV. The feline coronavirus FeCoV causes epizootic enteric disease in cats, and mutant FIPV viruses that arise within FeCoV-infected animals cause fatal systemic disease.

The HE glycoprotein is encoded by a gene found in the MHV group of coronaviruses, but not in the HCoV-229E or IBV groups. Phylogenetic analysis of coronavirus HE gene suggests that BCoV and HCoV-OC43 are more closely related to each other than to MHV. The organization of the polymerase gene with two large slightly overlapping ORFs in different reading frames joined by a pseudoknot is conserved among all coronaviruses and shared by toroviruses such as Berne virus (BEV) and *Arteriviridae*. In addition, BEV encodes a protein with significant homology to HE of coronaviruses and influenza C virus.

Recombination between genomes of related coronaviruses can occur in experimentally inoculated cell cultures or animals, and also during natural outbreaks of disease in the natural host. Recombinants between IBV strains have been isolated from infected birds. Nucleotide sequencing shows that one biotype of feline coronavirus that causes epizootic disease is a recombinant between canine coronavirus and the other biotype of feline coronavirus. Mutants of MHV derived from persistently infected murine cell lines have acquired the ability to infect nonmurine cells. These observations indicate that there may be naturally occurring interactions between the genomes of coronaviruses from different species.

Epidemiology

Most coronaviruses cause epidemic or epizootic disease in only one species. Because of the great antigenic variability among coronavirus strains and because many coronaviruses replicate only in epithelia where protective immunity is relatively short-lived, reinfection is common. Infection is often inapparent, and virus particles or antigens and subsequent seroconversion are observed in healthy individuals. The viruses are enzootic or endemic in their host species, causing sporadic disease and seasonal outbreaks when enough susceptibles are available. Adults with acute, self-limited, inapparent infection transmit virus to neonates that develop clinical disease. Coronavirus diseases are more severe in neonatal animals than adults, either because preexisting immunity to related virus strains moderates infections in adults, or because the immature immune system permits higher levels of virus replication. In immunocompromised hosts, infection may be inapparent but virus shedding may be prolonged. Such individuals serve as reservoirs of virus. For example, enterotropic MHV strains are endemic in colonies of laboratory mice, sustained both by persistent infection of immunocompromised nude mice and by the continuous availability of new susceptibles due to birth or importation.

Outbreaks of coronavirus diseases are often seasonal. In humans, coronaviruses cause 15–30% of colds, predominantly during the winter months, and outbreaks of different human coronaviruses alternate at 2–3 year intervals. Outbreaks of BCoV-induced dysentery in cattle also occur in the winter. Severe enteritis due to TGEV or BCoV infection of suckling pigs or calves, respectively, occurs seasonally in correlation with breeding cycles. Outbreaks of IBV-induced respiratory disease in chickens can occur at

any time, but are most common during the winter. Stress can exacerbate coronavirus-induced diseases. Mice with inapparent MHV infection can develop hepatitis if they are subjected to immunosuppression, thymectomy, transplanted tumors or infection with unrelated organisms. Cattle with inapparent BCoV infection may develop respiratory disease during shipping.

Coronaviruses have broad geographic distribution. Human infections with viruses related to HCoV-229E or to HCoV-OC43 occur worldwide, as do IBV, TGEV, BCoV, FCoV and CCoV infections. Occasionally, strains of these viruses that cause unusual manifestations of disease arise and spread locally, and some of these viruses become very widely distributed. For example, IBV strains that cause severe nephropathy arise and spread locally. PRCoV, which causes a mild epizootic respiratory infection in swine, has arisen several times in Europe and in the USA from TGEV by means of a very large deletion in the S glycoprotein. The epizootic spread of PRCoV in European pigs has apparently acted as a natural vaccine that has decreased the incidence of serious TGEV-induced enteric disease in piglets.

Pathology

Most coronaviruses cause only respiratory or enteric disease in one host species. These viruses generally replicate only in respiratory or enteric epithelial cells, where the apical membranes express specific glycoprotein receptors for the viruses. Coronaviruses are shed in respiratory secretions and/or feces. Some coronaviruses, such as some MHV strains, FIPV and rabbit coronavirus, cause disseminated disease and can replicate in macrophages, hepatocytes, neurons, glial cells, endothelial cells, kidney epithelium, lymphocytes, urogenital tract and/or myocardium. In general, coronavirus titers in the respiratory or enteric tract rise during the first 3–5 days postinoculation, and recovery of infectious virus from an immunocompetent host is usually not possible after 10–14 days, although viral antigens and RNA may continue to be detectable for several weeks. Infectious coronavirus can be shed for months by immunocompromised hosts. Although most coronaviruses do not persist in immunocompetent hosts, coronaviruses such as the neurotropic MHV-JHM strain can sometimes be detected in the brain months or years after inoculation. Reverse transcriptase–polymerase chain reaction (RT-PCR) can detect HCoV-229E and/or HCoV-OC43 RNA in up to 40% of brains from patients with neurological diseases and from healthy individuals, but the significance of this observation for human disease is unknown.

Coronavirus-induced lesions vary markedly depending upon the virus strain, dose and tissue tropism and the genetic background of the host. Intestinal infections with BCoV, MHV, TGEV, FeCoV, TCoV and CCoV cause loss of apical epithelial cells of the intestinal villi and shortening and broadening of the villi. Some enterotropic coronaviruses cause necrotizing enterocolitis, particularly in young animals, while others cause only watery diarrhea or inapparent enteric infection. Diarrhea is probably due to altered transport of fluids and electrolytes by the immature epithelial cells that cover the blunted villi. Intestinal absorption of certain sugars in TGEV-infected pigs remains altered for several days after the diarrhea has ceased. Mononuclear inflammatory cells infiltrate the lamina propria. Reinfection with a different strain of the virus generally causes more moderate disease than primary infection. However, kittens are more likely to develop FIP following their second infection with feline coronavirus, than after their first infection. Thus, an immunological response to the primary infection may somehow facilitate the later development of disseminated disease.

Human respiratory coronaviruses related to HCoV-229E or HCoV-OC43 infect the epithelial cells in the upper respiratory tract and cause colds. Infection of young asthmatic children can exacerbate wheezing, and lower respiratory tract coronavirus infection has occasionally been observed in adults. Reinfection is frequent, even in volunteers inoculated with the same strain of human coronavirus. Infection is usually demonstrated by RT-PCR or by rising serum antibody titers because primary isolation of these viruses from respiratory washings is difficult. It is not yet clear how many coronavirus strains cause human respiratory disease or whether coronaviruses may play a role in other human diseases.

IBV-induced respiratory diseases in chickens is of great economic importance. In addition, some IBV strains are nephrotropic and cause kidney disease, while others infect the oviduct and reduce egg laying. Recombinants between several IBV strains have been isolated from infected flocks.

Neurological disease and hepatitis can result from coronavirus infections. MHV strains cause local and/or systemic infections via respiratory or fecal/oral routes. While some MHV strains are strictly enterotropic, most strains infect both the respiratory and enteric tracts. Some MHV strains causes focal hepatitis, acute encephalitis, and subacute or chronic focal demyelinating disease in the murine brain and spinal cord. Cell fusion, necrosis and infiltration with mononuclear cells are observed in acutely infected tissues, depending upon the strain of virus and strain of mice. Susceptibility to MHV is affected by at least

three murine genes that determine the virus receptor isoforms expressed, the yield of infectious virus and the ability to generate monocyte procoagulant activity, a prothrombinase in the coagulation pathway, in response to MHV infection. Mutations in the MHV and TGEV S glycoproteins can alter tissue tropism, virulence and persistence. HEV causes respiratory infection and outbreaks of encephalomyelitis in suckling pigs, and virus infection of neurons that innervate the stomach causes vomiting and wasting syndrome.

Unusual syndromes associated with coronaviruses include feline infectious peritonitis, rabbit myocarditis and rat sialodacryadenitis. FECV causes enteritis in kittens and inapparent infection in adult cats, while the closely related FIPV can cause infectious peritonitis, with ascites, wasting and death in a small percentage of infected cats. A cardiotropic rabbit coronavirus causes dilated myocardiopathy and death within 7–12 days after intravenous inoculation of rabbits with serum from an infected rabbit. Rat coronaviruses infect the respiratory tract, salivary and lacrimal glands, causing sialodacryadenitis, and can also infect the urogenital tract, interfering with breeding.

Immune Responses

Infection of respiratory or enteric tracts of adults results in antiviral antibodies in the serum, secretory antibody in the respiratory and enteric tracts, colostrum and milk, and development of virus-specific T cells. These immune responses are useful for diagnostic purposes, terminate infection, and probably ameliorate subsequent infections, but they do not necessarily prevent reinfection. In newborns, passive oral immunization with neutralizing antibody can sometimes prevent fatal coronavirus enteritis. Because coronavirus diseases are generally most severe in newborns that do not respond well to active immunization, an attractive strategy to protect these newborn animals that is being explored is to vaccinate pregnant dams in order to maximize antiviral antibodies in the colostrum and milk.

Coronaviruses can sometimes infect cells of the immune system and may modulate cytokines and immune responses to unrelated immunogens. Infected macrophages can spread infection to distant tissues. Mononuclear cell infiltrates in infected tissues consist of macrophages, plasma cells, and CD4+ and CD8+ lymphocytes. Infection of glial cells with MHV can upregulate expression of major histocompatibility complex (MHC) class I or II antigens, making these cells potential targets for cytotoxic T cells. MHV infection causes thymic atrophy, and the virus can also infect B lymphocytes. Polyclonal B cell activation and hypergammaglobulinemia are associated with FIPV infection. Cheetahs, which have little polymorphism in their MHC genes, have a much higher fatality rate after feline coronavirus infection than cats.

Prevention and Control of Coronavirus Diseases

Because of the economic importance of coronavirus diseases of domestic animals, modified live vaccines against IBV, TGEV, BCV, CCV and FIPV have been developed. However, they do not provide solid protection from infection with wild-type coronaviruses. Other approaches to protection or control of coronavirus diseases include use of recombinant S proteins, synthetic peptides that mimic neutralization epitopes, passive immunization with antibody against S glycoproteins, and treatment with interferon α or monoclonal antireceptor antibody. Improvement of vaccines will require understanding of coronavirus virulence factors, strain variation and mechanisms of immunopathology.

Future Perspectives

Full-length cDNA copies of coronavirus genomes and a way to express them to obtain infectious virions are needed to investigate coronavirus replication and pathogenesis. Analysis of the complex synthesis, processing and functions of the coronavirus polymerase peptides will provide unique insight into mechanisms of RNA recombination, tools for coronavirus genetics, and possibly new targets for drugs to inhibit coronavirus replication. Improvements in diagnostic tests to identify coronavirus infections in humans and animals will elucidate the epidemiology of these viruses and may implicate coronaviruses in the etiology of additional diseases. The characterization of coronavirus–host interactions during persistent infection *in vitro* and *in vivo* will provide additional insight into coronavirus epidemiology and pathogenesis. Further understanding of virus variants and host responses to coronavirus infections of the respiratory and enteric tracts may lead to improved coronavirus vaccines.

See also: **Arteriviruses (*Arteriviridae*); Toroviruses (*Coronaviridae*); Immune response: Cell mediated immune response, General features; Respiratory viruses; Recombination of viruses; Rhinoviruses (*Picornaviridae*).**

Further Reading

Brian DA and Spaan WJM (1998) Recombination and coronavirus defective interfering RNAs. *Semin. Virol.* 8: 101.

Holmes KV and Lai MMC (1996) *Coronaviridae* and their replication. In: Fields BN, Knipe DM, Howley PM *et al Fields Virology*, 3rd edn, p. 1075. Philadelphia: Lippincott-Raven.

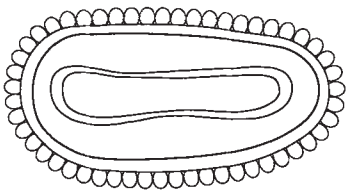
Lai MMC and Cavanagh D (1997) The molecular biology of coronaviruses. *Adv. Virus Res.* 48: 1.

Lane T E and Buchmeier MJ (1997) Murine coronavirus infection: a paradigm for virus-induced demyelinating disease. *Trends Microbiol.* 5: 9.

McIntosh K (1996) Coronaviruses. In: Fields BN, Knipe DM, Howley PM *et al Fields Virology*, 3rd edn, p. 1095. Philadelphia: Lippincott-Raven.

Rossen J W, Horzinek MC and Rottier PJ (1995) Coronavirus infection of polarized epithelial cells. *Trends Microbiol.* 3: 486.

COWPOX VIRUS (*POXVIRIDAE*)



D Baxby and **M Bennett**, Departments of Medical Microbiology and Veterinary Pathology, University of Liverpool, Liverpool, UK

History

The first published account of human and bovine cowpox is Edward Jenner's 'Inquiry' of 1798. He described the clinical signs of cowpox in both species, and how infection in humans with *Variolae vaccinae* ('known by the name of the cowpox') provided protection against smallpox. At that time smallpox killed 200 000–600 000 people each year in Europe and caused up to 30% of all deaths in city children. Jenner's observations, despite the concern of some over the consequences of inoculating bovine material into humans, soon led to the use of smallpox vaccine around the world. However, not until Pasteur's work nearly 100 years later was the principle of immunization used again, and it was Pasteur who suggested that all such immunizations be called 'vaccines' to honour Jenner.

Although Jenner's first vaccines came from cattle, later material was sometimes derived from horses, and the origins of modern vaccinia virus (smallpox vaccine), are unknown. Differences between cowpox and vaccinia viruses were first reported in 1939, since when further biological and genetic studies have confirmed that vaccinia virus is a separate species and not simply a mutant of cowpox virus or a recombinant of smallpox and cowpox viruses.

Even Jenner had difficulty finding cowpox, and cowpox virus is certainly not now endemic in cattle. It is now accepted that cowpox virus is endemic in rodents, with cattle and humans acting as accidental hosts. However since the 1970s, the domestic cat has been the animal most frequently seen with cowpox.

Taxonomy and Classification

Cowpox virus is a member of the genus *Orthopoxvirus* in the subfamily *Chordopoxvirinae* of the *Poxviridae* family; the international reference strain, Brighton, was isolated in 1937 from farm workers in contact with cattle. Cowpox virus can be differentiated from other orthopoxviruses by a combination of biological tests, including the ability to produce hemorrhagic pocks on chorioallantoic membranes, the production of A-type inclusions (ATI) in infected cells, and its 'ceiling temperature' (40°C, the highest temperature at which it will replicate), by minor antigenic differences, and by a variety of molecular assays including restriction enzyme digestion, particularly with Hind III, and sequencing or restriction endonuclease digestion of polymerase chain reaction (PCR) products from, for example, the ATI and fusion protein genes.

Not all strains of cowpox virus are identical and some, isolated from unusual hosts, have been referred to in the past as 'cowpox-like' viruses. However, they are clearly strains of cowpox virus, and probably represent geographic or host-range variants.

Properties of the Virion

Cowpox virus has a typical orthopoxvirus morphology, and is indistinguishable from vaccinia virus by electron microscopy. Virions are brick shaped, approximately 300 × 200 × 200 nm in size, and may be enveloped. The virion consists of a biconcave core, and within each concavity lies a lateral body (**Fig. 1**).

Further Reading

Brian DA and Spaan WJM (1998) Recombination and coronavirus defective interfering RNAs. *Semin. Virol.* 8: 101.

Holmes KV and Lai MMC (1996) *Coronaviridae* and their replication. In: Fields BN, Knipe DM, Howley PM *et al Fields Virology*, 3rd edn, p. 1075. Philadelphia: Lippincott-Raven.

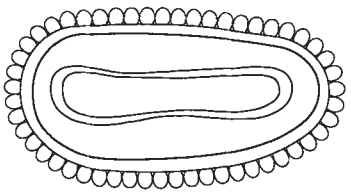
Lai MMC and Cavanagh D (1997) The molecular biology of coronaviruses. *Adv. Virus Res.* 48: 1.

Lane T E and Buchmeier MJ (1997) Murine coronavirus infection: a paradigm for virus-induced demyelinating disease. *Trends Microbiol.* 5: 9.

McIntosh K (1996) Coronaviruses. In: Fields BN, Knipe DM, Howley PM *et al Fields Virology*, 3rd edn, p. 1095. Philadelphia: Lippincott-Raven.

Rossen J W, Horzinek MC and Rottier PJ (1995) Coronavirus infection of polarized epithelial cells. *Trends Microbiol.* 3: 486.

COWPOX VIRUS (*POXVIRIDAE*)



D Baxby and **M Bennett**, Departments of Medical Microbiology and Veterinary Pathology, University of Liverpool, Liverpool, UK

History

The first published account of human and bovine cowpox is Edward Jenner's 'Inquiry' of 1798. He described the clinical signs of cowpox in both species, and how infection in humans with *Variolae vaccinae* ('known by the name of the cowpox') provided protection against smallpox. At that time smallpox killed 200 000–600 000 people each year in Europe and caused up to 30% of all deaths in city children. Jenner's observations, despite the concern of some over the consequences of inoculating bovine material into humans, soon led to the use of smallpox vaccine around the world. However, not until Pasteur's work nearly 100 years later was the principle of immunization used again, and it was Pasteur who suggested that all such immunizations be called 'vaccines' to honour Jenner.

Although Jenner's first vaccines came from cattle, later material was sometimes derived from horses, and the origins of modern vaccinia virus (smallpox vaccine), are unknown. Differences between cowpox and vaccinia viruses were first reported in 1939, since when further biological and genetic studies have confirmed that vaccinia virus is a separate species and not simply a mutant of cowpox virus or a recombinant of smallpox and cowpox viruses.

Even Jenner had difficulty finding cowpox, and cowpox virus is certainly not now endemic in cattle. It is now accepted that cowpox virus is endemic in rodents, with cattle and humans acting as accidental hosts. However since the 1970s, the domestic cat has been the animal most frequently seen with cowpox.

Taxonomy and Classification

Cowpox virus is a member of the genus *Orthopoxvirus* in the subfamily *Chordopoxvirinae* of the *Poxviridae* family; the international reference strain, Brighton, was isolated in 1937 from farm workers in contact with cattle. Cowpox virus can be differentiated from other orthopoxviruses by a combination of biological tests, including the ability to produce hemorrhagic pocks on chorioallantoic membranes, the production of A-type inclusions (ATI) in infected cells, and its 'ceiling temperature' (40°C, the highest temperature at which it will replicate), by minor antigenic differences, and by a variety of molecular assays including restriction enzyme digestion, particularly with Hind III, and sequencing or restriction endonuclease digestion of polymerase chain reaction (PCR) products from, for example, the ATI and fusion protein genes.

Not all strains of cowpox virus are identical and some, isolated from unusual hosts, have been referred to in the past as 'cowpox-like' viruses. However, they are clearly strains of cowpox virus, and probably represent geographic or host-range variants.

Properties of the Virion

Cowpox virus has a typical orthopoxvirus morphology, and is indistinguishable from vaccinia virus by electron microscopy. Virions are brick shaped, approximately 300 × 200 × 200 nm in size, and may be enveloped. The virion consists of a biconcave core, and within each concavity lies a lateral body (**Fig. 1**).

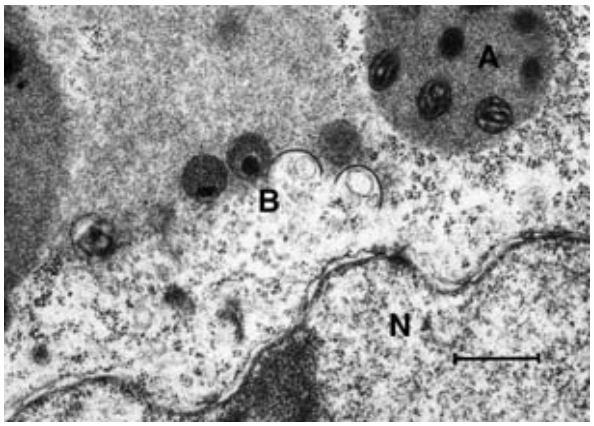


Figure 1 Thin section of a chick chorioallantois cell infected with cowpox virus. A, A-type inclusion containing mature virions; B, B-type inclusion showing virions in various stages of assembly; N, nucleus. Bar = 500 nm.

The whole is surrounded by an outer membrane covered in short spicules. The core contains DNA and virus-encoded enzymes.

Virions released naturally from the cell are wrapped in an envelope derived from the host cell's Golgi membranes. This envelope is very labile and often lost during manipulation. Most virions involved in cell-to-cell spread within an infected animal are probably enveloped (extracellular enveloped virus; EEV), but virus released by cell lysis (intracellular naked virus; INV) is also infectious, and INV, particularly within protective A-type inclusions, is probably important in spread from animal to animal. Because most laboratory stocks of cowpox are prepared by cell disruption, they consist almost entirely of INV, and the biology of EEV has been less well studied.

Negatively stained preparations of INV usually show two types of particle, an M (mulberry) form, the surface of which is covered in randomly arranged tubules, and a C (capsule) form which has a capsule and a dark, homogenous center. Both forms are infectious and their different appearances are due to different degrees of dehydration during preparation.

Properties of the Genome

The genome consists of linear double-stranded (ds) DNA with covalently linked inverted repeats at the termini. The cowpox virus genome is the largest of all the orthopoxviruses with a total of approximately 220 kbp (140 MDa). The central portions are highly conserved, but more variation occurs towards either end. Digestion with Hind III clearly differentiates cowpox viruses from other known orthopoxviruses, but isolates, particularly geographically distinct strains, do vary, and digestion of genomic DNA with other enzymes often reveals much greater differences between strains. However, the genome is fundamentally very stable and no changes in restriction enzyme profile have been seen over numerous passages and many years. Differences in the restriction enzyme profiles of different strains can therefore be used in epidemiological studies to help determine the source of an outbreak.

The main exception to this genomic stability is the frequent mutations which give rise to the 1% white pocks on chorioallantoic membranes. In the Brighton strain this mutation involves the deletion of 32–39 kbp DNA from the right hand end of the genome and its replacement by a nonhomologous copy of DNA from the left hand end. The size of the transposed fragment varies (5–50 kbp) but the net effect maintains the general symmetry of the termini, which is probably important for replication.

Several portions of the cowpox virus genome have been sequenced including the extreme termini, the hemagglutinin gene, a gene responsible for the major protein in A-type inclusions, a putative serine protease inhibitor, and a gene involved in host cell range.

Properties of Proteins

The size of the cowpox virus genome, and comparison with vaccinia virus, suggest that it could encode up to 200 proteins, although not all, for example hemagglutinin and thymidine kinase, are essential for replication. General comparison of proteins from different orthopoxviruses has helped to establish the separate identity of cowpox virus. However, many proteins are shared, and the many enzymes encoded by vaccinia virus have their homologues in cowpox virus.

Interest has recently focused on (1) proteins concerned with attributes possessed by cowpox but not vaccinia virus, such as the ability to grow on Chinese hamster ovary (CHO) cells, to produce A type inclusions (ATI) and in analysis of white pock mutation, and (2) proteins involved in evasion of the host's immune response.

Growth of cowpox virus in CHO cells is dependent on production of a 77 kDa protein, which inhibits apoptosis. These cells rapidly undergo apoptosis when infected with wild-type vaccinia virus, but not with a recombinant expressing this protein. Formation of ATI involves the production of two late proteins. One, of 160 kDa, is the major component of the inclusion itself. The other, probably a virus surface component, is required for occlusion of virions into the inclusion. Mutation to the white

pock character is associated with loss of a gene (*CmrA*), homologous to the *B13R* of vaccinia virus, which encodes a 38 kDa protein, one of the most abundant early gene products. The CmrA predicted amino acid sequence is similar to that of inhibitors of serine proteases (SERPIN) which are involved in blood clotting, and has *in vitro* serine protease inhibitor activity causing inhibition of both apoptosis and production of interleukin 1β (through inhibition of caspase 1) and inhibition of granzyme B, a serine protease released by cytotoxic T lymphocytes. Other cowpox proteins which may be involved in inhibition of aspects of the host's immune response include soluble tumor necrosis factor receptors (CmrB and CrmC), interferon receptors, and a complement-binding protein.

Physical Properties

Little work has been done directly on the physical properties of cowpox virus which are usually assumed to be similar to those of vaccinia virus and infectious ectromelia virus. The lipid envelope of cowpox virus is labile and readily lost by mechanical stress, but is not essential for virus infectivity. The virus is generally very hardy, and can survive for months in dry scab material at room temperature, and indefinitely at −70°C. It is readily inactivated by moist heat at 65°C, and by hypochlorites, phenolics and detergents, but less effectively by alcohols.

Replication

The mechanisms of DNA replication and temporally regulated transcription and translation of cowpox virus mRNA are largely similar to those of vaccinia virus (*see also:* **Vaccinia virus**). Virus replication takes place in the cytoplasm, and virus assembly occurs in areas known as B-type inclusions (**Fig. 1**). Like vaccinia virus, cowpox virions can leave the cell as individual naked (unenveloped) particles by lysis of the cell membrane or as enveloped particles by fusion with Golgi and the outer cell membranes. Cowpox differs from vaccinia virus in that its genome also encodes for large proteinaceous inclusions known as A-type inclusions, into which virions are incorporated (**Fig. 1**). These inclusions are released by cell lysis, and are thought to act as protective packets which aid survival of the virus outside the animal and therefore increase the chance of spread to another host on fomites.

Geographic and Seasonal Distribution

Confirmed isolations of cowpox virus have been made only in Europe and adjoining regions of Asia. Cowpox in domestic cats and humans is mostly seen in the late summer and autumn. This seasonal variation probably reflects the increased prevalence of infection in the reservoir hosts at this time of year. Bovine cowpox is so rare that no data exist to assess any seasonal effects on incidence.

Host Range and Virus Propagation

Cowpox virus has a wide host range both *in vivo* and *in vitro*. It is important to distinguish between reservoir hosts in which the virus is maintained, and accidental (indicator) hosts (**Table 1**; see Epidemiology). Cowpox virus causes natural infections in cattle, humans, domestic cats and dogs, and a variety of zoo animals. During an outbreak at Moscow Zoo, virus was also isolated from laboratory rats used to feed the big cats. Surveys of wildlife have demonstrated infection in ground squirrels and gerbils in Turkmenia, and voles and mice in Western Europe (see Epidemiology.) Mice, guinea pigs and rabbits have been experimentally infected.

Cowpox virus can be isolated and propagated on the chorioallantoic membrane of hens' eggs, but, unlike vaccinia virus, does not grow in feather follicles of adult chickens. It can be propagated in a variety of cell cultures derived from human, simian, bovine, feline, murine and rabbit tissues. Cowpox virus, unlike vaccinia virus, grows in CHO cells.

Genetics

Cowpox virus produces red, hemorrhagic pocks on the chorioallantoic membrane with about 1% white pock mutants. These breed true and the change in pock character reflects deletion and transposition events near the termini of the genome. White pock mutants also lack an antigen, designated 'd', and are better able to grow on arginine-deprived cells than is the parent virus. Other properties which are independently inherited, and vary between individual strains include relative haemagglutinin production, presence of virions in A-type inclusions, resistance to heat, minor differences in ceiling temperature for growth, and pathogenicity for new-born mice and chick embryos. These properties have been used to identify individual isolates of cowpox virus, and may be useful in epidemiological studies. Orthopoxviruses undergo genetic recombination, for example producing hybrids of cowpox and smallpox viruses.

Evolution

Little is known of the evolution of cowpox virus or orthopoxviruses as a whole. Serologic and cross-hybridization studies show that all orthopoxviruses

Table 1 Animals in which natural cowpox has been reported

Probable reservoir hosts	*Distribution*
Ground squirrel (*Citellus fulvus*)	Continental Europe and adjoining states of CIS (former USSR)
Giant gerbil (*Rhombomys opimus*)	Continental Europe and adjoining states of CIS (former USSR)
Root vole (*Microtus oeconomus*)	Continental Europe and adjoining states of CIS (former USSR)
Bank vole (*Clethrionomys glareus*)	UK, Continental Europe and adjoining states of CIS (former USSR)
Field vole (*Microtus agrestis*)	UK, Continental Europe and adjoining states of CIS (former USSR)
Woodmouse (*Apodemus sylvaticus*)	UK, Continental Europe and adjoining states of CIS (former USSR)
Indigenous accidental hosts	Cases detected per annum in UK
Cat	40–60
Human	1–2
Cow	None since 1976
Dog	(Only one case reported)
Nonindigenous accidental hosts[a]	
Felidae[b]	
Elephant	
Okapi	
Rhinoceros	
Anteater	

[a]Infection recognized in captive species in zoos etc. in areas where cowpox is endemic. Infection has not been reported in the animals' natural habitats, where cowpox does not circulate.
[b]Including cheetah, lion, puma, ocelot, panther and lynx.

are closely related, but each species can be readily differentiated by biological properties and genome structure. Cowpox virus isolates are genetically stable. Recent isolates often have near-identical restriction enzyme profiles to strains isolated many years ago, and the Brighton strain behaves the same now as when first isolated over 60 years ago. However, isolates of cowpox virus do differ in restriction enzyme pattern and minor biological characteristics, and generally greater differences occur between geographically distinct isolates (for example, the Brighton and Moscow Zoo strains). Furthermore, whereas British isolates are all very similar, greater variation exists among Continental isolates and this may reflect a central European origin for cowpox virus, and divergence as individual strains spread out into different host reservoirs and geographical areas.

Serologic Relationships and Variability

There is extensive antigenic crossreactivity within the genus *Orthopoxvirus* although minor differences may allow differentiation of some species. No serological differences have been reported among cowpox virus strains apart from the loss of 'd' antigen from white pock mutants.

Epidemiology

Cowpox virus is rarely isolated from cattle, which serologic surveys show not to be the reservoir host. Most human infection cannot be traced to contact with infected cattle, and over half the recent human cases seen in Britain have been traced to contact with infected cats. The domestic cat, although the species in which cowpox is most frequently diagnosed in Britain, is not the reservoir host because although cat-to-cat transmission can occur, antibody to cowpox virus is rare in cats. There is no evidence that cowpox virus can become endemic in any of the zoo animals which have been infected, but with increasing reliance on zoo populations for the survival of many species, these outbreaks may cause conservation problems.

Cowpox virus has been isolated from and antibody detected in ground squirrels and gerbils (*Rhombomys, Citellus* and *Meriones*) in Eurasia, voles (*Microtus*) in the Russo-Finnish border; antibody and virus DNA has been detected in wild voles and mice (*Apodemus* and *Microtus*) in Britain (**Table 1**). This, together with the restricted geographic range of the virus, the seasonal incidence of feline cowpox and several reports of human cowpox at the site of a rodent bite, all support the theory that cowpox virus is endemic in local rodent populations. **Figure 2**

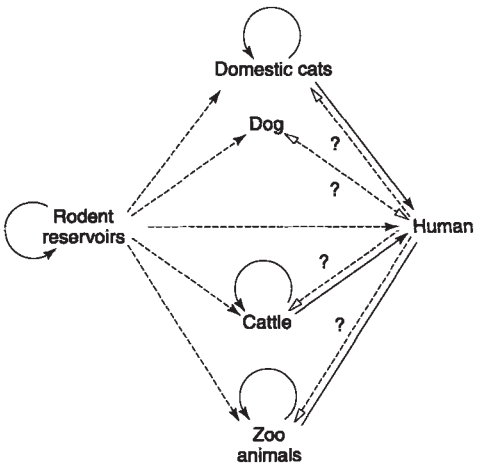


Figure 2 The probable epidemiology of cowpox. Solid lines indicate known routes of transmission, dotted lines indicate probable transmission, and dotted lines with ? indicate possible transmission. In all cases transmission might be direct or via fomites.

summarizes the probable epidemiology of cowpox virus.

Transmission and Tissue Tropism

Little is known about the routes of transmission of cowpox in endemic rodent populations. Voles and mice can be infected experimentally by both skin and oronasal inoculation, and arthropod vectors may play a role in mechanical transmission in wild colonies. Inoculation by either route can cause viremia and systemic infection, with virus detected particularly at the sites of inoculation, in lymphoid tissue and in lung. Rodent infections may persist for several weeks, although the role of possible persistent infection in the epidemiology of cowpox is unknown.

Among the occasional, or 'accidental', hosts of cowpox virus, the most frequent route of infection appears to be through the skin, probably through a cut or abrasion. Domestic cats, however, can be infected experimentally by oronasal inoculation, and respiratory spread may have been involved in some outbreaks of cowpox in big cat collections. Virus replication in cattle and humans is mainly limited to the epidermis at the site of entry, and possibly also to draining lymph nodes. In cats, virus can be isolated not only from skin lesions but also from lymphoid, lung and turbinate tissue. Skin inoculation of cats is followed by virus replication both at the site of entry and in draining lymph nodes, there is development of a viremia, and virus can be isolated from the white cell fraction of blood, from the spleen and other lymphoid organs. After about 7–10 days, virus can be detected in the epidermis leading to the development of secondary skin lesions. The viremia in cats appears to last 1–8 days, and no virus has been isolated from cats after the skin lesions have healed, which may take 5–6 weeks.

Pathogenicity

It is not known if different strains of cowpox virus vary in pathogenicity for cattle or humans. In a small-scale experiment, no differences in infectivity or ability to cause a primary lesion in domestic cats could be detected between the Brighton strain, and isolates from a cheetah and a domestic cat. Differences in pathogenicity of different strains do exist for newborn laboratory mice and chick embryos, but these differences are not associated with ability to infect various accidental hosts and it is not known if they have any significance in the maintenance of different strains of cowpox virus in different reservoir hosts.

Wild-type virus outgrows the white pock mutants and is more pathogenic for laboratory animals. Other virulence factors are discussed above. Given the large number of virulence genes encoded by cowpox virus, the large differences in pathogenicity (see Clinical Features, below) of the virus in different hosts, and particularly its apparently low pathogenicity in wild rodents are noteworthy.

Clinical Features of Infection

In cattle, cowpox virus causes teat lesions, but little apparent systemic disease. Human infection is also characterized by a single skin lesion, usually on a hand or the face, but is often accompanied by systemic signs such as nausea, fever and lymphadenopathy, and children may be hospitalized (**Fig. 3**). Spread of skin lesions in humans is usually the result of direct transmission, for example from hand to face, but multiple lesions may also occur if there is a pre-existing skin condition. Death is rare, but has been associated with underlying immunosuppression.

Cowpox in domestic cats is a more severe disease than in cattle or humans. There is usually a history of a single primary lesion, generally on the head or a forelimb, but by the time the cat is presented for veterinary attention widespread skin lesions have usually developed. The primary lesions vary enormously in character and secondary bacterial infection is common. The widespread secondary lesions first appear as small erythematous macules, which develop into papules and ulcers over several days. These scab over, and the cat usually recovers within 6–8 weeks. Cats may be slightly pyrexic in the early stages of the

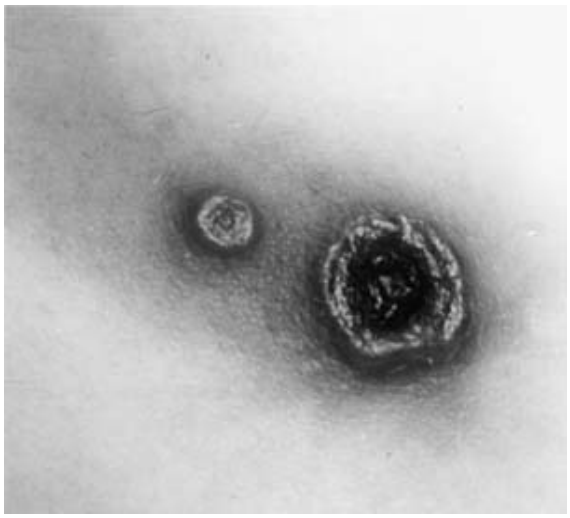


Figure 3 Human cowpox. Although most patients have only one lesion which is seen when it has developed to a hard black eschar, this illustration shows a primary lesion at the early eschar stage (right) and a secondary lesion at the earlier vesicular stage. (Reproduced from Baxby D *et al.* (1994) *Br. J. Dermatol.* 131: 598, with permission of Blackwell Science Publishers.)

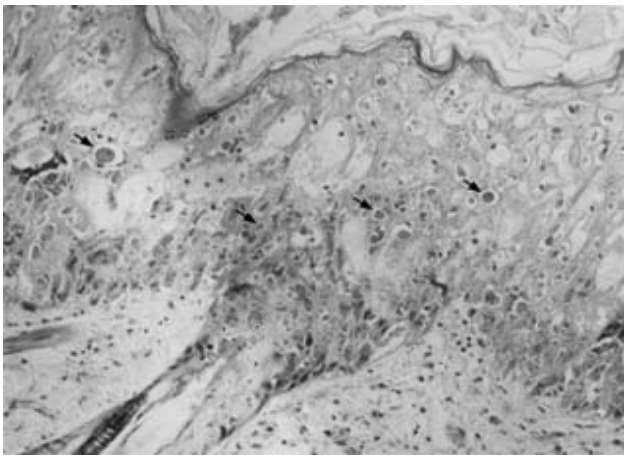


Figure 4 Microscopic section of a cowpox lesion in the skin of a cat, showing hypertrophy, hyperplasia, leukocyte infiltration and A-type inclusions (some indicated). (Reproduced from Baxby D *et al.* (1994) *Br. J. Dermatol.* 131: 598, with permission of Blackwell Science Publishers.)

disease, and some show signs of mild upper respiratory disease. More severe illness such as large non-healing lesions or pneumonia usually, but not always, results from secondary bacterial infection or immunosuppression (for example by concurrent feline leukaemia virus or feline immunodeficiency virus infection). In cheetahs, viral pneumonia is common, and cowpox has a higher mortality rate in most nondomestic felids.

Pathology and Histopathology

The skin lesions associated with human and feline cowpox are generally typical of those expected of an orthopoxvirus infection, developing through papule, vesicle, pustule, ulcer and healing stages, although macroscopic vesicles often quickly ulcerate because of abrasion or, in the case of domestic cats, because the epidermis is too thin in most areas to support a vesicle. Microscopic examination shows hypertrophy and hyperplasia of infected cells, multilocular vesicle formation, large, intracytoplasmic eosinophilic inclusion bodies (A-type inclusions) (**Figs. 1** and **4**) in epithelial cells and a vigorous polymorph infiltration of the dermis. In rodents, similar microscopic changes can be seen, although gross lesions may be limited to mild swelling. Immunostaining demonstrates virus antigen in epithelial cells of the skin, hair follicles and sebaceous glands, and in dermal macrophages.

Even in the lungs and turbinates of cats from which large amounts of virus can be isolated, there are often no gross lesions, and microscopic lesions may not be found. In cats showing clinical signs of pneumonia there is often an interstitial pneumonia, and, again, eosinophilic inclusions can be seen in infected cells. The tonsils and lymph nodes of infected cats and rodents contain many large reactive follicles, and immunostaining may demonstrate antigen in macrophage-like cells. Some follicles may have large necrotic centers suggesting that virus replication is occurring here.

After 3 days growth on the chorioallantoic membrane of 12-day chick embryos, cowpox virus causes hemorrhagic pocks approximately 2 mm in diameter, with a few (usually 1%) white pocks. Microscopically, the red pocks consist of ectodermal proliferation and hypertrophy with many cells containing A-type inclusions, and extensive edema and hemorrhage into the mesoderm. The histopathology of white pocks is similar but consists of more inflammatory infiltration and no hemorrhage.

Immune Response

Relatively little work has been done on the immune response to cowpox virus. Antibody can be detected by ELISA, immunofluorescence, virus neutralization (VN) with or without complement, complement fixation, and hemagglutination–inhibition (HAI) tests. HAI antibody can be detected before VN antibody, and in cattle begins to decline after about six months. HAI antibody is therefore more useful for

the diagnosis of acute infections than VN antibody, and in epidemiological studies indicates more recent infection than VN antibody alone.

Virus neutralizing antibody to EEV is detectable before VN antibody against INV, as has been described for vaccinia virus in mice. VN antibody to EEV is particularly important in protection as most cell-to-cell spread is by EEV. Thus an animal vaccinated with inactivated INV may have high INV-neutralizing antibody levels, which is what most VN assays measure, but little antibody to EEV, and may succumb to live virus challenge.

As in vaccinia and ectromelia virus infections, cell-mediated immunity must also play an important role in protection against cowpox virus, but very little work has been published specifically on the cell-mediated immunity to cowpox. A possible delayed-hypersensitivity response has been reported in cats.

Prevention and Control

As the source of infection is not usually known, and infection in humans or domestic animals is uncommon, measures to prevent infection are generally not warranted. Vaccination against smallpox is no longer routine, but in any case would probably not protect against the development of a skin lesion, although it might reduce the severity of systemic illness. Vaccinia virus infection of cattle and humans often causes lesions and disease similar to cowpox. Vaccination is not recommended for laboratory workers in the UK. Vaccinia virus does not grow well in cats, and its efficacy as vaccine in felids is therefore doubtful.

Although cowpox virus can cause quite severe human disease, it does not appear to be very infectious. Human-to-human spread of cowpox has not been reported (unlike for vaccinia virus). Most recent human cases of cowpox have been traced to contact with infected cats, but we have found no cases of cat-to-human transmission when cowpox has been first diagnosed in the cat. Simple hygiene – washing hands after handling the cat, keeping the cat or scab material away from cuts and the eyes – seems adequate to prevent transmission to humans, although special measures might be taken for the very young, elderly or immunosuppressed. Similarly, if an outbreak occurs in cattle, the main route of spread among the cows is on milking equipment, and simple hygiene should suffice to control spread.

Future Perspectives

Cowpox virus is one of several orthopoxviruses which have wildlife reservoirs. For example, monkeypox virus is endemic in squirrels, and raccoonpox and Californian volepox viruses each circulate in their eponymous hosts. Buffalopox virus is now regarded as a variant of vaccinia virus and may also have a wild animal reservoir in India. Occasionally these and other orthopoxviruses may infect other, accidental, hosts, such as domestic animals – there are reports of uncharacterized orthopoxviruses being isolated from horses, for example, in Africa, North America and Australia – or humans, for example in China. Study of the epidemiology of cowpox virus is therefore useful as a model for orthopoxvirus maintenance in a wildlife reservoir host and the mechanisms of transmission from animals to humans, especially in light of the proposed use of recombinant vaccinia viruses in wildlife.

See also: **Epidemiology of viral diseases; Immune escape mechanisms; Pathogenesis: Animal viruses; Smallpox and monkeypox viruses (*Poxviridae*); Vaccinia virus (*Poxviridae*); Zoonoses.**

Further Reading

Baxby D, Bennett M, Getty B (1994) Human cowpox 1969–93; a review based on 54 cases. *Br. J. Dermatol.* 131: 598.

Baxby D and Bennett M (1997) Cowpox: a re-evaluation of the risks of human infection based on new epidemiological information. *Arch. Virol.* Suppl. 13: 1.

Bennett M, Caskell CJ, Baxby D *et al* (1990) Feline cowpox virus infection. *J. Small Anim. Pract.* 31: 167.

Meyer H, Ropp S, Esposito J *et al* (1999) Poxviruses. In: Warnes A and Stephenson J (eds) *Methods in Molecular Biology: Diagnostic Virology Protocols*, p. x. Totowa, NJ: Humana Press, (in press).

Moss D (1996) Poxviridae; the viruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fundamental Virology*, 3rd edn, p. 1163. Philadelphia: Lippincott-Raven.

Smith GL *et al* (1997) Vaccinia virus immune evasion. *Immunol. Rev.* 159: 137.

COXSACKIEVIRUSES (*PICORNAVIRIDAE*)

Bruno Pozzetto and **Odette G. Gaudin**, Laboratory of Virology, Faculty of Medicine Jacques Lisfranc, University of Saint-Etienne, Saint-Etienne, France

History

Descriptions of a disease resembling epidemic myalgia presumably caused by viruses of the coxsackie group have been reported in different Scandinavian areas since the second half of the 19th century. The first coxsackievirus was isolated only in 1948 in New York state (USA) by Dalldorf *et al*, who were investigating an outbreak of paralytic poliomyelitis in the village of Coxsackie. They discovered a new group of viruses pathogenic for suckling mice. The observation led to the widespread use of suckling mice in the investigation of the viral etiology of poliomyelitis-like illnesses. The conditions of their isolation led some investigators to believe that the new viruses were only 'common fellow travellers of poliovirus'. But it rapidly became clear that these viruses had a full range of morbidity.

Taxonomy and Classification

Coxsackieviruses belong to the *Picornaviridae* family, genus *Enterovirus*, group of human enteroviruses. They contain a single plus-strand of RNA protected by an icosahedral capsid exclusively constituted of proteins. The viral particule contains no enzyme and no envelope. Its size is 22–30 nm. Coxsackieviruses are divided into two subgroups: coxsackievirus A (CA) with 23 serotypes 1–24 (23 is missing) and coxsackievirus B (CB) with six serotypes 1–6. This classification is based on the histopathologic lesions in suckling mice (see under Pathology and Histopathology below).

It was noticed early on that pathogenic features for suckling mice are not a definite characteristic. For instance, strains of CA23 have been recognized as being variants of echovirus 9 and some strains of CA24 have been temporarily classified as echovirus 34. Recent developments in molecular biology have contributed to the remodeling of the currently admitted classification. According to RNA analysis, four clusters have been proposed for human enteroviruses among which CA are scattered over three of them (**Table 1**).

Geographic and Seasonal Distribution

Coxsackieviruses are present worldwide, each serotype being found in a given area. More information has been gathered on CB than CA, owing to the somewhat tedious procedure for isolating the latter. Climate has a major role in the epidemiological pattern. In temperate countries, the circulation of coxsackievirus culminates in summer and autumn. In tropical regions, they occur all the year round in an endemic mode. In so-called 'Mediterranean' climates, they are distributed at a low endemic level with a summer–fall increase. They circulate together with other enteroviruses.

Host Range and Virus Propagation

Coxsackieviruses commonly cause diseases in humans. A virus very similar to CB5 has been recovered from pigs. Chimpanzees can also be infected by coxsackieviruses. They usually develop a subclinical infection, but CA7 can produce paralysis in monkeys.

Suckling mice can be experimentally infected by several parenteral routes: 1-day-old newborn mice are required for maximum pathogenic expression for CB. For CA, the susceptibility of the suckling mice extends to the first 4 days of life. Weaning mice can be infected by mutants of CB causing pancreatitis or myocarditis. Suckling merions are also susceptible.

Coxsackievirus can be grown in a number of primary tissue culture systems or continuous cell lines derived from primate (human or monkey) tissue: primary monkey kidney or embryonic human kidney, human heteroploid cells such as KB, HeLa and Hep2, or monkey kidney cell lines such as Vero and BGM. Replication results in a typical cytopathic effect with cell ballooning associated with a dendritic aspect. The virus cycle is completed within 7–8 h and takes place exclusively in the cytoplasm. Rapid cell lysis occurs.

CB induces a chronic infection in human embryonic lung fibroblasts. Some CA serotypes cannot be grown on cells, especially CA1–6. Primary or cell lines originated from mice are not susceptible *in vitro*. Cell susceptibility is linked to the presence of cell membrane receptors for the virus. It has been shown recently that the major cell receptor for CB is a 46 kDa protein that also mediates the attachment of adenoviruses 2 and 5 and has been called coxsackievirus and adenovirus receptor (CAR). The gene coding for this protein is located on chromosome 21. Some CA and CB strains are also able to bind to another receptor – the complement regulatory protein

Table 1 Classification of coxsackieviruses within the *Enterovirus* genus (according to Puli *et al* (1995) *Virology* 212: 30 and Pöyry *et al* (1996) *J. Gen. Virol.* 77: 1699

Clusters	*Coxsackieviruses*	*Other enteroviruses*
A	A2, 3, 5, 7, 8, 10, 12, 14, 16	Enterovirus 71
B	A9	Enterovirus 69
	B1, 2, 3, 4, 5, 6	All echoviruses except types 22 and 23
C	A1, 11, 13, 15, 17–22, 24	Poliovirus 1–3
D	None	Enteroviruses 68 and 70
Undetermined	A4, 6	—

Table 2 Identified cellular receptors of coxsackieviruses

Cell receptor	*Coxsackieviruses*	*Other viruses competing with the same receptor*
CAR	Coxsackieviruses B	Adenoviruses 2 and 5
DAF (CD55)	CB-3, CA-21	Echoviruses 6, 7 and 12
ICAM-1	CA-13, 15, 18, 20, 21	Major group rhinoviruses
$\alpha_v\beta_3$ integrin	CA-9	—

decay accelerating factor (DAF) – that has been shown to be the major receptor of echoviruses types 6, 7 and 12. The $\alpha_v\beta_3$ integrin (vitronectin receptor) is involved in the entry of CA9 in host cells whereas CA types 13, 15, 18, 20 and 21 use ICAM-1 as cellular receptor (**Table 2**). These data suggest that many coxsackieviruses use multiple receptor molecules for their cell entry mechanisms.

Strains of CB agglutinate human cord blood type 0 erythrocytes. The structure and location of the hemagglutinins are unknown.

Genetics

The coxsackieviruses whose full-length sequence is available (CB1, 3, 4, 5, CA9, 21) show a genomic structure similar to that of the other members of the genus *Enterovirus*, especially poliovirus, the most extensively studied. Briefly, the genome is about 7500 bases and is flanked by two nontranslating poly(A) regions (NTR) 5′ and 3′. A small protein, VPg, is linked to the 5′-NTR (**Fig. 1**). In contrast with most eukaryotic messenger RNA molecules, the 5′ NTRs of enteroviruses contain internal ribosomal entry sequences (IRES) so that ribosomes may bind in the absence of a free 5′ capped terminus. The RNA acts as an mRNA which encodes directly a high-molecular-weight polyprotein, p220, consisting of three segments coding for the three precursor proteins, namely P1, P2 and P3. A cascade of cleavages carried out by virus-encoded serine proteases (2A and 3C) leads to 11 polypeptides among which are the final structural proteins of the capsid. These structural proteins are derived from the P1 segment (mol. wt 97 000) of p220 (**Fig. 1**).

The capsid surface of the dense virion is a combination of 60 protomers of four viral proteins (VP) each, numbered from 1 to 4. In coreless particles (empty capsids), only three proteins are present: VP0, 1 and 3. The incorporation of the RNA genome to the empty capsid leads to the provirion. VP0 is the precursor protein for VP2 and 4. The autolytic cleavage of VP0 into VP2 and VP4 stabilizes the capsid structure and leads to the infectious virion. VP4 is an internal protein linked to the genome. The epitopes which bind neutralizing antibodies are mainly present on VP1. Nevertheless, minor epitopes are also present on VP2 and VP3. The structures responsible for adsorption of the virus to the cell receptor (see above)

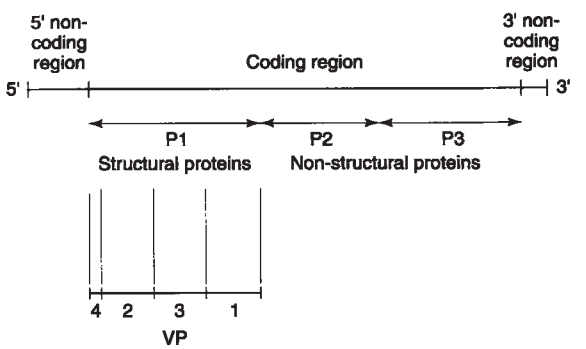


Figure 1 General organization of the genomic RNA of coxsackieviruses.

are buried within a kind of 'canyon' with VP1 on its edges. Defects in the RNA are frequent in the so-called 'defective particles'. Therefore the population of viral particles that finally burst out of the lysed cell is heterogeneous. It contains a mixture of at least the four types described above.

Evolution, Serologic Relationships and Variability

Minor antigenic drifts are currently observed in laboratory isolates and virions can exist as quasi-species in the same organism, as generally happens with other RNA viruses. Although no antigenic shifts comparable with the influenza A virus have been observed, the appearance of a variant of CA24 causing a 'new' disease consisting of a hemorrhagic conjunctivitis (see under Clinical Features of Infection below) may question this.

As a general rule, the 23 serotypes of CA and the six serotypes of CB are antigenically unrelated. The neutralization test is the reference procedure for identification of isolates: serum raised against one serotype does not crossneutralize the others. But limited neutralization sometimes occurs between related serotypes. For instance, CA3 and CA8 show antigenic crossing, as do CA13 and CA18.

Epidemiology

Coxsackieviruses demonstrate the same epidemiological pattern as the other enteroviruses. Each year, whatever the climate or geographic area, a human community experiences at least one, and often several, coxsackievirus infections.

Age is an important risk factor. Young children are the reservoir of the virus. In low socioeconomic conditions babies become infected during the first months of life. As economic development increases, the first contact with the virus may occur later during childhood or even later.

Coxsackieviruses are present in the environment. Many reports regarding isolation of CB from surface or waste waters, soils, raw vegetables and shellfish have shown that these viruses are a major public health problem. The spread of the virus from infected members in a community is due to the feces which contaminate the environment. As children with silent infections excrete the virus in their feces for several weeks, they play the role of an incessant source of virus. The poorer the sanitation, the higher the contamination level of the environment.

Transmission and Tissue Tropism

Coxsackieviruses have the same routes of transmission as polioviruses: the oral route via dirty hands and polluted water and food. Transmission by aerosol is possible when pulmonary syndromes are present, during an outbreak of conjunctivitis, or merely from subjects with subclinical infection when the virus is present in the throat during the incubation period.

Coxsackieviruses affect a wide range of susceptible tissues and organs: striated muscles, myocardium, brain and spinal cord, pancreatic islets, lung, skin and conjunctivae. Mutants with special pathogenic properties have been reported, for example cardiotropic and pancreatropic CB for mice. Much effort has been devoted to the characterization of the genetic determinants responsible for this virulence.

Pathogenicity

After entry by the oral route, the virus is thought to follow the same path as poliovirus: multiplication in the throat and small intestine and in the lymph nodes draining the primary sites of multiplication. Then, via the bloodstream or lymphatic drainage system, the virus reaches the target organs. In the lower intestine, the virus enters the Peyer patches through the M cells. Its multiplication in the absorptive cells has been shown in animal tissues *in vitro* but evidence in humans is lacking.

Most infections are clinically silent. The reason why some individuals exhibit a higher susceptibility to the virus than others is still largely a mystery. Several factors may increase susceptibility: previous immune status, immune deficiency restricted to some strains, higher pathogenic potency of mutants, genetic HLA constitution and/or hormonal interactions with viral multiplication. The last factor would explain the sex ratio of 1.5–2 males for every female when clinical expression of the infection is considered.

Clinical Features of Infection

A large panel of acute diseases are due to coxsackieviruses and there is much evidence for their causative role. The relationship between a serotype and a disease is generally not clear to the clinician, except under epidemic circumstances. Moreover it is difficult to distinguish between coxsackievirus infections and other nonpolio enterovirus infections when clinical symptoms only are considered. Another common clinical characteristic of enterovirus infections is the frequency of featureless diseases (febrile illness, resolutive rash, flu-like syndrome). During the last two decades, persistent pathologic disorders have been attributed to these viruses; their actual role in

such processes is still disputed although more and more evidence tends to accredit this concept, as is discussed below.

Much more information is available for CB than CA viruses, owing to the easier isolation of the former. For the latter, attention has been focused on those CA serotypes that multiply easily in cultured cells, such as CA7, 9, 16, 21 and 24. Coxsackieviruses induce protean clinical pictures depending on the target organ.

Nervous system

CA7 and 9 and CB1–6 have been infrequently involved in polio-like paralysis. Encephalitis, ataxis and paralysis of the cranial nerves are rare. Both CA and CB are responsible for aseptic meningitis, although echoviruses are more frequently the cause of meningitis than are coxsackieviruses.

Skin and mucosa

Herpangina is common in school age children. Clear reddish vesicles are distributed on the moderately inflamed mucosa of the throat, tonsils, soft palate and tongue. It appears as a self-limited stomatitis. CA1–6, 8, 10 and 22 have been isolated from the vesicles. Hand-foot-and-mouth disease consists of vesicles on the palms of the hand and soles of the foot, but also on the limbs and trunk, sometimes associated with stomatitis. The main serotypes involved are CA4, 5, 9, 10 and 16 and CB2 and 5. CA16 is responsible for the majority of outbreaks. Herpes-like vesicular rashes have been described, and CB1 has been isolated from the vesicular fluid. Rubella-like rashes are often associated with other more typical clinical symptoms.

Striated muscles

Pleurodynia, also known as 'epidemic myalgia' or 'Bornholm disease' (from the name of a Danish island where an outbreak gave the opportunity for a well-documented clinical description in 1933) consists of a sudden pain in the chest (due mostly to the involvement of the diaphragm) and a general malaise associated with headache, sore throat and fever. It is common in summer camps for children and teenagers.

CB are also associated with acute myositis and rhabdomyolysis. A mouse model of myositis with CB1 has been described.

Prolonged myalgia was described several decades ago in patients during convalescence from CA infections. A new concept has recently arisen, the 'post-viral fatigue syndrome' (or 'myalgic encephalomyelitis'), which consists of prolonged muscular fatigability (>6 months) often associated with other functional and/or psychological symptoms. The causative role of CB is still largely disputed and some herpetoviruses are also candidates.

Heart

CB are one of the main etiologic agents of acute myocarditis, pericarditis and myopericarditis. The association has been strengthened by the finding, by *in situ* hybridization, of viral RNA sequences in the myocardium of patients who had died from acute heart failure. In neonates, the disease is often fatal whereas the prognosis is usually milder in adolescents and adults.

Dilated cardiomyopathy (DCM), a chronic heart disease characterized by ventricular congestion leading to heart failure and for which the only remedy may be heart transplantation, has also been tentatively linked to a persistent CB infection. A mouse model using CB3 supports this hypothesis. In humans, the role of CB in DCM is suggested by high antibody titers (IgM included) against CB and by the detection of CB RNA by hybridization or polymerase chain reaction (PCR) in the myocardium of patients. The pathogenesis of the disease involves genetic, virological and autoimmune factors. The viral persistence might be related to an impaired replication of the virus illustrated by production of positive and negative RNA genomic strands in equal amount.

Alimentary tract and liver

Acute abdominal pains have been associated with CB, mimicking appendicitis in childhood. A few cases of hepatitis associated with CB have also been described, especially in neonates. Gastroenteritis is a frequent prodromic symptom of coxsackievirus infections. However, these viruses cannot be considered as main etiologic agents of viral diarrhea, owing to their low level of multiplication in the intestinal tract.

Pancreas

There are strong arguments in favor of the involvement of CB in type I insulin-dependent diabetes mellitus, including a mouse model using CB1 and 4. The disease is thought to result from several factors acting together: a genetic predisposition, the multiplication of a diabetogenic strain of CB in the islets of Langerhans and antigen mimicry between CB proteins and cell components of the pancreas, both of them resulting in cell lysis. Rather than a persistent infection, it may be the summation of repeated enteroviral infections, some of which occurred during intrauterine life, which leads to the last event responsible for the chronic insulin deficiency.

Respiratory tract

Coxsackieviruses may be responsible for infections of the respiratory tract: CA9 and 16 have been associated with pneumonia, CA21 and 24 with the common cold and CB2, 3, 4 and 5 with upper and lower respiratory tract infections. They mimic respiratory infections due to myxo-, rhino- and coronaviruses. Although occurring all through the year, the enteroviral origin of these infections may be evoked when observed during the warm season ('summer grippe').

Eye

A new nosological entity which arose in 1969–1970 and consisted of conjunctivitis was first described in South West Asia and was introduced into the Americas in 1981. A variant of CA24 was recognized as the etiologic agent of this acute conjunctivitis. It is clinically less severe than acute hemorrhagic conjunctivitis due to enterovirus 70, the subconjunctival hemorrhages being moderate. No paralysis has been reported from the areas where the main outbreaks occurred, a clinical pattern distinctive of the enterovirus 70 disease.

Coxsackievirus, pregnancy and the neonatal period

The risk of abnormalities in the fetus of pregnant women exposed to coxsackieviruses seems low, although the long-term consequences of such infections are difficult to evaluate. When the disease develops in an infant within the first 2 or 3 weeks of life, the virus is generally transmitted during or immediately after delivery. Infection in the infant ranges from mild to severe, even fatal. Meningitis, myocarditis, pneumonia, encephalitis, hepatitis and pancreatitis may be encountered. It should be noted that severe nosocomial outbreaks of coxsackieviruses have been reported in the neonatal period.

Pathology and Histopathology

The incubation period varies from 2 days (conjunctivitis) to 1 month or more, the mean time being 10–14 days. Information on the histopathological lesions comes mainly from animal models which have been extensively described (**Fig. 2**). A review of histopathological lesions observed in more than 400 isolations from patients in a UK hospital reports a wide range of lesions. The classical distinction between CA and CB is somewhat confused by the fact that a wide range of lesions can be found regardless of the subgroup. However, there are significant variations in the frequency of the main types of lesions.

With CA, polymyositis is found predominantly, as expected from previous data, with the thoracic, abdominal and thigh muscles being the most severely affected. Encephalitis and poliomyelitis are also found, as well as brown fat necrosis and myocarditis, but in a much smaller proportion than in animals infected with CB. In contrast, encephalitis, poliomyelitis, brown fat necrosis, endomyopericarditis and pancreatitis are the main lesions observed in animals infected with CB. Myositis is rare and, when present, it is much more limited. These considerations may explain the finding that the usual signs of CB infection in mice are spasticity and fine tremors rather than the flaccid paralysis commonly observed with CA infection. Wherever the sites of histological lesions are, inflammatory foci are invaded by numerous mononuclear cells.

In experimentally infected susceptible mice using a cardiotropic strain of CB3 by the intraperitoneal route, the virus can be detected in the myocardium as soon as two days postinfection; foci of necrosis associated to calcifications and to infiltrates of lymphocytic cells are present within one week. Inflammation and necrosis peak during the second week; then, these lesions decrease and are progressively replaced by fibrosis. During the acute phase of myocarditis, infectious virions can be recovered from the heart. At the end of the first month, a chronic myocarditis, characterized by an interstitial fibrosis and a mild infiltrate of mononuclear cells, can occur in some strains of mice; at this stage, no infectious virus can be cultured but RNA genome is detected by *in situ* hybridization in a small proportion of myocytes around the foci of fibrosis. Specific cytotoxic T cells and autoantibodies seem to play a leading part in the constitution of both acute and chronic lesions. Similar findings have been described in other experimental murine models using CB strains infecting peripheral muscles or pancreas.

Immune Response

Half a century of investigations has brought definite evidence that the control of and protection against viral infection is mainly related to neutralizing antibodies. Such antibodies mainly result from silent infections. They are considered to be lifelong, although the conditions for this may vary largely in space and time. Naturally acquired immunity declines when the current circulation of the virus decreases within a community.

Specific mucosal immunoglobulin A plays a protective role, especially in the gut. The presence of specific immunoglobulin M has been demonstrated by various technical approaches. However, their signifi-

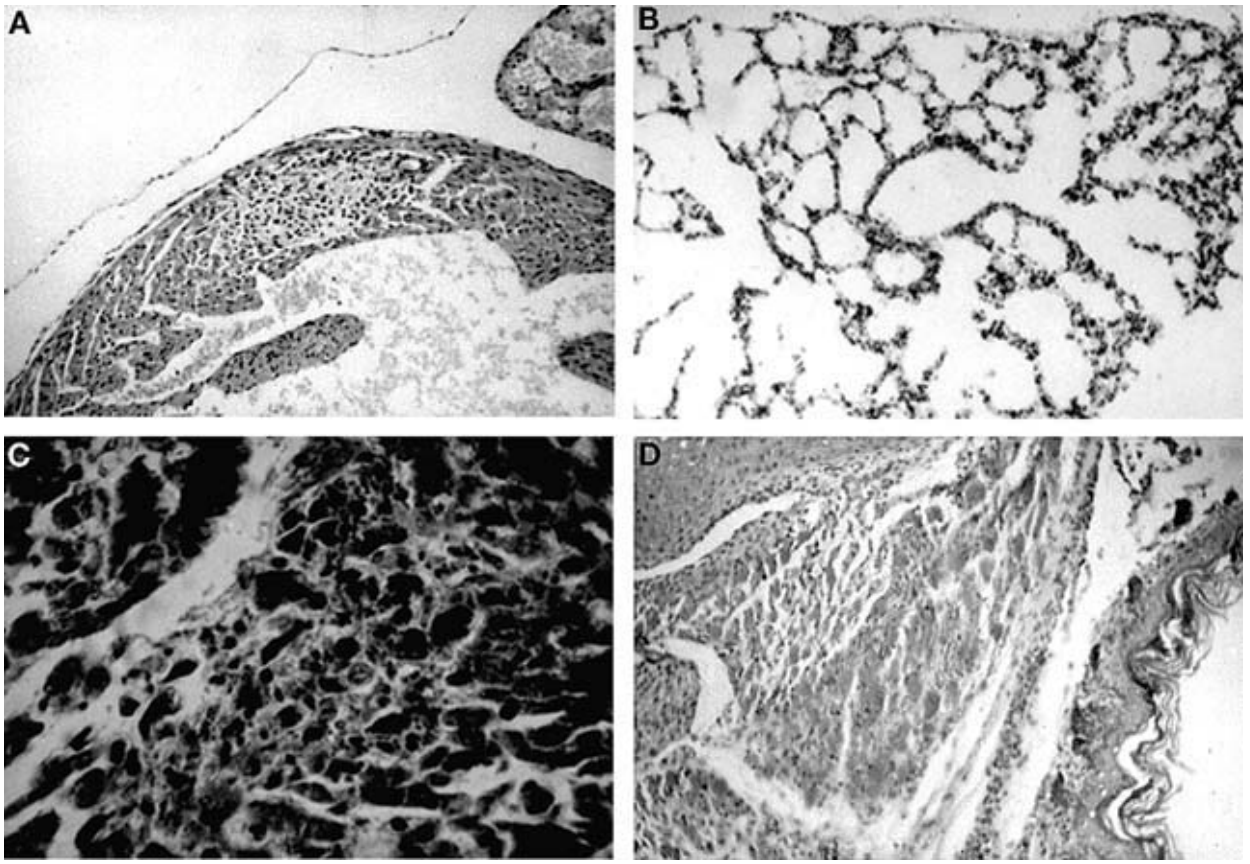


Figure 2 Tissue damage caused in baby mice by a freshly isolated strain of coxsackievirus B (**A–C**) or coxsackievirus A (**D**). (**A**) A large necrotic focus in the atrial myocardium (×100); (**B**) steatitis in the interscapular brown fat pad (×250); (**C**) a ventricular focus of necrotic cardiomyocytes (×450): note the dense mononuclear infiltrate in the infected area. (**D**) Intercostal muscles with diffuse hyalinization of myocytes and mild inflammatory infiltrate (×250).

cance as signs of a current or recent infection is still disputed, owing to the lack of data on their duration in different clinical situations.

Other aspects of immunity are still debated. The target of neutralizing antibodies is limited to epitopes on the surface of the virus. The total constitutive proteins of the capsid represent less than 50% of the total proteins encoded by the genome. Is there a host reaction against the nonstructural proteins? In the inflammatory foci seen in infected tissue in human and murine diseases, mononuclear cells are predominant; specific cytotoxic T cells probably play a key role in the control of infection. As discussed above, autoimmunity is probably involved in the genesis of tissue damage observed in chronic infections.

The immune response to coxsackieviruses is thought to be mostly type specific. However, frequent antigenic crossings of neutralizing, precipitating, complement-fixing antibodies are encountered when sera from infected patients are investigated, which evokes the possibility of common epitopes for subgroups or serotypes. Previous studies have identified a group-specific epitope located on VP1 which is common to most of the members of the enterovirus genus, including CA and CB. Similarly, T cells primed with a given serotype are able to proliferate *in vitro* in the presence of other serotypes of enterovirus.

Prevention and Control

No specific prevention by vaccines is presently available or planned for the near future. Preventive measures include a general hygiene policy, but this cannot avoid transmission through a community, especially among children. As the virus is mainly transmitted by dirty hands or contact with contaminated water or food, particular attention must be paid to individual hand washing and sanitary control of swimming pools and meals. Bathing in natural surface waters (rivers, ponds) should be avoided. Sea resorts may be at risk when waste waters are admitted into the sea at the shoreline without previous treatment. Eating raw shellfish collected in such places is a great risk.

Disinfectants have to be chosen carefully for institutions for children (hospitals, kindergartens, camps) because coxsackieviruses are resistant to many common ones, such as alcohols, quaternary ammonium compounds and chlorhexidine. They are stable at pH values ranging from 3.0 to 10.0. Chlorine is recommended for cleaning bathrooms and other surfaces. For hand disinfection, chlorinated or iodinated disinfectants should be used. For instruments, disinfectants containing aldehydes (formaldehyde or glutaraldehyde) are adequate, provided that the instruments have been cleaned before immersion in the decontaminating bath and the contact is for over 30 min.

Exposure to temperatures above 50°C destroys coxsackieviruses in a few minutes, but the presence of Ca^{2+} or Mg^{2+} can protect against heat inactivation. Viruses are highly resistant in feces, where adventitious substances reduce the efficiency of the disinfectant which does not easily penetrate the fecal material. Feces can remain at room temperature for weeks without substantial loss of infectivity.

No antiviral agent is available for therapy. γ-Globulins have proven useful in a limited number of cases.

Future Perspectives

The role of coxsackievirus has been well established in acute diseases, especially in communities of children. However, the concept of persistent infection, which is essentially related to adults, has been introduced more recently. It has been developed thanks to the tools of molecular biology. The demonstration of RNA sequences in tissues may provide a valuable argument for persistence, but will not elucidate the immunological mechanisms that allow this persistence. Increased knowledge of all facets of immunity is needed to understand these mechanisms. The wide use of PCR in the routine diagnosis of enterovirus infections will also help to identify the pathological role of those CA that cannot be cultivated in cell culture.

As development of vaccines is improbable, owing to the numerous serotypes of coxsackievirus, total control of the infection is not a realistic aim. However, if the role of CB in chronic infection of the heart was fully confirmed, a vaccine directed against the main serotypes of this subgroup could be designed. Two kinds of antiviral agents have been proposed: components acting on the initial phases of the lytic cycle (Win compounds) and protease inhibitors active against the two serine proteases 2A and 3C. They have still to prove their efficiency *in vivo/e2>*.

See also: **Echoviruses (*Picornaviridae*); Enteroviruses (*Picornaviridae*): Animal and related viruses, Human enteroviruses (serotypes 68–71); Eye infections; History of virology: General; Polio, coxsackie, echo and other enteroviruses; Persistent viral infection; Polioviruses (*Picornaviridae*): General features, Molecular biology; Quasispecies; Viral receptors.**

Further Reading

Bendinelli M and Friedman H (1988) *Coxsackievirus. A General Update.* New York: Plenum Press.

Hyypiä T and Stanway G (1993) Biology of coxsackie A viruses. *Adv. Virus Res.* 42: 343.

Hyypiä T, Hovi T, Knowles NJ and Stanway G (1997) Classification of enteroviruses based on molecular and biological properties. *J. Gen. Virol.* 78: 1.

Melnick JL (1991) Enteroviruses. In: Evans AS (ed.) *Viral Infections of Humans,* 3rd edn, p. 191. New York: Raven Press.

Minor PD, Morgan-Capner P and Schild GC (1995) Enteroviruses. In: Zuckerman AJ, Benatuala JE, Pattison JR (eds.) *Principles and Practice of Clinical Virology,* 3rd edn, p. 417. London: John Wiley.

Rotbart HA (1995) *Human Enterovirus Infections.* Washington: American Society of Microbiology Press.

Creutzfeld-Jakob Disease Virus *see* **Prions**

Cricket paralysis Virus *see* **Picornaviruses – insect**

CRYPTOVIRUSES (*PARTITIVIRIDAE*)

Robert G Milne and **Cristina Marzachi**, Istituto di Fitovirologia Applicata, CNR, Torino, Italy

History

In 1968 and 1969, Pullen at Rothamsted in England showed that all apparently healthy plants examined of seven species of beet (*Beta*) contained small isometric virus-like particles (VLPs) which were inherited through the seed. The particles could not be horizontally transmitted, and the carrier plants could not be freed from the VLPs by heat therapy; therefore they were suspected of being obligatory constituents of all beet plants. However, Kassanis, White and Woods in 1977 found a few beets not containing VLPs, and this stimulated them to purify the virus, study some of its properties, and name it beet cryptic virus (BCV). During this period, in the same laboratory, two other kinds of VLP were found with similar properties. They were named vicia cryptic virus (VCV) and ryegrass spherical virus (later renamed ryegrass cryptic virus, RGCV).

In 1981 members of the Turin plant virus group showed that a further VLP with 'cryptic' properties, named carnation cryptic virus (CCV), contained double-stranded (ds) RNA – at this time novel for small plant viruses. Soon they reported that alfalfa, red clover, white clover and *Festuca pratensis* also contained small isometric dsRNA-containing VLPs, and that RGCV likewise contained dsRNA.

Working independently in Japan, Natsuaki and colleagues at Utsunomiya discovered 'temperate' VLPs containing dsRNA, in several hosts including alfalfa, white clover, beet, radish and spinach. Collaborating, in 1984, the Torino and Utsunomiya groups found that they had been describing similar or identical viruses, and it became clear that cryptic viruses (now more correctly designated cryptoviruses) were a coherent group with some remarkable properties, which are or appear to be the following.

The particles are isometric, approximately 30 or 38 nm in diameter, and the genome consists of dsRNA in two, possibly three linear segments, each of about 1.3–2.3 kbp (mol.wt $0.9–1.5 \times 10^6$). At least in WCCV1 these segments are encapsidated together. Virus concentration in plants is low to very low and there are no obvious signs of disease. There is no mechanical, vector or graft transmission, but transmission through ovules and pollen to the seed is very high. There is no movement from cell to cell except to daughter cells during cell division.

Subsequent work in the UK, Germany, Italy and Japan has shown that cryptoviruses are widespread among both dicotyledonous and monocotyledonous plant species. Some plants are host to several apparently unrelated cryptoviruses; for example, white clover contains three, beet three, hop trefoil three, red pepper two, carrot five, radish three, ryegrass probably three, and carnation probably two. These numbers are conservative.

In 1990, Flachmann, Beuther and Wiese in Germany found small isometric VLPs associated with dsRNAs in fir trees (*Abies*) of six different species. In *A. alba* and *A. homolepis*, there were three dsRNAs in the range 1.5–1.65 kbp. The particles appeared to be seed-transmitted to a high degree but not associated with any symptoms, and not, in preliminary tests, mechanically transmitted, so they were probably also cryptoviruses. Funding for this interesting work was discontinued when it became clear that forest die-back was not attributable to these agents.

In 1992, four major dsRNAs, each about 2 kbp in length, encapsidated in isometric VLPs 25 nm in diameter and located in the chloroplasts of the marine green alga *Bryopsis*, were reported, together with less stable 40 nm particles containing dsRNAs of about 4.5 kbp in size constantly located in the mitochondria of the same organism. These particles appeared to be cryptic-like viruses; dsRNAs of various sizes (but unknown significance) were found in most green, red and brown algae examined.

Taxonomy and Nomenclature

Until recently, these viruses were placed in the genus *Cryptovirus*, comprising subgroups I and II; these categories were then recast as the family *Cryptoviridae*, and the genera *Alphacryptovirus* and *Betacryptovirus*, respectively. Now, *Alphacryptovirus* and *Betacryptovirus* are recognized as two genera within the family *Partitiviridae*, which also includes certain fungal viruses with similar properties in the genera *Partitivirus* and *Chrysovirus*.

Alphacryptovirus, type species, white clover cryptic virus 1 (WCCV1)

The segmented dsRNA genome (two RNAs of about 2.0 and 1.7 kbp, and possibly a third RNA in some cases) is contained in capsids about 30 nm in diameter, rounded and without visible subunits, and built of one polypeptide of about 55 kDa. An RNA-dependent RNA polymerase (replicase) is associated

Table 1 Species and possible species of the genus *Alphacryptovirus*

Species	*Possible species*
Alfalfa cryptic 1	Carnation cryptic 2
Beet cryptic 1, 2, 3	Fescue cryptic
Carnation cryptic 1	Garland chrysanthemum temperate
Carrot temperate 1, 3, 4	Mibuna (*Brassica rapa* var. *laciniifolia*) temperate
Cucumber cryptic	Red pepper cryptic 1, 2
Hop trefoil cryptic 1, 3	Rhubarb temperate
Radish yellow edge	Santosai (*B. rapa* var. *amplexicaulis*) temperate
Ryegrass cryptic 1	White mustard cryptic
Spinach temperate	Amaryllis cryptic
Vicia cryptic	
White clover cryptic 1, 3	

Table 2 Species and possible species of the genus *Betacryptovirus*

Species	*Possible species*
Carrot temperate 2	Alfalfa cryptic 2
Hop trefoil cryptic 2	
Red clover cryptic 2	
White clover cryptic 2	

with the capsid. Particles of WCCV1 have a buoyant density in CsCl of 1.392 g ml^{-1}.

Betacryptovirus, type species, white clover cryptic virus 2 (WCCV2)

The segmented dsRNA genome (two RNAs of about 2.25 and 2.1 kbp) is contained in capsids about 38 nm in diameter, rounded, and with prominent morphological subunits, whose precise arrangement is unknown. An RNA-dependent RNA polymerase (replicase) is associated with the capsid. The particles have a buoyant density in CsCl of 1.375 g ml^{-1}.

A few *Alphacryptovirus* (WCCV1, CCV1, the three BCVs) have been examined in some detail, but no *Betacryptovirus* has yet received much attention. The presumed cryptoviruses of conifers have yet to be studied, classified and named. **Tables 1** and **2** list some cryptoviruses and place them provisionally in one of the two genera.

The fungal viruses in the genera *Partitivirus* and *Chrysovirus*, also members of the family *Partitiviridae*, have affinities with the *Cryptoviridae*, possess two dsRNA genome segments each of about 3.3–2.25 kbp, encapsidated separately within isometric particles 30–35 nm in diameter, with a single coat protein of 50–70 kDa. In both virus families, the RNA polymerase also behaves as a replicase.

Virus–Host Relationships

No cryptic virus has been found to infect more than one species of host plant, although the leguminous genera *Trifolium*, *Medicago* and *Vicia* have been found to harbor related viruses. The one known exception is BCV1 (or a closely similar virus) which has been found in both *Beta vulgaris* and *B. maritima* by the Rothamsted group of Antoniw and White. In Aschersleben, Germany, the group of Thomas Kühne and Andreas Stanarius found that BCV1 and BCV2 occur differentially in different cultivars of fodder beet, red beet and chard, all varieties of the *B. vulgaris* complex.

Cryptoviruses have been detected in all organs and tissues of their hosts, and there is no tissue specificity. VLPs, apparently cryptovirus particles, have been seen in thin sections of cells in both the cytoplasm and the nucleus. With radish yellow edge virus, cytoplasmic viroplasms have been reported.

Cryptoviruses have experimentally been found not to pass across grafts between carrier and noncarrier plants, even though other 'normal' viruses in a mixed infection did pass, and though graft transmissibility is one of the cardinal properties of conventional plant viruses. In other experiments using white clover, some plants originally carrying either WCCV1 or WCCV2 were found, after some months, to have sectored with respect to the virus: some sectors of the plants and their stolons were virus-free, while the rest contained undiminished amounts of virus. These results, and the fact that no means of experimental or natural horizontal transmission are known, suggest that the viruses lack a transport function that would carry them from cell to cell. In this model, the only way for the viruses to spread is for the pollen and/or ovule to infect the zygote, and for the virus to pass to both daughter cells at cell division. Sectoring would be the result of occasional passage of the virus to only one of two daughter cells in a meristem.

The Rothamsted group in England examined rates of transmission through pollen and ovule of a mixture of BCVs in beet and of VCV in *Vicia faba*. In both cases they found that when pollen from carrier plants fertilized flowers of noncarrier plants, about 40% of the progeny were carriers; in the reverse situation, about 80% of progeny were carriers, and when both parents were carriers, the transmission rate was 100%. Computer modeling showed that such transmission rates could result in almost the entire

population being carriers after 80 generations, and that no other means of spread need be postulated.

The great majority of cryptoviruses produce neither symptoms nor cytopathology in their hosts, but there are reports of some mild effects, probably not of commercial significance.

Serologic Relationships and Variability

Although cryptic viruses occur in very low concentration, their particles are rather robust and not, in principle, difficult to purify. Antigenicity of the capsid is high, and several antisera are available. It is to be noted, however, that the genomic dsRNA can also react with any anti-dsRNA antibody present in antisera. Unequivocal identification of the genomic dsRNA can always be achieved by combining temperature-gradient gel electrophoresis and immunoblotting using an anti-dsRNA serum.

No serological relationship has been found with a range of fungal viruses. Using immunoelectron microscopy and gel immunodiffusion tests, the following crossrelationships have been found between cryptoviruses.

- *Alphacryptovirus*. Hop trefoil cryptic virus 1 (HTCV1; from *Medicago lupulina*) is related, but not identical, to alfalfa cryptic virus 1 (ACV1; from *M. sativa*). WCCV1 (from *Trifolium repens*) is related to a morphologically similar unnamed cryptic virus in red clover (*T. pratense*). HTCV3 is related, but not identical, to VCV (from *Vicia faba*).
- *Betacryptovirus*. HTCV2 (from *M. lupulina*) is related to but not identical with WCCV2 (from *T. repens*) and also red clover cryptic virus 2 (from *T. pratense*); these last two viruses are also related to each other.

No serological relationship has been found between cryptoviruses in different genera, nor has relationship been detected between viruses hosted by unrelated plant species.

Molecular Biology

With WCCV1, BCV1, BCV2, BCV3 and ACV1, Northern hybridization has revealed no sequence homology between the different segments of each virus or between the genomes of the different viruses. Some sequence homology has been found between ACV1 and HTCV1, both alphacryptoviruses.

With both ACV1 and BCV1, the two genomic RNAs, translated in an *in vitro* wheat germ system after heat denaturation, produce two major polypeptides of about 67 and 52 kDa. In each case, the smaller peptide, product of RNA2, is precipitated by an antiserum raised against the viral particles and is believed to be the coat protein. The 67 kDa polypeptide, product of RNA1, is thought to be the replicase. With these two viruses, the proteins synthesized represent 82–95% of the coding capacity of the respective RNAs, suggesting that the genome segments are monocistronic.

Nucleotide sequences have been analyzed for BCV3, WCCV1, carrot temperate virus 1 (CTeV1) and radish yellow edge virus 1 (RYEV1). BCV3 RNA1 and RNA2 share the same 5′-terminal sequences, suggesting that, as described for reoviruses, these might play an important role during the replication/packaging process. Terminal sequences for WWCV1, CTeV1 and RYEV1 are not definitive and therefore this possibility cannot be generalized. BCV RNA2 is monocistronic, encoding a peptide of 54.9 kDa which accounts for about 90% of its total coding capacity. The peptides encoded by BCV3 RNA2 and WCCV1 RNA1 are not immunoprecipitated by the respective coat protein antisera, and contain polymerase consensus sequences; they probably represent the viral replicases. Thus with BCV3, RNA2 appears to encode the replicase, not the coat protein, whereas with ACV1, BCV1 and WCCV1 the replicase seems to be encoded by RNA1. Polymerase motifs are indeed found in RNA1 of WCCV1, CTeV1 and RYEV1.

Sequence homology at the amino acid level between the RNA-dependent RNA polymerases (RDRPs), as well as coat proteins, is relatively close (79% and 58%, respectively) between WCCV1 and CTeV1 and there is some homology (19% and 28%, respectively) between WCCV1 and RYEV1, while homology between any of these viruses and BCV3 is not significant.

RDRP activity has been detected in all cryptic viruses examined (WCCV1, WCCV2, CCV (probably a 1 + 2 mixture) and ACV1). The *in vitro* products of these polymerases are dsRNAs of genomic size; they react, in hybridization experiments, with the respective denatured genome segments. Thus the polymerases resemble those of some *Partitiviridae*, and their *in vitro* behavior suggests they are better defined as replicases rather than transcriptases. Of course *in vivo* activity must also include synthesis of mRNA.

Perspectives

Little work has been done on cryptoviruses because they are not of pressing practical importance. However, they are of some theoretical interest as they appear to represent the minimal 'self-sufficient' virus, able to operate using only two cistrons, those of the replicase and the coat protein. There is as yet no evidence for a specifically encoded transport function,

and there is apparently no movement from cell to cell; despite this, the viruses are obviously very successful, being encountered frequently where they are sought. Notably, however, they have not been found in some commonly used plant virus host plants such as *Chenopodium quinoa, Nicotiana tabacum* or *N. clevelandii*.

If indeed these viruses have no routine means to exit from their host species and infect another (sexually incompatible) species, they are also of evolutionary interest, as they may then represent cases of strict coevolution of the virus and its host. However, if it is confirmed that similar cryptoviruses are found in both conifers and angiosperms, then it would appear more likely that, at least on occasion, the viruses have been able to jump wide interspecies barriers. Their genomes, although incompletely characterized, are known to encode some of the amino acid sequence motifs conserved in other well-known virus families, suggesting that cryptic viruses have a common origin with these.

Cryptoviruses are of little direct economic importance and appear never to cause significant diseases. However, in mixed infections, the damage caused by a second (pathogenic) virus might be aggravated by the presence of one or more cryptic viruses. No such case is reliably reported. The unsuspected presence of cryptic viruses could (and sometimes has) confused the interpretation of serological or molecular assays designed to detect other viruses.

Further Reading

Accotto GP, Marzachi C, Luisoni E and Milne RG (1990) Molecular characterization of alfalfa cryptic virus 1. *J. Gen. Virol.* 71: 433.

Boccardo G, Lisa V, Luisoni E and Milne RG (1987) Cryptic plant viruses. *Adv. Virus Res.* 32: 171.

Flachmann M, Lesemann D, Franzel E and Koenig R (1990) Isometric virus-like particles in *Abies alba* Mill. and other *Abies* species: partial purification and improved detection by means of immuno-electron microscopy. *J. Phytopathol.* 129: 193.

Ishihara J, Pak JY, Fukuhara T and Nitta T (1992) Association of particles that contain double-stranded RNAs with algal chloroplasts and mitochondria. *Planta* 187: 475.

Natsuaki T, Natsuaki KT, Okuda S *et al* (1986) Relationships between the cryptic and temperate viruses of alfalfa, beet and white clover. *Intervirology* 25: 69.

Xie WS, Antoniw JF and White RF (1993) Nucleotide sequence of beet cryptic virus 3 dsRNA 2 which encodes a putative RNA-dependent RNA polymerase. *J. Gen. Virol.* 74: 1467.

CUCUMOVIRUSES (*BROMOVIRIDAE*)

Contents

General Features

Marilyn J. Roossinck, Plant Biology Division, The Samuel Roberts Noble Foundation, Ardmore, Oklahoma, USA

History

Cucumber mosaic virus (CMV), is the type member of the *Cucumovirus* genus. The first report of CMV was in 1916, as the causal agent of a mosaic disease in cucumber and muskmelon in Michigan, and in cucumber in New York. Before the advent of modern identification techniques such as nucleic acid hybridization or serology, plant viruses were named by disease type and host plant. More than 40 plant viruses of other names, most notably southern celery mosaic virus, were later proven to be CMV. Over the last 80 years, CMV has been identified as the causal agent of over 100 plant diseases, ranging from mild mosaic to severe necrosis and death.

Robinia mosaic virus (RoMV) was first described in 1935 as the causal agent of a mosaic disease in black locust. Peanut stunt virus (PSV) was identified in 1966 after a widespread epidemic of stunting in peanuts in the southeastern United States, and was recognized as a separate cucumovirus. Recent molecular analyses, as well as indications from earlier serology, clearly show that RoMV and PSV are the same species, and even though it was described first, RoMV is now designated the Robinia strain of PSV.

and there is apparently no movement from cell to cell; despite this, the viruses are obviously very successful, being encountered frequently where they are sought. Notably, however, they have not been found in some commonly used plant virus host plants such as *Chenopodium quinoa, Nicotiana tabacum* or *N. clevelandii*.

If indeed these viruses have no routine means to exit from their host species and infect another (sexually incompatible) species, they are also of evolutionary interest, as they may then represent cases of strict coevolution of the virus and its host. However, if it is confirmed that similar cryptoviruses are found in both conifers and angiosperms, then it would appear more likely that, at least on occasion, the viruses have been able to jump wide interspecies barriers. Their genomes, although incompletely characterized, are known to encode some of the amino acid sequence motifs conserved in other well-known virus families, suggesting that cryptic viruses have a common origin with these.

Cryptoviruses are of little direct economic importance and appear never to cause significant diseases. However, in mixed infections, the damage caused by a second (pathogenic) virus might be aggravated by the presence of one or more cryptic viruses. No such case is reliably reported. The unsuspected presence of cryptic viruses could (and sometimes has) confused the interpretation of serological or molecular assays designed to detect other viruses.

Further Reading

Accotto GP, Marzachi C, Luisoni E and Milne RG (1990) Molecular characterization of alfalfa cryptic virus 1. *J. Gen. Virol.* 71: 433.

Boccardo G, Lisa V, Luisoni E and Milne RG (1987) Cryptic plant viruses. *Adv. Virus Res.* 32: 171.

Flachmann M, Lesemann D, Franzel E and Koenig R (1990) Isometric virus-like particles in *Abies alba* Mill. and other *Abies* species: partial purification and improved detection by means of immuno-electron microscopy. *J. Phytopathol.* 129: 193.

Ishihara J, Pak JY, Fukuhara T and Nitta T (1992) Association of particles that contain double-stranded RNAs with algal chloroplasts and mitochondria. *Planta* 187: 475.

Natsuaki T, Natsuaki KT, Okuda S *et al* (1986) Relationships between the cryptic and temperate viruses of alfalfa, beet and white clover. *Intervirology* 25: 69.

Xie WS, Antoniw JF and White RF (1993) Nucleotide sequence of beet cryptic virus 3 dsRNA 2 which encodes a putative RNA-dependent RNA polymerase. *J. Gen. Virol.* 74: 1467.

CUCUMOVIRUSES (*BROMOVIRIDAE*)

Contents

General Features

Marilyn J. Roossinck, Plant Biology Division, The Samuel Roberts Noble Foundation, Ardmore, Oklahoma, USA

History

Cucumber mosaic virus (CMV), is the type member of the *Cucumovirus* genus. The first report of CMV was in 1916, as the causal agent of a mosaic disease in cucumber and muskmelon in Michigan, and in cucumber in New York. Before the advent of modern identification techniques such as nucleic acid hybridization or serology, plant viruses were named by disease type and host plant. More than 40 plant viruses of other names, most notably southern celery mosaic virus, were later proven to be CMV. Over the last 80 years, CMV has been identified as the causal agent of over 100 plant diseases, ranging from mild mosaic to severe necrosis and death.

Robinia mosaic virus (RoMV) was first described in 1935 as the causal agent of a mosaic disease in black locust. Peanut stunt virus (PSV) was identified in 1966 after a widespread epidemic of stunting in peanuts in the southeastern United States, and was recognized as a separate cucumovirus. Recent molecular analyses, as well as indications from earlier serology, clearly show that RoMV and PSV are the same species, and even though it was described first, RoMV is now designated the Robinia strain of PSV.

Tomato aspermy virus (TAV), the third member of the *Cucumovirus* genus, was first described in 1946. TAV is a common virus of chrysanthemums, and historically it was also known as chrysanthemum aspermy virus.

The cucumoviruses were among the first plant viruses to be purified, in the early 1960s, and a structural analysis of the virions was published in 1963. The RNA nature of the genome was described in the early 1970s, as well as the occasional incidence of satellite RNAs associated with CMV and PSV. Satellite RNAs were isolated from a strain of CMV causing an epidemic of severe necrosis and death in tomato in France in the early 1970s.

CMV was one of the earliest plant viruses for which cDNA clones capable of producing infectious transcripts were generated, in the late 1980s, and the molecular genetic analysis of the virus has progressed rapidly since then.

Taxonomy and Classification

The genus *Cucumovirus* is in the family *Bromoviridae*. All members of the family have a tripartite genome that is packaged in separate virions. The *Cucumovirus* genus consists of three species, CMV, PSV and TAV. The initial classification was based on serological data, but the species have also been distinguished by compatibility of RNAs 1 and 2. The RNA 3 of all members of the genus can be interchanged and still produce infection, whereas RNAs 1 and 2 must be from the same species. CMV is further divided into two subgroups (designated subgroup I and subgroup II) based on serology and nucleotide sequence similarity. PSV has been less thoroughly studied, but appears to have several subgroups, based on the same criteria as the CMV subgroup designations, and is more heterogeneous as a species in nucleotide sequence than the other cucumoviruses. TAV does not have any subgroup divisions, although only a few strains have been thoroughly studied.

Geographic and Seasonal Distribution

All three species of *Cucumovirus* are distributed worldwide. CMV, one of the most common plant viruses known, and by far the most thoroughly studied of the cucumoviruses, has been reported from temperate, subtropical and tropical climates. In temperate zones the virus apparently overwinters in trees or other perennial hosts. PSV has been predominantly isolated from temperate zones in the United States, eastern Europe, South America, and Japan, Korea and China. It has also been found in warmer climates in northern Africa. It has not been reported in Australia. The perennial host of PSV in many parts of the world is white clover, which it often infects asymptomatically. Most PSV epidemics in peanut in the southeastern United States were attributed to a common practice of planting white clover along the borders of peanut fields, providing a constant source of the virus. TAV is found in most places where chrysanthemums are grown. In general, the cucumoviruses do not replicate well at very high temperatures, and so may be less problematic in very hot weather.

Host Range and Virus Propagation

CMV has the broadest host range of any known virus, infecting about 1000 species of plants in 85 families, including herbaceous plants, shrubs and trees, and both monocots and dicots. Individual strains have a more limited host range, but they can be readily propagated experimentally in tobacco or cucurbits. PSV predominantly infects legumes, including leguminous trees such as black locust. Most strains can be propagated in tobacco, but a few strains do not infect tobacco, so cowpea is the preferred host for propagation. TAV is most commonly found in chrysanthemum and tomato, but has also been reported in other hosts, such as zinnia, pepper, chrysanthemum, hyacinth, and *Amaranthus* spp. It is dificult to mechanically transmit to chrysanthemum, but infects most tobacco experimentally, and *Nicotiana* spp. are good propagation hosts. Virus titers for all of the cucumoviruses generally peak about 2 weeks after initial infection, and propagations are usually done for 2–3 weeks. The virions are readily purified from homogenized and clarified infected plant material by high speed centrifugation, usually through a sucrose cushion. The virus can also be concentrated from clarified homogenate by precipitation with polyethylene glycol. Yields range from about 100 mg kg^{-1} to 2 g kg^{-1} of fresh tissue. The purified virus or purified viral RNA of all the cucumoviruses is readily transmitted by mechanical inoculation to experimental plants.

Genetics

The divided genome of the cucumoviruses allowed for substantial genetic analysis even before cDNA clones of the virus were obtained. The three genomic RNAs were isolated by gel electrophoresis separation and reassorted to determine various functions, and symptom and host range determinants were mapped to all three RNAs in this manner. *In vitro* translations of viral RNAs also helped elucidate functions. RNAs 1 and 2 were shown to encode the viral replication

components (the 1a and 2a proteins), and RNA 3 to encode two proteins, the 5′ open reading frame (ORF) encoding a protein (3a) of M_r about 30 000, and the 3′ ORF encoding the coat protein, translated from the subgenomic RNA 4. The 3a protein was later shown to be the movement protein, although other components of the virus are also involved in movement. Reassortants (or pseudorecombinants) also confirmed the species divisions.

RNA 3 could be exchanged between all the viruses, whereas RNAs 1 and 2 could not be exchanged between the species, implying a complex interaction between the 1a and 2a proteins. The construction of cDNA clones of all three genomic RNAs of several strains of CMV, PSV and TAV has allowed for more detailed genetic analyses.

Satellite RNAs

CMV and PSV can both harbor satellite RNAs. These small molecular parasites of the virus do not encode any proteins, but exert their (sometimes dramatic) biological effects with their RNA. They do not contain any regions of sequence similarity with the helper virus that are greater than about 10 nucleotides. The cucumovirus satellite RNAs are linear, highly structured RNAs ranging from about 330 to 400 nucleotides in length. There is no significant sequence similarity between the CMV satellite RNAs and the PSV satellite RNAs. They cannot be replicated by the heterologous helper virus, but the CMV satellite RNAs can be supported by TAV.

Evolution

The *Cucumovirus* genus, family *Bromoviridae* is a member of the alphavirus 'supergroup', that includes both plant and animal viruses. Two domains in the 1a protein are highly similar to methyltransferase- and helicase-like domains within the group. A third domain, in the 2a protein, is highly similar to the RNA-dependent RNA polymerases of the alphaviruses. Hence these viruses are believed to have a common ancestor, and it is postulated that they originated from an insect virus, and were transmitted and adapted to both plants and animals.

All of the cucumoviruses contain a second ORF in RNA 2, encoding the 2b protein. This ORF is not found in most of the other members of the *Bromoviridae* but it was recently described in one other member, tobacco streak virus, a member of the *Ilarvirus* genus, although it is not known if the 2b ORF is functional in tobacco streak virus. The 2b ORF presumably evolved by overprinting of the 2a ORF. The deduced amino acid sequences are much more divergent than those of other cucumoviral proteins. There is conservation of amino acid sequence of the 2b between strains and subgroups of CMV, and between PSV and TAV. However, there is virtually no similarity between CMV and PSV or TAV 2b, or between any of the cucumovirus 2b proteins and the tobacco streak 2b protein.

Table 1 Nucleotide sequence similarity between CMV subgroups, and the *Cucumovirus* species for each RNA

Virus[a]	*CMV (I)*	*CMV (II)*	*PSV*	*TAV*
RNA 1				
CMV (I)	100	78	68	70
CMV (II)		100	70	69
PSV			100	68
TAV				100
RNA 2				
CMV (I)	100	74	65	64
CMV (II)		100	66	66
PSV			100	64
TAV				100
RNA 3				
CMV (I)	100	78	64	65
CMV (II)		100	65	66
PSV			100	72
TAV				100

[a]Comparison were made between Fny-CMV (subgroup I), Q-CMV (subgroup II), J-PSV, and V-TAV (RNAs 1 and 2) or C-TAV (RNA 3).

Nucleotide sequence similarity of the *Cucumovirus* species varies for the different RNAs (**Table 1**). For RNA 1, there is about 78% identity between CMV subgroups, and 68–70% identity between species. For RNA 2, there is about 74% identity between CMV subgroups, and 64–66% between species. For RNA 3 there is 78% identity between CMV subgroups, and 64–72% between species. Hence, the RNA 1 sequences are somewhat more conserved than other portions of the genome, which could reflect less flexibility in the function of the 1a protein. Sequence similarity can be a good approximation of evolutionary relationships, but clearly a rule about levels of divergence necessary for species parameters is not easy to determine.

The ability of *Cucumovirus* species to exchange their RNAs 3 led to the speculation that reassortment may have been important in their evolution. Phylogeny estimations of individual ORFs show different topologies for each RNA, supporting this hypothesis (**Fig. 1**). The characterization of a naturally occurring reassortant, having RNAs 1 and 2 derived from PSV and RNA 3 derived from CMV is further evidence

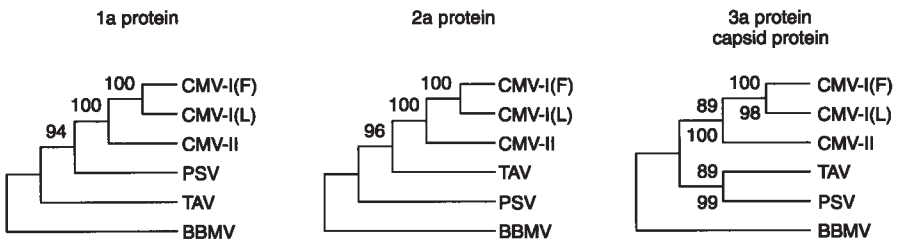


Figure 1 Phylogeny estimation of *Cucumovirus* spp, based on cladistic analysis of aligned amino acid sequences. Bootstrap values are given above the branch points. F, Fny strain of CMV; L, legume strain of CMV; BBMV, broad bean mottle virus, genus *Bromovirus*, family *Bromoviridae*, used as an outgroup.

that reassortment has probably played a pivotal role in the evolution of the cucumoviruses. The very broad host range and numerous strains of CMV indicate that it is a highly adaptable virus. The other members of the *Cucumovirus* genus have more narrow host ranges, but PSV probably has even greater sequence diversity than does CMV. This makes the *Cucumovirus* genus very attractive for studies on virus evolution, and recently it has been exploited as a model system for such studies.

The origin of the satellite RNAs has been the source of much speculation, but no concrete data have emerged to elucidate their evolutionary history. They often appear in experimental plants inoculated with virus alone, and hence appear to have mechanisms of transmission that are different from helper virus transmission. They have even been found in plants inoculated with *in vitro* generated transcript from cDNA clones, but only in greenhouse settings where wild-type virus was also present in nearby plants. They have not been found in transcript-inoculated plants kept under sterile conditions. A phylogeny estimation of satellite RNAs from numerous sources around the world shows essentially no branching, suggesting that they have arisen many times from a common or related source. The longevity of satellite RNA in plants in the absence of virus (25 days in one study with tomato) has added to the difficulty in determining their origins. Hybridization analysis of host nucleic acids has not detected any related sequences, but the sensitivity of these studies may have been too low to detect a single copy gene of the size of the satellite RNAs.

Due to their small size and ease of molecular manipulation, the satellite RNAs have provided a very useful model system to study RNA virus evolution. These studies have shown that selection for specific satellite RNAs in mixed infections is very strong, and that even though satellite RNAs may evolve to become more pathogenic, the selection for these pathogenic variants is at the level of other functions, and not pathology.

Serological Relationships and Variability

There is only very weak or no serological cross-reactivity between the *Cucumovirus* species. Within species, there are also considerable serological differences for CMV and PSV, especially when monoclonal antibodies are used, leading to the original subgroup designations for these viruses. Serological tests have been used in diagnosis of cucumoviruses, and enzyme-linked immunosorbent assay (ELISA) and immunodiffusion tests have been used most widely. The instability of the virions requires that they be fixed with formaldehyde before they can act as good immunogens. This instability also requires careful handling of samples prior to detection of virus, and nucleotide hybridization techniques are sometimes more reliable than serology for diagnostic detection.

There is a large amount of sequence variation among strains of CMV and PSV as assessed by nucleotide sequence analysis. In the case of CMV this is reflected in the variation of host range and symptoms induced by different strains. Sequence similarity between subgroups of CMV is about 75–80%, whereas within subgroups it is 95–99%. Not enough strains of PSV have undergone sequence analysis to determine variation with subgroups, but between major groups the sequence similarity is 78–80%. TAV strain variation has not been studied in any detail. Only RNA 3 has had more than one isolate that has been sequenced, and these are 99% similar.

Epidemiology

Strains of CMV isolated from different parts of the world have proven to be very similar, showing a somewhat uniform worldwide distribution of strains. The only exceptions are the IB strains, a subset of subgroup I strains found only in Asia. This suggests that CMV has been spread on numerous occasions, and may be due to a combination of the enormous host range of CMV, and the frequent worldwide transport of humans and their cultivated plants. PSV

isolates on the other hand, appear to be unique in distinct geographic locations, as assessed by serology and nucleotide sequence analysis, with the exception of the strains found in the eastern United States and in Japan. Hence, most of the worldwide distribution of PSV may have occurred some time ago and the strains have evolved further in their present locations.

Transmission and Tissue Tropism

The cucumoviruses are transmitted by aphid, grafting, dodder, seed and mechanical transmission. CMV is transmitted by more than 75 species of aphids. The specificity of aphid transmission for PSV and TAV has been less thoroughly studied. The viruses are not replicated in the insect vector, and are transmitted in a nonpersistent manner, although all instars of aphids can transmit virus. The transmission rate varies with the virus strain, aphid species, and host plant. The mechanism of aphid transmission and the number of particles transmitted are poorly understood. The coat protein is apparently the only viral component involved in aphid transmission. Recent studies with CMV have identified key amino acids in the coat protein that are essential in aphid transmission and the specificity of transmission by different aphid species.

Graft transmission of CMV has been reported in fruit trees; transmission by dodder (a parasitic plant) is common. Seed transmission varies from 0 to 100%, depending on the virus and the host plant. CMV can also be transmitted vertically from infected corms to new suckers in banana. Mechanical transmission is the most common method used experimentally. The infectivity of purified viral RNA by mechanical inoculation has greatly aided genetic studies of the virus.

The viruses replicate in the epidermal and mesophyllic cells of leaf tissue. They move systemically through the phloem, but it is not thought that they replicate in phloem tissue. Although the coat protein is required for both cell-to-cell and systemic movement, it is not known if the virus moves as a packaged virion or as some other RNA–protein complex. Once a plant is infected, newly formed leaves show symptoms of virus as they emerge, indicating meristematic infection. Mosaic patterns could be due to variable infection of meristematic cells.

Pathogenicity and Cytopathology

The symptoms induced by CMV vary from very mild mosaic to severe necrosis and death. Virus symptoms can appear on the leaves, stems, fruits, and flowers of infected plants. Distortions of leaf morphology are common, including rugosity, stunting, and curling. There is no convincing evidence to correlate severity of symptoms with virus titer. Many wild host plants do not show any symptoms. The presence of a satellite RNA can dramatically alter the symptoms induced by CMV. In most instances satellite RNA attenuates symptoms, but in some cases, most notably in tomato, a satellite RNA can result in chlorosis or systemic necrosis. Tomato necrosis has been cause for great concern in parts of southern Europe, where severe crop losses have resulted. PSV also induces a range of symptoms, from stunting to mosaic. It has also been implicated in decline of year-to-year persistence in white clover. In most cases the PSV satellite RNAs have little effect on the helper virus symptoms, but a few satellite RNAs can attenuate symptoms. TAV infection induces malformation of flowers in chrysanthemum and production of seedless fruit in tomato.

In experimentally infected plants, the cucumoviruses induce several cytoplasmic modifications. Cytoplasmic membranes, thought to be derived from the plasma membrane, proliferate. There is an increase in secondary vacuoles. Vesicles are formed along the tonoplast membrane. The chloroplast structure can be severely altered, through impairment of plastid differentiation. In some strains that induce chlorosis, chloroplasts are smaller and the grana are poorly developed. Mitochondrial structures also may be altered. Effects on chloroplasts and mitochondria appear to be host- and viral strain-specific. The nucleus does not appear to be affected by cucumovirus infection, although one report using tobacco protoplasts described a loss of heterochromatin and vacuolation of the nucleolus.

Inclusion bodies are sometimes seen in cucumovirus-infected plant cells. These are either amorphous or crystalline, and are apparently composed of viral particles. The crystalline structures are generally seen in the vacuoles, and can take a variety of shapes, whereas the amorphous inclusion bodies are usually found in the cytoplasm.

Prevention and Control

The most common means of control of the cucumoviruses are by using virus-free stocks for propagation, eradicating infected plants, and controlling the aphid vectors of the virus. Resistant plants have also been used, but they are not available for many hosts of CMV. Transgenic plants expressing the coat protein of CMV show resistance, and plants expressing portions of the 2a protein are sometimes immune to the virus, but this resistance is generally restricted to similar strains. The attenuating satellite RNAs of

CMV have also been used as biocontrol agents. These have been introduced both as inoculum and as transgenes. This has proven effective in the case of tomato, but the high degree of nucleotide sequence similarity between attenuating and pathogenic strains of satellite RNAs, along with their propensity to mutate, indicates that this method should be used with caution. Transgenic plants expressing viral genes or satellite RNAs have not been used commercially in the United States, but they have been used in other parts of the world. It remains to be seen how long this type of control will hold up under the strong selection pressure of field situations.

Future

The past decade has produced detailed information about the molecular biology of cucumoviruses, and the sequence analysis of numerous strains has allowed conclusions to be drawn about the evolutionary history of these viruses. A lot of experimental work has allowed for the identification of viral components involved in pathogenesis, and many symptom determinants have been mapped to specific amino acids or nucleotides. However, much of the work has focused only on CMV, and many intriguing questions remain. Almost nothing is known about the processes in the plants that allow the viruses to cause disease, or why disease is present in some hosts and not others. Very little is known about the distribution of these and other plant viruses in hosts other than crop plants. These are more difficult questions to answer experimentally, but they are crucial for long-term understanding and control of virus disease. Most control measures seek to reduce or eliminate the virus, and hence exert strong selection pressure on the virus to change to overcome the resistance. In the final analysis, as with all viruses, we may have to learn to live with them rather than try to get rid of them.

See also: **Alfamovirus and Ilarviruses (*Bromoviridae*); Bromoviruses (*Bromoviridae*); Defective interfering viruses; Pathogenesis: Plant viruses; Plant resistance to viruses: Engineered resistance, Natural resistance; Satellite RNAs and Satellite viruses; Vectors: Plant viruses.**

Further Reading

Francki RIB (1985) *The Plant Viruses*, Vol. I: *Polyhedral Virions with Tripartite Genomes*. New York: Plenum.

Kaper JM and Waterworth HE (1981) Cucumoviruses. In: Kurstak E (ed.) *Handbook of Plant Virus Infections and Comparative Diagnosis*, p. 258. North Holland: Biomedical Press.

Palukaitis P, Roossinck MJ, Dietzgen RG and Francki RIB (1992) Cucumber mosaic virus. *Adv. Virus Res.* 41: 281.

Roossinck MJ and White PS (1998) Cucumovirus isolation and RNA extraction. In: Foster GD and Taylor SC (eds) *Plant Virology Protocols, Methods in Molecular Biology*, p. 189. Totowa, NJ: Humana Press.

Molecular Biology

Marilyn J. Roossinck, Plant Biology Division, The Samuel Roberts Noble Foundation, Ardmore, Oklahoma, USA

Properties of the Virion

Cucumoviruses are icosahedral viruses ($T = 3$) of about 29 nm diameter. They consist of RNA (about 18%) and protein, with 180 copies of the coat protein per capsid. They do not contain any lipids or glycoproteins. The internal diameter of the virion is approximately 16.5 nm. The integrity of the capsid is maintained by electrostatic RNA–protein interactions, and digestion of the encapsidated RNA by ribonuclease results in complete degradation of the particle. The virions contain the genomic RNAs, encapsidated separately, as well as at least one subgenomic RNA, and more than one in some species. In addition, the satellite RNAs, when present, are encapsidated in the virion. Based on the molar ratio of viral RNA to satellite RNA, it is likely that some virions contain only multiple copies of satellite RNA. All of the particles have similar sedimentation properties, and hence it is not possible to separate them by physical means. The virion has not been amenable to detailed structural analysis by x-ray crystallography. This may be due to the mixture of particles of similar but not identical size, resulting in the formation of imperfect crystals. The particles of the three species of *Cucumovirus* cannot be distinguished by electron microscopy.

The virions can be readily dissociated into their component proteins and RNA by using high ionic strength buffers. These associations can be re-established by lowering the ionic strength, and the viral coat protein can encapsidate a number of other RNA species *in vitro*.

Properties of the Genome

The viral genome consists of three single-stranded, messenger sense RNAs, designated RNAs 1, 2 and 3, by diminishing size (**Fig. 1**). All the RNAs have a cap structure at the 5′ terminus, that is similar to host

CMV have also been used as biocontrol agents. These have been introduced both as inoculum and as transgenes. This has proven effective in the case of tomato, but the high degree of nucleotide sequence similarity between attenuating and pathogenic strains of satellite RNAs, along with their propensity to mutate, indicates that this method should be used with caution. Transgenic plants expressing viral genes or satellite RNAs have not been used commercially in the United States, but they have been used in other parts of the world. It remains to be seen how long this type of control will hold up under the strong selection pressure of field situations.

Future

The past decade has produced detailed information about the molecular biology of cucumoviruses, and the sequence analysis of numerous strains has allowed conclusions to be drawn about the evolutionary history of these viruses. A lot of experimental work has allowed for the identification of viral components involved in pathogenesis, and many symptom determinants have been mapped to specific amino acids or nucleotides. However, much of the work has focused only on CMV, and many intriguing questions remain. Almost nothing is known about the processes in the plants that allow the viruses to cause disease, or why disease is present in some hosts and not others. Very little is known about the distribution of these and other plant viruses in hosts other than crop plants. These are more difficult questions to answer experimentally, but they are crucial for long-term understanding and control of virus disease. Most control measures seek to reduce or eliminate the virus, and hence exert strong selection pressure on the virus to change to overcome the resistance. In the final analysis, as with all viruses, we may have to learn to live with them rather than try to get rid of them.

See also: **Alfamovirus and Ilarviruses (*Bromoviridae*); Bromoviruses (*Bromoviridae*); Defective interfering viruses; Pathogenesis: Plant viruses; Plant resistance to viruses: Engineered resistance, Natural resistance; Satellite RNAs and Satellite viruses; Vectors: Plant viruses.**

Further Reading

Francki RIB (1985) *The Plant Viruses*, Vol. I: *Polyhedral Virions with Tripartite Genomes*. New York: Plenum.

Kaper JM and Waterworth HE (1981) Cucumoviruses. In: Kurstak E (ed.) *Handbook of Plant Virus Infections and Comparative Diagnosis*, p. 258. North Holland: Biomedical Press.

Palukaitis P, Roossinck MJ, Dietzgen RG and Francki RIB (1992) Cucumber mosaic virus. *Adv. Virus Res.* 41: 281.

Roossinck MJ and White PS (1998) Cucumovirus isolation and RNA extraction. In: Foster GD and Taylor SC (eds) *Plant Virology Protocols, Methods in Molecular Biology*, p. 189. Totowa, NJ: Humana Press.

Molecular Biology

Marilyn J. Roossinck, Plant Biology Division, The Samuel Roberts Noble Foundation, Ardmore, Oklahoma, USA

Properties of the Virion

Cucumoviruses are icosahedral viruses ($T = 3$) of about 29 nm diameter. They consist of RNA (about 18%) and protein, with 180 copies of the coat protein per capsid. They do not contain any lipids or glycoproteins. The internal diameter of the virion is approximately 16.5 nm. The integrity of the capsid is maintained by electrostatic RNA–protein interactions, and digestion of the encapsidated RNA by ribonuclease results in complete degradation of the particle. The virions contain the genomic RNAs, encapsidated separately, as well as at least one subgenomic RNA, and more than one in some species. In addition, the satellite RNAs, when present, are encapsidated in the virion. Based on the molar ratio of viral RNA to satellite RNA, it is likely that some virions contain only multiple copies of satellite RNA. All of the particles have similar sedimentation properties, and hence it is not possible to separate them by physical means. The virion has not been amenable to detailed structural analysis by x-ray crystallography. This may be due to the mixture of particles of similar but not identical size, resulting in the formation of imperfect crystals. The particles of the three species of *Cucumovirus* cannot be distinguished by electron microscopy.

The virions can be readily dissociated into their component proteins and RNA by using high ionic strength buffers. These associations can be re-established by lowering the ionic strength, and the viral coat protein can encapsidate a number of other RNA species *in vitro*.

Properties of the Genome

The viral genome consists of three single-stranded, messenger sense RNAs, designated RNAs 1, 2 and 3, by diminishing size (**Fig. 1**). All the RNAs have a cap structure at the 5′ terminus, that is similar to host

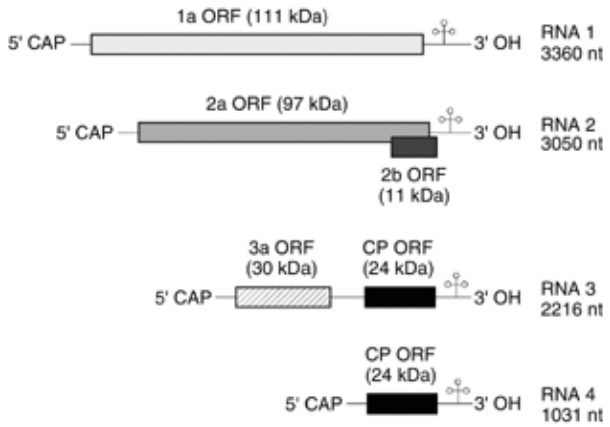


Figure 1 Viral genome of three single-stranded message-sense RNAs designated RNAs 1, 2 and 3 by diminishing size. RNA 4 is not required for infection.

messenger RNA cap structures. The 5′ noncoding regions of RNAs 1 and 2 are highly similar within a strain. The 3′ portion of all the RNAs is also highly similar within a strain, and can form a tRNA-like structure that can be aminoacylated with tyrosine. The sizes of the genomic RNAs and encoded proteins vary between species. Representative members of each species are listed in **Table 1**. RNA 1 is about 3400 nucleotides (nt) in length, and encodes a single protein (the 1a protein) of M_r 112 000. RNA 2 (about 3000 nt) encodes two open reading frames (ORFs) of about M_r 97 000 (2a) and M_r 11 000 – 17 000 (2b). The 2b protein is translated from a subgenomic RNA (RNA 4a) that may be encapsidated. The 2b ORF is found in all *Cucumovirus* members, but not in most of the other *Bromoviridae*. RNA 3 (2200 nt) encodes the M_r 30 000 3a protein, and the M_r 24 000 coat protein, that is translated from the subgenomic RNA 4. RNAs 3 and 4 are coencapsidated, but RNA 4 is not required to establish infection.

Defective RNAs have been isolated from cucumber mosaic virus (CMV) after high multiplicity of infection passage in tobacco plants. These do not appear to interfere with virus symptoms or titer, and hence are not really interfering RNAs. They are not supported by all hosts of the parent virus. They are generated by deletions of portions of the 3a protein coding region. An additional virus-derived RNA, known as RNA 5, is found in infected tissue and is encapsidated in some strains of CMV and TAV. RNA 5 is derived from the genomic RNA 3. Other smaller RNAs are also often seen in agarose gel electrophoresis of peanut stunt virus (PSV) and tomato aspermy virus (TAV) viral RNAs, but for the most part these have not been characterized.

Table 1 Sizes and M_r of *Cucumovirus* RNAs and proteins

Species/strain	*Fny-CMV (I)*	*Q-CMV (II)*	*J-PSV*	*TAV*[a]
RNA 1 (nt)	3360	3389	3355	3410
1a protein (M_r)	111 432	110 917	112 016	112 061
RNA 2 (nt)	3050	3035	2946	3074
2a protein (M_r)	96 709	94 439	93 520	93 509
2b protein (M_r)	12 749	11 579	17 946	15 277
RNA 3 (nt)	2216	2197	2186	2214
3a protein (M_r)	30 461	30 348	31 636	30 532
Coat protein (M_r)	24 243	24 243	24 179	25 753

[a]RNAs 1 and 2 and encoded proteins are given for V-TAV; RNA 3 and encoded proteins are given for C-TAV.

Satellite RNAs

Molecular parasites known as satellite RNAs are also found associated with CMV and PSV. These small RNAs range in size from about 330 to 400 nt. They do not encode any proteins, but can affect both virus titer and symptoms. Analysis of the secondary structure of a CMV satellite RNA responsible for necrosis in tomato (the D satellite RNA) demonstrated that a stem-loop in the 3′ portion of the RNA was highly conserved in the necrosis-inducing satellite RNAs, and contained the three nucleotides previously shown to be critical for necrosis (**Fig. 2**). Another domain in

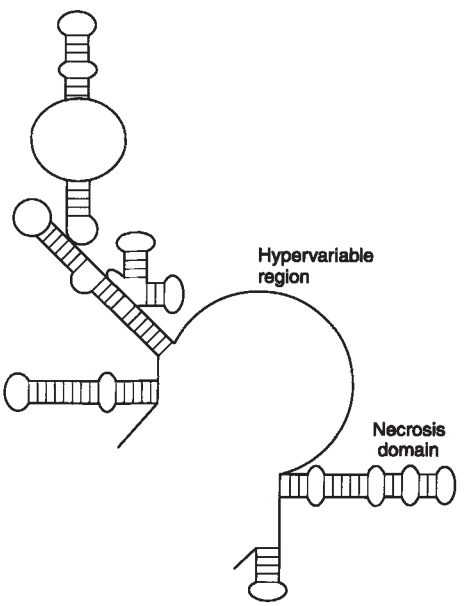


Figure 2 Secondary structure of CMV satellite RNAD *in planta*.

the D4 satellite, identified as the hypervariable domain because of the amount of variation both in wild-type isolates and in progeny of cDNA clones, is found in a nonstructured region of the RNA. The nucleotides responsible for other pathogenic phenotypes of satellite RNAs have also been identified, but structural analysis of these satellite RNAs *in planta* has not been done. The satellite RNAs have provided a model system for studying both the structure of biologically active RNAs and RNA evolution.

Properties of the Proteins

The 1a and 2a proteins are the only viral components required for replication. By analogy to related viruses, the 1a protein apparently encodes the methyltransferase and other activities responsible for capping the viral RNAs, in the amino portion of the protein. In the related animal alphavirus, Sindbis virus, this region is also required for initiation of (−) strand synthesis. There is a central domain in the 1a protein that is not well conserved, but that contains a large number of acidic amino acid residues in the cucumoviruses and in other related viruses. A nucleotide-binding domain is found adjacent to the acidic domain, and the carboxy-terminal portion contains the conserved elements of a helicase. The precise role of the helicase is not known. It may be required to resolve the secondary structure inherent in RNA molecules, or it may be involved in synthesis of (+) strand from a double-stranded replicative intermediate. The 2a protein contains the Gly-Asp-Asp domain conserved in many viral polymerases, but it too may have additional undefined functions. In the closely related brome mosaic virus (BMV), the amino-terminal domain is involved in interactions with the 1a protein. Antibodies to the methyltransferase, helicase and polymerase domains can all inhibit replication *in vitro*.

The 2b protein is the most recently described viral protein, although the open reading frame for the 2b protein was observed when the first strains were sequenced. It has been implicated in long distance movement and host range of the virus, but may have other unidentified functions.

The 3a protein potentiates the cell-to-cell movement of the virus. Like other plant viral movement proteins, the 3a protein presumably alters the plasmodesmata, allowing passage of molecules that are larger than the normal size exclusion limit. The CMV 3a protein produced in *Escherichia coli* has a cooperative single-stranded DNA and RNA binding activity, as well as GTP binding activity. However, all of the viral proteins have been implicated in either long distance or cell-to-cell movement of CMV in various hosts.

The coat protein is involved in encapsidation, movement and aphid transmission of the virus. In protoplast studies it also stabilizes the (+) sense viral RNA, perhaps by encapsidation. In addition, all the viral proteins have been implicated in the host range of various strains, and in pathogenicity.

Physical Properties

The purified virions have a relatively short half-life when stored at 4°C. For CMV, the virus titer drops by one half in about two weeks. This is probably due to the virion permeability to ribonucleases. The virion is unstable to freezing, but can be stored in 50% glycerol at −20°C. The virus is stable to short exposure to chloroform, and nonionic detergents such as Triton X-100, but not to phenol or ionic detergents such as sodium dodecyl sulfate (SDS). The viral RNA is readily purified from virions in the presence of SDS, ethylene diamine tetra-acetic acid (EDTA), and phenol, without the use of protease. The particles are negatively charged, with a pI of 4.75. They sediment as a single component with $s = 98.6$. The extinction coefficient at 260 nm is 5.

Replication

The viral replication complex consists of the 1a and 2a proteins, and host factors. Interactions between the 1a and 2a proteins, as well as 1a–1a interactions have been demonstrated using a yeast two-hybrid system. In the closely related bromoviruses a 1a–2a fusion, resembling the replication protein of tobacco mosaic virus, was only functional when additional 1a protein was added, indicating that the 1a–2a interacting complex was not sufficient for replication. Hence the replicase complex may contain a ratio of 1a to 2a of greater than 1:1, along with necessary host components. Replication occurs on cytoplasmic membranes, and may be associated with the rough ER, as replicase activity can be readily demonstrated in the membrane-bound polysome fraction of infected cells.

The cucumoviruses replicate by first synthesizing a (−) strand from the (+) strand template. Double-stranded (ds) RNA can be readily isolated from infected tissue, and this is presumably the replicative intermediate, although it is possible that (+) and (−) strand RNAs exist separately in the cell, and anneal upon purification. The 3′ terminus of the (−) strand contains an unpaired nontemplate G residue. In the satellite RNA the unpaired G is required for synthesis of (+) strand *in vitro*, suggesting that (+) strand synthesis begins internally with the second nucleotide

of the (−) strand. The 3′ terminus of the (+) strand of viral RNAs also contains a nontemplate nucleotide, an A residue, resulting in a CCA 3′ terminus that is similar to tRNA 3′ ends. The 3′ terminus of all of the cucumoviral satellite RNAs consists of 3 C residues. Deletion of the CCC in the (+) strand of a CMV satellite RNA resulted in repair *in vivo* and restoration of the wild-type sequence, indicating that nontemplated nucleotides can be added to the 3′ end of the (+) sense satellite RNAs as well. Hence both (+) and (−) strand synthesis may initiate internally, rather than at the precise 3′ end of the template.

The CMV and PSV satellite RNAs are found in large quantities as double-stranded RNA in infected cells, and were assumed to replicate in a similar manner to the viral RNAs. However, there have been some reports of multimeric forms of CMV satellite RNAs in infected cells, and it is possible that they utilize other replication strategies such as rolling circle replication.

The 5′ termini of the (+) strands of the genomic and subgenomic RNAs, as well as the satellite RNAs are modified by a 7MeGppp structure. Sindbis virus performs this function with the nsp1 protein. Nsp1 is analogous to the 1a protein of cucumoviruses, although details of the capping reactions may differ.

CMV was the first eukaryotic virus for which an RNA dependent RNA polymerase was isolated that was functional *in vitro*. This complex contained the viral 1a and 2a proteins, and only one apparent host protein. However, the viral replication by this complex *in vitro* did not duplicate the *in vivo* replication in quantities of (+) and (−) synthesis, and it now seems likely that other host factors are involved *in vivo*. Other purification schemes isolated a replicase complex that could only synthesize (−) strand from (+) strand template. These complexes could also use related viral RNAs as templates, but not cellular RNAs, suggesting that much of the specificity of replication may be in the (+) strand synthesis.

Characterization of Transcription and Translation

Transcription of subgenomic RNA 4 from the (−) strand of RNA 3 occurs from a promoter located in the intercistronic region. The minimal core promoter is also found upstream of the start of the 4a transcript, on RNA 2, but additional sequences in the intercistronic region affect levels of RNA 4 transcription in CMV. Most of the intercistronic region is also essential for accumulation of the (+) but not (−) strand of RNA 3 of CMV. RNA 4 is synthesized very efficiently, and accumulates to levels similar to the genomic RNAs. RNA 4a, the subgenomic RNA for the 2b protein, is found in much lower abundance in infected cells. The details of *cis*-acting sequences for transcription of the 4a RNA, as well as for transcription of subgenomic RNAs of other cucumoviruses have not been elucidated.

The level of viral proteins that can be detected in infected cells does not correlate with the steady-state level of the corresponding viral RNAs. In many hosts the genomic RNAs and RNA 4 are found in similar molar ratios, but the coat protein is the most abundant protein, usually easily detected by Coomassie blue staining of total cell extracts separated by gel electrophoresis. The 3a protein is much less abundant, and the 1a and 2a proteins can be difficult to detect at all in infected cells. Hence there appears to be a translational control of viral proteins. Since each virion requires 180 coat protein units, the coat protein is needed in much greater quantities than the other viral proteins. The replicase and movement proteins are needed in much smaller quantities, and higher concentrations of these proteins may actually prove toxic to the host cell. In viruses that produce polyproteins and hence equimolar levels of all proteins, the excess replicase proteins are often sequestered in inclusion bodies. The divided genome of the cucumoviruses has allowed them to evolve more efficient strategies for their protein synthesis.

Although a number of short open reading frames (ORFs) are found in the satellite RNAs, that can be translated *in vitro*, none of them are conserved among satellite RNA strains, and mutation of the AUGs of these ORFs does not affect satellite RNA infectivity or function. Hence they do not appear to be functional in plants.

No specific proteolysis of the viral proteins has been demonstrated, but western blot analyses of protein extracts from infected cells sometimes contain discreet bands not found in uninfected cell extracts, that react strongly with polyclonal antibody to the 1a protein. It is possible that a cleaved form of the 1a protein, along with intact 1a, is involved in the replication complex.

Genetic Mapping of Symptom Determinants

Before the availability of cDNA clones of the viral RNAs that were capable of producing infectious transcripts, numerous symptom determinants were mapped to specific viral RNAs by reassortment of gel-purified RNAs from strains exhibiting different phenotypes. These included determinants for chlorosis, which mapped to RNAs 2 and 3 in different virus strain/host combinations, determinants for necrotic

lesions in legumes, which also mapped to RNAs 2 and 3, and determinants for severe systemic infection, which mapped to RNA 1. In recent years, many of these determinants have been more precisely mapped using recombinant and mutant cDNA clones, and in several cases the precise amino acid responsible for symptom type has been identified. For example, the determinant for eliciting the hypersensitive response (necrosis) in cowpea was identified as a single amino acid in the 2a protein, whereas the determinant for chlorosis in tobacco was mapped to an amino acid in the coat protein. These studies have been useful, but they have not provided a clear picture of how the viral proteins and/or RNAs are interacting with the host to cause disease. This will require more work with the hosts, whose genetic systems are much more complicated. Over the next few years more information about the host's role in disease will undoubtedly become available, and a thorough understanding of virus–host interactions on a molecular level will allow for many practical applications.

See also: **Alfamovirus and ilarviruses (*Bromoviridae*); Bromoviruses (*Bromoviridae*); Defective interfering viruses; Pathogenesis: Plant viruses; Plant resistance to viruses: Engineered resistance, Natural resistance; Satellite RNAs and Satellite viruses; Vectors: Plant viruses.**

Further Reading

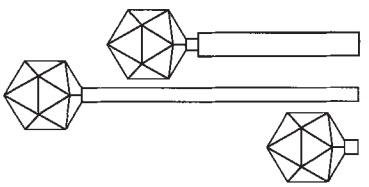
Francki RIB (1985) *The Plant Viruses*, Vol. I: *Polyhedral Virions with Tripartite Genomes*. New York: Plenum.

Kaper JM and Waterworth HE (1981) Cucumoviruses. In: Kurstak E (ed.) *Handbook of Plant Virus Infections and Comparative Diagnosis*, p. 258. North Holland: Biomedical Press.

Palukaitis P, Roossinck MJ, Dietzgen RG and Francki RIB (1992) Cucumber mosaic virus. *Adv. Virus Res.* 41: 281.

Roossinck MJ, Sleat D and Palukaitis (1992) Satellites of plant viruses; structures and biological effects. *Microbiol. Rev.* 56: 265.

CYANOPHAGES



Eugene L Martin and **Tyler A Kokjohn**, Department of Biological Sciences, University of Nebraska, Lincoln, USA

Introduction

The cyanophages are a group of DNA viruses that attack host organisms found within the cyanobacteria or blue-green algae, a group of oxygenic photosynthetic procaryotes. A continuing dichotomy has occurred as to the nomenclature and taxonomy of these microorganisms. Many of the existing genera and species were mainly described on the basis of morphological characteristics obtained from field-collected samples, and not from axenic cultures. It has been argued that in cell size and morphological complexity the blue-green algae more closely resemble the green algae and other microalgae than the other bacteria and that their dual photosystems are almost identical with that of the eucaryotic algae and higher green plants rather than with anything known in the anoxygenic photosynthetic bacteria.

Detailed physiological and genetic studies have been used to delineate this group of microorganisms more completely. Their multilayered cell wall closely resembles the cell wall structure of Gram-negative eubacteria. On the basis of 16S and 5S rRNA nucleotide base sequence data, the blue-green algal procaryotes have been assigned to the eubacteria, a group distinct from eucaryotes (e.g. algae) and the archaebacteria. Other characteristics that have been analyzed include analysis of pigments (chlorophyll *a* and phytobilins), chemoheterotrophic/photoheterotrophic capabilities, nitrogen-fixation ability, DNA base composition, DNA/DNA hybridization and modes of asexual and sexual reproduction. Of considerable importance is the fact that the basic cell structure of this group is procaryotic, which is synonymous with bacteria. Therefore, while blue-green algae and the cyanobacteria are reasonably compatible terms, cyanobacteria will be used here. In Volume 3 of *Bergey's Manual of Systematic Bacteriology*, Castenholz and Waterbury have divided the cyanobacteria into five morphological groups, which are summarized in **Table 1**. This is the classification scheme that will be used for this article.

lesions in legumes, which also mapped to RNAs 2 and 3, and determinants for severe systemic infection, which mapped to RNA 1. In recent years, many of these determinants have been more precisely mapped using recombinant and mutant cDNA clones, and in several cases the precise amino acid responsible for symptom type has been identified. For example, the determinant for eliciting the hypersensitive response (necrosis) in cowpea was identified as a single amino acid in the 2a protein, whereas the determinant for chlorosis in tobacco was mapped to an amino acid in the coat protein. These studies have been useful, but they have not provided a clear picture of how the viral proteins and/or RNAs are interacting with the host to cause disease. This will require more work with the hosts, whose genetic systems are much more complicated. Over the next few years more information about the host's role in disease will undoubtedly become available, and a thorough understanding of virus–host interactions on a molecular level will allow for many practical applications.

See also: **Alfamovirus and ilarviruses (*Bromoviridae*); Bromoviruses (*Bromoviridae*); Defective interfering viruses; Pathogenesis: Plant viruses; Plant resistance to viruses: Engineered resistance, Natural resistance; Satellite RNAs and Satellite viruses; Vectors: Plant viruses.**

Further Reading

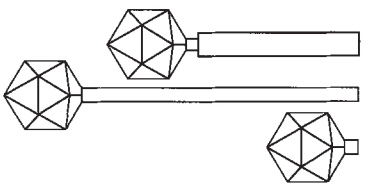
Francki RIB (1985) *The Plant Viruses*, Vol. I: *Polyhedral Virions with Tripartite Genomes*. New York: Plenum.

Kaper JM and Waterworth HE (1981) Cucumoviruses. In: Kurstak E (ed.) *Handbook of Plant Virus Infections and Comparative Diagnosis*, p. 258. North Holland: Biomedical Press.

Palukaitis P, Roossinck MJ, Dietzgen RG and Francki RIB (1992) Cucumber mosaic virus. *Adv. Virus Res.* 41: 281.

Roossinck MJ, Sleat D and Palukaitis (1992) Satellites of plant viruses; structures and biological effects. *Microbiol. Rev.* 56: 265.

CYANOPHAGES



Eugene L Martin and **Tyler A Kokjohn**, Department of Biological Sciences, University of Nebraska, Lincoln, USA

Introduction

The cyanophages are a group of DNA viruses that attack host organisms found within the cyanobacteria or blue-green algae, a group of oxygenic photosynthetic procaryotes. A continuing dichotomy has occurred as to the nomenclature and taxonomy of these microorganisms. Many of the existing genera and species were mainly described on the basis of morphological characteristics obtained from field-collected samples, and not from axenic cultures. It has been argued that in cell size and morphological complexity the blue-green algae more closely resemble the green algae and other microalgae than the other bacteria and that their dual photosystems are almost identical with that of the eucaryotic algae and higher green plants rather than with anything known in the anoxygenic photosynthetic bacteria.

Detailed physiological and genetic studies have been used to delineate this group of microorganisms more completely. Their multilayered cell wall closely resembles the cell wall structure of Gram-negative eubacteria. On the basis of 16S and 5S rRNA nucleotide base sequence data, the blue-green algal procaryotes have been assigned to the eubacteria, a group distinct from eucaryotes (e.g. algae) and the archaebacteria. Other characteristics that have been analyzed include analysis of pigments (chlorophyll *a* and phytobilins), chemoheterotrophic/photoheterotrophic capabilities, nitrogen-fixation ability, DNA base composition, DNA/DNA hybridization and modes of asexual and sexual reproduction. Of considerable importance is the fact that the basic cell structure of this group is procaryotic, which is synonymous with bacteria. Therefore, while blue-green algae and the cyanobacteria are reasonably compatible terms, cyanobacteria will be used here. In Volume 3 of *Bergey's Manual of Systematic Bacteriology*, Castenholz and Waterbury have divided the cyanobacteria into five morphological groups, which are summarized in **Table 1**. This is the classification scheme that will be used for this article.

Table 1 Taxa of the cyanobacteria

Order	*Distinguishing characteristics*	*Representative genera*
Chroococcales	Unicellular, reproduce by binary fission in one, two or three planes or by budding. Cells are coccoid to rod-shaped	*Chamaesiphon, Gloeobacter, Synechococcus, Synechocystis, (Microcystis), (Anacystis)*
Pleurocapsales	Reproduce by the formation of small spherical cells (baeocytes) produced through multiple fission, often colonial	*Dermocarpa, Xenococcus, Myxosarcina, Pleurocapsa*
Oscillatoriales	All filamentous forms which undergo binary fission in a single plane and produce 'vegetative' cells only. Heterocysts and akinetes do not occur	*Spirulina, Oscillatoria, Lyngbya, (Phormidium), (Plectonoma)*
Nostocales	Filamentous organisms dividing exclusively by binary fission in one plane only. Have the potential to produce heterocysts. Some possibility of false branching	*Anabaena, Aphanizomenon, Nostoc, Nodularia*
Stigonematales	Exhibit the highest degree of morphological complexity and differentiation. Filamentous with branching and heterocysts	*Fischerella, Chlorogloeopsis*

Summarized from Castenholz RW and Waterbury JG (1989) Cyanobacteria. In: Staley JT (ed.) *Bergey's Manual of Systematic Bacteriology*, vol. 3, p. 1710. Baltimore: Williams and Wilkins.

Types and Classification

The cyanophages that have been characterized possess a single piece of double-stranded DNA and the various types of head–tail morphology found for bacteriophages. Many of the cyanophages listed in **Tables 2** and **3** have been placed in the family *Myoviridae* (which includes phages with a central tube and a contractile sheath separated from the head by a neck), in the family *Siphoviridae* (which comprises phages with long, noncontractile tails) and in the family *Podoviridae* (which contains phages with short tails). The cyanophages that are specific for unicellular cyanobacteria (**Table 2**) all attack host organisms found in the order *Chroococcales*, whereas cyanophages that are specific for filamentous cyanobacteria attack host organisms found in the orders *Oscillatoriales* and *Nostocales* (**Table 3**). There appear to be minimal or no reports of cyanophages found for the unicellular order *Pleurocapsales* and the multicellular order *Stigonematales*. Within the last few years Kim and Choi isolated a new virus similar to AS-1. Recently, many new marine cyanophages have been characterized and will be described in the Ecology section.

Because the cyanobacteria occupy such a unique position among living organisms, a wide diversity of research has been pursued. In the 1990s the role cyanophages occupy in marine ecosystems has been thoroughly investigated. In addition to a survey of the ecological research, recent results will be examined in the areas of physiology–genetics and metabolism.

Ecology

Cyanobacteria are clearly of singular importance in many freshwater and marine ecosystems as both primary producers and nitrogen-fixing organisms. From the early 1960s through the 1980s extensive characterization of freshwater cyanophages which infect both unicellular and filamentous cyanobacteria occurred (**Tables 2** and **3**). A role for cyanophages as a major controlling agent of microbial community structure was hypothesized. As a result many early studies focused on using cyanophages as a means to control nuisance cyanobacterial (e.g. blue-green algal) blooms. Limited success has been achieved in this endeavor. Recent research has been directed toward the understanding of cyanophage distribution and dynamics in marine systems.

Suttle and Chan have noted that cyanobacteria of the genus *Synechococcus* are important primary producers that are responsible for considerable carbon fixation in the world's oceans. Three recent lines of research have determined that infective cyanophages for *Synechococcus* are extremely numerous in various coastal and offshore marine environments. The important aspects of each of these research groups will be described.

Using liquid dilution cultures Waterbury and Valois isolated a collection of 75 *Synechococcus* cyanophages from both coastal and offshore waters. From both types of samples, phage titers seasonably reached 10^3 and 10^4 viruses per ml. Representatives were found in each of the three families of tailed

Table 2 Cyanophages specific for unicellular cyanobacteria

Cyanophage	*Host range*	*Head diameter (nm)*	*Tail morphology*	*DNA G + C content (%)*	*Buoyant density in CsCl (g ml^{-3})*	*Temperature range of stability (°C)*	*pH range of stability*	*Mg^{2+} requirement*
SM-1	*Synechococcus elongatus* UTEX 563 *Microcystis aeruginosa* NRC-1	67	Collar, 6 nm long	66–67	1.480	4–40	5–11	No
SM-2	*Synechococcus elongatus* UTEX 563 *Microcystis aeruginosa* NRC-1	50–55	Noncontractile, 130–140 nm long	69–70	1.483	4–40	5–11	Yes
AS-1	*Anacystis nidulans* UTEX 625 *Synechococcus cedrorum* UTEX 1191	90	Contractile, 250 nm long	52–55	1.490	—	4–10	No
AS-1M	*Anacystis nidulans, Synechococcus cedrorum*	90	Contractile, 240 nm long	52–55	1.490	4–45	4–10	No
AS-2	*Anacystis nidulans, Synechococcus cedrorum, Synechococcus* sp. NRC-1	Not known	Not known	—	—	—	—	—
S-1	*Microcystis aeruginosa* NRC-1	50	Noncontractile, 140 nm long	70–74	1.501	—	—	—
S-2L	*Synechococcus* sp. 698	56	Noncontractile, 120 nm long	68.7	—	0–60	4–11	No
S-3L	*Synechococcus schmidlea*	81	Contractile, 161 nm long	67.3	—	0–45	6–11	Yes
S-4L	*Synechococcus* sp. 698	75	Noncontractile, 300 nm long	46	—	0–60	5–11	No
S-5L	*Synechococcus* sp. 698	80	Collar, 16 nm long	66	—	0–60	5–11	No

Data taken from Martin E and Benson R (1988) and Saffermann RS *et al.* (1983) Classification and nomenclature of viruses of cyanobacteria. *Intervirology* 19: 61. For further information concerning individual viruses, please consult these references.

Table 3 Cyanophages specific for filamentous cyanobacteria

Cyanophage	*Host range (members of genera)*	*Head diameter (nm)*	*Tail morphology*	*DNA G + C content (%)*	*Buoyant density in CsCl (g ml^{-3})*	*Temperature range of stability (°C)*	*pH range of stability*	*Mg^{2+} requirement*
LPP-1	*Lyngbya, Plectonema,* and *Phormidium*	60	Short, contractile	53	1.48	4–40	5–11	Yes
LPP-2	*Lyngbya, Plectonema,* and *Phormidium*	60	Short, contractile	52	1.48	4–40	5–11	Yes
LPP-3A	*Lyngbya, Plectonema,* and *Phormidium*	—	—	—	1.71	—	—	Yes
A-1(L)	*Anabaena* spp.	60–62	Long, contractile	37.3	—	—	—	Yes
A-4(L)	*Anabaena* spp.	56	Short	—	—	—	—	No
AC-1	*Anabaena* spp.	62.5	Short	—	—	—	—	—
A-2	*Anabaena* spp.	63	Long, contractile	50–52	—	—	—	—
AN-11 through AN-22	*Anabaena* spp., *Nostoc* spp.	50–63	Variable	37–41	1.498	—	—	Yes
N-1	*Nostoc muscorum* (now *Anabaena* spp.)	61	Long, contractile	50–52	—	—	—	—
NP-1T[a]	*Nostoc* spp., *Plectonema* spp.	76–80	None noted	72	1.730	4–64	6–11	No

Data taken from Martin E and Benson R (1988) and Saffermann RS *et al.* (1983) Classification and nomenclature of viruses of cyanobacteria. *Intervirology* 19: 61. For further information concerning individual viruses please consult these references.

[a] Data taken from Muradov MM *et al.* (1990) Comparative study of NP-1T cyanophages, which lysogenize nitrogen-fixing bacteria of the genera *Nostoc* and *Plectonema*. *Mikrobiologiya* 59: 819.

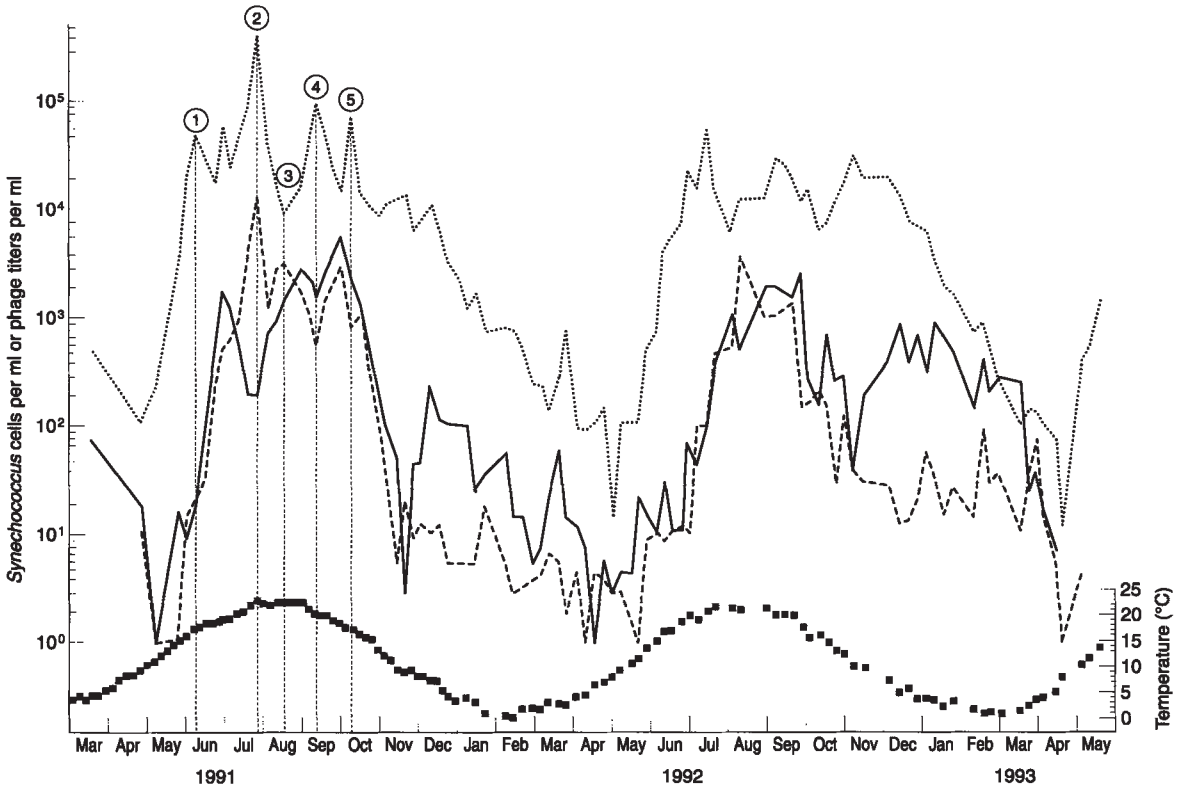


Figure 1 Annual cycle of *Synechococcus* spp. and *Synechococcus* phages in Woods Hole Harbor. Cell counts were determined by epifluorescence microscopy. Phage titers were determined by the most-probable-number technique in tissue culture wells with the host strains WH8018, isolated from Woods Hole in 1980, and WH8012, isolated from the Sargasso Sea in 1980. These strains were chosen because of their sensitivity to a broad spectrum of *Synechococcus* phages. ....., Natural *Synechococcus;* ——, phage titer using host 8018; -----, phage titer using host 8012; ■, temperature (°C). (From Waterbury and Valois (1993).)

phages, with the majority being in *Myoviridae*. Whereas some phage isolates only infected the host strain used for isolation, other phages infected as many as 10 of the 14 host strains. Preliminary characterization indicated that the isolates are virulent phages. Attempts to induce lysogeny in *Synechococcus* strains by using temperature shock, light shifts, ultraviolet and X-radiation, and mitomycin were not successful. The annual cycle of natural *Synechococcus* in Woods Hole Harbor waters is related to water temperature and is very repeatable (**Fig. 1.**). Over the 3 year period examined, cyanophages were present at concentrations approximately one order of magnitude less than the natural *Synechococcus* levels. At Points 1 to 5 in **Fig. 1** the percentage of the natural population that might be lysed by viral infection was measured and this ranged from 0.005% per day at the end of the spring 1991 bloom to 3.2% per day during a *Synechococcus* peak in July 1991.

Similar results were obtained from offshore samples collected from Slope Water south of Cape Cod and from the Gulf Stream: ranging from 0.6 to 2.0% per day. It was then determined that from the Woods Hole Harbor location that the majority of the *Synechococcus* population at the time of sampling was resistant to its co-occurring cyanophages. Waterbury and Valois summarized their results by stating that the acquisition and maintenance of this resistance enables marine *Synechococcus* communities to coexist with cyanophages abundant in seawater: 'As a consequence of this stable coexistence, *Synechococcus* cyanophages have little effect on the overall population densities of marine *Synechococcus* cells but should be important in determining and enhancing the clonal composition of these populations.' Apparently, cyanophages are maintained by infecting the small proportion of *Synechococcus* cells that are still sensitive to viral infection.

Suttle and Chan reported that cyanophages infecting marine *Synechococcus* cells were often very abundant and were found in every seawater sample across an inshore-to-offshore transect in the western Gulf of Mexico and during a 28 month study of

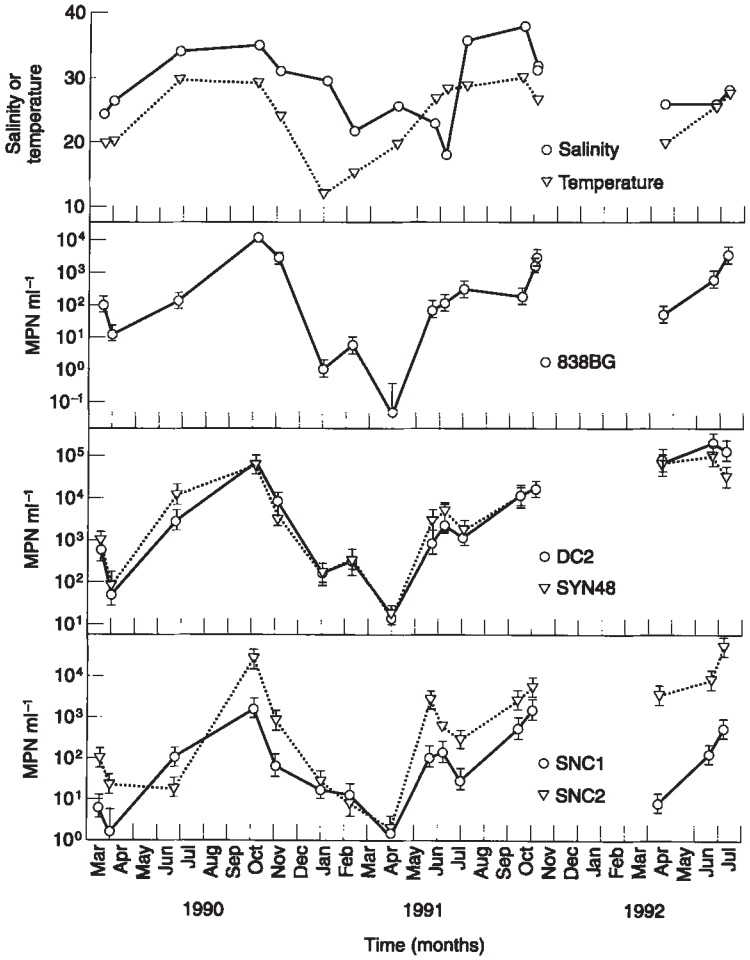


Figure 2 Temperature, salinity and most probable number (MPN) of cyanophages infecting five marine *Synechococcus* strains (838BG, DC2, SNC1, SNC2 and SYN48) in coastal sea water collected from the pier of the Marine Science Institute, Port Aransas, Texas. (From Suttle and Chan (1994).)

coastal seawater collected off the pier of the Marine Science Institute, Port Aransas, Texas. In the coastal seawater study (**Fig. 2**), cyanophages reached a level of 10^3 and 10^4 viruses per ml seasonably during the warmer months of the year, with the lowest concentrations occurring in the cooler water of the winter months. These results were very similar to the Woods Hole Harbor study of Waterbury and Valois (**Fig. 1**). Along the transect viruses infecting two strains of *Synechococcus* (DC2 and SYN48) ranged from a few hundred per ml at 97 m deep and 83 km offshore to 4×10^5 per ml near the surface within 18 km of the coast. Generally when the concentration of *Synechococcus* cells exceeded 10^3 per ml, the concentration of cyanophages increased markedly from 10^2 to $>10^5$ per ml. This suggested for these offshore samples a minimum host density was necessary for efficient viral replication. For most offshore samples the numbers of *Synechococcus* cells and infectious cyanophages were much lower than those found in coastal seawater samples. Therefore, the rate at which viruses contact host cells is less frequent, which in turn results in less selection for cyanophage-resistant strains.

Suttle and Chan further summarized their results in that 'balancing estimates of virus production with contact rates for the furthest offshore station required that most *Synechococcus* cells be susceptible to infection, and that the burst size be about 324 viruses per lytic event'. Therefore, the transport theory of Murray and Jackson used to calculate contact rates for the offshore *Synechococcus* cyanophage can not rely on a small subset of the host cell community being responsible for viral proliferation. For coastal or inshore marine *Synechococcus* populations, a different conclusion is projected. Because rates of viral decay from solar radiation are substantially reduced in the more turbid water, only about 1% of *Synechococcus* cyanophage contacts would result in

infection. This would coincide with the aforementioned work of Waterbury and Valois where they found the majority of inshore host cells isolated were resistant to the most abundant cyanophages. Suttle and coworkers have also used fluorescently stained cyanophages as probes to label, identify and enumerate specific strains of cyanobacteria in marine microbial communities.

The results of these two research groups indicate that cyanophages play an important role in carbon cycling in aquatic ecosystems by causing the lysis of key producer organisms. In effect, a portion of the system carbon held as biomass is converted to dissolved organic carbon in a short-circuiting of the flow from producers to higher trophic level consumers. While heterotrophic bacteria may recapture some of the dissolved organic carbon released by viral lysis, the precise fate of carbon and the effects on the microbial loop as a result of cyanophage activity is not known.

As previously mentioned, in many environments cyanobacteria and cyanophages will be exposed to very substantial levels of solar radiation. Experimental investigation has demonstrated that sunlight exposure rapidly inactivates cyanophages, suggesting that rates of production must be extremely rapid or that the viruses are capable of reactivation after infection of the host cell. Numerous studies have demonstrated that many bacteriophages are able to use host repair systems to eliminate DNA damage and resume replication, and these observations may explain the ability of cyanophages to persist in high-insolation environments. It is at present an open question as to whether cyanophages possess unique host-independent DNA repair genes analogous to those described for bacteriophage T4 and phycovirus PBCV-1.

Wilson and coworkers have also made substantial contributions to the study of cyanobacteria–cyanophage interactions in marine ecosystems. Five marine cyanophages from the *Myoviridae* and *Siphoviridae* families were isolated from three oceanographic sites. These sites included coastal water from the Sargasso Sea, Bermuda, Woods Hole Harbor and coastal water off Plymouth Sound, UK. While measurement of cyanophage numbers was not the primary objective of this study, the cyanophage titers were of same order of magnitude as for previous reports in the same oceanographic sites. All five cyanophages were propagated on *Synechococcus* sp. WH7803, the viral DNA extracted and purified, and restriction endonuclease digestion run. The *Myoviridae* isolates from Bermuda and the English Channel had highly related restriction patterns, as did the DNA from the *Styloviridae* isolates from Bermuda and the English Channel. The DNA of the *Myoviridae* Woods Hole isolate had a unique restriction pattern. The three *Myoviridae* isolates had a genome size of 80–85 kb and the genome size of *Siphoviridae* was in the range of 90–100 kb. The authors remarked that they were surprised to find such highly related DNA profiles from two cyanophage families obtained from the subtropical waters off Bermuda and the more temperate waters of the English Channel. This suggested that certain types of marine cyanophages have a wide distribution pattern comparable to those observed for certain freshwater cyanophages. The *Myoviridae* and *Styloviridae* revealed a clear difference in polypeptide profiles. However, all of the five cyanophages had a major (probably structural) protein in the 53–54 kDa range.

Wilson, Carr and Mann next turned their focus to the effect that phosphate status had on cyanophage infection in the marine *Synechococcus* sp. WH 7803. This cyanobacterial strain contains phycoerythrin and is considered to be a major primary producer in oligotrophic subtropical and tropical oceanic ecosystems. One contributing factor in the control of primary productivity is the availability and flux of nitrogen and phosphate. Other researchers working with viruses that replicated in a marine coccolithophorid found that phosphate limitation but not nitrate limitation affected viral propagation. It was suggested that this was due to the high nucleic acid:protein ratio found for viruses. Wilson, Carr and Mann examined the effects phosphate limitation has on the infection kinetics of a *Myoviridae* cyanophage S-PM2 that was isolated from coastal waters near Plymouth. S-PM2 was propagated on *Synechococcus* sp. WH 7803 under either phosphate-deplete or phosphate-replete conditions. When the growth of the host cells in phosphate-deplete medium was followed after cyanophage infection, an 18 h delay in cell lysis was observed when compared with a phosphate-replete control. Host cultures grown at two different rates with the same nutritional conditions both lysed 24 h postinfection, ruling out growth rate as the factor in the delay of cell lysis.

While phage adsorption kinetics showed no differences under phosphate-deplete and phosphate-replete conditions, there was an apparent 80% decrease in burst size under the phosphate-deplete growth conditions. When the proportion of infected cells that lysed was determined, 9.3% of phosphate-deplete infected cells lysed, in contrast to 100% of the phosphate-replete cells. This suggested that the majority of the cyanophages established lysogeny in response to the phosphate-deplete conditions. Kokjohn and coworkers supported these findings when they observed that a natural phage isolate exposed to

starved cells developed a pseudolysogenic relationship with a naturally occurring aquatic host strain *Pseudomonas aeruginosa*. Wilson and his coworkers concluded: 'These data strongly support the concept that the phosphate status of the *Synechococcus* cell will have a profound effect on the eventual outcome of phage–host interactions and therefore exert a similarly extensive effect on the dynamics of carbon flow in the marine environment.'

Physiology–Genetics

Khudyakov and Wolk have studied *Anabaena* sp. strain PCC 7120, which is a filamentous cyanobacterium that fixes nitrogen under aerobic conditions. In the presence of oxygen the cells are deprived of fixed nitrogen, heterocysts are formed at semiregular intervals along the filaments and in turn provide a microaerobic environment, ATP and reductant for nitrogenase. It was found that the highly pleiotropic, transposon-generated mutant AB22 of the aforementioned host cell exhibited slow growth, the inability to differentiate heterocysts and resistance to cyanophage A-4 (L). Sequencing of the flanking DNA showed that the transposon had disrupted the *hanA* gene which encodes the *Anabaena* HU protein. Therefore, the histone-like HU protein was essential for cyanophage A-4 (L) sensitivity of *Anabaena* sp. strain PCC 7120. Subsequently, Wolk and coworkers found that a modified lipopolysaccharide (LPS) may prevent cyanophage infection of *Anabaena* vegetative cells and the formation of a functional heterocyst envelope. LPS is a major component of the outer membrane of Gram-negative bacteria and consists of a core polysaccharide, an O polysaccharide and lipid A. The O polysaccharide (e.g. O antigen) was the LPS portion that, when altered via mutation, determined the susceptibility of *Anabaena* sp. strain PCC 7120 to infection by cyanophages A-1 (L) and A-4 (L).

Sarma and Singh, working with cyanophage N-1 and the nitrogen-fixing cyanobacterium *Nostoc muscorum* ISU (ATCC 27893), have isolated and characterized temperature-sensitive (ts) mutants of N-1. Treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was used to generate the mutants. These ts-mutants constitute a class of conditional lethal mutants which behave as lethal at the 40°C restrictive temperature but, when shifted to a permissive 28–30°C temperature, replicate as normal wild-type phages. All the ts-mutants were more sensitive to germicidal ultraviolet light (emission peak at 253.7 nm) and to ethylenediaminetetraacetic acid (EDTA) than was the wild type.

Some other recent work with *Nostoc muscorum* ISU and cyanophage N-1 has followed host–phage interactions under osmotic stress. Osmotic stress (0.1 mol l^{-1} NaCl for 2–8 h) extended the latent period and significantly reduced the burst size. The deployment of an osmotolerant host and betaine improved the virus yield. Additionally, it has been shown for a strain of *Anacystis nidulans* resistant to cyanophage AS-1, that the resistant strain had some degree of protection from ultraviolet B light (at a wavelength of 290–320 nm). Finally, the activity of a temperate marine cyanophage, ms-1 of *Synechococcus* sp. NKBG 042902, was induced by copper. Chromium, lead, cobalt and zinc were not effective as inducers. The results suggested that copper could be a potential trigger for changes in the cyanobacterial population in the marine environment.

Metabolism

Mendzhul and coworkers have published a series of papers which determine the effect that the replication of cyanophage LPP-3 in the filamentous cyanobacterium *Plectonema boryanum* has on the activity of various host enzymes. The activity of glutamate dehydrogenase and glutamate synthetase was intensified in viral infected cells by 135 and 220%, respectively. Isozymes of glutamate synthetase were purified from both infected and noninfected cells. The chemical and physical properties of these isozymes were different, indicating cyanophage development could cause specific modifications of cell enzymes. In a comparable study of proteinases, new proteinases were found to appear immediately after cyanophage adsorption. The level of alanine hydrogenase fell by 50% after viral infection. It was found that this enzyme also varied in its physicochemical properties from a comparable enzyme found for noninfected host cells. Using the host filamentous cyanobacterium *Anabaena variabilis* and cyanophage A-1, the dynamics of the amino acid pool in the host was studied. Two peaks in the amino acid pool occurred after 0 and 2 h of infection. These were postulated to result not only from proteolysis and degradation of cytoplasmic membranes but also from the enhancement of virus-determined processes of amino acid biosynthesis. Many other aspects of changes in metabolism upon viral infection were also studied by this research group.

Concluding Remarks

The cyanobacteria with their procaryotic structure and oxygenic photosynthesis occupy a unique position among living organisms. These characteristics closely relate them not only to the eubacteria, but also to eucaryotic algae and plants. Over the last 5 or 6

years research has continued to investigate the genetics, physiology and metabolism, primarily for cyanophages which replicate in both unicellular and filamentous freshwater cyanobacteria; however, the major impetus of the latest research has shifted to the role cyanophages occupy in marine ecosystems. Cyanophages are of critical importance in that they can cause substantial lysis of primary producer organisms. This in turn can exert an extensive effect on the dynamics of carbon flow in many of the marine environments studied. It is hoped that many of the aspects of the research described here can continue to come together to give a more interconnected and complete understanding of the interactions between cyanophages and cyanobacteria.

See also: **Algal viruses (*Phycodnaviridae*); Phage taxonomy and classification.**

Further Reading

Martin E and Benson R (1988) Phages of cyanobacteria. In: Calendar R (ed.) *The Bacteriophages*, vol. 2, p. 607. New York: Plenum Press.

Mendzhul M, Koltukova N, Lysenko T, Shainskaya O and Perepelitsa S (1995) Effect of reproduction of cyanophage LPP-3 on activity of glutamic dehydrogenase and glutamine synthetase in the cells of cyanobacterium *Plectonema boryanum*. *Ukr. Biokhim. Zh.* 67: 33.

Suttle C and Chan A (1994) Dynamics and distribution of cyanophages and their effect on marine *Synechococcus* spp. *Appl. Environ. Microbiol.* 60: 3167.

Waterbury J and Valois F (1993) Resistance to co-occurring phages enables marine *Synecchococcus* communities to coexist with cyanophages abundant in seawater. *Appl. Environ. Microbiol.* 59: 3393.

Wilson W, Carr N and Mann N (1996) The effect of phosphate status on the kinetics of cyanophage infection in the oceanic cyanobacterium *Synechococcus* sp. WH 7803. *J. Phycol.* 32: 506.

Xu X, Khudyakov I and Wolk P (1997) Lipopolysaccharide dependence of cyanophage sensitivity and aerobic nitrogen fixation in *Anabaena* sp. strain PCC7120. *J. Bacteriol.* 179: 2884.

CYPOVIRUSES (*REOVIRIDAE*)

Serge Belloncik, University of Quebec, Armand-Frappier Institute, Laval, Quebec, Canada

History

The observation of abnormal presence of dense inclusion bodies in the midgut of the silkworm *Bombyx mori* led a Japanese scientist, in 1938, to the first diagnosis of cytoplasmic polyhedrosis viruses (CPVs). Later, it was demonstrated that several economically important insects collected in nature or reared in the laboratory, could be infected by different CPVs. Historically, the prevention of CPV infections was the main research objective. Later, however, important fundamental data in virology as well as in molecular and cell biology, such as on the CAP structure of messenger RNA, host–virus relationship, viral persistence and crystallization of proteins were obtained from researches on CPVs. Recently, the potential of these viruses for insect pest control was also demonstrated.

Geographic and Seasonal Distributions

No particular geographic and seasonal distribution has been noted. CPVs are isolated from insects in various climatic and ecological conditions in different geographical areas and all through the year as long as the insect is physiologically active.

Taxonomy and Classification

The cytoplasmic polyhedrosis viruses (CPVs) have double-stranded (ds) RNA and are members of the *Cypovirus* genus within the *Reoviridae* family. Classification of these viruses is determined according to the electrophoretic pattern of the ten viral dsRNA segments on a 3% polyacrylamide gel. Twelve types of CPVs were originally classified, and more recently, two additional types have been reported. Intra-type virus differentiation can be detected by modification of polyacrylamide concentrations of migration gels. Cypoviruses are named according to the type and the insect host, e.g. *Bombyx mori* CPV1 (BmCPV-1), *Euxoa scandens* CPV5 (EsCPV-5), *Spodoptera exempta* cypovirus 12 (SexmCPV-12) etc.

Properties of the Virion

Occluded and non-occluded virions are identical (**Fig. 1**). The icosahedral virus particle has a diameter of

years research has continued to investigate the genetics, physiology and metabolism, primarily for cyanophages which replicate in both unicellular and filamentous freshwater cyanobacteria; however, the major impetus of the latest research has shifted to the role cyanophages occupy in marine ecosystems. Cyanophages are of critical importance in that they can cause substantial lysis of primary producer organisms. This in turn can exert an extensive effect on the dynamics of carbon flow in many of the marine environments studied. It is hoped that many of the aspects of the research described here can continue to come together to give a more interconnected and complete understanding of the interactions between cyanophages and cyanobacteria.

See also: **Algal viruses (*Phycodnaviridae*); Phage taxonomy and classification.**

Further Reading

Martin E and Benson R (1988) Phages of cyanobacteria. In: Calendar R (ed.) *The Bacteriophages*, vol. 2, p. 607. New York: Plenum Press.

Mendzhul M, Koltukova N, Lysenko T, Shainskaya O and Perepelitsa S (1995) Effect of reproduction of cyanophage LPP-3 on activity of glutamic dehydrogenase and glutamine synthetase in the cells of cyanobacterium *Plectonema boryanum*. *Ukr. Biokhim. Zh.* 67: 33.

Suttle C and Chan A (1994) Dynamics and distribution of cyanophages and their effect on marine *Synechococcus* spp. *Appl. Environ. Microbiol.* 60: 3167.

Waterbury J and Valois F (1993) Resistance to co-occurring phages enables marine *Synecchococcus* communities to coexist with cyanophages abundant in seawater. *Appl. Environ. Microbiol.* 59: 3393.

Wilson W, Carr N and Mann N (1996) The effect of phosphate status on the kinetics of cyanophage infection in the oceanic cyanobacterium *Synechococcus* sp. WH 7803. *J. Phycol.* 32: 506.

Xu X, Khudyakov I and Wolk P (1997) Lipopolysaccharide dependence of cyanophage sensitivity and aerobic nitrogen fixation in *Anabaena* sp. strain PCC7120. *J. Bacteriol.* 179: 2884.

CYPOVIRUSES (*REOVIRIDAE*)

Serge Belloncik, University of Quebec, Armand-Frappier Institute, Laval, Quebec, Canada

History

The observation of abnormal presence of dense inclusion bodies in the midgut of the silkworm *Bombyx mori* led a Japanese scientist, in 1938, to the first diagnosis of cytoplasmic polyhedrosis viruses (CPVs). Later, it was demonstrated that several economically important insects collected in nature or reared in the laboratory, could be infected by different CPVs. Historically, the prevention of CPV infections was the main research objective. Later, however, important fundamental data in virology as well as in molecular and cell biology, such as on the CAP structure of messenger RNA, host–virus relationship, viral persistence and crystallization of proteins were obtained from researches on CPVs. Recently, the potential of these viruses for insect pest control was also demonstrated.

Geographic and Seasonal Distributions

No particular geographic and seasonal distribution has been noted. CPVs are isolated from insects in various climatic and ecological conditions in different geographical areas and all through the year as long as the insect is physiologically active.

Taxonomy and Classification

The cytoplasmic polyhedrosis viruses (CPVs) have double-stranded (ds) RNA and are members of the *Cypovirus* genus within the *Reoviridae* family. Classification of these viruses is determined according to the electrophoretic pattern of the ten viral dsRNA segments on a 3% polyacrylamide gel. Twelve types of CPVs were originally classified, and more recently, two additional types have been reported. Intra-type virus differentiation can be detected by modification of polyacrylamide concentrations of migration gels. Cypoviruses are named according to the type and the insect host, e.g. *Bombyx mori* CPV1 (BmCPV-1), *Euxoa scandens* CPV5 (EsCPV-5), *Spodoptera exempta* cypovirus 12 (SexmCPV-12) etc.

Properties of the Virion

Occluded and non-occluded virions are identical (**Fig. 1**). The icosahedral virus particle has a diameter of

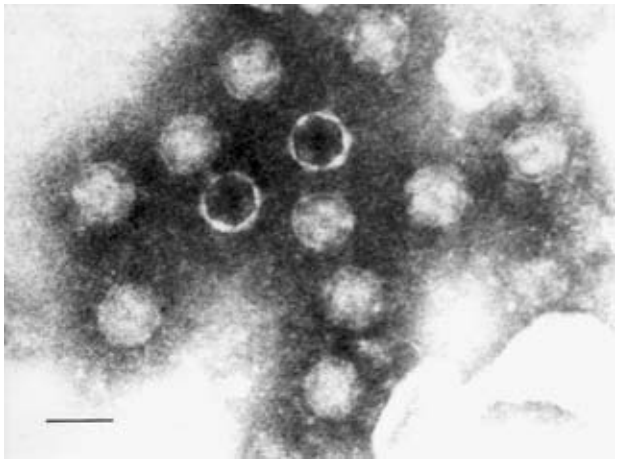


Figure 1 Electron micrographs of negatively stained cytoplasmic polyhedrosis virus particles. Bar = 70 nm.

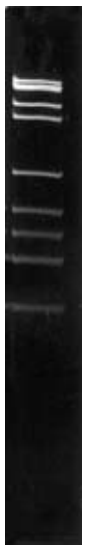


Figure 2 Electrophoretic pattern of *B. mori* CPV (type 1) RNA (7.5% polyacrylamide gel).

50–70 nm and one hollow spike located on each of the 12 vertices of the shell. The absence of a double-shelled structure differentiates the capsid of CPV from other *Reoviridae*. The molecular weight of the virus particles is approximately 50×10^6. The buoyant density in CsCl is 1.44 and 1.30 g cm^{-3} for the virion and empty viruses, respectively. The $s_{20,w}$ is approximately 420 (virions) and 260 (empty particles). Between 25 and 30% by weight of the virion is composed of three to five proteins (mol.wts 27×10^3 – 151×10^3). A transcriptase not requiring activation with proteolytic enzyme, capping enzymes, nucleotide phosphohydrolase, exonuclease and hemagglutinin are also components of the virus particles.

Properties of the Genome

Ten segments of dsRNA characterize the CPV genome (**Fig. 2**) and represent 25–30% of the weight of the virus. The molecular weights of the segments are from 0.3×10^6 to 2.7×10^6 making a total of 15×10^6. Reassortant between CPVs has not yet been isolated. The genome of different CPVs is stable. However, some others present a high degree of predictable or unpredictable genome variability and instability. A viable 11th 'dwarf gene' which is a deletion of gene 10 has been reported.

The coding assignment of each segment has been studied. Gene 10 codes for the polyhedrin, gene 1 for polymerase and methyltransferase, segments 2, 3, 4 and 6 for structural proteins and segments 5, 7, 8 and 9 for nonstructural proteins.

Results published in recent years have demonstrated that mutations at the N-terminal region of the polyhedrin gene are responsible for changing the shape of polyhedra whereas mutations around the C-terminal portion are related to modification of the crystallization pattern and the intracellular localization of the polyhedra.

The genome content of G+C is 36–42%. The plus strand (mRNA) of each strand is methylated and capped at the 5′terminus. Each segment is transcribed independently having a separate transcription enzyme system. The CPV genome and dsRNA–protein complex were visualized by electron microscopy after disruption of viral particles by physical and chemical treatments.

Properties of Polyhedra

A crystalline matrix protein (polyhedrin) constituting the polyhedra is mainly synthesized late during the viral infection. The spacing of the cubic lattice ranges between 4.1 to 7.4. The molecular weight of the polyhedrin is in the range 25 000–37 000. An alkaline protease associated with polyhedra produced in larvae was reported in cell cultures. The polyhedrin is glycosylated. The polyhedra has a buoyant density in CsCl of 1.28 g cm^{-3} and may occlude from one to several thousand virions. The size and shape (cubic, icosahedral) of the polyhedron varies according to virus strain and the insect cell (**Fig. 3**).

Physical Properties

Ultraviolet radiation inactivates virus infectivity and disrupts the structure of virus particles. The infectivity of CPVs is retained for weeks at 25°C. However, treatment of virions at 80°C or 60°C for 10–15 min or 140–160 min, respectively, inactivates this infectivity. The viral polyhedra as well as virions are resistant to

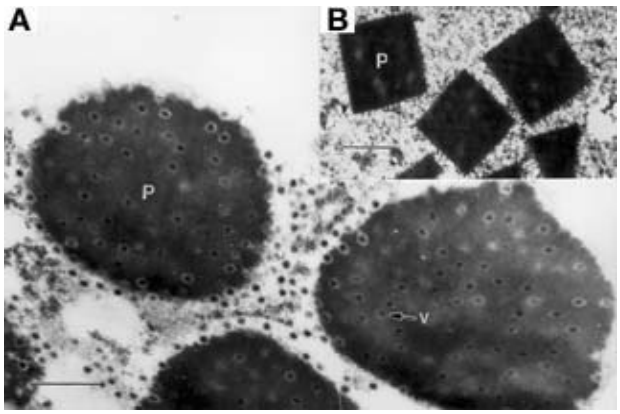


Figure 3 Electron micrgraphs of inclusion bodies of *B. mori* (**A**) and *E. scandens* (**B**) CPVs. V, virion; P, polyhedra. Bar = 2 μm.

low temperatures. The infectivity of polyhedra and virions is retained for many years at −20°C. The enzymatic activity is not affected by repeated freezing and thawing. The virus particles are resistant to ether, trypsin, chymotrypsin, RNase A, DNase, phospholipase C and deoxycholate and are disrupted by treatment with sodium dodecyl sulfate.

It is important to note that several physical as well as chemical factors which reduce the viral infectivity by acting on the integrity of the virus particles could promote and activate CPV infection in insects.

Evolution

No systematic studies have been conducted on the evolution of cytoplasmic polyhedrosis viruses. As stated here, the structure of the CPV virus particle differs from those of vertebrate or other insect reoviruses. According to some speculation on the evolution of viruses, the polyhedrin could be equivalent to the second capsid of reovirus particles or the second reovirus capsid could be a residue of CPV polyhedrin after evolution. Numerous CPVs are noted in nature and several strains of one CPV such as *B. mori* CPV are documented. Some CPV genomes are stable but others demonstrate predictable and arbitrary genomic deletions as well as the appearance of a viable dwarf gene after passages in the insect of some other CPVs. Mutations may explain the evolution of CPVs. The isolation of more than 10 dsRNA segments from insects demonstrates that mixed infections of larvae with several CPV types are frequent in nature. Genetic re-assortment of viral RNA segments between CPVs could be as for other reoviruses a plausible mechanism but it has not been demonstrated during the evolution of cytoplasmic polyhedrosis viruses.

Serologic Relationship

No serological classification of CPVs is available. The classification of cytoplasmic polyhedrosis viruses into types and intratypes is based on the electrophoretic pattern of the RNA segments. No antigenic relationship has been demonstrated between viruses belonging to distinct types using polyclonal and monoclonal antibodies and different serological techniques, but crossreactions between CPVs from a same intratype can occur.

Host Range, Virus Propagation and Epidemiology

Cytoplasmic polyhedrosis viruses have a wide host range and have been isolated from more than 250 species of insects, such as Lepidoptera, Diptera, Hymenoptera and Coleoptera. A CPV of one insect can replicate in other insect species. One CPV strain may therefore be propagated by different hosts. Mixed infections of larvae with several CPV types or strains have been noted in nature. Moreover, insect infection with an exogenous CPV could activate replication of an endogenous CPV strain. However, no crossinfection between insect genera has been documented. CPV infection of invertebrates other than insects and the crustacean *Simocephalus expinosus* has not be noted. Vertebrates, plants or prokaryote organisms are also not susceptible to CPV infection.

The transmission of the viral infection from one larva to another occurs mainly through contaminated food plants. The viral polyhedra are excreted in soil or on plants with the feces or cadavers of the infected larvae. Another mode of transmission of virus infection in nature is by spoilage of the egg surface laid by contaminated females. The polyhedra are ingested by larvae when feeding on contaminated plants or when hatching from spoiled eggs. On some occasions, the dissemination of the viral polyhedra could be achieved by birds, wind, running water or parasites. Latency of CPV in insects and vertical transmission of CPV through the eggs are suspected in the transmission from one generation to another and the persistence of the viral infection in insect populations (see later in this entry).

Morphogenesis and Cytopathology

The liberation of the virions from the polyhedra occurs in the insect midgut by the action of alkaline juice. Approximately 3 h after adsorption of the virus to the gut epithelium and penetration in the cell, new virions are observed in the cytoplasm. Small micronet structures represent the virogenic stroma or viro-

plasms and are the first sign of viral infection. Many viroplasms fuse in structures where the RNA, thin filaments of proteins and granules represent the site of assemblage of capsids, core and virions. It is assumed that the dsRNA is associated with subviral cores. Similar evolution is noted if the virions are injected intraperitoneally into the insect. Crystallization of the polyhedrin occurs about 15 h post-infection when a certain amount of this protein is synthesized. No incorporation of material other than homologous virus particle is noted. The signal that controls the start of polyhedrin crystallization is still unknown. Antigenic modification of the polyhedrin, as earlier reported, or a critical quantity of polyhedrin may be the signal. The insect cell could be involved in the initiation of the crystal formation since abnormal crystallization of expressed polyhedrin in *Escherichia coli* has been obtained. The presence of virions is not essential for normal formation of polyhedra, in contrast with previous assumptions, since no virus is present in the nuclear polyhedra of the A strain of *B. mori* CPV. Moreover, normal crystallization of CPV polyhedrin and formation in the nucleus of polyhedra containing no virus are obtained following the expression of *E. scandens* CPV polyhedrin gene by the *B. mori* baculovirus expression vector.

The shape of the polyhedra depends on different factors. Polyhedra of some CPVs, such as those of *E. scandens* and *Trichoplusia ni* are 99% spherical in larvae but 100% cubical in cell culture. The polyhedra of the A strain of *B. mori* CPV are cubical in shape in both the midgut and cell culture, whereas those of strain H of *B. mori* and *H. armigera* are spherical and, on rare occasions, cubical in any cell system *in vivo* or *in vitro*. Several reports describe the presence of a proportion of malformed polyhedra and fragmentation of inclusion bodies during a normal viral infection. In addition, several polyhedra shapes were obtained when abnormal crystallization of polyhedrin occurred in CPV-infected cells that had been gamma irradiated or treated with fungal toxin.

Therefore, it is strongly believed that the structure and shape of the inclusion body crystal as well as the crystallization of the polyhedrin could in some cases be controlled by the virus and in some others by the cell or some unknown interaction mechanism between the virus and the cell. There is a close interrelationship between the host cell and the CPV strain. Different cell culture conditions that influence the cell metabolism and other cellular factors are crucial for normal crystallization of the polyhedrin. A comparison of results obtained from *in vivo* and *in vitro* viral infections leads to the conclusion that the cubical-shaped polyhedra represent the final stage of maturation of the inclusion bodies when optimal conditions are met and no external and internal factors interrupt the crystallization process. The other shapes observed can be considered as intermediate forms between the mature polyhedra and the pro-polyhedron stage.

At an advanced stage of CPV infection, the midgut is hypertrophied and milky-white in color. The cytoplasm is filled with polyhedra, 0.5–10 μm, containing one to more than thousands of virions. Usually, the inclusion bodies of the posterior part of the midgut are smaller than those of the anterior part. The size and the shape of the polyhedra are also variable according to the location and the CPV strain. Icosahedral, hexahedral, pyramidal, hexahedral, acicular/hexahedral, acicular/conglomerate, acicular and amorphous shapes were described for the strains I, H, P, A, B, B_1, B_2, C_1 and C_2 respectively. Locations of the polyhedrin are strictly cytoplasmic for I, H and P strains, strictly nuclear for A strain and both cytoplasmic and nuclear for the remaining strains. The complete viral morphogenesis is noted only in the midgut cells of the insect and almost exclusively in cylindrical cells. Goblet and differentiated cells are sometimes infected. Variable amounts of non-occluded virions are also noted in the cell cytoplasm at the end of the viral morphogenesis. The infected cells are released in the feces and contaminate by means of the food other larvae. Concomitant passage of non-occluded virus particles to newly generated cells contribute to persistence of the CPV infection in the larva and the amplification of the virus. If the larvae and pupae survived from infection, viral particles can be detected in these insects. Despite the restriction of complete viral infection to the midgut cells, the virions can be detected in the hemolymph of the larvae. However, the cytopathology is limited to the midgut cells where, except for the A, B and C strains, changes are not detected in the nuclei. In the cytoplasm, abundant endoplasmic reticulum and microvilli are degraded.

More information on the CPV morphogenesis was obtained in infected cell cultures of different origins. In particular CPV infection is characterized by no shut-off of cellular protein synthesis and no lysis of cells. Moreover, it has been demonstrated that interactions between the viral genome and the cell culture conditions could influence the crystal formation and shape of the polyhedra.

Transmission and Persistence of CPV Infection

The posterior portion of the midgut is infected first followed by the anterior part and at an advanced stage of infection, the cytoplasm of epithelial midgut

cells. When the cells are filled with polyhedra, diarrhea occurs. Infected cells desquame and viral polyhedra are excreted in insect feces and in this way the viral infection is propagated among the larvae. In the meantime non-occluded virus particles pass through plasma membrane, from infected to newly differentiated regenerated cells. This mechanism allows the persistence and amplification of the virus in insects. CPV are also transmitted from one generation to another. This vertical transmission is partially understood. Infected adults can propagate the viral polyhedra and contaminate the eggs. However, numerous data suggest trans-ovum transmission as well as latency of the CPV. Several physical (temperature, UV), chemical (formalin, diet components), stress or genetic factors which promote outbreaks of cytoplasmic polyhedrosis virus infections have permitted the further investigation of more mechanisms of viral propagation and persistence.

In insect tissue culture no lytic effect of CPV replication has been noted. Infected cell lines can be maintained *in vitro* for several months without lysis of the cells. Foci of CPV-infected cells are often noted as the result of non-occluded virus propagation from one cell to an adjacent cell and multiplication of infected cells. Persistence of viral infection in a cell line has been proved for some CPVs. *Heliothis armigera* CPV infection was detected for more than 100 passages during which time polyhedrin was continuously expressed and polyhedra were formed.

Replication

The complete replication cycle of CPVs can be demonstrated in susceptible insects and various insect cell cultures. Virions contain transcriptase which is not sensitive to actinomycin D. This enzyme acts on the viral dsRNA and no proteolytic or heat treatment of the virions is required. No replication is noted in vertebrates. Other information on the biochemistry of CPV replication has also been obtained from *in vitro* experiments.

When nucleic acid replication is investigated in infected larvae, single-stranded (ss) RNA (15 and 22S) related to the mRNA transcripts of the viral genome is synthesized. CPV transcriptase is active only in intact virions. When mRNA synthesis occurs, it has been suggested that the genome strand moves to the spike during which the mRNA is produced and excreted through the spike. The virion is able to synthesize several segments of RNA simultaneously. Control of the quantity of each polypeptide is achieved by a higher transcription rate of the smaller segments. Transcription is stimulated by a methyl-group donor, *S*-adenosylmethionine (SAM), and 'cap' formation requiring other enzymes present in CPV particles is mandatory for further synthesis of CPV mRNA. Limitation of replication and mRNA synthesis are therefore independent of the cellular enzymes. However, CPV replication may somehow be limited and controlled by the availability of nucleotide triphosphate and SAM in the cell.

The mechanism of genomic RNA synthesis and assembly of dsRNA with proteins is not known in detail. mRNA could, as found for other *Reoviridae*, serve as a template for the synthesis of the complementary strand. Active synthesis of viral dsRNA (15 and 22S) is detected in infected midgut and some reports have stated that viral RNA synthesis takes place in the nucleus. These latter observations have still to be confirmed.

Kinetic studies of protein and antigen synthesis conducted in either larvae or cell cultures give similar results. Viral antigens are detected as early as 4–8 h after infection and the polyhedrin is synthesized 9 h after infection. In a cell culture system, seven viral polypeptides are present after 24 h of CPV infection. Polyhedrin is produced later than the other proteins and increases from 8 to 96 h after infection and this production rate remains high even if the infected cells are subcultured. It is suggested that regulation of polyhedrin viral mRNA synthesis may occur. However, it has also been demonstrated that infected cells are constantly reinfected by the virus and that a number of viral amplification cycles occur. This could also contribute to the constant and persistent increase in polyhedrin synthesis. Nucleic acid and protein metabolism of CPV-infected larvae is affected. However, no shut-off of host protein synthesis occurs in the cell culture system during viral replication despite excessive viral protein synthesis in the cells.

Results of experiments using cell culture systems complement our knowledge on CPV replication and demonstrated the complexity of the multiplication of these viruses. To conclude this section, it is important therefore to point out that CPV replication is dependent on several external and cellular factors which have different enhancing or inhibitory effects on viral synthesis. CPV replication is optimal between 25° and 28°C, and temperatures above 31°C are detrimental. For instance, a temperature of 37°C inhibits all viral synthesis and this is most probably due to inactivation of the transcriptase. Interesting features are observed at the low incubation temperature of 4°C: viral infectivity is not affected and, moreover, dsRNA is synthesized but without any expression of polyhedrin and viral proteins.

Mixed infection by different types of CPV or insect viruses is a frequent phenomenon. Interference between the replication of two CPV types is observed

but co-infection of a single cell by two types of CPV is also found. On the other hand, a large increase in polyhedrin synthesis is the result of simultaneous replication of CPV and the chilo iridescent virus (CIV). Therefore more investigations are needed for a better understanding of interactions at the molecular level between CPV and other insect virus replication steps.

Several lepidopteran cell lines support the entire replication cycle of different CPVs. Internalization of CPV particles was demonstrated in different insect or vertebrate cell lines. Abortive viral replications were also demonstrated. We must, in the light of the new information on CPV replication, reconsider the criteria for detecting CPV infection. The manifestation of different viral synthesis and replication steps rather than the presence of polyhedra may be a more appropriate approach to the assessment of the viral host range and specificity.

Pathogenicity: Virus–Host Interactions

The pathogenicity for an insect of CPVs depends on different factors. Among these factors, the virus strain and the insect are the most important. In addition, other environmental factors influence the viral pathogenicity and the nature of the virus–host interactions. Several CPVs are extremely and more infectious than other insect viruses, such as the baculoviruses. The lethality of CPVs is also variable and depends on the virus strain. Some strains are highly lethal. However, it is a characteristic of CPV infection to be chronic rather than lethal. It is important to point out that the lethality of a particular CPV may be different in laboratory conditions from that in nature where the insect is subjected to numerous environmental stresses.

In the insect, antagonism between infective CPV strains either of the same type or one inactivated and one infective have been demonstrated. Furthermore, during mixed CPV infection the polyhedra of each strain are located in different cells. However, in cell culture infection of a single cell by two different CPVs has been reported. It is not clear how the interaction between CPV strains occurs. Competition at the cell receptors or metabolic pathway level could explain the antagonism effects.

If the virions (non-occluded or alkali-liberated from the polyhedra) are injected into the hemocele of the larvae, the pathogenicity of CPV is the same as for the ingested polyhedra. Despite the presence of the virion in the hemolymph, the complete infection is located in the columnar cells and on rare occasions in the goblet cells. After differentiation of the regenerative cells located in the basal region, these cells are susceptible to complete CPV. Pathogenicity of CPV for the embryo has also been demonstrated.

Insect resistance to CPVs has been documented. As for baculoviruses, old larvae are less susceptibile to CPVs. Molting has also been demonstrated to have an inhibitory effect on the development of CPV. Research using the silkworm showed that two strains of the silkworm could be resistant or susceptible to a CPV infection. The resistance was demonstrated to be under either a multifactorial genetic or a dominant major gene control system. In some rare instances resistance to CPV infection was acquired following repeated application of the virus. Several exogenous factors can affect the pathogenicity and the virus–host relationship. Among these factors treatment of larvae with different chemicals (formalin, chemical insecticides, H_2O_2, disodium salts, dietary components), radiations and low temperatures enhance the susceptibility of the insects to CPV. However, microbial toxins and high temperature reduce the incidence of the virus in insect colonies.

Typical features commonly related to CPV pathogenicity are the alterations of insect functions. Among the metabolic alterations, are decreases in the amounts of proteins and nucleic acid of CPV-infected hemolymph. Amino acid levels decrease except for the glycine. In infected midgut, uric acid and ammonia increase. A reduction of midgut pH was also noted.

Nucleic acid, mostly RNA synthesis in infected cells has been studied by several authors. No major modification of nucleic acid DNA synthesis in infected and healthy midgut cells was demonstrated. Mitochondrial and ribosomal RNA are decreased.

The integrity of the midgut is crucial for the absorption of nutrients. Therefore, the metabolic functions of different organs and tissues may be affected, as a consequence of nutritional deficiency although complete normal viral replication occurs only in the midgut.

Physiological alterations occurring in CPV-infected insects are the consequence of the metabolic modifications. Higher virus concentrations combined with early larval stage result in greater effects. Extended instar duration, decrease of length of the larvae and the weight of the pupae as well as less lipid and proteins are common. Even though the larval stages are extended the damages caused by the infected insects are lower. The breakdown of lipid reserves of CPV-infected larvae results in an increase in oxygen uptake. Adult and larval pigmentations are reduced either because of tyrosine deficiency resulting from less amino acid being available in infected hemolymph or because of hormonal imbalance which affects tyrosinase activity. Moreover, numerous pupae are deformed and the surviving adults have difficulty in

emerging. Malformation of adults reduces the mating ability. Poorly developed oocytes have been noted, and fecundity and the viability of the offspring are reduced.

Interestingly, a consequence of the metabolic and physiologic alterations is the reaction of CPV-infected insects to other pathogens and detrimental environmental conditions. It was demonstrated by several authors that CPV-infected larvae, compared to healthy populations, are less resistant to hibernation which results in a high larval mortality rate during the winter.

Furthermore, synergistic interactions between CPV and chemical insecticide (permethrin) as well as CPV and baculoviruses are the result of a decrease of the resistance of CPV-infected insects to these chemicals and pathogens. When bacteria such as *Pseudomonas maltophilia, Bacillus subtilis, E. coli, Staphylococcus epidermis* are present as contaminants the physiological alterations and mortalities noted when insects are infected by CPV are enhanced significantly. Synergistic interactions have also been shown in relation to insect mortality between CPV and *Bacillus thuringiensis* treatments both in the laboratory and in the field.

Prevention, Control and Practical Applications

The cytoplasmic polyhedrosis viruses have been studied intensively mainly because they were considered as noxious microorganisms and contaminants of reared insect colonies. Therefore, most efforts were first given toward the prevention and control of CPV infections. Cross-protection of insects with inactivated CPVs and the exposure of larvae at high temperature have been shown successfully to control the viral infection. Moreover, the provision of optimal nutritional and environmental conditions contribute to maintaining healthy colonies by controlling the CPV expression in insects. However, despite these demonstrations and the possibility of a vertical transmission, the systematic disinfection of eggs and the equipment with chemicals such as hypochloride and formalin solutions are to date the only techniques used to prevent CPV propagation in reared insect colonies.

On another hand, development of viral insecticides has been based on several characteristics of the cytoplasmic polyhedrosis viruses. Numerous virus particles are protected inside the polyhedra from detrimental conditions of the environment. The CPVs could be lethal, are highly infectious, persistent and induce physiological and metabolic alterations in insects. Furthermore, combinations of CPV with other microbial pathogens or chemicals and environmental factors contribute to a decrease of insect populations over one to several generations. However, CPVs are slow-acting viruses and most of the field applications were conducted in forests where a certain level of damage is tolerable. Considering the viral persistence their use in stable ecosystems such as the forests is the more suitable stable ecosystem to be protected.

The taxonomic relatedness of CPVs to vertebrate viruses, such as reoviruses, could represent a handicap for their use as viral insecticides. Therefore, biosafety testing of CPVs has been conducted in several laboratories. Numerous vertebrate hosts, such as mice, rabbits, avian embryos, fish etc., as well as on vertebrate and mosquito cell lines demonstrated no viral pathogenicity or replication of CPVs for these nontarget organisms.

The *Dendrolimus spectabilis* (DsCPV) has been commercialized, as Matsukemin product, in Japan and the efficacy of several other CPVs such as those of *Lymantria dispar* and *fumida, Thaumetopoea pityocampa, Choristoneura fumiferana, Dendrolimus pini* and *Trichoplusia ni* have been shown to have promise as viral insecticides when used alone or combined with *B. thuringiensis*.

Future

Since the first isolation of cytoplasmic polyhedrosis viruses as contaminants of beneficial insects their importance has been demonstrated in fundamental research in the fields of biology, virology, biochemistry and molecular biology as well as in a variety of applied research programs. The virulence of CPV combined with important physiological and metabolic disturbances produced in the insect as a consequence of CPV infection has renewed interest in CPVs as biological control agents. Furthermore, the peculiar interactions between the insect cell and the CPV, the interaction of the viral infection and different endogenous as well as exogenous factors for an insect cell host and latency phenomena could serve in the future as important and interesting models for the study of other microbial pathogenesis.

See also: **Reoviruses (*Reoviridae*): General features, Molecular biology, Plant reoviruses.**

Further Reading

Aruga H and Tanada Y (eds) (1971) *Cytoplasmic polyhedrosis virus of the silkworm*. Tokyo: University of Tokyo Press, 234 pp.

Belloncik S (1996) Interactions of cytoplasmic polyhedrosis viruses with insects. *Adv. Insect Physiol.* 26: 235.
Belloncik S and Mori H (1998) Cypoviruses. In Ball and Miller (eds) *The Insect Viruses*, pp. 337–369.
Hukuhara T and Bonami JR (1992) Reoviridae. In: Bonami JR and Adams JR (eds) *Atlas of Invertebrate Viruses*, p. 394. Boca Raton; CRC Press.
Payne CC (1981) Cytoplasmic polyhedrosis viruses. In: Davidson EW (ed.) *Pathogenesis of Invertebrate Microbial Diseases*, p. 61. Allandheld, Osmun, Totowa, New Jersey.
Payne CC and Mertens PPC (1984) Cytoplasmic polyhedrosis viruses. In: Joklik WK (ed.) *The Reoviridae*, p. 425. New York: Academic Press.

CYTOKINES AND CHEMOKINES

Diane E. Griffin, Department of Molecular Microbiology and Immunology, Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland, USA

Introduction

Cytokines are small soluble proteins produced by a wide variety of cells and are important mediators of cell-to-cell communication. Production of these biologically potent proteins is particularly associated with generation of the innate and acquired immune responses. Cytokines regulate both the initiation and maintenance of immune responses and some also have direct antiviral effects. Chemokines (*chemo*tactic cyto*kines*) regulate infiltration of leukocytes into infected tissues. Both cytokines and chemokines share the properties of pleiotropy and redundancy. Each cytokine and chemokine has more than one function and any one function can be performed by more than one of these soluble factors.

Cytokines can act at a distance, but usually act in the areas in which they are produced and mediate their effects by binding to small numbers of high affinity receptors on responding cells. Cytokine receptors have a modular design consisting of two or more chains, each with a single transmembrane-spanning domain. Individual components may participate in formation of the receptor for more than one cytokine. Chemokine receptors are single G-protein-coupled molecules with seven transmembrane domains. The biologic function of many cytokines and chemokines is regulated in part by a short half-life for both the protein and the mRNA, but also by the production of circulating cytokine inhibitors such as soluble forms of the cytokine receptor or biologically inactive forms of the cytokine itself. A number of viruses, particularly in the herpesvirus and poxvirus families, encode inhibitory homologues of cytokines, chemokines or their receptors, attesting to their importance for antiviral immunity.

Overview of Cytokine Networks (Fig. 1)

Cytokines associated with the innate immune response are those induced early after infection by virus replication *per se*. One of the most important of these early components of the immunologically nonspecific response are type 1 (α and β) interferons (IFNs) which are generally synthesized and released by infected cells. IFN-α/β can induce an antiviral state in surrounding cells and can also influence the characteristics of the subsequent immune response. In addition, phagocytic cells, responsible for processing and presentation of viral antigens to initiate the antigen-specific immune response, produce a variety of cytokines, including IFN-α/β. These antigen-presenting cells, generally dendritic cells and monocyte/macrophages, may or may not be infected or produce infectious virus. Important cytokines and chemokines produced by antigen-presenting cells in the initial phases of the response to many viral infections are tumor necrosis factor (TNF), monocyte chemotatic proteins (MCP), interleukin (IL)-1, IL-6, IL-12 and IL-18. These soluble factors promote the activation of T cells and recruitment of inflammatory cells into areas of virus replication. IL-12 stimulates natural killer (NK) cells to produce IFN-γ, promotes differentiation of CD8+ cells to cytotoxic T lymphocytes, and influences the early development and differentiation of CD4 T cells toward a delayed type hypersensitivity cellular immune response (type 1).

CD4 and CD8 T lymphocytes are the effectors of the specific cellular immune response and function primarily through synthesis of cytokines. T cells do not produce cytokines constitutively, but upregulate expression in response to stimulation through the T cell receptor. CD8 T cells are stimulated by viral

Belloncik S (1996) Interactions of cytoplasmic polyhedrosis viruses with insects. *Adv. Insect Physiol.* 26: 235.
Belloncik S and Mori H (1998) Cypoviruses. In Ball and Miller (eds) *The Insect Viruses*, pp. 337–369.
Hukuhara T and Bonami JR (1992) Reoviridae. In: Bonami JR and Adams JR (eds) *Atlas of Invertebrate Viruses*, p. 394. Boca Raton; CRC Press.
Payne CC (1981) Cytoplasmic polyhedrosis viruses. In: Davidson EW (ed.) *Pathogenesis of Invertebrate Microbial Diseases*, p. 61. Allandheld, Osmun, Totowa, New Jersey.
Payne CC and Mertens PPC (1984) Cytoplasmic polyhedrosis viruses. In: Joklik WK (ed.) *The Reoviridae*, p. 425. New York: Academic Press.

CYTOKINES AND CHEMOKINES

Diane E. Griffin, Department of Molecular Microbiology and Immunology, Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland, USA

Introduction

Cytokines are small soluble proteins produced by a wide variety of cells and are important mediators of cell-to-cell communication. Production of these biologically potent proteins is particularly associated with generation of the innate and acquired immune responses. Cytokines regulate both the initiation and maintenance of immune responses and some also have direct antiviral effects. Chemokines (*chemo*tactic cyto*kines*) regulate infiltration of leukocytes into infected tissues. Both cytokines and chemokines share the properties of pleiotropy and redundancy. Each cytokine and chemokine has more than one function and any one function can be performed by more than one of these soluble factors.

Cytokines can act at a distance, but usually act in the areas in which they are produced and mediate their effects by binding to small numbers of high affinity receptors on responding cells. Cytokine receptors have a modular design consisting of two or more chains, each with a single transmembrane-spanning domain. Individual components may participate in formation of the receptor for more than one cytokine. Chemokine receptors are single G-protein-coupled molecules with seven transmembrane domains. The biologic function of many cytokines and chemokines is regulated in part by a short half-life for both the protein and the mRNA, but also by the production of circulating cytokine inhibitors such as soluble forms of the cytokine receptor or biologically inactive forms of the cytokine itself. A number of viruses, particularly in the herpesvirus and poxvirus families, encode inhibitory homologues of cytokines, chemokines or their receptors, attesting to their importance for antiviral immunity.

Overview of Cytokine Networks (Fig. 1)

Cytokines associated with the innate immune response are those induced early after infection by virus replication *per se*. One of the most important of these early components of the immunologically nonspecific response are type 1 (α and β) interferons (IFNs) which are generally synthesized and released by infected cells. IFN-α/β can induce an antiviral state in surrounding cells and can also influence the characteristics of the subsequent immune response. In addition, phagocytic cells, responsible for processing and presentation of viral antigens to initiate the antigen-specific immune response, produce a variety of cytokines, including IFN-α/β. These antigen-presenting cells, generally dendritic cells and monocyte/macrophages, may or may not be infected or produce infectious virus. Important cytokines and chemokines produced by antigen-presenting cells in the initial phases of the response to many viral infections are tumor necrosis factor (TNF), monocyte chemotatic proteins (MCP), interleukin (IL)-1, IL-6, IL-12 and IL-18. These soluble factors promote the activation of T cells and recruitment of inflammatory cells into areas of virus replication. IL-12 stimulates natural killer (NK) cells to produce IFN-γ, promotes differentiation of CD8+ cells to cytotoxic T lymphocytes, and influences the early development and differentiation of CD4 T cells toward a delayed type hypersensitivity cellular immune response (type 1).

CD4 and CD8 T lymphocytes are the effectors of the specific cellular immune response and function primarily through synthesis of cytokines. T cells do not produce cytokines constitutively, but upregulate expression in response to stimulation through the T cell receptor. CD8 T cells are stimulated by viral

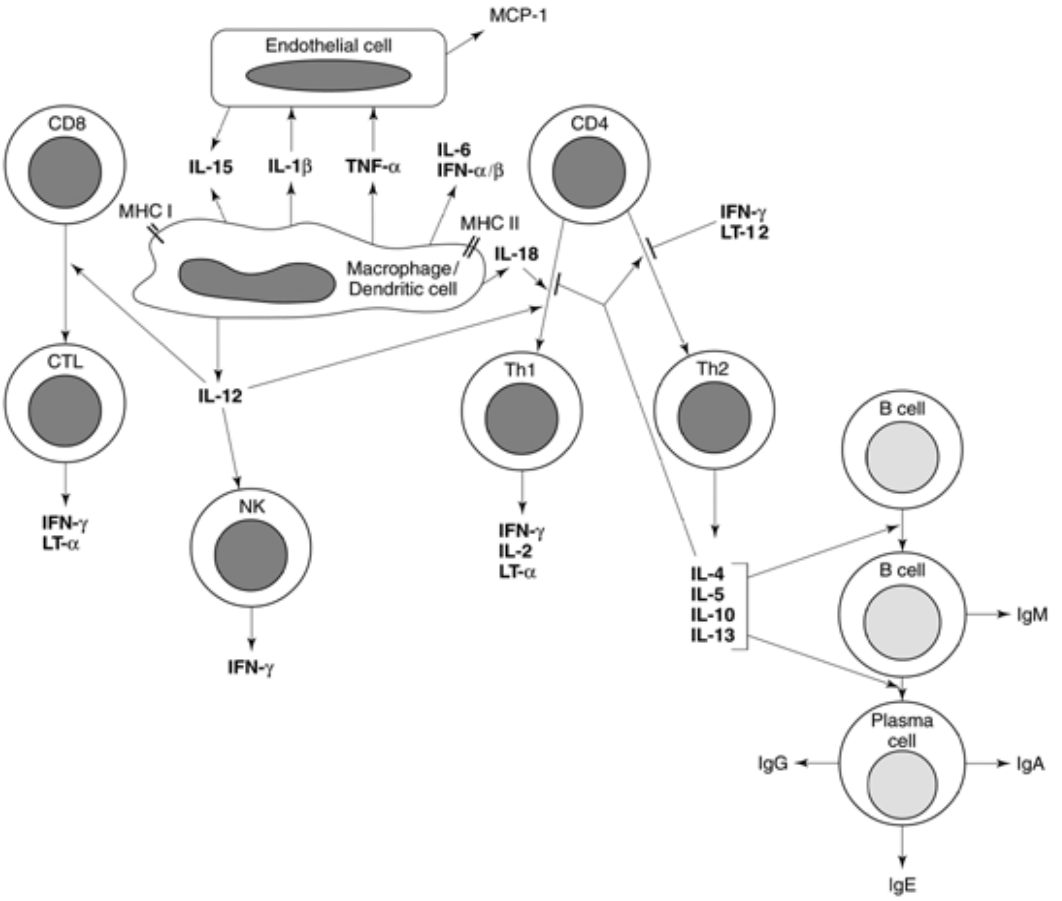


Figure 1 Overview of cytokine networks important for innate and acquired antiviral immune responses.

antigens presented in association with class I major histocompatibility (MHC) antigens. This type of stimulation is most likely to occur if the virus replicates in the antigen presenting cell since antigens presented in association with MHC class I must be processed by proteasomes in the cytosol. CD8 T cells often have cytotoxic activity, but also produce substantial amounts of IFN-γ and lymphotoxin (LT). CD4 T cells are stimulated by viral antigens presented in association with class II MHC antigens. Processing of antigens for presentation in association with MHC class II occurs through an endosomal pathway, therefore, virus replication is not required. Activated CD4 T cells produce IL-2, a T cell growth factor which supports proliferation of both CD4 and CD8 T cells.

With further development of the virus-specific immune response, CD4 T cells tend to differentiate into type 1 or type 2 helper T (Th) cells. Type 1 CD4 T (Th1) cells develop under the influence of IL-12 and produce primarily IFN-γ and TNF-β (LT-α) and are associated with classic delayed type hypersensitivity or cellular immune responses. Type 2 CD4 T cells develop under the influence of IL-4 and produce primarily IL-4, IL-5, IL-10 and IL-13 and are associated with strong antibody responses to viral antigens. Cytokines produced by T cells are necessary for B cell development and for the switch from B cells producing IgM to plasma cells producing more mature forms of immunoglobulin (Ig) such as IgG, IgA and IgE. Both Th1 and Th2 responses are associated with mononuclear cell infiltrates into areas of virus replication, but the cytokines being produced in those sites are distinct.

Brief Description of Cytokine Families

TNF

The TNF family has three members: TNF-α, TNF-β/LT-α and LT-β. These cytokines have approximately 30% homology and share the same cellular receptors. Activated macrophages are the major source of TNF-α and T cells are the major source of lymphotoxin.

TNF-α is synthesized as a proprotein (26 kDa) which is membrane-bound and cleaved by a specific multi-domain cell-surface metalloprotease to yield a 17 kDa protein. The active form of the soluble protein is a cone-shaped trimer which can bind to either of two TNF receptors (TNFR1, p55 and TNFR2, p75). Binding of TNF to TNFR1 induces NFκB and can trigger apoptosis. Homotrimers of LT-α also bind to TNFR1 and TNFR2, but LT-β reacts with a separate member of the TNF receptor family, the LT-β receptor. TNF interacts with a wide variety of cells systemically and locally and induces proinflammatory cellular responses such as expression of adhesion molecules, release of cytokines, chemokines and procoagulatory substances, synthesis of acute phase proteins and production of fever. TNF also has some antiviral activity and is an important early participant in the immune response to infection. In bacterial infections, TNF is one of the mediators of endotoxin-induced (septic) shock. TNF and LT also play an important role in development of lymphoid tissues since mice with deletions of these genes do not have the normal lymphoid architecture.

IFN-γ

IFN-γ is a glycosylated protein of 25 kDa that is produced by NK cells and by type 1 CD4 and CD8 T cells (immune IFN). Although IFN-γ has some antiviral activity it is structurally unrelated to IFN-α/β and uses a distinct receptor. However, IFN-γ shares some components of the intracellular signaling pathways for type 1 IFN. The biologically active form of IFN-γ is a dimer. A primary role for IFN-γ is the activation of macrophages increasing phagocytosis, tumoricidal properties and intracellular killing of pathogens, particularly bacteria and fungi. IFN-γ induces macrophage production of a variety of inflammatory mediators and reactive oxygen and nitrogen intermediates. IFN-γ increases expression of high-affinity immunoglobulin F_c receptors on phagocytes, which increases recognition of opsonized microorganisms by these cells. IFN-γ also increases expression of MHC antigens by macrophages and this facilitates antigen presentation to T cells. The IFN-γ cell surface receptor is composed of two chains, IFN-γR1 (α) and IFN-γR2 (β). The α (R1) chain is sufficient for binding, but the β (R2) chain is required for signaling and receptor complex formation. T cell responsiveness is regulated by receptor expression. Both Th1 and Th2 cells express IFN-γR1, but only Th2 cells express IFN-γR2. Therefore, Th1 cells produce, but do not respond to, IFN-γ whereas Th2 cells respond to, but do not produce this cytokine. Macrophages express both receptor chains and are a primary target of IFN-γ activity.

IL-1

The IL-1 family has three members: IL-1α, IL-1β and IL-1 receptor antagonist (IL-1Ra). IL-1α and IL-1β have only distantly related amino acid sequences, but similar structure, and recognize the same receptors. IL-1Ra binds the IL-1 receptor without transducing a signal and this binding blocks the activities of IL-1α and IL-1β. Activated monocytes and macrophages are the major source of IL-1, although many other cells can produce this cytokine. IL-1β is synthesized as a precursor protein of 31 kDa that is processed by the caspase interleukin-1β-converting enzyme (ICE, caspase-1) to the active secreted 17 kDa form. IL-1 acts systemically, as well as locally, and can produce fever, sleep and anorexia, frequent symptoms of viral infection. Hepatocytes are among the cells that produce IL-1Ra as a part of the acute-phase response to inflammation and infection, presumably to control the effects of IL-1. There are two IL-1 receptors but only IL-1RI transduces an activation signal to responding cells. IL-1RII appears to function as a decoy receptor that regulates IL-1 activity by binding the cytokine without transducing a signal.

IL-2

IL-2 is a unique 15 kDa cytokine produced primarily by activated type 1 CD4 T cells. IL-2 plays an important role in supporting proliferation of activated helper T cells, cytotoxic T cells, B cells, macrophages and NK cells. For stimulated CD4 cells IL-2 acts in an autocrine fashion since Th1 cells can both produce IL-2 and upregulate high affinity IL-2 receptors. The high affinity IL-2R consists of three chains, IL-2Rα, β and γ, which are expressed on the surface of activated T cells. IL-2Rα is a unique protein whereas IL-2Rβ and IL-2Rγ are members of the hematopoietic growth factor receptor superfamily. Members of this superfamily share a 210 amino acid domain in the extracellular region of the molecule. This domain contains a distinct cysteine and tryptophan motif at the N-terminus and a Trp-Ser-X-Trp-Ser motif at the C-terminus. The whole domain is folded into two barrel-like structures with the ligand-binding region between. In addition to the IL-2 receptor this receptor superfamily includes components of receptors for cytokines IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, granulocyte macrophage–colony stimulating factor (GM-CSF), and granulocyte–colony stimulating factor (G-CSF). Signal transduction is through the IL-2Rβ chain. Intermediate affinity IL-2R$\beta\gamma$ receptors are expressed on monocytes and NK cells. IL-2Rγ (γ

constant) also participates in the formation of the multichain IL-4, IL-7, IL-9 and IL-15 receptors. Defects in expression of this molecule lead to severe combined immunodeficiency in humans. Binding of IL-2 to the high affinity receptor leads to T cell proliferation.

IL-4

There are five members of the IL-4 family of cytokines: IL-3, IL-4, IL-5, IL-13 and GM-CSF. These cytokines have approximately 30% protein sequence homology, have similar α-helical structure, are linked in the same chromosomal region and are produced by type 2 CD4 T cells. IL-3 and GM-CSF are two of the four major human myeloid growth factors which also include G-CSF and M-CSF. IL-3 is produced by activated T cells and binds to a heterodimeric receptor. The β subunit of the IL-3R is shared with the receptors for IL-5 and GM-CSF.

IL-4 and IL-13 have similar effects on B cells and monocytes. Both serve as a costimulating factor for B cell proliferation, drive activated B cells into immunoglobulin secretion and, in human B cells, induce class switching to IgG_4 and IgE. IL-4 is regarded as an anti-inflammatory cytokine because it antagonizes the effects of IFN-γ on macrophages and downregulates type 1 CD4 T cell responses. IL-13 induces monocyte proliferation and monocytosis *in vivo*. IL-4 is important for promoting the differentiation of CD4 T cells to type 2 cytokine production. IL-4 and IL-13 are produced by type 2 CD4 T cells. IL-4 is also produced by a subset of CD4 T cells that are $NK1.1^+$, by type 2 CD8 T cells and by mast cells, basophils and eosinophils.

IL-5

IL-5, along with IL-3 and GM-CSF, acts as an eosinophil stimulating factor and its receptor shares a common β chain with these cytokines. The high affinity IL-5R has a unique α chain. IL-5 induces proliferation and differentiation of eosinophil progenitors whereas IL-3 and GM-CSF probably act at earlier stages of development. IL-5 is produced by type 2 CD4 and CD8 T cells and NK cells. IL-5 also induces antigen-stimulated B cells to differentiate into immunoglobulin-secreting plasma cells and enhances secretion of IgA.

IL-6

IL-6 is a member of a family of cytokines and neuronal differentiation factors that includes leukemia inhibitory factor, oncostatin M, IL-11 and ciliary neurotrophic factor. The receptors for these cytokines share the transmembrane protein gp130 which transduces the cytokine signal. IL-6 is a functionally diverse cytokine produced by macrophages and monocytes early in the response to infection. It is an important inducer of hepatic synthesis of acute phase proteins, supports proliferation of B cells and has an important role in production of IgA.

IL-7

IL-7 is a 15 kDa protein that is produced primarily by epithelial cells and supports the growth and development of immature B and T cells, but is unnecessary for the development of NK cells. IL-7 is produced by bone marrow stromal cells, thymic epithelial cells, keratinocytes and intestinal epthelial cells and supports lymphocyte development in these locations. In the thymus IL-7 induces rearrangement of germline T cell receptor genes by upregulating expression of the RAG-1 and RAG-2 recombinases. The α chain of the IL-7 receptor is a member of the hematopoietic growth factor family and associates with the IL-2Rγ chain to form a functional high affinity IL-7R.

IL-10

IL-10 is an 18 kDa polypeptide that forms homodimers. It is predicted, on the basis of sequence, to be a member of the 4α-helix bundle family of cytokines. It is produced by a wide variety of cells including type 2 CD4 and CD8 cells, B cells, mast cells, macrophages and keratinocytes. Epstein–Barr virus encodes a viral homologue of IL-10 that is functionally active. The activities of IL-10 are diverse. IL-10 inhibits cytokine production by Th1 cells, NK cells and macrophages. In macrophages it suppresses the synthesis of TNF-α, IL-1α, IL-6, IL-8, GM-CSF and G-CSF while increasing the production of IL-1Ra. In contrast, IL-10 stimulates proliferation and differentiation of activated B cells and T cells and of mast cells. The IL-10R is structurally related to the IFN-γR.

IL-12

IL-12 is a structurally unique heterodimeric cytokine composed of two disulfide-linked 35 kDa and 40 kDa polypeptides. The 40 kDa protein conveys receptor binding activity and is constitutively produced by many cells. Only the heterodimer is biologically active so regulation of synthesis of the 35 kDa chain controls IL-12 activity. IL-12 is produced by macrophages and dendritic cells and stimulates the production of IFN-γ by NK and T cells. It is essential for differentiation of CD4 T cells into Th1 cells. At least two classes of the IL-12 receptor exist. The IL-12R β1 chain is expressed in both Th1 and Th2 cells whereas the β2 chain is expressed only in Th1 cells. Both β1 and β2 are necessary for IL-12 responsiveness and for T cell

differentiation so only potential Th1 cells respond to this cytokine. Both IL-12R β1 and β2 subunits are related to the gp130 group of cytokine receptors. Homodimers of p40 bind with high affinity to the IL-12R, transduce no signal and block the effect of the heterodimer.

IL-15

IL-15 is a novel cytokine functionally related to IL-2, but without significant IL-2 sequence homology. It binds to a receptor with the β and γ chains of the IL-2R, but has its own unique α chain. IL-15 induces T cell proliferation, enhances NK cell function, is a potent T lymphocyte chemoattractant and stimulates production of IL-5. Unlike IL-2 it is produced primarily by monocyte/macrophages and endothelial cells.

IL-18

IL-18, also known as IFN-γ-inducing factor, is an 18 kDa cytokine produced by macrophages. IL-18 shares structural features with the IL-1 family of proteins and functional properties with IL-12. It is synthesized as a proprotein that, like IL-1β, is cleaved by caspase 1 (ICE) to its biologically active form. Like IL-12, IL-18 stimulates IFN-γ production by T cells, NK cytotoxicity and T cell proliferation.

Chemokines and Chemokine Families

The chemokine superfamily includes a large number of structurally related, basic, heparin-binding, small-molecular-weight (8–10 kDa) proteins that have potent chemotactic and immunoregulatory functions. Chemokines attract specific, but overlapping, populations of inflammatory cells and can be divided into subfamilies based on the spacing of the first two of four conserved disulfide-forming cysteine residues. The subfamilies have different biologic activities. CXC or α-chemokines are the primary attractants for neutrophils. CC or β-chemokines modulate responses of monocytes, macrophages, lymphocytes, basophils and eosinophils. Some variations on these two chemokine themes have been reported. For instance, lymphotactin, a γ chemokine, has only one set of cysteines and is chemotactic for lymphocytes. Neurotactin is a CXXXC or δ-chemokine and is unique in that it is a type 1 transmembrane protein with the chemokine domain on top of an extended mucin-like stalk.

α-chemokines

The first CXC chemokine to be identified was platelet factor 4 (PF4). PF4 is stored in the granules of platelets which contain two additional α-chemokines, platelet basic protein and the related connective tissue activating peptide III. The best characterized α-chemokine is IL-8 which is chemotactic for neutrophils. Other α-chemokines with chemotactic activity for neutrophils include macrophage inflammatory protein (MIP)-2, neutrophil activating protein-2, GRO-α, GRO-β, GRO-γ, epithelial cell-derived neutrophil activating protein and granulocyte chemotactic protein-2. Sequence identities between these various family members range from 24% to 46%. Interferon inducible protein (IP)-10 is a structurally similar, but functionally distinct CXC chemokine since it is an attractant for monocytes and T cells rather than granulocytes.

β-chemokines

The first of the monocyte/lymphocyte chemotactic factors to be identified were MIP-1α and MIP-1β. However, the best characterized chemokine is monocyte chemoattractant protein (MCP)-1. MCP-1 is produced by endothelial cells in response to IL-1β and TNF-α and functions as a dimer. MCP-1 attracts and activates monocytes, NK cells, CD4 and CD8 T cells and is a potent histamine-releasing factor for basophils. MCP-2 and MCP-3 are structurally and functionally related chemokines. Other CC chemokines include monocyte chemotactic and activating factor, RANTES and I309. Sequence identities between family members are 29–71%. These chemokines activate adhesion molecules in the integrin family thereby inducing or increasing adhesion to endothelial cells or extracellular matrix proteins further promoting development of an inflammatory response. Two novel, related β chemokines, MCP-4 and eotaxin, are preferential chemoattractants for eosinophils. Eotaxin is produced by epithelial and endothelial cells, often in concert with IL-5.

Chemokine receptors

Chemokine receptors are 7-transmembrane, G protein-coupled molecules that transduce intracellular signals resulting in upregulation and activation of adhesion molecules. In general, the α-chemokine receptors (CXCR) are distinct from the β receptors (CCR). Each receptor can bind multiple chemokines within its class and each chemokine can bind multiple receptors. Therefore, the specificity of cellular attraction to a site and subsequent activation is probably achieved by selective mixtures of chemokines and cellular receptors. These molecules serve as secondary

receptors for entry of human immunodeficiency virus into T cells and monocytes.

See also: **Autoimmunity; Interferons: General features, Therapy of aids and cancer; Pathogenesis: Animal viruses; Vaccines and immune response.**

Further Reading

Boehm U, Klamp T, Groot M and Howard JC (1997) Cellular responses to interferon-gamma. *Annu. Rev. Immunol.* 15: 747.

Brown MA and Hural J (1997) Functions of IL-4 and control of its expression. *Crit. Rev. Immunol.* 17: 1.

Del Prete G (1998) The concept of type-1 and type-2 helper T cells and their cytokines in humans. *Int. Rev. Immunol.* 16: 427.

Dinarello CA (1996) Biologic basis for interleukin-1 in disease. *Blood* 87: 2095.

Sugamura K, Asao H, Kondo M *et al* (1996) The interleukin-2 receptor gamma chain: its role in the multiple cytokine receptor complexes and T cell development in XSCID. *Annu. Rev. Immunol.* 14: 179.

Taga T and Kishimoto T (1997) Gp130 and the interleukin-6 family of cytokines. *Annu. Rev. Immunol.* 15: 797.

Trinchieri G (1998) Immunobiology of interleukin-12. *Immunol. Res.* 17: 269.

CYTOMEGALOVIRUSES (*HERPESVIRIDAE*)

Contents

General Features (Human)

Edward S. Mocarski Jr., Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California, USA

History

Cytomegaloviruses, the prototype members of the *Betaherpesvirinae* subfamily of the *Herpesviridae* family, are widely distributed in nature. All animal species have an associated cytomegalovirus (CMV) which exhibits exquisite species-specificity. These viruses infect for life and are readily isolated from saliva or salivary gland explants, a feature that resulted in the assignments of their earlier name, salivary gland viruses. Intermittent, lifelong shedding in saliva and urine have long revealed a persistent/latent lifestyle typical of all herpesviruses, although CMV recurrence and shedding is not associated with any sort of recurrent disease. CMV infection is typically of little consequence to the host, despite sporadic shedding throughout life: it is only in immunocompromised hosts that the virus is recognized as a major opportunist and a significant cause of disease. The impact of human CMV in allogeneic organ and bone marrow transplant recipients and in patients with acquired immune deficiency syndrome (AIDS) has made this virus the subject of intense study. Immune surveillance plays a critical role in maintaining viral latency and suppressing viral replication. Although reactivation in the immunocompromised host is important, this virus was first recognized because of its etiologic role in congenital disease, and it remains the major infectious cause of deafness in the USA. Infection of the fetus is a consequence of primary infection of the mother and occurs in 0.5–1% of all live births. Neurological damage due to congenital CMV ranges from mild learning or hearing disabilities to a catastrophic central nervous system damage. The involvement in cytomegalic inclusion disease along with the cytomegalic (enlarged cell) cytopathology caused by the virus led to the adoption of the current name for this virus. Although a medically important virus, human CMV is a classic opportunist, with almost all infections remaining subclinical even in highly susceptible populations.

Cytomegaloviruses infecting animal species, such as mice, rats, guinea pigs and rhesus macaques, have been used as surrogates for experimental investigation of viral pathogenesis and the virus–host interaction. They were first recognized early in the twentieth century when guinea pigs were experimentally infected with a filterable agent that caused cytopathol-

Molecular biology; Latency; Pathogenesis: Animal viruses; Persistent viral infection; Transplantation and virus infections; Varicella-Zoster virus (*Herpesviridae*): General features, Molecular biology.

Further Reading

Britt WJ and Alford CA (1996). Cytomegalovirus. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 2493. Philadelphia: Lippincott-Raven.

Chee MS, Bankier AT, Beck S *et al* (1990) Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. *Curr. Top. Microbiol. Immunol.* 154: 125.

Gibson W, Welch AR and Hall MRT (1995) Assemblin, a herpes virus serine maturational proteinase and new molecular target for antivirals. In: Craik C (ed.) *Perspectives in Drug Discovery and Design*, p. 413. The Netherlands: ESCOM Science.

Mocarski ES (1996) Cytomegaloviruses and their Replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology* 3rd edn, p. 2447. Philadelphia: Lippincott-Raven.

Stinski MF (1997) Cytomegaloviruses. *Intervirology* 39: p. 293.

Roizman B (1996) Herpesviridae. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology* 3rd edn, p. 2221. Philadelphia: Lippincott-Raven.

Animal Cytomegaloviruses

Donald J Alcendor and **Gary S Hayward**, Departments of Pharmacology and Oncology, Johns Hopkins School of Medicine, Baltimore, USA

History

In addition to human cytomegalovirus or salivary gland virus (HCMV or HHV-5) and the equivalent mouse virus (MCMV), closely related species with typical cytomegalovirus-like characteristics have been described from rats (RCMV), guinea pigs (GpCMV) and other rodents, and from both Old and New World monkeys (SCMV). GpCMV was originally recognized in 1920 and served as a model system for the biology and pathogenicity of HCMV disease for many years. Host-specific SCMV isolates have been reported from almost all major nonhuman primate groups, including gorilla, chimpanzee, bonobo, baboon, rhesus and other macaques, African green monkey, spider monkey, owl monkey, capuchin and marmoset.

Classification and Evolution

All of the animal CMVs described to date are members of the family *Herpesviridae* and belong to the *Betaherpesvirinae* subfamily of herpesviruses. Based on the relatively high (G+C) content of their DNA molecules and their adaptability to grow in fibroblasts in cell culture, most if not all of the traditionally recognized animal CMVs are likely to be evolutionarily more similar to the beta-1 subgroup that includes HCMV and MCMV than to the (A+T)-rich beta-2 subgroup exemplified by HHV-6. The International Committe on Taxonomy of Viruses has now classified betaherpesviruses into three distinct genera, *Cytomegalovirus* (HCMV-like), *Muromegalovirus* (MCMV-like) and *Roseolovirus* (HHV-6, HHV-7), but it is likely that distinctions between the first two taxons at least will become murky when molecular genetic criteria (i.e. gene content and DNA and protein sequence similarities) are applied to those CMV species that infect other mammalian and primate hosts. The genomes of prototype species of all three taxons, including HCMV (1989; 229354 bp; X17403), MCMV (1996; 230278 bp; U68299) and HHV-6 (1995; 159321 bp; X83413) as well as HHV-7 (1996; 144861 bp; U43400), have all been completely sequenced.

All formally recognized betaherpesviruses are listed in **Table 1**. However, it should be noted that there are two very different viruses (with somewhat different gene content) that are both known as rat cytomegalovirus (RCMV). Two CMV-like viruses have also been recently reported to be associated with fatal hemorrhagic disease in young African and Asian elephants, but current genetic analysis suggests that these two viruses are distinct in the two groups of animals and from all three of the currently recognized taxons. Another CMV-like virus, sometimes referred to as 'Stealth virus', has been reported to have been isolated from a human patient with central nervous system neuropathy, but genetic analysis has revealed that this virus contains UL34, UL35 and SSB genes that are virtually identical to those of several characterized SCMV isolates from African green and Vervet monkeys (AgCMV). The AgCMV isolate 'Colburn' was also originally thought to have been of human origin, but whether either is truly a human isolate rather than a contaminant from primary monkey cell cultures is subject to some doubt. Based on genome analyses, both the bovine herpesvirus BHV-4 and the two equine herpesviruses EHV-2 and EHV-5, which were all originally thought to be

Table 1 Known betaherpesviruses

Host group	*Host species*	*Common name*	*Classification*
Old World primates	Human	HCMV	Human herpesvirus 5
	Human	HHV-6A, 6B	Human herpesvirus 6A, 6B
	Human	HHV-7	Human herpesvirus 7
	Bonobo	BoCMV	
	Chimpanzee	ChCMV	
	Baboon	BbCMV	
	Rhesus	RhCMV	Cercopithecine herpesvirus 8
	African Green	AgCMV	Cercopithecine herpesvirus 5
	Vervet	SA6	Cercopithecine herpesvirus 3
	Vervet	SA15	Cercopithecine herpesvirus 4
New World primates	Marmoset	MsCMV	Callitrichine herpesvirus 2
	Owl monkey	HV Aotus type 1	Aotine herpesvirus 1
	Owl monkey	HV Aotus type 3	Aotine herpesvirus 3
	Cebus	CeCMV	
	Capuchin	AI-5, AP18	Cebine herpesvirus
Rodent	Mouse	MCMV	Murid herpesvirus 1
	Rat	RCMV-1	Murid herpesvirus 2
	Rat	RCMV-2	
	Guinea pig	GpCMV	Caviid herpesvirus 2
	Squirrel	SqCMV	Sciurid herpesvirus
Other	Pig	PCMV	Suid herpesvirus 2
	African elephant	HV Loxodonta	Elephantid herpesvirus 1

The betaherpesvirus family contains three formally defined taxons at present: namely, 3.1.21, *Cytomegalovirus* (e.g. HCMV); 3.1.2.2. *Muromegalovirus* (e.g. MCMV); and 3.1.2.3 *Roseolovirus* (e.g. HHV-6, HHV-7)

cytomegaloviruses, have had to be reclassified into the gamma-2 (rhadinovirus) class of herpesviruses. No animal equivalents of HHV-6 or HHV-7 have yet been identified, but they are expected to exist.

Typically, betaherpesviruses have no more than 45 of their 110–180 genes in common with the alpha (HSV, VZV) and gamma (EBV) classes of herpesviruses. Another 35 genes appear to be beta-specific and are likely to be shared by all CMVs and the HHV-6-like viruses, whereas most of the remaining genes are unique to the different species of CMV. As judged by the presence of several large families of related genes in HCMV and MCMV, it appears that the beta-1 group viruses, which possess by far the largest of all herpesvirus genomes, underwent a rapid genomic expansion in the early stages of mammalian evolutionary radiation. In contrast, the beta-2 herpesviruses (HHV-6 and HHV-7) can be considered to be mini-CMVs that lack most of the repeated gene families and the S-segment of the genome. Unlike other herpesviruses, none of the betaherpesviruses is known to encode either a thymidine kinase enzyme or the A-subunit of ribonucleotide reductase, although they do encode uracil DNA gylcosylase and a dUTPase, and HCMV encodes a novel phosphotransferase (UL97) activity that is the target for the effective anti-CMV agent ganciclovir and its derivatives. Like all herpesviruses, the animal CMVs are expected to encode the typical six core DNA replication proteins of herpesviruses: DNA polymerase (POL), DNA primase (PRI), helicase (HEL), single-stranded DNA-binding protein (SSB), polymerase processivity factor (PPF) and primase accessory factor (PAF).

Virion Structure

Animal CMVs are structurally similar to other members of the *Herpesviridae*, as described elsewhere in this volume, and are essentially indistinguishable from HCMV at the electron microscopic level. However, many of the virion proteins of each species display characteristic size variations after PAGE analysis. Large nuclear and cytoplasmic inclusion bodies detectable in lytically infected cells by light microscopy are a hallmark of the group.

Genome Structure

The intact genome size of all known CMV DNA molecules lies between 210 and 240 kb and most have a (G+C) content of around 58%, in contrast to the

much smaller genome size of 145 to 166 kb and 46% (G+C) content of HHV-6 and HHV-7. Structurally, two distinct CMV genome types can be discerned. The New World monkey SCMV genomes (HVaotus types 1 and 3) and HCMV represent one type, with both having large internal inverted repeats that generate four isomeric arrangements of the L (long) and S (short) unique segments similar to the pattern observed in HSV. On the other hand, at least one Old World monkey SCMV studied namely AgCMV (Colburn), and several rodent CMVs (MCMV, RCMV and GpCMV) have essentially linear non-inverting DNA molecules. HHV-6 and HHV-7 both lack the entire 40 kb S-segment region of HCMV and the L-segment is bounded by large (8 kb) direct terminal repeats containing telomere-like motifs. MCMV has a similar sized gene block in place of the S-segment, but this region has very little organizational resemblance or homology to that of HCMV. Large blocks of genes at the left and right ends of the L-segment of each genome are also unique to each of the three taxons. Current evidence also suggests that the gene segments mapping to the right of and adjacent to the major immediate-early (MIE) region of several Old World SCMV genomes differ dramatically both from each other and from the equivalent regions in HCMV.

Both DNA sequence and amino acid analysis indicates that individual species of beta-1 CMVs from different animal hosts are highly diverged in accordance with their host phylogeny, although different genes diverge at very different rates. For example, in a highly conserved region between HCMV and the African green monkey AgCMV, the major single-stranded DNA binding proteins (UL57, SSB) have 72% overall amino acid identity, whereas a group of four adjacent betaherpesvirus-specific glycoproteins mapping to the left of the major immediate early (MIE) region (UL118–UL121) vary from as much as 35% identity overall to as little as 15% at one end only. Similarly, the betaherpesvirus-specific IE2 (UL122) immediate early regulatory proteins of HCMV and AgCMV have 58% amino acid identity, but only over the C-terminal half of the protein. This is a commonly observed level of variation in herpesviruses, with the two primate gamma-1 class viruses EBV (human) and HVpapio (baboon), for example, showing 37% and 68% overall amino acid identity between their EBNA-2 and the Bcl2-like proteins, which are both unique to that subgroup of herpesviruses. Nucleotide sequence differences between homologous CMV genes frequently reach the 20–30% level, even in closely related species such as African green monkey and rhesus, or human and chimpanzee, etc. In fact the DNA molecules of individual isolates of each CMV species are almost always distinguishable on the basis of multiple restriction fragment length polymorphisms stemming from overall intraspecies nucleotide variations of up to 3–4%.

The three prototype betaherpesviruses contain several interesting and in some cases taxon-specific genes related to known cellular genes that appear to be used primarily for evasion of immune responses. For example, HCMV encodes four GDP-coupled seven-membrane spanning receptors (GCR), at least one of which (US28) functions as a broad spectrum chemokine receptor, and three glycoproteins (US3, 10 and 11) that together function to destabilize or inhibit cellular HLA-mediated responses. HHV-6 and MCMV each encode one GCR protein. Both HCMV and MCMV also each encode diverged homologues of an HLA-I protein (inserted at different loci in their genomes) and MCMV also encodes a functional β-chemokine protein. HHV-6 (but not HHV-7) encodes an REP (name of an early protein required for replication) protein apparently captured from adeno-associated virus (AAV), and HHV-6, HHV-7 and RCMV, but not MCMV, encode OX-2 related proteins.

Epidemiology and Physical Properties

Features of host range, virus propagation, virus transmission, tissue tropism, pathogenicity, histopathology and immune responses, together with the physical properties and assembly pathways of the virions, and a number of biochemical properties of their structural proteins, closely resemble those described elsewhere in this volume for HCMV and MCMV.

Replication Strategies

All herpesviruses have enveloped icosahedral capsids and replicate in the nucleus, with expression of several immediate early regulatory proteins preceding expression of the early proteins, including the biochemical functions, viral DNA and late structural proteins. In cell culture, fully permissive host cell types for HCMV are restricted almost exclusively to diploid human fibroblasts (plus some astrocytomas and perhaps smooth muscle cells and differentiated macrophages), although even in fibroblasts fresh clinical isolates usually need to be adapted for efficient growth by multiple rounds of successive passaging (which is accompanied by gene losses at the L-segment unique-repeat boundaries). Laboratory strains of HCMV replicate their DNA efficiently by 72 h in human fibroblasts, but express only the MIE

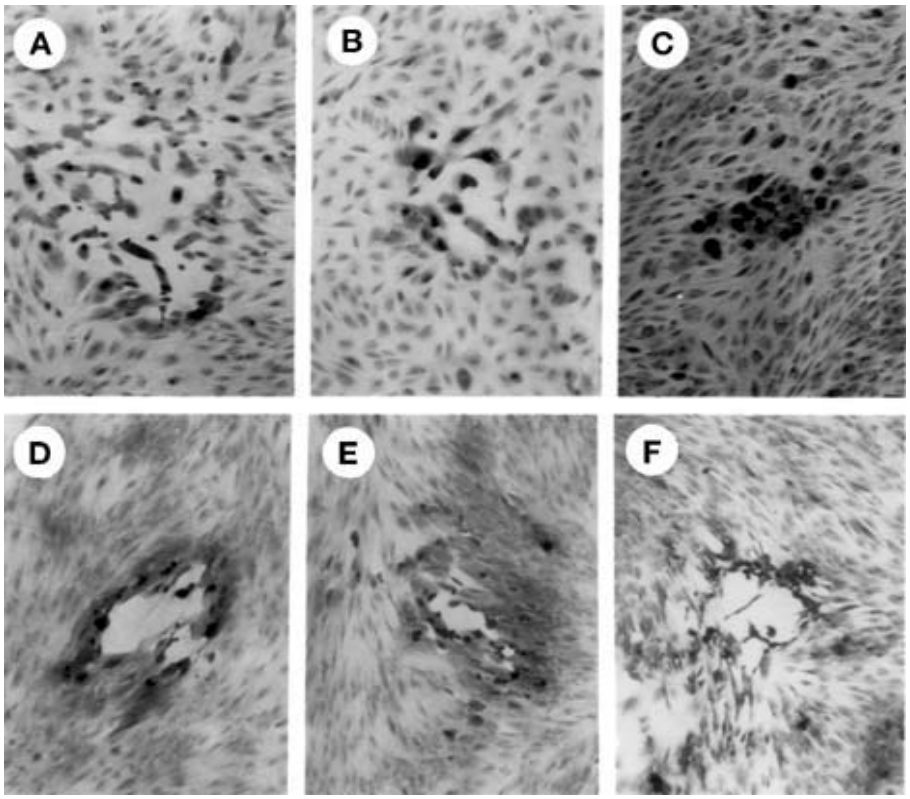


Figure 1 Infection of human diploid fibroblasts (HF cells) with both human and nonhuman primate cytomegaloviruses. HF cells at passage level 15 were infected and examined daily for cytopathic effects. All infections produced characteristic cytomegalic cells in culture. (**A**) HCMV Towne strain, plaque at 7 days after infection; (**B**) African green monkey CMV (AgCMV), plaque at 8 days after infection; (**C**) baboon CMV (BbCMV), plaque at 11 days after infection; (**D**) chimpanzee CMV (ChCMV), plaque at 14 days after infection; (**E**) rhesus CMV (RhCMV), plaque at 14 days after infection; (**F**) Cebus CMV (CeCMV), plaque at 8 days after infection.

proteins and fail to synthesize any viral DNA in rodent or monkey fibroblasts, whereas AgCMV, RhCMV, BaCMV, ChCMV and CeCMV all replicate their DNA and form plaques in both human and simian fibroblasts (**Fig. 1**). In contrast, MCMV successfully carries out viral DNA synthesis in human, monkey and rodent fibroblasts in cell culture. Most transformed human cell types are nonpermissive for HCMV replication, with the block being between DNA entry and synthesis of MIE mRNA. However, some differences between HCMV and African green monkey SCMV have been observed here. For example, HCMV fails to synthesize its MIE proteins in infected human (NTera) or mouse (F9) teratocarcinoma stem cells, in human 293 or NBE cells or in mouse L-cells. In contrast, AgCMV does produce its MIE proteins in infected NTera, 293 and NBE cells, but not in F9 or L-cells. The NTera stem cells can become permissive for both HCMV MIE expression and infectious virus production after differentiation with retinoic acid (RA). Similarly, the F9 cells treated with RA induce AgCMV but not HCMV MIE expression. These biological differences appear to coincide with structural differences in the organization of *cis*-acting transcriptional control elements and adjacent accessory domains located both upstream of, and in the large first intron of, the MIE genes among the different species of old world primate CMVs. While a major *in vivo* site for quiescent inactive infection for both HCMV and MCMV is believed to involve monocytes, many other sites of inapparent noncytopathic infection occur *in vivo* (especially in vascular tissue) and in salivary gland stromal cells for rodent CMVs, but the true site of reactivatable latent state infection has not yet been deciphered for any CMV species.

Control of Gene Expression

The lytic cycle pathway of gene expression for animal CMVs follows the typical herpesvirus cascade of immediate early mRNA and proteins followed by activation of delayed early then late class genes. Similarly organized MIE transcription units, which

encode regulatory proteins that trigger the lytic cycle, have been characterized in HCMV, ChCMV, AgCMV, RhCMV, BaCMV, GpCMV, RCMV, MCMV, and HHV-6 and HHV-7. In each case, these transcription units are oriented leftwards at genome map position 0.7 and produce several multiply-spliced mRNAs whose expression is controlled by powerful upstream *cis*-acting enhancer control regions. By far the most predominant viral mRNAs synthesized after infection of permissive cells in the presence of cycloheximide are these MIE mRNAs, which are also the only HCMV or AgCMV mRNAs and proteins produced after infection of nonpermissive rodent fibroblasts. At least two types of phosphorylated nuclear regulatory proteins are encoded by the MIE transcription unit. These include the highly abundant 72 kDa acidic IE1 (UL123) nuclear protein and the less abundant 86 kDa IE2 (UL122) DNA-binding trans-activator-repressor protein. Not only do the IE2 proteins stimulate transcription of downstream HCMV, SCMV and MCMV promoters and non-specifically transactivate heterologous promoters, but they also specifically downregulate their own MIE promoters (negative autoregulation). The IE1 proteins of AgCMV and MCMV are much larger than in HCMV (94 kDa and 97 kDa, respectively) because of expanded C-terminal glutamic acid-rich domains.

Interestingly, in contrast to the rest of the viral genome, the IE1 and the IE2 coding regions of the MIE transcription units in HCMV, AgCMV, MCMV, RCMV, HHV-6 and HHV-7 (and probably all other betaherpesviruses) are highly CpG suppressed, which suggests that they are accessible to cellular methylation events at some stage of the viral life cycle in which all other viral genes are transcriptionally silent. The 490 amino acid IE1 proteins have barely detectable amino acid homology (15% identity) between HCMV and AgCMV or RhCMV and much less between the primate and MCMV and RCMV versions, although all of the betaherpesvirus IE1 proteins (except for HHV-6 or HHV-7) have a highly acidic glutamic acid-rich C-terminus. In contrast, the 570 amino acid IE2 proteins of HCMV, AgCMV, RhCMV, MCMV, RCMV, and their much larger counterparts in HHV-6 and HHV-7, all exhibit between 25 and 58% amino acid identity over the C-terminal 270 amino acid conserved DNA-binding domain.

The upstream MIE promoter-enhancer regions that serve to sense the intranuclear environment and control entry into or out of the lytic cycle, are large and complex noncoding DNA sequence domains that consist of often multicopy, high-affinity binding sites for numerous constitutive or inducible cellular transcription factors. In HCMV, ChCMV, AgCMV, RhCMV, BaCMV, GpCMV and MCMV these sites include response elements for cyclic AMP (CRE, PKA), phorbol esters (TPA, PKC) and retinoic acid (RA), together with recognition motifs for SRF, ETS, CREB, SP-1, AP-1 and NFκB. Even among the five Old World primate CMVs that have been examined, the organization of these sites differs significantly and the number and pattern of the adjacent tandemly-repeated 15 and 30 bp high-affinity overlapping NFI plus YY1 motifs also differ greatly. A diagram illustrating differences in the organization of BENT DNA, NF1, enhancer (ENH), minimal promoter (MIN) and intron (INT) domains from the upstream control regions of the MIE genes of two Great Ape and three Circopithecine CMVs is presented in **Fig. 2.** Curiously, the organization of the MIE enhancer in RCMV seems to be quite different from and simpler than that in MCMV. The existence or function of other specific regulatory proteins that are common to HCMV and HHV-6, such as the UL36-37 proteins, the US22 family and the UL82-83 matrix phosphoproteins, the UL84 replication-associated protein, and also the HCMV-specific US3 protein and its novel complex NFκB containing IES enhancer, have not yet been evaluated in any of the animal CMVs.

The lytic cycle DNA replication origins (ORI-LYT) are all located to the right of and adjacent to the single-stranded DNA-binding protein gene (SSB, UL57) near the center of the HCMV, AgCMV and MCMV genomes, but again they differ significantly in structural organization and apparently cannot be complemented by each other's replication protein machinery. Although the lytic origin in HHV-6 and HHV-7 is located at the equivalent site in the genome, its structure and the UL9-dependent mode of DNA replication appear to be quite different from that in the CMVs. It is still unknown whether betaherpesviruses also use or need a latency-state plasmid DNA replication mode like that of Epstein–Barr virus.

Future Perspectives

Persistent and inapparent infection with members of the CMV family is probably virtually ubiquitous in most individuals of all mammalian species. The biological and pathological properties of animal CMVs have attracted the interest of herpesvirologists and clinicians interested in CMV disease in patients with acquired immune deficiency syndrome (AIDS) and those who have had organ transplants, probably not so much because of serious morbidity or economic consequences of these infections in their own hosts, but more often as models for latency and pathogenesis or immunological responses in HCMV. Mouse, rat, rhesus and baboon CMVs have all been

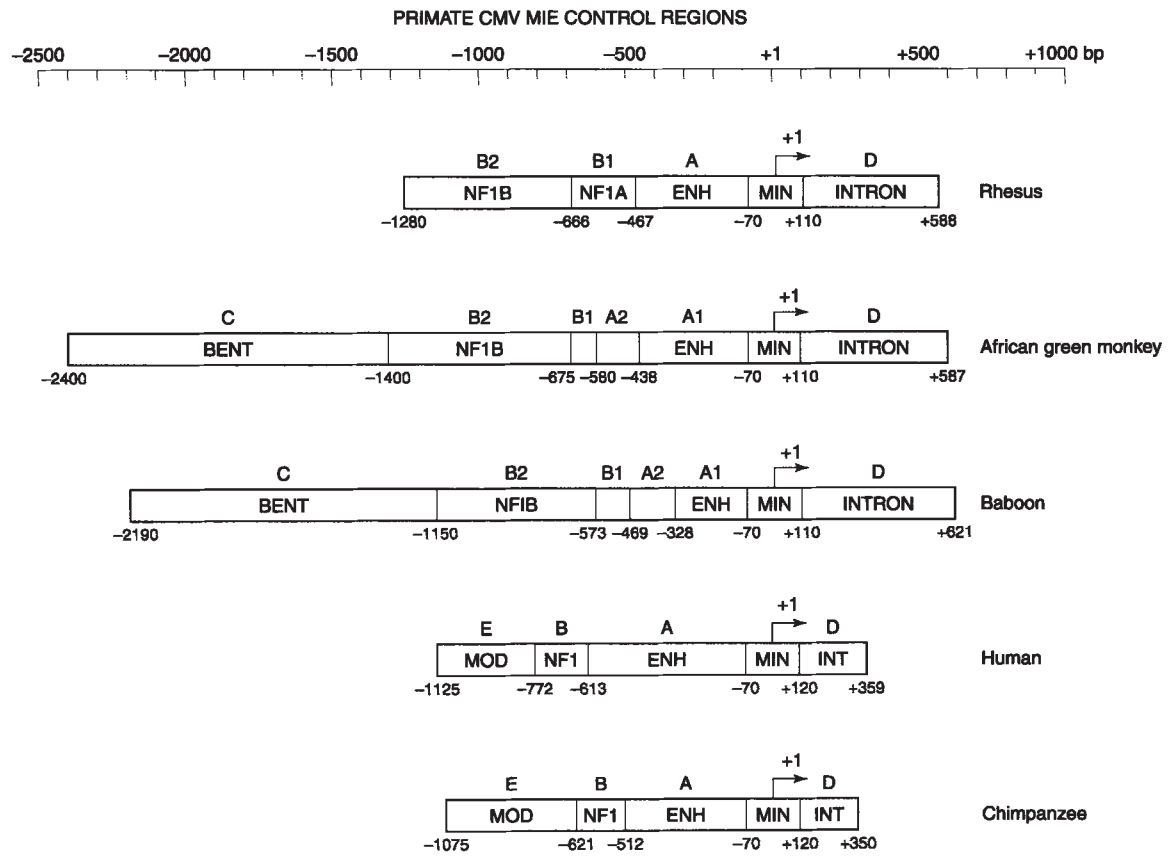


Figure 2 Upstream control regions in the major immediate early genes of five human and nonhuman primate cytomegaloviruses. The diagram illustrates the respective size and organization of domains containing specific transcription factor-binding motif patterns. These include clusters of nuclear factor 1 (NF1) and YY1 sites, the distal enhancer containing multiple CRE, SRE and NFκB-sites (ENH), the proximal promoter and TATATAA box region that includes the start site of transcription +1 (arrowed), and the 5′ half of the large first intron (INTRON). Where appropriate additional upstream AT-rich (BENT) and modulatory (MOD) regions are also included. Domain boundaries are designated as + or − nucleotide positions with reference to the start site of transcription (+1) and given in base pairs. The domains have also been designated A through E with subcategories 1 or 2 in the case of the NF1 and ENH blocks. (With permission from *Journal of Virology* (1996), **70**: 8590–8605 and American Society of Microbiology.)

investigated as models for virus reactivation, and for control of acute and chronic disease by anti-CMV agents (such as ganciclovir) in association with allografted organ transplants or immunosuppression. There is also an expanding interest in questions related to whether, or how extensively, different CMVs can cross host species barriers with possible pathological consequences, which is likely to raise concerns and place some additional focus on baboon and pig CMV, for example, as the prospects of xenografted organ transplants grow. Recently, animal CMVs have also had comparative value in molecular genetics, and biochemical analysis of both their similarities to and differences from HCMV. Current basic scientific interest in genomic evolution (made possible by large-scale DNA sequencing), as well as the role of transcriptional gene regulation mechanisms in determining cell tropism and the switching between latent and lytic cycle virus–cell interactions pathways, may lead to an expansion of these types of studies with animal CMVs in the next few years.

See also: **Persistent viral infection; Cytomegaloviruses (*Herpesviridae*): General features (human), Molecular biology (human), Murine cytomegaloviruses; Cowpox virus (*Poxviridae*).**

Further Reading

Gibson W (1991) Cytomegalovirus protein structure and function. In: Landini MP (ed.) *Proceedings of the 3rd International Cytomegalovirus Workshop*, p. 357. Amsterdam: Elsevier.

Hayward GS, Ambinder R, Ciufo D, Hayward SD and LaFemina RL (1984) Structural organization of human herpesvirus DNA molecules. *J. Invest. Dermatol.* 83: 29.

Isom HC and Yin CY (1990) Guinea-pig cytomegalovirus gene expression. *Curr. Top. Microbiol. Immunol.* 154: 101.

Kaplan AS (1973) *The Herpesviruses,* chapts 12 and 13. London: Academic Press.

LaFemina RL and Hayward GS (1980) Structural organization of the DNA molecules from human cytomegaloviruses. In: Jaenisch R, Fields J and Fox F (eds) *Animal Virus Genetics.* ICN-UCLA Symposium on Molecular Biology, vol. 18, p. 39. New York: Academic Press.

Staczek J (1990) Animal cytomegaloviruses. *Microbiol. Rev.* 54: 247.

Murine Cytomegaloviruses

John Staczek, Louisiana State University Medical Center, Shreveport, Louisiana, USA

History

Cytomegalia refers to the enlargement of a cell. The discovery of the viral etiology of cytomegalia in the mouse salivary gland preceded the isolation of the virus by 19 years. The mouse salivary gland virus, commonly called mouse (or murine) cytomegalovirus (MCMV), is a surrogate for studying human cytomegalovirus (HCMV)-associated pathogenesis.

Classification

Classification by the International Committee on Taxonomy of Viruses (ICTV) places MCMV in the family *Herpesviridae,* subfamily *Betaherpesvirinae,* genus *Muromegalovirus,* type species mouse cytomegalovirus 1. Herpesviruses are designated by serial arabic number and the family or subfamily of the natural host. Thus, MCMV is designated Murid herpesvirus 1, but its most commonly used synonym is mouse cytomegalovirus.

Properties of Virion

MCMV virions have typical herpesvirus morphology. The virions have a buoyant density of 1.20 g cm^{-3}. Their nuclear membrane-derived envelope is sensitive to denaturation by detergents, pH, heat and desiccation. Viral glycoprotein spikes extending through the envelope are linked to the nucleocapsid via tegument proteins. The icosahedral nucleocapsid, containing 162 capsomeres, surrounds an ultraviolet radiation-sensitive, linear, double-stranded DNA.

Properties and Organization of Genome

General comments concerning the DNA sequence and genomic organization of the Smith strain of MCMV are presented. It is suggested that the reader refer to Rawlinson *et al* for a more detailed description of the DNA sequence and compilation of open reading frames (ORFs).

DNA sequencing of the Smith strain genome reveals 230 278 bp. Buoyant density and sequence analyses denote an overall 58.7% G+C content distributed with areas of major (155–163 kb) and minor (1–17 kb and 204–221 kb) G+C content. Coding regions have a higher G+C content than noncoding regions. CpG dinucleotides occur throughout the genome at frequencies expected for random associations between mononucleotides, except for a CpG deficiency that is evident in the major immediate early (MIE) gene region.

DNA isolated from purified virions contains a single 3′ nucleotide extension that may facilitate circularization to a prereplicative intermediate. Herpesvirus genomes are grouped (A–F) according to sequence reiterations greater than 100 base pairs. Based on this scheme, the MCMV genome, consisting primarily of unique sequences, is catalogued with the group F genomes. However, clusters of 31-bp direct repeats delimit each terminus of the genome, and additional, nonhomologous, intra- and intergenic direct repeats punctuate the centralized unique region. Inverted repeats, present in the origin of replication and upstream of several ORFs, do not mediate isomerization.

Families of homologous genes are a general feature of betaherpesviruses. MCMV has six gene families, two of which (m02 and m145) are unique and four of which are homologous to HCMV families US22, UL25, UL82 and to the G protein-coupled receptors (GCRs). The functions of the gene families are generally unknown. However, the M25 protein has homology to eukaryotic nucleolins and, therefore, may interact with chromatin.

Genetics

Restriction length fragment polymorphisms among MCMVs isolated from wild-type mice indicate widespread heterogeneity in genomic composition which is reflected in peptide analyses using polyclonal sera recovered from infected wild mice. Strain variation influences disease presentation and recovery. Variation in the nonapeptide of the immunodominant

receptors for entry of human immunodeficiency virus into T cells and monocytes.

See also: **Autoimmunity; Interferons: General features, Therapy of aids and cancer; Pathogenesis: Animal viruses; Vaccines and immune response.**

Further Reading

Boehm U, Klamp T, Groot M and Howard JC (1997) Cellular responses to interferon-gamma. *Annu. Rev. Immunol.* 15: 747.

Brown MA and Hural J (1997) Functions of IL-4 and control of its expression. *Crit. Rev. Immunol.* 17: 1.

Del Prete G (1998) The concept of type-1 and type-2 helper T cells and their cytokines in humans. *Int. Rev. Immunol.* 16: 427.

Dinarello CA (1996) Biologic basis for interleukin-1 in disease. *Blood* 87: 2095.

Sugamura K, Asao H, Kondo M *et al* (1996) The interleukin-2 receptor gamma chain: its role in the multiple cytokine receptor complexes and T cell development in XSCID. *Annu. Rev. Immunol.* 14: 179.

Taga T and Kishimoto T (1997) Gp130 and the interleukin-6 family of cytokines. *Annu. Rev. Immunol.* 15: 797.

Trinchieri G (1998) Immunobiology of interleukin-12. *Immunol. Res.* 17: 269.

CYTOMEGALOVIRUSES (*HERPESVIRIDAE*)

Contents

General Features (Human)

Edward S. Mocarski Jr., Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California, USA

History

Cytomegaloviruses, the prototype members of the *Betaherpesvirinae* subfamily of the *Herpesviridae* family, are widely distributed in nature. All animal species have an associated cytomegalovirus (CMV) which exhibits exquisite species-specificity. These viruses infect for life and are readily isolated from saliva or salivary gland explants, a feature that resulted in the assignments of their earlier name, salivary gland viruses. Intermittent, lifelong shedding in saliva and urine have long revealed a persistent/latent lifestyle typical of all herpesviruses, although CMV recurrence and shedding is not associated with any sort of recurrent disease. CMV infection is typically of little consequence to the host, despite sporadic shedding throughout life: it is only in immunocompromised hosts that the virus is recognized as a major opportunist and a significant cause of disease. The impact of human CMV in allogeneic organ and bone marrow transplant recipients and in patients with acquired immune deficiency syndrome (AIDS) has made this virus the subject of intense study. Immune surveillance plays a critical role in maintaining viral latency and suppressing viral replication. Although reactivation in the immunocompromised host is important, this virus was first recognized because of its etiologic role in congenital disease, and it remains the major infectious cause of deafness in the USA. Infection of the fetus is a consequence of primary infection of the mother and occurs in 0.5–1% of all live births. Neurological damage due to congenital CMV ranges from mild learning or hearing disabilities to a catastrophic central nervous system damage. The involvement in cytomegalic inclusion disease along with the cytomegalic (enlarged cell) cytopathology caused by the virus led to the adoption of the current name for this virus. Although a medically important virus, human CMV is a classic opportunist, with almost all infections remaining subclinical even in highly susceptible populations.

Cytomegaloviruses infecting animal species, such as mice, rats, guinea pigs and rhesus macaques, have been used as surrogates for experimental investigation of viral pathogenesis and the virus–host interaction. They were first recognized early in the twentieth century when guinea pigs were experimentally infected with a filterable agent that caused cytopathol-

ogy in salivary glands. Many cytomegaloviruses, including human and murine CMV, were isolated in the 1950s when cell culture became widely available. Due to the ease of culture, congenital disease was quickly associated with *in utero* infection in humans. The importance of CMV as a human pathogen increased as immunosuppressive post-transplant therapies and AIDS, as well as other immunodeficiency states, have come to the forefront of medicine. These conditions predispose individuals to disease during primary infection or, once primary infection has resolved, to disease following reactivation of latent infection. CMV is known to be transmitted by direct contact with bodily secretions, including saliva, urine, breast milk and semen, as well as by blood transfusion and organ transplantation. The large reservoir of seropositive, latently infected individuals, which range from 40 to 100% of the population depending on age and geographic location, contributes to the medical importance of the virus. The replication, biology and pathogenesis of CMV have been covered in several reviews listed at the end of this article.

Taxonomy and Classification

The formal designation given by the International Committee on Taxonomy of Viruses to human CMV is human herpesvirus 5 (HHV-5), a name that is not in common usage. Human CMV is the prototype of the genus *Cytomegalovirus*. Cytomegaloviruses share many characteristics with other herpesviruses, including typical virion morphology and large, linear DNA genome, as well as common biological properties such as persistence and latency in the host. These viruses have distinguishing characteristics as well, such as their well-established tropism for salivary glands, restriction to the host species of origin and slow growth in culture. These differentiating characteristics define the *Betaherpesvirinae* subfamily. In addition to the cytomegaloviruses, genome sequence analysis established that the betaherpesviruses include two lymphotropic human herpesviruses, HHV-6 (genus *Roseolovirus*) and HHV-7. Large-scale nucleotide sequence analysis of human CMV, murine CMV, HHV-6 and HHV-7 demonstrated a collinear arrangement of genes on the viral genomes and a significant level of similarity. Human CMV carries over 200 genes, of which 60–70 are similar among betaherpesviruses and 35–40 are common to all mammalian herpesviruses. Cytomegaloviruses, but not HHV-6 or HHV-7, share common growth characteristics and cytomegalic cytopathology. The distribution of this type of virus in nature is very broad, and examples have been found in every mammalian species in which an example has been sought.

Human CMV does not share significant genome nucleotide sequence homology with any of the other human herpesviruses or with animal cytomegaloviruses. The host serologic response does not detect significant differences between human CMV strains. Strains have been differentiated by several nucleic acid-based methods including polymorphism in DNA restriction enzyme fragments as well as sequence variation in DNA polymerase chain reaction (PCR) products from known variable viral genes, such as that encoding envelope glycoprotein (g) B (UL55). Both of these methods reveal the presence of numerous strains circulating in all human populations. Reports have suggested that certain gB genotypes might be associated with more severe disease in immunocompromised individuals, raising the possibility that some strains of CMV are more virulent than others; however, this suggestion has not been generally confirmed. Early evidence based on DNA–DNA reassociation kinetics, suggesting that strains of CMV exhibit only 80–90% sequence homology, has been explained by more recent genome sequencing efforts. Significant sequence variation due to deletions of up to 15 000 bp has been found in some laboratory strains of virus, such as AD169 and Towne vaccine strain (passage 125) relative to clinical strains. It has recently become clear that AD169 and Towne that have been propagated in different locations for extended periods may vary considerably in their growth and genome properties. These have been thought to have accumulated in the course of serial high multiplicity passage of laboratory strains as they were developed to be used as live-attenuated vaccines. Individual low passage or fresh isolates of CMV seem to retain a much higher level of similarity to each other and have not been observed to change following limited passage in cell culture. Currently, isolates of CMV are commonly differentiated either by restriction fragment polymorphism or by direct DNA sequence analysis of DNA PCR products from gB or other highly variable genetic loci.

Geographic and Seasonal Distribution

Geographic, but not seasonal, variation in CMV transmission is known to occur. As is the case with other herpesviruses spread by direct contact and without much morbidity, the virus is distributed worldwide and appears in even the most remote populations. In urban environments, some regions of developed countries and all less developed countries, a high proportion (80–100%) of the population is infected early in life, whereas in rural and suburban

settings of North America, Australia and Europe a smaller proportion (40–60%) of the population is infected, with significant transmission occurring in young adults. Infection rates are clearly related to close contact amongst individuals. There is little evidence for point sources in the epidemiology of CMV infections. Transmission of CMV between young children or from young children to adults is efficient, reaching rates of 25–50% per year in some child homecare or daycare settings. Daycare providers and parents of young children have an increased risk of CMV infection, although good personal hygiene clearly reduces this risk. Very high levels of virus in urine and saliva of young children may play an important role in these transmission patterns. Simply informing individuals of the usefulness of good hygiene can have a dramatic impact on transmission rates in young adults.

Host Range and Virus Propagation

The only natural host for human CMV is humans, and human fibroblasts are used to propagate this virus. Chimpanzee fibroblasts are also permissive for CMV replication, but cells from other primates or animal species are not. As a result of host restriction, little experimental work with human CMV has been undertaken in a host animal and most work relies on surrogate studies of animal CMVs, such as those infecting mice, rats, guinea pigs or rhesus macaques. In addition to a strict host species restriction, only certain types of human cells are permissive for CMV. The virus exhibits a cell type and differentiation requirement and replicates in differentiated, but not undifferentiated or malignant, cell types. In laboratory practice, human CMV is isolated and propagated using secondary cultures of human fibroblasts derived from fetal lung or newborn foreskin. Fibroblasts and stromal cells from any tissue source appear to be permissive. Prolonged (3–4 weeks) culture may be required to isolate virus from infected tissues or fluids, although, once isolated, infectivity of strains that grow in culture may be directly quantified by plaque assay on human fibroblast cell monolayers. Other primary cell types of epithelial, endothelial and monocyte/macrophage origin exhibit more limited permissiveness for viral replication *in vitro*, despite evidence that these cell types are permissive and are involved in the host. During infection of cultured cells, which is prolonged even with laboratory strains, infected cells enlarge and develop both nuclear and cytoplasmic inclusions that confer an 'owl's eye' appearance typical of cytopathology used for histopathological identification of virus-infected tissues in the host. Virus maturation begins with the encapsidation of DNA in the nucleus followed by acquisition of an envelope and release into the culture fluid of infected fibroblasts by 3–4 days after infection. Undifferentiated, malignant and almost all tested immortal human cell lines are nonpermissive for CMV replication. For unknown reasons, human fibroblasts immortalized by the human papillomavirus E6/E7 oncogenes are fully permissive and certain glioblastoma and astrocytoma tumor cell lines are semipermissive for virus replication. Replication of human CMV in differentiated monocyte/macrophages is thought to be important in virus dissemination and persistence in the host, but these cells exhibit striking variations in permissiveness, depending upon the conditions which are used to support their growth and differentiation.

Genetics

Based on genome sequence polymorphisms, a large number of CMV strains circulate worldwide. The difficulty in propagating recent isolates in human fibroblasts led to the dependence on selected laboratory strains for studies of virus biology and genetics. Although the genotype of a virus isolate is stable over short-term passage in culture, as well as during transmission from person to person, high multiplicity passage in fibroblasts appears to drive the generation of nucleotide sequence deletions and duplications such that dramatic sequence differences are obvious in laboratory strains as compared to fresh isolates. Efficient growth in fibroblasts is one common characteristic of laboratory strains. Strains of CMV exhibiting more efficient growth on cultured endothelial cells or fibroblasts have been derived from single isolates, suggesting that there may be a genetic basis for adaption to certain cell types. The significance of adaption in cell culture to the biology of CMV in the host remains unclear.

The only human CMV genome to have been completely sequenced is strain AD169, and this strain has a 230 283 bp genome with over 200 predicted genes. An additional 13 000–15 000 bp are present in fresh isolates but are apparently replaced by duplication within the AD169 or Towne (passage 125) genomes during serial propagation in culture. The original isolate of AD169 dates to the 1950s but was not preserved. Towne (passage 125) and Towne (passage 36) were noted to have different restriction maps when analyzed but the extent of genome rearrangement that characterizes the high passage virus only became evident when regions were sequenced. Significantly, 70 out of over 200 genes in the human CMV genome are already known to be dispensable for growth in cultured fibroblasts. These

genes may carry out functions that are important for growth, dissemination, tissue tropism or pathogenesis in the host. Such functions have now been unambiguously demonstrated to occur through studies comparing the growth of murine CMV mutants in cultured cells and mice.

Several CMV gene functions have been predicted based on their sequence similarity to genes of other herpesviruses or of mammals. The 35–40 genes that exhibit a significant level of predicted amino acid similarity to genes in all herpesviruses of mammals play roles in viral DNA replication and virion structure. The genes in human CMV that bear resemblance to host genes include homologues of a major histocompatibility class I antigen, α chemokines, a β chemokine receptor and other G-protein coupled receptors, a T cell receptor δ chain and a soluble tumor necrosis factor/nerve growth factor receptor.

Evolution

All mammalian herpesviruses apparently share a common progenitor because of limited shared protein sequence similarity. Based on comparisons of 16 mammalian herpesvirus genomes that have been completely sequenced, betaherpesviruses appear to represent the oldest type of herpesvirus. The evolution of herpesviruses has proceeded along with speciation of vertebrates, but with some substantial and abrupt substitutions such that ongoing exchange of genetic information with the host is likely to have contributed substantially over time to the genome complement in any particular virus. CMV has certainly acquired genes from the host after it came into existence as a virus. While a systematic study of evolution continues due to the availability of rapid DNA sequencing methods, the large size of CMV genomes continues to make this a difficult undertaking.

Epidemiology

ased on the facts that humans are the only known reservoir of this virus, that efficient person-to-person transmission results in widespread distribution in the population, and that disease is a relatively rare outcome of infection, human CMV has achieved the status of a successful pathogen. The epidemiology of human CMV disease follows the pattern of an endemic infectious disease agent, although medically significant disease only occurs in the face of diminished host immune surveillance. The occurrence of a significant number of long-term serologically disparate couples suggests that virus transmission is not always assured and may require either large amounts of virus or some breech of epithelium. Virus infection certainly reaches all strata of society. Although hygiene is important, the age of acquisition and the overall percentage of individuals who are infected are influenced by as yet incompletely characterized social behavioral patterns. Crowded living conditions, breast feeding and certain sexual practices are all known to be important components of transmission via infectious bodily secretions. Modern medical practices such as blood transfusion and organ transplantation have clearly brought notoriety to CMV. Importantly, CMV is very unstable in the environment, suggesting that all transmission is via direct contact or very recently contaminated fomites. Following primary infection, CMV replicates at the site of infection as well as in leukocytes, which are likely to be responsible for dissemination of virus to other tissues, including salivary glands and kidneys. Cell-free virus shedding from epithelial cells into saliva and urine may go on for months to years and, once controlled, reactivation results in sporadic shedding throughout life. Congenital or neonatal acquisition of CMV results in a longer time course and greater levels of shedding compared with acquisition later in life. During pregnancy, where primary infection remains a major medical concern because of congenital disease, an estimated 2–2.5% of all women become infected during each gestation, and those who care for young, CMV-shedding infants or toddlers may encounter a tenfold greater risk of infection. Spread to toddlers can occur in any setting where another infected child is present, particularly in any daycare setting. While 0.5 and 1% of all newborns are infected *in utero*, less than 10% of these show any symptoms of CMV disease and very few suffer the severe consequences of cytomegalic inclusion disease. Another 10–60% of newborns, depending on undetermined factors, become infected within the first 6 months of life but only premature infants may be at risk of disease. Although neonatal infection occurs, medically significant sequela, such as hearing or learning disabilities, are uncommon in this population. Congenital infection and perinatal infection of premature infants remain the most important childhood diseases associated with CMV and account for the importance of CMV as the major infectious cause of hearing loss.

From puberty to about 30 years of age, increased sexual activity as well as direct contact with young infants correlate strongly with increasing infection levels. Virus is found free in saliva and urine as well as in cells shed with cervical secretions, breast milk and semen. Individuals at any age are susceptible to infection via blood transfusion or organ transplantation, where seropositive blood and solid organs are of

greatest risk to seronegative recipients. Seroconversion rates in transfusion recipients suggest that 3–15% of seropositive units of blood may transmit and all seropositive solid organs are likely to transmit. Individuals receiving multiple units or large volumes of blood are most likely to become infected. Reactivation of latent virus and reinfection with additional strains are both important in the epidemiology of CMV disease in immunocompromised hosts; however, disease in these situations is typically less severe than following primary infection.

Primary infection with CMV results in lifelong latency in a population of mononuclear leukocytes. There is little chance that any individual ever clears this virus completely. Thus, all individuals who have been infected at any time are potential sources of virus in a wide range of settings for the remainder of their lives.

Transmission and Tissue Tropism

Human CMV may be transmitted by direct person-to-person contact at any time shedding is occurring, but immune status dictates the likelihood of disease following transmission. There is no seasonal variation in infection rates. Primary infection of an immunocompetent host results in a viremia which disseminates virus to organs, including the salivary glands and kidneys. The more immunocompromised a host, the greater the likelihood of spread to additional organs, with or without disease. Congenital transmission may occur throughout gestation following primary maternal infection, although some believe disease risk is greatest during the first half of pregnancy. Seropositive mothers who reactivate virus during pregnancy are less likely to transmit virus because their own adaptive immune response probably reduces the virus load. Congenital infection is a consequence of CMV crossing the placenta, possibly via carriage in maternal leukocytes or replication in trophoblasts. Virus is also shed in cervical secretions and this site can contribute to neonatal infection during natural childbirth.

Direct contact, blood transfusion and organ transplantation may be responsible for transmission at all other ages. Transfusion of whole blood to premature infants and immunocompromised children is restricted because of the high level of risk. Once infected, these children shed for longer periods and at higher levels than immunocompetent children. CMV-seronegative and leukocyte-depleted blood products are widely used to prevent infection in highly risky situations. Virus infection of either immunocompetent or immunocompromised individuals leads to prolonged replication in ductal cells of salivary glands and kidney tubules without any indication of organ dysfunction. Direct histology, antigen detection, *in situ* hybridization and cytopathology have shown that CMV infection proceeds progressivly to infect additional tissues, depending on the immune status of the host.

CMV remains latent in all immunocompetent hosts following primary infection and for this reason transmission via organ transplantation or blood transfusion can occur throughout life. Owing to the fact that only a small proportion of healthy seropositive individuals are able to transmit virus at any particular time, sporadic reactivation in the donor may be a factor in transmission patterns. Although polymorphonuclear leukocytes, endothelial cells and monocytes are all important sites contributing to viremia in immunocompromised individuals, only monocytes and their progenitors have been implicated as sites of long-term persistence and latency. The numbers of CMV positive, latently infected cells in peripheral blood or bone marrow have been estimated to be of the order of 0.01% of total mononuclear cells.

Pathogenicity

The widespread prevalence of CMV in the absence of significant disease is evidence that this virus is an effective pathogen exhibiting low virulence for a host with an intact immune system. Life-threatening diseases follow infection in the fetus or reactivation in individuals with compromised cellular immunity, and define the spectrum of this opportunistic pathogen. Damage to the fetus or immunocompromised host ranges from focal damage in a single site, such as retinitis in patients with AIDS, or pneumonitis in bone marrow allograft recipients, to disseminated multiorgan disease. Disease caused by CMV is a complicated process that may include some contribution by the immune response itself. The demonstration of CMV histopathology and replication at a site are not always indicative of an ongoing disease process. The interplay between the virus and host, both with regards to tissues and immunity, is intricate and little is known about the role of particular viral gene products as virulence determinants. How much each virus strain differs in virulence remains an important question. As is the difficulty with many human pathogens, CMV cannot be readily studied in any experimental animal and the similarity between the pathogenesis of human CMV and animal CMVs remains difficult to qualify.

Clinical Features of Infection

Infection of the normal host is rarely associated with disease. Most children and adults do not appear to

suffer any disease. An infectious mononucleosis-like syndrome, characterized by fever, malaise, myalgia, liver dysfunction and heterophil antibody-negative lymphocytosis, may follow several weeks after exposure in a small proportion of individuals and may last for several weeks. This disease occurs and was first noticed in transfusion recipients and denoted post-perfusion syndrome. It transpires in the face of an aggressive humoral response but a decrease in CD4+ T cells. Cell-mediated immunity appears to be depressed by CMV infection. Chronic diseases that have been associated with long-term CMV infection include atherosclerosis and restenosis that follows coronary angioplasty, but the role of any ubiquitous virus such as CMV in such diseases remains conjectural.

Congenital CMV disease typically follows a subclinical primary infection of an expectant mother. Disease is much less likely to follow reactivated maternal infection, although virus transmission occurs. Perinatal infection can result in a similar disease pattern, but at a much lower frequency. While 0.5–1% of infants are infected *in utero*, fewer than 10% suffer disease. Cytomegalic inclusion disease is the worst manifestation of congenital CMV, representing an extremely broad spectrum of different syndromes, including petechia, hepatosplenomegaly, jaundice, microencephaly and other serious neurological damage. A majority of symptomatic infants suffer subclinical disease with slowly developing hearing loss and mental retardation. Cytomegalic inclusion disease is the most significant disease caused by CMV, and seems to be a manifestation of widespread viral growth before the immune system has had a chance to develop. Congenitally infected neonates may present with rashes, hepatitis, pneumonitis and gastroenteritis, in addition to long-term neurological damage affecting hearing and IQ. The risk of a similar generalized disease in premature, low birth weight infants who are transfused with CMV-contaminated blood products has led to the use of screened products in this population.

CMV poses a great risk to the immunocompromised host. Primary infection results from transfer of CMV-positive transplants or blood and reactivated infection results when latent virus reactivates due to immunosuppression. In solid organ transplant settings, the transplanted organ is likely to be a target of viral disease and a CMV seronegative individual who receives a seropositive organ is at greatest risk. In bone marrow allografting, the serological status of the recipient seems to be the primary determinant of disease, with seropositive recipients being more likely to suffer the consequences of disease. Disease is often targeted to the transplanted solid organ, and may include pneumonitis, hepatitis, gastroenteritis and other organ-specific diseases. Although prophylaxis for CMV with antiviral drugs and antibodies prevents serious disease in many solid organ transplant settings, CMV remains a serious consequence in other settings. Interstitial pneumonia remains a prominent disease in both bone marrow allograft and heart–lung recipients, despite efforts to control CMV infection with antivirals. In many of the situations where CMV is a major problem, viral immunity is either severely depressed or nonexistent. Adoptive transfer of autologous cytotoxic T cell lines into bone marrow allograft recipients can prevent disease. The humoral response is often intact, and even elevated, in immunocompromised hosts, but to little avail. The experience with immunocompromised hosts reinforces the importance of the cellular immune response in preventing virus reactivation and systemic dissemination in normal hosts.

CMV is one of the most common opportunistic infections in individuals with T cell deficiency and patients with AIDS, where the likelihood of CMV disease correlates with a decrease in CD4+ helper T cell function. Prior to the advent of highly active antiretroviral therapy to control human immunodeficiency virus (HIV) infection, CMV was one of the most common AIDS-defining pathogens. Retinitis, with progressive blindness, and gastroenteritis were the two most common diseases. It remains to be seen what will happen in individuals as HIV resistance to these therapies develops.

Serological diagnosis is widely used to assess past infection, and detection of viral antigen in leukocytes, bronchial lavage, semen or infected tissue is used to assess the level of active infection. The presence of high levels of viral DNA in peripheral blood cells or plasma has also been used to detect active infection. There are no reliable serological assays to assess recent infections, although all classes of antibodies rise during primary infection. Histopathology and virus isolation are used to detect CMV in tissues of bodily fluids, but the presence of virus does not always indicate an ongoing disease process.

Pathology and Histopathology

Historically, diagnosis of CMV infections has focused on identifying cells in infected tissues with obvious CMV cytopathology where the combination of nuclear and cytoplasmic inclusions confers an 'owl's eye' appearance on infected cells. Similar inclusions occur in cultured cells late in productive infection and define sites of viral capsid and virion maturation. CMV-infected cells are many times larger than uninfected cells. In most organs, the virus infects

and causes cytopathology in epithelial, endothelial and reticular cells.

The widespread application of monoclonal antibody and nucleic acid probe technologies has expanded our understanding of the distribution of virus in an infected host and has revealed many cells that become infected with CMV but do not become enlarged or show outward cytopathology. Infection, particularly in an immunocompromised host, is more widespread and involves a greater spectrum of organs than originally anticipated based on histopathology. Virus cytopathology is limited to the salivary glands and kidney tubules, where ductal epithelium is infected and is responsible for the production of virus in saliva and urine. The disseminated infection that occurs in an immunocompromised host results in virus cytopathology in a wide range of tissues in addition to those that are the target of disease, including blood cells, intestines, liver, lungs, pancreas, ovaries, skin and bone. While the brain is the primary site of severe cytomegalic inclusion disease, and a range of mild to severe disruption of brain function may accompany infection, many other organs may be infected in this setting as well. Infection of the inner ear is a common sequel of congenital infection, and direct destruction of cochlear duct cells is related to hearing loss. In AIDS patients, CMV and CMV plus HIV can be found in brain as well as broadly distributed throughout vascularized organs.

Immune Response

Little is known about the innate immune response to CMV, although natural killer cells can recognize and destroy virus-infected cells and the virus encodes a function that modulates this response. The adaptive cellular immune response has long been recognized as critical for clearance of viral infection and resolution of disease. T cell-deficient individuals are predisposed to CMV disease. CMV has provided one indication of the importance of the cytotoxic T cell response because virus-encoded functions directly reduce major histocompatibility class I-mediated antigen presentation. A key piece of evidence supporting the role of CMV-specific T cells that have cytotoxic T cell function has to do with their successful transfer and therapeutic efficacy in humans. A role for humoral antibody is less clear, although passive antibody has been associated with better control of CMV in a number of settings, such as neonatal infection and solid organ transplantation. The neutralizing antibody response to a major envelope glycoprotein, gB, has been correlated with protection in some settings.

Humoral and cellular immunity persists for years after primary infection. The long-term response to certain viral antigens may be favored and may be a better choice for detection of immunity. Antibodies to certain structural antigens such as tegument proteins and nonstructural proteins are excellent markers of past infection in the population, although they have little role in protective immunity. Certain viral tegument proteins appear to be very important targets of the cell-mediated immune response and to mediate protective immunity by cytotoxic T cells.

The immune response does not completely protect from reinfection with different virus strains, a situation that is well documented in sexually active homosexual men and recipients of solid organ transplants or of high volume blood transfusions. In these cases reinfection clearly occurs in the presence of respectable levels of humoral and cellular immunity.

The contribution of the immune response or immunopathology to CMV has been widely suspected but poorly documented. Pathogenesis of important diseases such as pneumonitis may involve an immune component that leaves the host manifesting a damage-inducing response to virus or viral antigens. The modulation of the immune response by CMV is also a widely acknowledged characteristic, particularly in relation to mononucleosis; however, a mechanism remains to be identified. Immunocompromised patients supporting high levels of CMV infection also have circulating immune complexes that may contribute to diseases such as glomerulonephritis.

Prevention and Control

Neither treatment nor prophylaxis has eliminated the risk of CMV disease in susceptible individuals, although the medical importance of this virus continues to stimulate identification and development of new strategies for control. Latency complicates prophylaxis and provides an almost ubiquitous reservoir. The major population of patients that benefit from therapy include immunosuppressed and immunodeficient patients. These individuals have difficulty with drug toxicity due to the numbers of other therapeutics they encounter. Nucleoside, nucleotide and pyrophosphate analogues that inhibit the viral DNA polymerase have been the most widely used therapeutics to date. Several other viral gene products have been targeted with other antiviral candidates in preclinical and clinical development.

CMV is not susceptible to the nucleoside analogue acyclovir except at very high concentrations (100 μm) because it does not encode a thymidine kinase necessary for drug phosphorylation, but is highly

sensitive to a compound, ganciclovir (9-[2-hydroxy-1-(hydroxymethyl)ethoxymethyl]guanine, DHPG), that is structurally related to acyclovir but acts via a poorly understood process whereby another viral kinase (UL97) is responsible for its phosphorylation. Ganciclovir is administered parenterally or, at very high doses, orally and can control CMV infections. Because of the chronic immunocompromised settings where CMV emerges as a pathogen, prolonged treatment is often necessary and this leads to the emergence of drug-resistant mutants due to mutations in the UL97 kinase or the DNA polymerase. Another parenterally administered nucleotide analogue, cidofovir ((S)-1-[3-hydroxy-2-(phosphonylmethoxy)propyl]cytosine), has also been licensed for use against CMV, but it shows a very similar tendency towards resistance. Foscarnate (phosphonoformate, PFA), a pyrophosphate analogue that is also administered parenterally, is used as an alternative to ganciclovir or cidofovir when resistance emerges. Due to the prolonged time of treatment and emergence of resistant strains, effective nontoxic treatments are still greatly needed to treat CMV disease.

Pooled human γ globulin, which has a respectable titer of antibodies to CMV, has been in use as a prophylactic therapy in transplant settings to reduce the incidence of CMV disease alone and in combination with antiviral compounds. Monoclonal antibodies that have a strong neutralizing capability have been tested but have thus far failed to show the benefit ascribed to human γ globulin. Vaccines for CMV were initially developed in the 1970s, and one, Towne 125, has undergone almost continuous evaluation since its initial characterization. This vaccine induces cell-mediated and humoral immunity to CMV, and, when evaluated in a solid organ transplant setting, was attributed with reduction in disease severity. Due to the fact that immunity induced by Towne 125 is not as strong as that induced by natural infection, recombinant chimeric viruses that combine segments of the Towne vaccine genome and a low passage strain, Toledo, have been made to test for immunogenicity. Vaccines consisting of the envelope gB subunit have also been produced by recombinant DNA methods and have been shown to induce strong humoral immunity in preclinical evaluation. Universal vaccination before the child-bearing years is the only way to control congenital disease and other diseases that are caused by this virus.

Future Perspectives

Despite the reduced impact of CMV in AIDS due to combination antiretroviral therapies, this virus remains one of the most important opportunist pathogens. The virulence determinants that create the pathogenic signature of CMV will be better understood, as will the role of the host immune response as a contributor to disease. Additional viral targets for antiviral intervention will continue to be described, and latency and reactivation should continue to be better understood. The contribution of CMV to any of a number of chronic diseases, such as atherosclerosis, will hopefully continue to be evaluated on a firmer scientific basis. Finally, the components of the cellular immune response that provide protective immunity against CMV disease and the viral functions that modulate host immune clearance will continue to be better understood.

See also: **Cytomegaloviruses (*Herpesviridae*): Molecular biology (human); Animal cytomegaloviruses; Human immunodeficiency viruses (*Retroviridae*): General features; Herpesviruses 6 and 7 – human (*Herpesviridae*); Herpesvirus 8 – Human (*Herpesviridae*); Immune response: Cell mediated immune response; General features; Transplantation and virus infections.**

Further Reading

Alford CA and Britt WJ (1996) Cytomegalovirus. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology* 3rd edn, p. 2493. Philadelphia: Lippincott-Raven.

Ho M (1991) *Cytomegalovirus: Biology and Infection* 2nd edn. New York: Plenum Press.

Mocarski ES Jr (1996) Cytomegaloviruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology* 3rd edn, p. 2493. Philadelphia: Lippincott-Raven.

Molecular Biology (Human)

Wade Gibson, Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

Properties of the Virion

Typical of the herpesvirus group, the virion of human cytomegalovirus (HCMV, human herpesvirus 5) is approximately 230 nm in diameter and is composed of a DNA-containing nucleocapsid, surrounded by a less structured tegument layer, and bounded by a trilaminate membrane envelope. The nucleocapsid is icosahedral; has an outside diameter of approximately

sensitive to a compound, ganciclovir (9-[2-hydroxy-1-(hydroxymethyl)ethoxymethyl]guanine, DHPG), that is structurally related to acyclovir but acts via a poorly understood process whereby another viral kinase (UL97) is responsible for its phosphorylation. Ganciclovir is administered parenterally or, at very high doses, orally and can control CMV infections. Because of the chronic immunocompromised settings where CMV emerges as a pathogen, prolonged treatment is often necessary and this leads to the emergence of drug-resistant mutants due to mutations in the UL97 kinase or the DNA polymerase. Another parenterally administered nucleotide analogue, cidofovir ((S)-1-[3-hydroxy-2-(phosphonylmethoxy)propyl]cytosine), has also been licensed for use against CMV, but it shows a very similar tendency towards resistance. Foscarnate (phosphonoformate, PFA), a pyrophosphate analogue that is also administered parenterally, is used as an alternative to ganciclovir or cidofovir when resistance emerges. Due to the prolonged time of treatment and emergence of resistant strains, effective nontoxic treatments are still greatly needed to treat CMV disease.

Pooled human γ globulin, which has a respectable titer of antibodies to CMV, has been in use as a prophylactic therapy in transplant settings to reduce the incidence of CMV disease alone and in combination with antiviral compounds. Monoclonal antibodies that have a strong neutralizing capability have been tested but have thus far failed to show the benefit ascribed to human γ globulin. Vaccines for CMV were initially developed in the 1970s, and one, Towne 125, has undergone almost continuous evaluation since its initial characterization. This vaccine induces cell-mediated and humoral immunity to CMV, and, when evaluated in a solid organ transplant setting, was attributed with reduction in disease severity. Due to the fact that immunity induced by Towne 125 is not as strong as that induced by natural infection, recombinant chimeric viruses that combine segments of the Towne vaccine genome and a low passage strain, Toledo, have been made to test for immunogenicity. Vaccines consisting of the envelope gB subunit have also been produced by recombinant DNA methods and have been shown to induce strong humoral immunity in preclinical evaluation. Universal vaccination before the child-bearing years is the only way to control congenital disease and other diseases that are caused by this virus.

Future Perspectives

Despite the reduced impact of CMV in AIDS due to combination antiretroviral therapies, this virus remains one of the most important opportunist pathogens. The virulence determinants that create the pathogenic signature of CMV will be better understood, as will the role of the host immune response as a contributor to disease. Additional viral targets for antiviral intervention will continue to be described, and latency and reactivation should continue to be better understood. The contribution of CMV to any of a number of chronic diseases, such as atherosclerosis, will hopefully continue to be evaluated on a firmer scientific basis. Finally, the components of the cellular immune response that provide protective immunity against CMV disease and the viral functions that modulate host immune clearance will continue to be better understood.

See also: **Cytomegaloviruses (*Herpesviridae*): Molecular biology (human); Animal cytomegaloviruses; Human immunodeficiency viruses (*Retroviridae*): General features; Herpesviruses 6 and 7 – human (*Herpesviridae*); Herpesvirus 8 – Human (*Herpesviridae*); Immune response: Cell mediated immune response; General features; Transplantation and virus infections.**

Further Reading

Alford CA and Britt WJ (1996) Cytomegalovirus. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology* 3rd edn, p. 2493. Philadelphia: Lippincott-Raven.

Ho M (1991) *Cytomegalovirus: Biology and Infection* 2nd edn. New York: Plenum Press.

Mocarski ES Jr (1996) Cytomegaloviruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology* 3rd edn, p. 2493. Philadelphia: Lippincott-Raven.

Molecular Biology (Human)

Wade Gibson, Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

Properties of the Virion

Typical of the herpesvirus group, the virion of human cytomegalovirus (HCMV, human herpesvirus 5) is approximately 230 nm in diameter and is composed of a DNA-containing nucleocapsid, surrounded by a less structured tegument layer, and bounded by a trilaminate membrane envelope. The nucleocapsid is icosahedral; has an outside diameter of approximately

110 nm; and is made up of four principal protein species that are organized into 162 capsomeres (150 hexamers + 12 pentamers) and 320 'triplexes'. The capsomers are approximately 20 nm in length and 15 nm in diameter; have a channel about 3 nm in diameter that is open at the exterior end; possess favored cleavage planes along the longitudinal axis; and have short spicule-like protrusions, probably representing the triplexes, extending out symmetrically and resulting in a pinwheel appearance of the capsomere viewed end on. The tegument region is approximately 50 nm thick and contains six highly abundant protein species, five of which are phosphorylated. The virion envelope is estimated to be 10 nm thick and contains at least ten protein species, nine of which are known to be glycosylated. The nature of the virion DNA and proteins is described below. In addition to these molecules, the virion is reported to contain the phospholipids phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol; the polyamines spermidine and spermine; several enzymatic activities, including protein kinase, DNA polymerase, DNase, and topoisomerase II; and several host cell proteins, including PP1 and PP2A phosphatases, and a 45 kDa cellular-actin-related protein.

Properties of the Genome

The genome of HCMV is composed of a linear, double-stranded DNA molecule whose M_r of $\approx 150 \times 10^6$ is 50% larger than that of herpes simplex virus (HSV), and the largest of the characterized herpes group viruses. It is a class E isomerizing genome, like that of HSV, consisting of a long unique (U_L) and a short unique (U_S) sequence, both of which are flanked by much shorter inverted repeat sequences that enable U_L and U_S to invert relative to each other and give rise to the four structural isomers of the genome found in virions. The origin of DNA replication (*ori*-Lyt) has been localized to a 3–4 kb region near the center of U_L. Like *ori*-L, one of three duplicated origin sequences in the HSV genome, the HCMV *ori*-Lyt region is located adjacent to the promoter for the early, single-strand DNA-binding protein, DB140 (UL57). HCMV *ori*-Lyt includes a 2.4 kb sequence which has no homology with other described viral DNA replication origins and contains duplications, inversions, consensus binding sequences for the cyclic-AMP response element and other transcription factors, and 23 copies of the sequence AAAACACCGT that is conserved near the homologous *ori*-Lyt of simian CMV. The complete nucleotide sequence of the HCMV strain AD169 genome has been determined (EMBL data base accession number X17403), analyzed and reviewed. It is 229 354 bp in length, has a G + C content of 57.2 moles percent, and contains approximately 200 open reading frames (ORFs) at least 300 bp in length. Lower-passage strains (e.g. Toledo) contain additional ORFs (see Gene Bank accession numbers U33331 and U33332) and are ≈15 kb larger than strain AD169. Sequence analyses have revealed that the HCMV genome has the comparatively unusual feature of containing nine families of closely related genes that tend to be clustered as homologous blocks of ORFs. Most of these encode known (e.g. RL11, US6 and US27 families) or predicted (e.g. US2) glycoproteins. Taken together these gene families represent 26% (59 728 bp) of the AD169 DNA sequence and may, in part, account for the comparatively large size of the HCMV genome. It is also notable that the HCMV genome contains homologues of at least three cellular genes that code for surface receptors: class I major histocompatibility (MHC) antigens, T cell receptor gamma chains (TCR_γ), and G protein-coupled receptors (GCRs).

Properties of the Proteins

HCMV proteins share the general pattern of expression characteristic of the herpesvirus group. Immediate early (α), early (β) and late (γ) proteins are synthesized sequentially from corresponding mRNAs whose transcription is regulated in a temporal cascade (see Characterization of Transcription below). Immediate early proteins are required to regulate transcription from their own promoters and those of subsequently expressed genes. Early proteins include many of the enzymes and regulatory factors needed to carry out the synthesis of progeny DNA and proteins. Late proteins include most of the virion structural proteins. Members of all three classes have been described for HCMV, but only a small number of its more than 200 potential proteins have been identified. Those that have been reported include both nonvirion and virion species and their properties are briefly described below.

Nonvirion Proteins

Many of the recognized nonvirion proteins are made at early times after infection and localize to the nucleus. The first of these to appear is the major immediate early protein (MIEP or IE1, UL123), a 72 kDa phosphoprotein that together with products of a second immediate early gene, IE2 (UL122), can transactivate CMV early promoters. The IE2 gene gives rise to a family of proteins, ranging in size from 23 to 86 kDa, that are generated by differential RNA

splicing and translational start sites. IE2 proteins can repress the immediate early promoter by acting on a sequence immediately upstream of the transcription initiation site. Other immediate early proteins are encoded by ORFs IRS1, TRS1 and UL36–38 and can act synergistically with IE1 and the US3 protein to regulate both cellular and virus gene transcription.

The early proteins include the 140 kDa viral DNA polymerase (UL54), a 140 kDa nuclear DNA-binding protein referred to as DB140 or CMV ICP8 (UL57), and a set of closely related nuclear phosphoproteins (UL112). The DNA polymerase, like its homologue in other herpesviruses, has an associated 3′-specific exonuclease activity. DB140 is the HCMV homologue of HSV ICP8, one of seven HSV proteins essential for viral DNA replication. Like HSV ICP8, the DB140 gene is located adjacent to the origin of viral DNA replication, and the protein localizes to discrete foci within the nucleus of infected cells and accumulates in the cytoplasm under conditions of inhibited viral DNA synthesis. There is also a set of four early, nuclear, DNA-binding phosphoproteins (34, 43, 50 and 84 kDa) that are related to each other by their common N-terminal sequence; appear to be produced by alternate splicing; have expression regulated at both the transcriptional and post-transcriptional level; and are differentially phosphorylated. Some similarity between these proteins and Epstein–Barr virus nuclear antigen (EBNA-1) suggests that they may have a role in DNA replication or gene regulation.

Another nonvirion nuclear DNA-binding phosphoprotein is distinguished by its high abundance and its kinetics of synthesis. This ≈52 kDa species, referred to as DB52 or ICP36, is the product of ORF UL44. On the basis of its reduced but not eliminated synthesis under conditions of inhibited viral DNA replication, DB52 is a delayed-early or γ_1 protein. Expression of this gene is under different promoter control at early and late times of infection, and there is evidence that its expression is also post-transcriptionally regulated. DB52 and its simian CMV homologue DB51 bind to DNA *in vitro* and *in vivo* and associate with the viral DNA polymerase to enhance its activity, analogous to the HSV UL42 protein. Two other proteins that appear to function in viral DNA replication or processing are a phosphotransferase (UL97 protein) and an apparent homologue of the bacteriophage DNA cleavage/packaging enzyme, terminase (UL89 protein).

Several other nonvirion HCMV proteins have recently been shown to interfere with the class I MHC antigen presentation system (i.e. US11 and US6 proteins). These proteins are thought to help the virus-infected cell escape recognition and destruction by immune surveillance, and provide a mechanism for virus persistance in the immune-competent host.

Virion Proteins

HCMV virions contain a minimum of 23 protein species that differ somewhat in size and relative amount from strain to strain. Characteristics of these proteins, including size, charge, carbohydrate and phosphate content, and deduced location in the particle, are summarized in **Table 1**, along with a descriptive name and the ORF encoding each.

The capsid shell is composed of four abundant protein species referred to as the major capsid protein (MCP), the minor capsid protein (mCP), the mCP-binding protein (mC-BP) and the smallest capsid protein (SCP). Formation of the capsid shell is coordinated by precursors of an internally located maturational proteinase (assemblin) and assembly protein (pAP), which serve a scaffolding role and are present in nascent intranuclear capsids (e.g. B-capsid) and in noninfectious enveloped particles (NIEPs, see below), but not in virions. These internal scaffolding proteins are the only abundant capsid proteins known to be post-translationally modified (**Table 1**).

There are seven proteins provisionally designated as virion tegument constituents. The largest of these, called the high molecular weight protein (HMWP), forms a heterotrimer with another tegument protein called the HMWP-binding protein (hmw-BP), and is thought to be the HCMV homologue of the HSV UL36 protein, VP1,2, which binds to the cleavage/packaging sequence of HSV DNA. Neither the HMWP nor its binding protein is detectably phosphorylated or glycosylated. The basic phosphoprotein (BPP or pp150) and the upper matrix (UM or pp71) and lower matrix (LM or pp65) proteins account for the greatest protein mass of the tegument constituents and are highly phosphorylated, both *in vivo* and *in vitro*, by the virion-associated protein kinase. The BPP is distinguished among the virion proteins by having the highest density of *O*-linked *N*-acetylglucosamine residues, which are attached at a single site or tight cluster of sites. The upper matrix protein is a transacting inducer of immediate early gene transcription that may be functionally analogous to the *α-trans*-induction factor of HSV; the lower matrix protein appears to be an important component in cellular immunity and there are reports that it has an associated protein kinase activity. Several much lower-abundance species have also been found associated with HCMV capsid/tegument structures, including the UL56 protein.

The virion envelope contains at least ten protein species, of which nine are known to be glycosylated.

Table 1 Proteins of the virion

Descriptive name	M_r[a]	*Modification*[b]	*Charge*[c]	*ORF*[d]
Capsid				
Major capsid protein (MCP)	153	—	N	UL86
(Assembly protein) (AP)[e]	(36)	P, C	B	UL80.5
mCP-binding protein (mC-BP)	35	—	N	UL46
Minor capsid protein (mCP)	34	—	B	UL85
Proteinase assemblin (A, An, Ac)[f]	28	—	A	UL80a
Smallest capsid protein (SCP)	8	—	B	UL48/49
Tegument				
High mol. wt protein (HMWP)	212	—	N	UL48
Basic phosphoprotein (BPP; pp150)	149	G, P	B	UL32
HMWP-binding protein (hmw-BP)	115	—	N	UL47
80K	80	P		
Upper matrix protein (UM; pp71)	74	P	N	UL82
Lower matrix protein (LM; pp65)	69	P	B	UL83
24K (pp28)	24	P	B	UL99
Envelope				
gB (gcI)	102	G, P, C	N	UL55
Acidic glycoprotein (AGP)	145	G	A	
gB_N (gcI)	130	G	A	UL55
gH (gcIII)	84	G	N	UL75
gp70	70	G	A	
gB_C	62	G, P	A	UL55
gp57 (gcII)	57	G	A	
Integral membrane protein (IMP)	43			UL100
GCR33	44	G	B	UL33
GCR27	42	G	B	US27

[a] Values given determined by SDS-PAGE and are $\times 10^3$.
[b] G, glycosylation; P, phosphorylation; C, proteolytic cleavage.
[c] Relative net charges were determined by 2-D PAGE (charge/size). N, neutral with respect to the major capsid protein, which is approximately neutral in such separations; A, acidic with respect to the major capsid protein; B, basic with respect to the major capsid protein.
[d] Open reading frame, where known.
[e] Present as a component of immature capsids and noninfectious enveloped particles (NIEPs), but absent from virions.
[f] Assemblin (A) is autoproteolytically concerted to an amino (A_n) and carboxyl (A_c) half.

The high degree of size and charge heterogeneity among this group has complicated their analysis. Three of the HCMV envelope glycoproteins are homologues of HSV glycoprotein B (gB). HCMV gB is synthesized as a 130–160 kDa, cotranslationally *N*-glycosylated precursor that undergoes at least four further modifications: (1) its 17–19 predicted *N*-linked oligosaccharides are converted from nontrimmed to trimmed high mannose forms, and then a portion are further modified to complex structures; (2) *O*-linked carbohydrates are added; (3) the C-end of the molecule is phosphorylated; and (4) the protein is cleaved between Arg460 and Ser461 to yield an intramolecularly disulfide-linked heterodimer composed of a highly glycosylated 115–130 kDa N-terminal fragment (gB_N), and a less extensively modified 52–62-kDa C fragment (gB_C). This gB_N–gB_C heterodimer can form intermolecular disulfide crosslinks, yielding a complex with the composition $(gB_N–gB_C)_2$. A fourth HCMV envelope glycoprotein is the homologue of HSV gH. HCMV gH is an 86 kDa species that is less extensively *N*-glycosylated than gB and its oligosaccharides are not processed beyond the high mannose structure, gH has been proposed to mediate virus–host membrane fusion during the initial steps of virus infection, and its intracellular trafficking associates covalently with another HCMV glycoprotein (gL, UL115 protein).

Two other envelope proteins and two disulfide-linked glycoprotein complexes have been described.

Glycoprotein complex II (gcII) is composed of proteins with M_rs of 50–52 kDa and >200 kDa. These proteins have similar peptide maps indicating that they are closely related, and the 52 kDa component is comparatively highly *O*-glycosylated and sialylated. Glycoprotein complex III (gcIII) is composed of two glycoproteins, 145 and 86 kDa. An additional highly sialylated, *O*-glycosylated virion glycoprotein, gp145, differs from the 145 kDa gcIII protein in that it is not disulfide linked. The relative amount of this protein is more strain-dependent than those of gB and gH. Another envelope protein that may be a component of the gcII complex is referred to as the integral membrane protein (IMP, gM, UL100 protein). It is the homologue of HSV UL10, has an M_r of approximately 45 kDa, five potential *N*-glycosylation sites and eight possible membrane-spanning domains.

Physical Properties

Infectivity of HCMV is eliminated by exposure to 20% ether for 2 h, by heating at 65°C for 30 min, by exposure to ultraviolet light (4000 ergs s^{-1} cm^{-2}; 400 μJ s^{-1} cm^{-2}) for 4 min, by acidic pH (<5) and by treatment with low concentrations of either ionic or nonionic detergents. Infectivity is also reduced following sonication, pelleting by ultracentrifugation, and repeated cycles of freezing and thawing.

Replication

General features of the HCMV replication cycle are typical of the herpesvirus group. Following virus adsorption and entry into the cell, the DNA is transported to the nucleus where it is replicated and packaged into preformed capsids. Maturation of the nucleocapsid involves acquisition of the tegument constituents followed by envelopment, which has been observed at both nuclear and cytoplasmic membranes.

DNA Replication

HCMV DNA is replicated in the nucleus. Parental DNA appears to circularize soon after entering the cell, but maximal rates of progeny DNA synthesis do not occur until approximately 3 days after infection. The 'endless' concatameric nature of progeny DNA is consistent with a rolling circle mechanism of DNA replication. However, the finding that viral DNA synthesis proceeds bidirectionally from *ori*-Lyt under conditions of ganciclovir [9-(1,3-dihydroxy-2-propoxymethyl) guanine, (DHPG)]-inhibited replication is not easily reconciled with this model. Unit length genomic DNA is released from the concatamers by cleavage at a specific site located in the 'a' repeat elements, 30–35 bp from a herpesvirus group-common cleavage/packaging region referred to as the 'herpes pac homology' sequence.

Characterization of Transcription

Transcription of the HCMV genome is temporally regulated and the pattern and control of gene expression shares many features with that of HSV. The first class of mRNAs made are transcribed from input DNA and are referred to as immediate early (IE) or αRNAs. Their expression requires no preceding viral protein or DNA synthesis, and they are operationally defined as those RNAs made in the presence of inhibitors of protein synthesis. The most abundant IE RNAs are transcribed from a region between 0.732 and 0.751 map units, which contains ORF UL122 and exons UL123EX2–4. These transcripts are related to each other by alternate splicing patterns and encode IE1 and multiple forms of IE2. The promoter region for this transcription unit is one of the strongest known, presumably due to its high content of consensus binding sequences for host cell transcription factors such as nuclear factor 1, CAAT-binding protein, and SP1; and the large number of repeat sequences that contain cyclic-AMP response elements and which may interact with additional DNA-binding proteins. Transcription from this region is enhanced by a virion tegument phosphoprotein, referred to as the upper matrix protein or pp71 (UL82), whose function appears to be analogous to that of the HSV *α-trans*-induction factor. Lower abundance IE RNAs arise from ORFs IRS1, TRS1 and UL36–38.

Expression of early or βRNAs requires the preceding synthesis of immediate early proteins, but does not require viral DNA synthesis. Thus, early RNAs are made in the presence of inhibitors of viral DNA synthesis, but not in the presence of inhibitors of protein synthesis. Both immediate early and early genes appear to be transcribed by host cell RNA polymerase II. Expression of late or γRNAs requires the preceding synthesis of both immediate early and early viral proteins, as well as synthesis of viral DNA. The possibility that post-transcriptional events may be involved in controlling HCMV RNA processing and translation, is suggested by the presence of some 'early' RNAs that are not translated until 'late' times of infection.

Characterization of Translation

HCMV RNAs are translated by the host cell protein synthesizing system. Virus-specific proteins that may

augment the host system (e.g. initiation or elongation factors) have not yet been identified. Each viral protein species appears to be encoded by a unique mRNA. Cells infected with HCMV do not show the generalized early shutoff of cell protein synthesis that occurs in HSV-1 infected cells. There are, nevertheless, very early changes in the metabolism of HCMV-infected cells that include: stimulated transcription of the genes for heat shock protein 70, ornithine decarboxylase, thymidine kinase, and creatine kinase; decreased transcription of the fibronectin gene; and changes similar to those induced by the G protein signaling pathway, such as decreased intracellular Ca^{2+} stores and increased levels of intracellular cAMP.

Post-translational Modifications

Modifications of HCMV proteins include phosphorylation, glycosylation and proteolytic cleavage. Phosphorylation has been detected on immediate early (e.g. IE1 and IE2), early (e.g. UL112) and late (e.g. γ_1 nonvirion UL44 protein; γ_2 virion tegument (UL32, UL82, UL83) and capsid (UL80.5)) proteins, all of which localize to the infected-cell nucleus. At least some of these phosphorylations are likely to be catalyzed by reported virus-coded protein kinases. Glycosylation typifies many of the virion envelope glycoproteins and includes both *N*-linked (high mannose and complex structures) and *O*-linked oligosaccharides. The only nonenvelope virion protein that has been demonstrated to be glycosylated is the tegument basic phosphoprotein, which has *O*-linked *N*-acetylglucosamine. Glycosylation of HCMV proteins is most likely carried out by host cell systems. The third modification that has been described for HCMV proteins is proteolytic cleavage. There are two maturational endoproteolytic cleavages, in addition to glycoprotein signal sequence cleavages. The first of these cuts between Arg460 and Ser461 in the envelope glycoprotein gB and is catalyzed by a calcium-dependent cellular, furin-like proteinase. The other is a cleavage between residues Ala308 and Ser309 of the capsid assembly protein precursor (UL80.5 protein). This later cleavage is catalyzed by a genetically related, virus-encoded proteinase, assemblin (UL80a protein), and is essential for the production of infectious virus.

Cytopathology

The mechanisms of cell recognition, binding and penetration by HCMV are not yet understood, but there are at least four proteins that may be involved: (1) β_2-microglobulin is present on virions and may interact with cell surface major histocompatibility proteins; (2) virion envelope glycoprotein B binds to a 30 kDa cell protein that may be a receptor; (3) virion envelope glycoprotein H interacts with a 90 kDa cell protein that may also be involved in binding or penetration; and (4) a highly *O*-glycosylated envelope glycoprotein complex (gC-II) is able to bind heparin, and this interaction could promote early virus-cell interaction as proposed for cellular heparin sulfate and HSV-1.

Assembly of the capsid and DNA replication and packaging take place in the nucleus. As with HSV, the tegument constituents are presumed to be added to the maturing nucleocapsid in the nucleus, and envelopment to occur as the tegument-coated capsid passes through the inner nuclear membrane. The presence of nonenveloped capsids appearing to bud through tubule or vesicle membranes in the infected-cell cytoplasm suggests the possibility of additional or alternate envelopment mechanisms for HCMV.

The cytopathic effects of HCMV are distinctive. Early after infection a transient cell rounding occurs, followed by overall enlargement (cytomegalia) and by the appearance of basophilic intranuclear inclusions, which gradually enlarge to fill and distort the nucleus into an elongated or kidney-shaped form late in infection. These intranuclear inclusions contain viral DNA, proteins and capsids and are thought to be nucleocapsid assembly sites. Late in infection the cytoplasm contains numerous enveloped and nonenveloped capsids, large (>500 nm 'black holes') and smaller (250–500 nm 'dense bodies') electron-dense aggregates, and a large eosinophilic spherical region adjacent to the nucleus that exhibits strong F_C binding.

Although the mechanism by which HCMV is released from the infected cell is not known, the process results in three kinds of enveloped particles being freed into the growth medium of cultured fibroblasts: (1) virions, and two aberrant particles referred to as (2) noninfectious enveloped particles (NIEPs), which closely resemble virions in structure and composition, but have no DNA and contain the capsid assembly protein (UL80.5 protein) that is absent from virions, and (3) dense bodies, which are solid, 250–600 nm spherical aggregates of lower matrix protein (pp65 or UL83 protein) that contain no DNA and are surrounded by an envelope layer.

See also: **Cytomegaloviruses (*Herpesviridae*): General features (human), Animal cytomegaloviruses; Epstein-Barr virus (*Herpesviridae*): General features, Molecular biology; Herpes simplex viruses (*Herpesviridae*): General features,**

Molecular biology; Latency; Pathogenesis: Animal viruses; Persistent viral infection; Transplantation and virus infections; Varicella-Zoster virus (*Herpesviridae*): General features, Molecular biology.

Further Reading

Britt WJ and Alford CA (1996). Cytomegalovirus. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 2493. Philadelphia: Lippincott-Raven.

Chee MS, Bankier AT, Beck S *et al* (1990) Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. *Curr. Top. Microbiol. Immunol.* 154: 125.

Gibson W, Welch AR and Hall MRT (1995) Assemblin, a herpes virus serine maturational proteinase and new molecular target for antivirals. In: Craik C (ed.) *Perspectives in Drug Discovery and Design*, p. 413. The Netherlands: ESCOM Science.

Mocarski ES (1996) Cytomegaloviruses and their Replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology* 3rd edn, p. 2447. Philadelphia: Lippincott-Raven.

Stinski MF (1997) Cytomegaloviruses. *Intervirology* 39: p. 293.

Roizman B (1996) Herpesviridae. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology* 3rd edn, p. 2221. Philadelphia: Lippincott-Raven.

Animal Cytomegaloviruses

Donald J Alcendor and **Gary S Hayward**, Departments of Pharmacology and Oncology, Johns Hopkins School of Medicine, Baltimore, USA

History

In addition to human cytomegalovirus or salivary gland virus (HCMV or HHV-5) and the equivalent mouse virus (MCMV), closely related species with typical cytomegalovirus-like characteristics have been described from rats (RCMV), guinea pigs (GpCMV) and other rodents, and from both Old and New World monkeys (SCMV). GpCMV was originally recognized in 1920 and served as a model system for the biology and pathogenicity of HCMV disease for many years. Host-specific SCMV isolates have been reported from almost all major nonhuman primate groups, including gorilla, chimpanzee, bonobo, baboon, rhesus and other macaques, African green monkey, spider monkey, owl monkey, capuchin and marmoset.

Classification and Evolution

All of the animal CMVs described to date are members of the family *Herpesviridae* and belong to the *Betaherpesvirinae* subfamily of herpesviruses. Based on the relatively high (G+C) content of their DNA molecules and their adaptability to grow in fibroblasts in cell culture, most if not all of the traditionally recognized animal CMVs are likely to be evolutionarily more similar to the beta-1 subgroup that includes HCMV and MCMV than to the (A+T)-rich beta-2 subgroup exemplified by HHV-6. The International Committe on Taxonomy of Viruses has now classified betaherpesviruses into three distinct genera, *Cytomegalovirus* (HCMV-like), *Muromegalovirus* (MCMV-like) and *Roseolovirus* (HHV-6, HHV-7), but it is likely that distinctions between the first two taxons at least will become murky when molecular genetic criteria (i.e. gene content and DNA and protein sequence similarities) are applied to those CMV species that infect other mammalian and primate hosts. The genomes of prototype species of all three taxons, including HCMV (1989; 229354 bp; X17403), MCMV (1996; 230278 bp; U68299) and HHV-6 (1995; 159321 bp; X83413) as well as HHV-7 (1996; 144861 bp; U43400), have all been completely sequenced.

All formally recognized betaherpesviruses are listed in **Table 1**. However, it should be noted that there are two very different viruses (with somewhat different gene content) that are both known as rat cytomegalovirus (RCMV). Two CMV-like viruses have also been recently reported to be associated with fatal hemorrhagic disease in young African and Asian elephants, but current genetic analysis suggests that these two viruses are distinct in the two groups of animals and from all three of the currently recognized taxons. Another CMV-like virus, sometimes referred to as 'Stealth virus', has been reported to have been isolated from a human patient with central nervous system neuropathy, but genetic analysis has revealed that this virus contains UL34, UL35 and SSB genes that are virtually identical to those of several characterized SCMV isolates from African green and Vervet monkeys (AgCMV). The AgCMV isolate 'Colburn' was also originally thought to have been of human origin, but whether either is truly a human isolate rather than a contaminant from primary monkey cell cultures is subject to some doubt. Based on genome analyses, both the bovine herpesvirus BHV-4 and the two equine herpesviruses EHV-2 and EHV-5, which were all originally thought to be

Hayward GS, Ambinder R, Ciufo D, Hayward SD and LaFemina RL (1984) Structural organization of human herpesvirus DNA molecules. *J. Invest. Dermatol.* 83: 29.

Isom HC and Yin CY (1990) Guinea-pig cytomegalovirus gene expression. *Curr. Top. Microbiol. Immunol.* 154: 101.

Kaplan AS (1973) *The Herpesviruses,* chapts 12 and 13. London: Academic Press.

LaFemina RL and Hayward GS (1980) Structural organization of the DNA molecules from human cytomegaloviruses. In: Jaenisch R, Fields J and Fox F (eds) *Animal Virus Genetics.* ICN-UCLA Symposium on Molecular Biology, vol. 18, p. 39. New York: Academic Press.

Staczek J (1990) Animal cytomegaloviruses. *Microbiol. Rev.* 54: 247.

Murine Cytomegaloviruses

John Staczek, Louisiana State University Medical Center, Shreveport, Louisiana, USA

History

Cytomegalia refers to the enlargement of a cell. The discovery of the viral etiology of cytomegalia in the mouse salivary gland preceded the isolation of the virus by 19 years. The mouse salivary gland virus, commonly called mouse (or murine) cytomegalovirus (MCMV), is a surrogate for studying human cytomegalovirus (HCMV)-associated pathogenesis.

Classification

Classification by the International Committee on Taxonomy of Viruses (ICTV) places MCMV in the family *Herpesviridae,* subfamily *Betaherpesvirinae,* genus *Muromegalovirus,* type species mouse cytomegalovirus 1. Herpesviruses are designated by serial arabic number and the family or subfamily of the natural host. Thus, MCMV is designated Murid herpesvirus 1, but its most commonly used synonym is mouse cytomegalovirus.

Properties of Virion

MCMV virions have typical herpesvirus morphology. The virions have a buoyant density of 1.20 g cm^{-3}. Their nuclear membrane-derived envelope is sensitive to denaturation by detergents, pH, heat and desiccation. Viral glycoprotein spikes extending through the envelope are linked to the nucleocapsid via tegument proteins. The icosahedral nucleocapsid, containing 162 capsomeres, surrounds an ultraviolet radiation-sensitive, linear, double-stranded DNA.

Properties and Organization of Genome

General comments concerning the DNA sequence and genomic organization of the Smith strain of MCMV are presented. It is suggested that the reader refer to Rawlinson *et al* for a more detailed description of the DNA sequence and compilation of open reading frames (ORFs).

DNA sequencing of the Smith strain genome reveals 230 278 bp. Buoyant density and sequence analyses denote an overall 58.7% G+C content distributed with areas of major (155–163 kb) and minor (1–17 kb and 204–221 kb) G+C content. Coding regions have a higher G+C content than noncoding regions. CpG dinucleotides occur throughout the genome at frequencies expected for random associations between mononucleotides, except for a CpG deficiency that is evident in the major immediate early (MIE) gene region.

DNA isolated from purified virions contains a single 3′ nucleotide extension that may facilitate circularization to a prereplicative intermediate. Herpesvirus genomes are grouped (A–F) according to sequence reiterations greater than 100 base pairs. Based on this scheme, the MCMV genome, consisting primarily of unique sequences, is catalogued with the group F genomes. However, clusters of 31-bp direct repeats delimit each terminus of the genome, and additional, nonhomologous, intra- and intergenic direct repeats punctuate the centralized unique region. Inverted repeats, present in the origin of replication and upstream of several ORFs, do not mediate isomerization.

Families of homologous genes are a general feature of betaherpesviruses. MCMV has six gene families, two of which (m02 and m145) are unique and four of which are homologous to HCMV families US22, UL25, UL82 and to the G protein-coupled receptors (GCRs). The functions of the gene families are generally unknown. However, the M25 protein has homology to eukaryotic nucleolins and, therefore, may interact with chromatin.

Genetics

Restriction length fragment polymorphisms among MCMVs isolated from wild-type mice indicate widespread heterogeneity in genomic composition which is reflected in peptide analyses using polyclonal sera recovered from infected wild mice. Strain variation influences disease presentation and recovery. Variation in the nonapeptide of the immunodominant

immediate early protein, pp89, presented by major histocompatability complex (MHC) class I molecules confers escape from antigen-specific cytotoxic T lymphocytes (CTLs). The Cmv1 locus located on chromosome 6 of the mouse determines the host's susceptibility/resistance phenotype.

Replication

Strategy of replication of nucleic acid

The presence of a singular, unpaired 3′ nucleotide extension on the linear DNA molecule provides an opportunity for the formation of a circular prereplicative intermediate. Semiconservative replication occurs by way of a rolling-circle model. The MCMV replicator region (oriLyt) contains 1.7 kbp and is analogous to (but not identical to and cannot substitute for) its HCMV counterpart. The oriLyt is composed of AT- and GC-rich regions, many repeat sequence elements and arrays of closely spaced direct repeats.

Characterization of transcription

MCMV transcription is temporally ordered and regulated in a cascade fashion. The time-dependent appearance of transcripts relative to the initiation of infection and replication of genomic DNA, and the use of inhibitors of transcription, translation and DNA replication, allow division of the transcription into immediate early (IE), early (E) and late (L) phases.

IE transcripts are made from three regions which are similar to three of the four IE regions of HCMV. The most abundant IE mRNAs originate from the MCMV MIE region (0.769–0.818 map units). Transcription from the MCMV MIE is similar to that found in HCMV MIE transcription: transcripts from both viral genomes are colinear, have matching splicing patterns and are under the influence of a strong enhancer. The MIE contains three transcriptional units. The *ie1* gene contains four exons and is transcribed from right to left from the very strong MIE enhancer. The *ie2* gene is located on the opposite side of the enhancer and is transcribed in the opposite direction from *ie1*. The *ie3* gene contains exons 1, 2, and 3 of *ie1* spliced to a fifth exon located downstream of exon 4; thus, this transcript is a hybrid (*ie1/ie3*). The IE1 and IE2 regions are CpG-suppressed and possibly accessible to cellular methylation enzymes during the replication cycle. The enhancer associated with the MIE is composed of five 94-bp tandem repeats containing many viral and cellular transcriptional regulator binding sites responsive to p21ras, NF kappa B, AP1, SP1, ATF and p53. Expression from the MCMV MIE promoter appears to be less species-specific than its HCMV counterpart. The other IE transcription regions in MCMV include ORFs M36–M38 and m143. In contrast to the HCMV *UL37* gene, the M37 ORF does not contain consensus splice donor or acceptor sites. The m143 ORF is similar to the HCMV TRS1 region.

Early and late phase transcripts originate from transcription units located throughout the genome. In general, these transcripts are not spliced. The exceptions are transcripts originating from ORFs m133/m132, which encode the *sgg1* gene required for virus replication in the salivary glands, and M33, which encodes the MCMV homologue to the cellular G protein-coupled receptors (GCR). However, all transcripts are modified post-transcriptionally by capping and polyadenylation.

Characterization of translation

After the transcription of viral genes, the translation of viral messenger RNAs occurs on cellular ribosomes. Currently, much effort is focused on the identification of viral polypeptides, post-transcriptional modifications, and sequence homology to other cytomegalovirus proteins.

Properties of Proteins

Many MCMV structural and nonstructural proteins are functionally homologous to those in other herpesviruses. Most of these proteins contain significant sequence similarity with HCMV.

Proteins with regulatory functions

Several MCMV regulatory genes are homologous to HCMV and HSV-1 genes. MCMV M69 is homologous to HCMV UL69 and HSV-1 ICP27. MCMV ORFs M121 and M116 have sequence similarity to HSV-1 transactivator ICP0. The MIE phosphoprotein (pp89) transactivates heterologous promoters, including the NF-kappa B p105/p50 promoter, the proto-oncogene c-*fos*, and the SV40 early promoter, and interacts synergistically with MCMV IE3 to transactivate the early gene, *e1*. The 88-kDa IE3 protein is homologous to HCMV UL122, autoregulates the *ie3* promoter, and is a major transactivator of early and late genes.

Proteins involved with genome replication and repair

MCMV has homologues for six of seven HSV-1 origin-dependent replication proteins. These proteins are the DNA polymerase, the polymerase accessory protein, the helicase complex (containing three proteins) and the alkaline nuclease. Homologous en-

zymes involved in nucleotide metabolism and repair include ribonucleotide reductase (M45), uracil-DNA glycosylase (M114), dUTPase (M27), pyruvoyl decarboxylase (M77), and phosphotransferase (M97). MCMV does not encode a thymidine kinase or an origin-binding protein.

Structural proteins

It is predicted that MCMV encodes 33–35 structural proteins including the major capsid protein (M86), the large tegument protein (M48), a minor capsid protein (M46), the upper (M82) and lower (M83) matrix proteins, large (M32) and small (M99) phosphoproteins, and a fused protease-capsid assembly protein precursor (M80). The 28-kDa matrix phosphoprotein induces a strong humoral immune response during MCMV infection of mice.

Other proteins

The M84 ORF encodes a nonstructural protein with homology to HCMV UL83 and UL84. M44 ORF encodes a DNA-binding protein. The M103 gene product is an immunoreactive protein that is homologous to HCMV UL103. The M97 protein is a phosphotransferase.

Glycoproteins

There are 64 genes that potentially encode glycoproteins. MCMV glycoproteins with homology to HCMV glycoproteins have been identified by sequence similarity, potential transmembrane hydrophobic sequences, and potential N-linked glycosylation sites. MCMV homologues to HSV-1 glycoproteins include gB (M55), gH (M75), gM (M100) and gL (M115). Antibodies to MCMV gL or gH do not exhibit neutralizing activity in a plaque reduction assay.

Assembly Site, Uptake, Release, Cytopathology

Nucleocapsid self-assembly occurs in the nucleus. Concatemeric viral DNA is cleaved and packaged into preassembled capsids. The association of the nucleocapsids with the nuclear membrane is facilitated by the viral tegument proteins. Viral glycoproteins are targeted to the nuclear membrane and aid in virion assembly. The virion buds out into the cisternae of the Golgi apparatus where further modifications of the glycoproteins may occur.

Infected cells display a cytopathology that is characterized by the enlargement (cytomegaly) of the cell. The enlarged cells have characteristic intranuclear inclusion bodies, marginated chromatin, and large cytoplasmic vacuoles.

Evolution

Comparisons between MCMV sequences and nucleotide sequences of herpes simplex type 1 (HSV-1), equine herpesvirus type 1 (EHV-1), Epstein–Barr virus (EBV), and channel catfish virus (CCV) reveal significant differences in the overall arrangement of genes and nucleotide composition. Yet, MCMV has similarity at the nucleotide level with HCMV. MCMV and HCMV homologues are encoded in the central portion of both genomes and possess either regulatory or structural functions.

Host Range and Virus Propagation

MCMV infects wild or commercially-raised mice. In Australia 80–90% of wild mice (*Mus domesticus*) are seropositive for MCMV. Seroprevalence varies significantly with time and host density. Multiple strains of MCMV coexist in individuals or populations. The incidence of infection in field mice is higher than among commercially available strains. Successful infection of commercially bred mice depends on virus strain, dosage site of inoculation and genetic susceptibility of the mouse.

Most laboratory strains of MCMV are descendants from the original Smith strain isolated in 1934. Initially serially propagated in mice as a homogenate of infected salivary gland tissue, these strains replicate efficiently in cultures of 13–16-day mouse embryos, in epithelial cells of tracheal ring explant cultures, or in continuous murine cell lines. MCMV also replicates in hamster, rabbit, monkey, and sheep cell cultures. Passage of MCMV in mouse tissue culture quickly attenuates its virulence, which may be restored following *in vivo* passage.

Transmission

MCMV is recovered from saliva, urine and sperm of infected mice. Caged weanling mice are infected through contact. Therefore, environmental and sexual contact are probable routes for MCMV transmission.

Pathogenicity

The virus is not highly pathogenic in the immunocompetent host. Experimental pathogenicity of MCMV depends on several factors including dosage, virus strain, genetic susceptibility of the mouse, site of inoculation, and age of the mouse. Immunocompetent mice manifest minor illness when inoculated with a

sublethal LD50. Virus continually passaged in tissue culture loses virulence. Commercially available mice may be genetically susceptible, moderately resistant, or resistant.

Virus titers following an intraperitoneal infection peak between days 5 and 7. Initial virus replication occurs in peritoneal macrophages and mesothelial cells and virus is subsequently disseminated by mononuclear cells to the liver and the spleen. Virus amplification in the reticuloendothelial organs precedes secondary viremia. Infected mononuclear cells infiltrate organs to initiate organ-specific infection. Resolution of the productive infection need not clear virus from organs which become chronically infected. Chronic infections may be persistent or latent. Persistently infected mice shed virus from saliva and urine, and virus may be recovered from explants of organ tissues in a time-dependent manner. The persistent infection wanes, and virus proliferation segues into a state of molecular latency in which the copy number of viral genomes per organ reflects the initial viral burden. In latent infection, viral footprints are detected by molecular analyses, or infectious virus is recovered after immunosuppression or by explantation/cocultivation/transplantation of infected tissues on permissive cell lines or into severe combined immunodeficient (SCID) mice. Primary infections in younger mice are more likely to establish latent infections than in older mice. In germ-free and specific pathogen-free mice, the indigenous bacterial flora influences the establishment of organ-specific latency. The extent of organ-specific, latent MCMV burden in immunocompetent, but not immunocompromised, mice reflects virus amplification during primary infection and augurs reactivation.

Pathology and Histopathology

During a generalized infection, MCMV infects many organs and tissues including the salivary glands, lungs, pancreas, liver, spleen, adrenal glands, cardiac muscle, striated muscle, and, to a lesser extent, lymphoid tissue and kidney. Thymic involution occurs as a result of infection, but the thymus does not support virus replication. Pathologic changes occur in the central nervous system only after intracerebral inoculation. The acinar cells of the salivary gland are the site for persistent MCMV infection. Whether a clear distinction between low level persistence and true molecular latency has been made in published reports is problematic. Organs suspected of harboring latent, but not lytic (persistent), virus include the spleen, kidneys, heart, liver, brain, salivary glands and lungs. Organ-specific endothelial cells, infiltrating T lymphocytes, macrophages and/or peripheral blood mononuclear leukocytes are candidates for the reservoirs of latent virus.

MCMV Infections as Models of Human Infection

There is no specific disease associated with MCMV infection of wild mice. Sublethal dosages of virus inoculated intraperitoneally into commercial mice cause a variety of generalized and organ-specific symptoms. MCMV infections of mice are used as models for HCMV disease. A variety of mouse tissues and organs can be selectively inoculated and the pathology of the resulting infection can be studied with respect to histology and immune responses.

Embryonic development

MCMV infection of pregnant mice causes a decrease in litter size and an increase in stillborn fetuses. Since virus is not recovered from stillborn or newborn mice, it is presumed that the virus cannot pass the placental barrier and that infection of pregnant mice indirectly influences fetal development. Microinjection directly into the endometrial lumina, or the transplantation of blastocyst stage embryos infected *in vitro*, increases the incidence of fetal wastage; however, the embryos are not infected. After day 7.5 of gestation, embryos support infection. Infecting the conceptus at mid- or late-gestation causes microphthalmia, cortical atrophy and cystic lesions in a time-dependent manner. Embryonic mesenchymal, neuronal and endothelial cells support MCMV replication. Mesenchymal cells are the primary target of infection and subsequent spreading to the eyes and brain induces disrupted organogenesis.

Lung infections

Intranasal inoculation of immunocompetent mice with small doses of MCMV produces a generalized, subclinical infection without pneumonitis, whereas larger dosages effect a fatal, interstitial pneumonitis characterized by macrophage infiltration and accumulation of proteinaceous material in pulmonary lesions. When administered intraperitoneally, virus is not recovered from the lungs unless mice are treated with cytokines. Athymic mice develop severe pneumonitis following intranasal inoculation.

Inner ear infection

Experimental inoculation of MCMV through the anterior coronal suture results in perilabyrinthitis in the cochlea.

Eye infections

Intraperitoneal inoculation is not necessarily associated with overt ocular disease, but virus is recovered from explanted tissues of the eye or optic nerve. In natural killer cell-depleted BALB/c mice inoculated intraperitoneally, virus is recovered from blood lymphocytes, macrophages, granulocytes and plasma, but not the optic nerves or trigeminal ganglia. Recovery of virus from the eyes of mice inoculated intravenously requires immunosuppression prior to infection. In normal mice, direct intraocular inoculation of MCMV produces pathology on the eyelid, conjunctiva and cornea. The virus spreads to the ocular muscles and chorioretinal layer before establishing a generalized infection. Supraciliary or subretinal inoculation of mice immunosuppressed by methylprednisolone administration, radiation treatment, or retrovirus-induced immunodeficiency (MAIDS) results in virus replication and destruction of the retina. Systemic administration of interleukin 2 (IL-2), but not IL-12, mitigates MCMV-associated retinitis in mice with MAIDS, and the adoptive transfer of MCMV-immune lymph node cells confers protection from retinitis. Upon immunosuppression, latent virus is reactivated and recovered from ocular and nonocular tissues.

Cardiac infections

Myopericarditis and dystrophic cardiac calcification are associated with MCMV inoculation of the heart. Uninfected recipients of hearts heterotropically transplanted from infected donors develop a fatal, generalized infection.

Arteritis

Genetically resistant or susceptible mice feeding on commercial or atherogenic diets develop MCMV-associated inflammatory lesions in the aorta and pulmonary artery after MCMV exposure.

Cerebral infections

Severe ependymitis, encephalitis and cerebral malformation are sequelae to intracerebral inoculation of MCMV into suckling mice. Inoculation of virus into the cerebral ventricles of embryos disrupts neuronal migration and causes neuronal loss. In tissue culture, interferon γ (INF-γ) treatment of permissive MCMV-infected-microglial cells suppresses virus infection.

Lethal hepatitis

Intraperitoneal inoculation with a lethal dose of MCMV is followed by a marked increase in serum transaminase levels and severe liver pathology.

Skin infections

Following intravenous inoculation, lesions on the mouth, ears, tail and feet are associated with vascular damage, edema, cellular infiltration, and swollen endothelial cells. Upon histologic examination, infected cells are detected in the dermis, but not the epidermis. However, antibodies against the MCMV 98-kDa structural protein are cross-reactive with the stratum spinosum and the outer sheath of hair follicles.

Response to Infection

Host immune response

In general, mice are genetically susceptible, moderately resistant, or resistant to MCMV infection. The genetic resistance locus (Cmv1) spans 0.4 map units on chromosome 6 of the mouse and is tightly linked to genes *Ly49* and *Nkrp1* of the natural killer complex. Some strains of mice primarily use CD8+ T cells to control MCMV replication, whereas others use natural killer cells. In mice moderately resistant (C57BL/10) or resistant (CAB/CaH) to MCMV infection, natural killer cells mediate viral clearance without a requirement for CD4+ or CD8+ T cells, although increased numbers of CD4+ T cells and activated macrophages are detected. Natural killer cells in SCID mice devoid of functional T and B cells mediate MCMV-associated pathology. In susceptible mice (BALB/c or A/J), CD8+ T cells primarily limit viral replication. CD4+ T cells expressing mRNAs for IFN-γ, IL-2 and IL-4 are required for antibody responses and delayed type hypersensitivity in all mice regardless of genetic background. Viral clearance is also influenced by organ-specific mechanisms. For example, clearance of virus from salivary glands requires a subset of CD4+ T cells, but not CD8+ T cells. MCMV resistance in the spleen of C57BL/6 mice is natural killer-cell perforin-mediated, whereas natural killer cell-associated IFN-γ regulates MCMV synthesis in the liver.

Initial MCMV infection induces IFNs, IL-12 and tumor necrosis factor (TNF), followed by increased levels of IL-1α, IL-6 and glucocorticoid early in infection. IFN-α induces natural killer cell blastogenesis and cytotoxicity, but not IFN-γ production by natural killer cells. IL-12 induces natural killer cell-associated IFN-γ production. Pretreatment of mice with IFN-α and IFN-γ significantly reduces mortality. IFN-α- and IFN-γ-associated reduction of MCMV replication in tissue culture correlates with the decreased availability of NF-κ B, which, in turn, leads to a cascade of reduced (via unused NF-κ B elements located in the MIE enhancer) IE mRNA,

pp89 production, E transcription, viral DNA synthesis, and nucleocapsid formation.

MCMV immune modulation

MCMV infection induces a generalized immune suppression, but enhances autoimmunity and exacerbates graft-versus-host disease. Immune suppression correlates with the mouse's genetic phenotype (susceptibility). Sublethal infection of susceptible mice reduces the number of myeloid and erythroid progenitor cells in the bone marrow, decreases leukocyte counts in the peripheral blood, and reduces (by 80–90%) the number of thymocytes recovered from the thymus. The bone marrow atrophy and loss of cortical thymocytes is not due to lytic infection of bone marrow cells or thymocytes, but may be associated with increased maturation and migration of bone marrow cells to the sites of virus infection or Fas-mediated apoptosis (in thymocytes or bone marrow cells). Thymic involution coincides with bone marrow hypoplasia, but the two appear to be independent events. Myelosuppression is restored after treatment with the immunomodulator AS101 [ammonium trichloro (dioxyethylene *O-O'*)tellurate].

Autoimmunity is a common manifestation of cytomegalovirus infections. Thymic involution may impair the elimination of autoreactive T cells, thus promoting autoimmunity. CD4+-dependent antibody profiles are altered showing increases in serum immunoglobulin (reactive with autologous tissues and ovalbumin) levels in general and 6–20-fold increases in IgG_3 and IgG_{2b} levels specifically. Antibodies with specificity toward the 98-kDa structural protein are neutralizing, but are also cross-reactive with mouse neurofilament protein (NFP), which is a component of the central and peripheral nervous systems, and to a lesser extent to the stratum spinosum of the skin and the outer sheath of hair follicles. Concurrent MCMV infection in bone marrow transplants exacerbates the host-versus-graft reaction.

MCMV encodes genes whose proteins are immune modulators. The nonapeptide YPHFMPTNL from the immunodominant immediate early protein (pp89) elicits a protective MHC class I H-$2L^d$-restricted CD8+ T-lymphocyte response. During a permissive infection, several MCMV early-gene functions from a 6.8-kb region within the *HindIII* E restriction fragment prevent transport of the trimolecular complex of H-$2D^b$ from the endoplasmic reticulum-*cis* Golgi compartment and independently prevent maturation of the class I heavy chains to the fully glycosylated forms. Degradation of class I molecules is not affected. The decrease in MHC class I expression on the surface of the infected cell prevents priming of the antigen-specific helper and cytotoxic T cells, thereby protecting the infected cell from the effects of cytotoxic T cells. Treatment with IFN-γ causes a 25-fold increase in the presentation of MHC class I molecules containing the pp89-derived nonapeptide; the excess molecules apparently become surface-exposed, thereby restoring susceptibility to primed T-cells. In viruses recovered from wild mice populations, variation in the immediate early-CTL epitope in 19 of 27 virus isolates potentially explains why CTLs primed against some isolates did not react with cells infected by heterologous MCMV isolates.

Additional immune modulators include a MHC class I homologue (m144), which potentially disrupts the presentation of peptides by native MHC class I molecules to cytotoxic T cells, and MCMV gp34, which binds to MHC class I complexes, thereby counteracting the retention of the complex in the endoplasmic reticulum-*cis* Golgi compartment. It is thought that presentation of the m144 homologue and the gp34–MHC class I complex on the surface of the infected cell is necessary for defense against attack by natural killer cells. MCMV also encodes a Fc receptor homologue which is expressed and glycosylated into 86–88-kDa and 105-kDa forms. And, lastly, several MCMV genes have homology to the beta class of chemotactic cytokines.

Prevention and Control

Immunization with vaccines remains the mainstay for prophylactic prevention of virus-associated diseases. As a model for HCMV vaccines, MCMV vaccines are readily available as one passage in tissue culture is sufficient to attenuate the virus. However, reversion occurs after passage *in vivo*. Sodium periodate-inactivated virus is innocuous, elicits a strong humoral response and protects mice from a lethal challenge for a three-week period. The administration of the CTL-specific nonapeptide from the immediate early pp89 protects mice; however, variability in this sequence in wild-type virus may render this strategy ineffectual. Intradermal DNA immunization with pp89 provides variable protection from lethal challenge and interferes with virus replication.

Combination immunotherapy with IFN-γ/TNF-α increases infected mouse survival rates and decreases virus replication *in vitro*, but not *in vivo*. Systemic immunotherapy with IL-2, but not IL-12, reduces the frequency of MCMV-induced retinitis in mice with MAIDS. Administration of the octapeptide thymic humoral factor (THF-γ 2) enhances the immunocompetence of spleen cells and restores MCMV-suppression of T cell mitogenic responses and IL-2 secretion.

Immunotoxins containing MCMV-specific IgG linked to deglycosylated ricin A chain or gelonin (ribosome-inactivating enzyme) inhibit MCMV replication. Immunomodulators such as BCH-527 (the hydrochloride salt of D-alanyl L-glutamine), lactoferrin, polyinosinic:polycytidylic acid (poly(I:C)), GLA-60 (synthetic lipid A subunit analogue), and iota-carrageenan (i-CAR) mediate viral spread by modulating the immune response.

Several antiviral drugs, including ganciclovir (GCV and its derivatives SR3772 and SR3773), BMS-180194, HPMPA, HPMPC and cidofovir are effective at reducing MCMV replication and extending the survival of MCMV-infected immunodeficient mice. Virus cross-resistance to the antivirals correlates with mutated viral DNA polymerase and not the phosphorylation of the drug (MCMV does not encode a thymidine kinase). Drug-resistant viruses are often markedly attenuated in their ability to kill SCID mice. Combined GCV/THF-γ 2 therapy significantly reduces MCMV in the salivary glands of intraperitoneally infected mice. GCV and immunotoxin synergistically extend survival time of infected SCID mice.

Future

The most striking advantage of the murine cytomegalovirus system is its potential for answering questions concerning the molecular biology of cytomegalovirus infection while simultaneously providing an *in vivo* system for testing molecular hypotheses in a naturally occurring biological context. Numerous homologies to HCMV bespeak the usefulness of *in vivo* MCMV infections as a surrogate for HCMV infections.

See also: **Cytomegaloviruses (*Herpesviridae*): Animal cytomegaloviruses, General features (human), Molecular biology (human); Epstein-Barr virus (*Herpesviridae*): Molecular biology; Herpes simplex viruses (*Herpesviridae*): General features, Molecular biology.**

Further Reading

Osborn JE (1982) CMV-herpesviruses of mice. In: Foster HL, Fox JG and Small JD (eds) *The Mouse in Biomedical Research*, Vol. II, p. 267. New York: Academic Press.

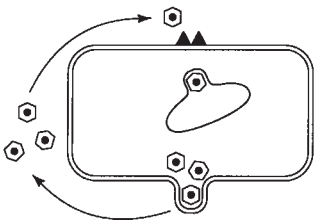
Rawlinson WD, Farrell HE, Barrell BG (1996) Analysis of the complete DNA sequence of murine cytomegalovirus. *J. Virol.* 70: 8833.

Roizman B (1996) *Herpesviridae*. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Field's Virology*, 3rd edn, p. 2221. Philadelphia: Lippincott-Raven.

Staczek J (1990) Animal cytomegaloviruses. *Microbiol. Rev.* 54: 247.

D

DEFECTIVE INTERFERING VIRUSES



Laurent Roux, Department of Genetics and Microbiology, University of Geneva Medical School, Geneva, Switzerland

History

In 1943, Henle and Henle reported the decreased infectivity for mice of influenza virus stocks obtained after a series of undiluted passages in embryonated chicken eggs. In the early 1950s, von Magnus showed that such undiluted passages generate incomplete virus particles capable of limiting the growth of infectious virus (hence exhibiting interference). This first characterization was soon followed by similar reports by Mims, on the one hand, and Cooper and Bellet, on the other, dealing respectively with Rift Valley fever virus and vesicular stomatitis virus (VSV). In the late 1950s, Cooper and Bellet went so far as to assign interference to sedimentable particles, but failed to identify them as antigenically related to VSV. From the mid-1960s on, the characterization of other positive- and negative-stranded RNA virus defective particles continued. In 1970, a review by A. Huang and D. Baltimore set the basic definition of defective interfering (DI) particles and emphasized their widespread occurrence. Since then, DI particles have been described for almost all the known DNA and RNA viruses, including plant and even fungal viruses.

Structure

DI particles have the same protein composition as their homologous nondefective 'parents', often called (St). However, they differ from the St particles in the primary structure of their genome. As emphasized later, DI genomes lack part of the genetic information. They may or may not serve as coding sequences. However, they always conserve the *cis*-sequences needed for replication initiation (origins of replication), sometimes present in more than one copy, and sequences involved in encapsidation. Foreign sequences can also be inserted. DI particles can sometimes be separated from St particles on the basis of size, when the size of the particle closely corresponds to the size of the genome (for instance rhabdovirus), or on the basis of particle density differences (changes in nucleic acid to protein ratios). Often, however, only viral stocks enriched in DI particles are available owing to the size heterogeneity of the virus particles.

Generation of DI Genomes

DI DNAs very likely arise from various recombinational events not necessarily linked to genome replication, and which result in deletion, tandem duplication, insertion of host DNA and polymerization of small monomer sequences. DI RNAs have been proposed to arise almost exclusively during genome replication by a mechanism of 'leaping polymerase' consisting of polymerase stop/fall off or slippage/reinitiation events (**Fig. 1**). In this model the replicase complex moves with the nascent RNA still attached to it. Depending on where reinitiation takes place, and on the number and the direction of the leaps, the resulting molecules can be of the copy-back type, with more or less intramolecular inverted complementary sequences (A), of the internal deletion type (B), and of the duplication type (C). Multisteps (B) or (C) and combinations of steps (B) and (C) can, moreover, lead to various mosaic types. Insertion of host RNA is also observed, especially in plant DI RNA. The frequency at which the polymerase leaps and resumes its synthesis is unknown. The probability for this exercise to be successful in producing a viable DI genome has been estimated for VSV to range in the order of 10^{-7} to 10^{-8} per genome replication.

Defectiveness

The DI genomes contain interrupted or rearranged open reading frames. They partly or completely lack the full coding capacity of the viral genome. They are

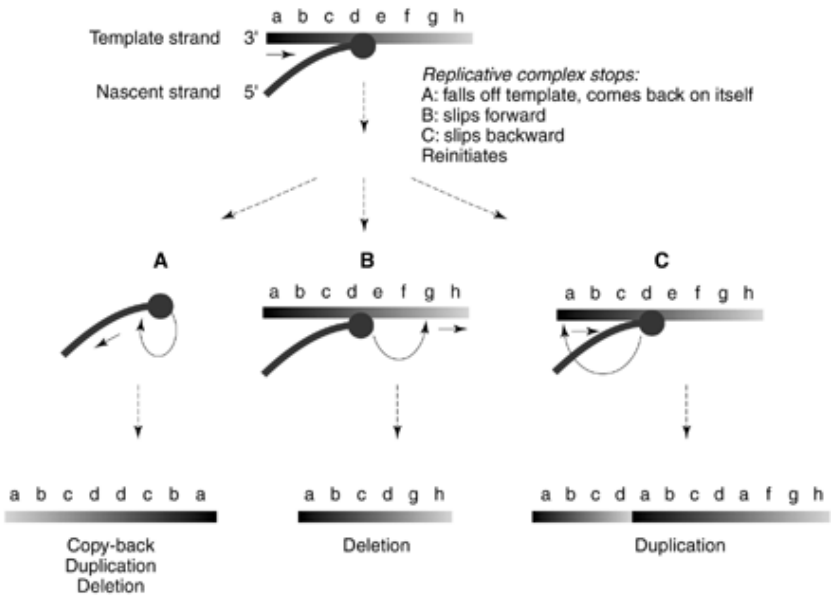


Figure 1 Defective RNA genome generation.

therefore defective, and depend for their replication and for their propagation (formation of virus particles) on the functions provided by the homologous standard virus (helper). Co-infection of cells with DI and standard particles is therefore essential for DI particle multiplication. Consequently, low-multiplicity infections, and particularly plaque purification, represent conditions which decrease, and potentially eliminate, DI particles from a viral preparation.

Interference

As stated earlier, the generation of a defective genome is likely to represent a rare event. This event would never be seen unless it was successfully amplified. During this amplification step the defective genome is preferentially replicated over the nondefective genome. This ability to replicate efficiently at the expense of the nondefective genome is called interference. The mechanisms of interference are not completely understood. They obviously change depending on the specificity of the viruses, and appear to be also affected by the host cell types. In general, interference involves an early step in genome replication, and can be pictured as a competition for limiting replication 'factors' (viral replicase, encapsidation proteins, host cell factors). Reiterated origins of replication or encapsidation sites on DI DNAs, presence of higher affinity sites for the replicase or for the encapsidation on both positive and negative polarity DI RNAs, and shorter length of the replicating units, higher availability for replication of molecules not involved in transcription, have been shown or postulated as taking part in the interference mechanism.

Defective Interfering versus Defective Viruses

Based on the outcome of experimental co-infections of defective with nondefective viruses, a distinction has been made between defective interfering or defective noninterfering particles, according to the ability of the defective viruses to selectively restrict nondefective virus replication. This distinction may not apply during the first events following the generation of a defective genome. As this is bound to be a rare event, then an interference mechanism has to be invoked any time this defective genome is amplified to the point it can be detected, or become predominant.

Cyclic Variations of Defective Interference

The dependence of DI genome replication on functions provided by the nondefective genome on the one hand, and the interference exhibited by DI genome on the other hand, result in out-of-phase cyclic variations of both DI and St genome replication. As illustrated in **Fig. 2**, efficient St genome replication must precede extensive DI genome replication. This in turn establishes conditions of high interference which results in

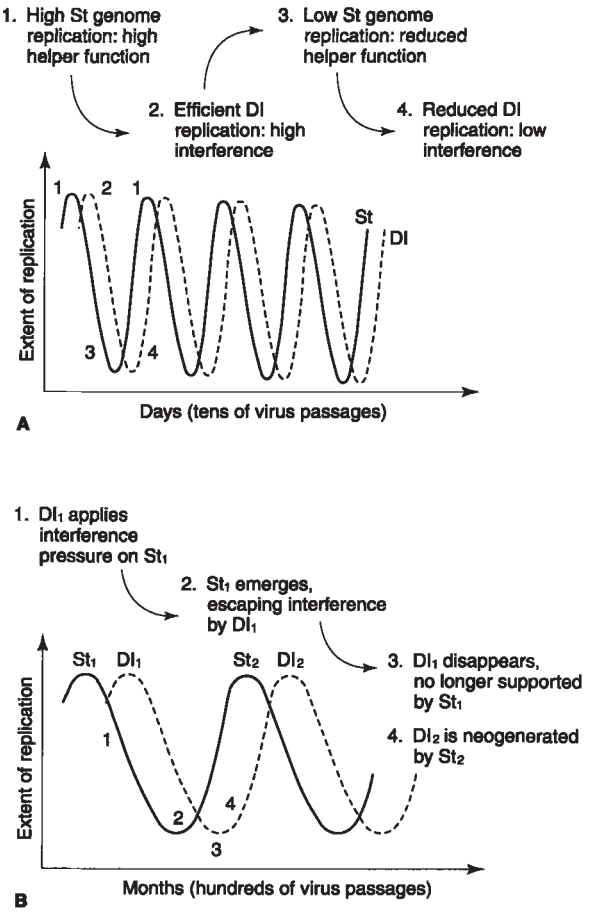


Figure 2 Cyclic variation of DI replication in (**A**) days, and (**B**) months.

inhibition of St genome replication. Decrease of helper function availability leads to DI genome replication dampening, and therefore to release from interference, allowing efficient St genome replication to resume. These cyclic variations have been observed in serial passages of St and DI viruses in cell culture as well as in persistent infections. The periodicity of a complete cycle is generally a matter of days or of a few serial passages.

Assay for DI Particles

DI particles can be detected by physical separation on velocity or density gradients when applicable (see above, Structure). The presence of subgenomic nucleic acids in viral stocks or in infected cells (distinct from viral messengers) can also be diagnostic. The ability to decrease the infectivity of a viral stock or to protect infected cells from the lytic infectious virus (see below, Biological Effects) are used in various biological assays to estimate quantitatively and qualitatively the DI particle composition of a viral stock. These assays, although appropriate to characterize DI particle preparations, are generally not sensitive enough to exclude, when negative, the presence of DI particles in a viral preparation. The test that still remains the most dependable to assess presence or absence of DI particles consists of multiple independent serial undiluted passages. It is based on the observation that a viral stock contaminated with an undetectable amount of DI particles will, on subsequent independent serial passages, promote in each series amplification of the same contaminating DI particles. A DI particle-free stock, on the other hand, will yield in each series different DI particles or different sets of DI particles.

Biological Effects

DI particles have been shown to modulate the course of an infection. In cell culture, attenuation of the cytopathic effects is the most frequently described, and DI particles can promote cell survival and establishment of persistent infections. As far as negative-stranded RNA viruses are concerned, copy-back DI RNAs appear to prevent induction of apoptosis through a mechanism which has still to be unravelled, in which a certain category of small leader RNAs may participate. A possible role of the matrix M protein, the concentration of which is decreased to the point where viral assembly and budding at the cell surface is highly diminished in the presence of DI genomes, has also been considered. DI interfering ability selectively allows emergence of St virus (St2) which escapes interference (**Fig. 2B**). St2, resistant to interference, is selectively amplified over St1, still sensitive to interference, and soon becomes predominant. It loses its ability to support DI1, which therefore disappears. St2 will generate its own DI2, which in turn will favor emergence of a new St variant. Thus, DI viruses serve as mutational drivers favoring virus evolution, through cycles of high and low interference whose periodicity is this time measured in months or in hundreds of viral passages (compare **Fig. 2A** and **2B**).

DI Particles in Experimental Animals

DI particles are generated and amplified in the whole animal as well as in cell culture. They can change the symptoms of viral infection from rapid death to slow, progressing paralysis. They can sometimes fully protect the animals from an otherwise lethal infection. Interference is likely to be involved in this modulation of symptoms, but other phenomena like

increased interferon induction and immune response modulation are reported.

DI Particles in Natural Infections

Involvement of DI particles in natural infections is still poorly documented. This is partly because the experimental results supporting the strong potential for infection modulation of DI particles have not been fully recognized, as detection of DI particles in natural infections is not straightforward. Unpredictable cyclic variations in DI replication, efficiency of DI replication changing with the types of infected tissues, and potent interfering ability associated with poor DI particle replication are all factors which undoubtedly make DI particle detection difficult *in vivo*. Last but not least, virus isolation, which is used to characterize the infectious agent associated with a disease, often represents conditions (low multiplicity of infection) known to impair drastically DI particle replication.

Nevertheless, association of a chicken influenza virus strain, efficiently producing DI particles, with an epidemic of low morbidity and low mortality, and conversely, a high-mortality epidemic associated with a strain free of DI particles, have been reported. Murine and feline leukemia virus strains causing immunedeficiency syndromes are shown to contain predominantly replication-defective viral genomes before onset and during the development of the disease. The pathogenicity of some bovine and swine pestiviruses has clearly been associated with presence of DI RNAs in the animals. For the bovine viral diarrhea virus (BVDV), a pestivirus of the same family as hepatitis C virus, the presence of a particular DI RNA can turn noncytopathic virus into a fatal infectious agent. In plants, at least three examples of DI RNAs are described to be involved in infection modulation. Interestingly, depending on the types of viruses, DI RNAs can either attenuate or exacerbate the symptoms.

DI RNAs are identified in stool and blood samples of humans suffering from hepatitis A virus, an infection known to be rather moderate and prolonged. DI particles are identified in measles virus-attenuated vaccine preparations which have been, and are being, widely and successfully used (raising the question of DI particle participation in vaccine attenuation). Measles viruses defective in viral assembly are currently found associated with human subacute sclerosing panencephalitis (SSPE). The brain cells of SSPE patients were, moreover, shown to harbor many species of measles virus copy-back DI RNAs. Direct amplification of a portion of the HIV *tat* gene from infected patients demonstrates that about a third of the sequences correspond to defective *tat* function. Moreover, human immunodeficiency virus (HIV)-1 sequences isolated from a cohort of six blood or blood product recipients infected with one donor all contained a similar deletion in the *nef* gene. Remarkably, all the patients harboring this deleted viral genome remained free of HIV-related diseases 12–16 years after infection, suggesting that this defective species of HIV genome is responsible for this decreased pathology. Epstein–Barr virus (EBV) replicative infections developing in human epithelial lesions involve a deleted rearranged form of EBV DNA (het DNA). This het DNA is associated, in experimental infections, with disruption of latency and persistent productive infections. Specific identification of viral hepatitis B (HBV) genomes containing an interrupted precore antigen (HBeAg) coding sequence in patients dying from fulminant hepatitis suggests that such defective genomes may be responsible for the exacerbation of the disease. This contrasts with more recent data reporting experimental evidence for the existence of DI-like viruses in HBV human chronic carriers; fluctuations between these naturally occurring core internal deletion variants and helper HBV in three chronic carriers were reminiscent of the cycling phenomenon in other DI viral systems.

Future Perspectives

Defective interfering particles are ubiquitous in the realm of animal and plant viruses. In experimental conditions they appear as necessary companions of their nondefective homologues. Capable of affecting the extent of viral growth, the course of viral infections, and serving as selective pressure to drive mutational changes, they can be seen as natural regulators of virus evolution. The demonstration of their participation in natural infections, and of their ability to affect the course of diseases, constitutes a challenge for the years to come. As pointed out by the few examples listed earlier, their direct detection in infected tissues will certainly be needed to assert their involvement in natural infections. The availability of sensitive detection techniques (like polymerase chain reactions), allowing direct observation of viral genomes without the possible distortion of virus isolation, bears great hope. More than giving increased insights into the physiopathology of viral infections, in times where the modifications of the viral genomes represent an imperative step in the generation of viral recombinant vaccines or of appropriate vectors for gene targeting, DI viral genomes represent natural versions of defective genomes that can serve as model tools for creation of more adapted vectors.

See also: **Influenza viruses (*Orthomyxoviridae*): General features, Molecular biology, Structure of antigens; Interference; Pathogenesis: Animal viruses; Vesicular stomatitus viruses (*Rhabdoviridae*).**

Further Reading

Barrett ADT and Dimmock NJ (1986) Defective interfering viruses and infection of animals. *Curr. Top. Microbiol. Immunol.* 128: 55.

Perrault J (1981) Origin and replication of defective interfering particles. *Curr. Top. Microbiol. Immunol.* 93: 151.

Roux L, Simon AE and Holland JJ (1991) Effects of defective interfering viruses on virus replication and pathogenesis. In: Shatkin A (ed.) *Advances in Virus Research,* vol. 40, p. 181. New York: Academic Press.

DENGUE VIRUSES *(FLAVIVIRIDAE)*

Duane J Gubler, Division of Vector-Borne Infectious Diseases, Fort Collins, Colorado, USA

History

Dengue fever is a very old disease; the earliest record of a dengue-like illness found to date is in a Chinese encyclopedia of disease symptoms and remedies, first published during the Chin Dynasty (265–420 AD) and formally edited in 610 AD (Tang Dynasty) and again in 992 during the Northern Sung Dynasty. There are reports of epidemics of dengue-like illnesses in the French West Indies in 1635 and in Panama in 1699. By the late 1700s, the disease had a worldwide distribution in the tropics, with epidemics of a clinically compatible disease occurring in 1779 in Batavia (Jakarta), Indonesia and Cairo, Egypt, and in 1780 in Philadelphia, Pennsylvania, USA. From the late 1700s to World War II, repeated epidemics of dengue-like illness occurred in most tropical and subtropical regions of the world at 10- to 30-year intervals. There is no documentation, however, that dengue viruses were responsible for all of these epidemics because diagnosis was based only on clinical reports. Clinical descriptions of some early epidemics were compatible with chikungunya virus infection, which has a transmission cycle similar to dengue. It is likely that epidemic chikungunya did occur, but recent data show that the dengue viruses, not chikungunya, were responsible for the majority of epidemics in the past 50 years.

The virus etiology of dengue fever was not documented until 1943–1944, when Japanese and American scientists simultaneously isolated the viruses from soldiers in the Pacific and Asian theaters during World War II. Albert Sabin isolated dengue viruses from soldiers who became ill in Calcutta, India, New Guinea and Hawaii. The viruses from India, Hawaii and one strain from New Guinea were antigenically similar, whereas three other strains from New Guinea appeared to be different. These viruses were called dengue 1 (DEN-1) and dengue 2 (DEN-2) and designated as prototype viruses (DEN-1, Hawaii, and DEN-2, New Guinea C). The Japanese virus, isolated by Susumu Hotta, was later shown to be DEN-1. Two more serotypes, called dengue 3 (DEN-3) and dengue 4 (DEN-4), were subsequently isolated by William Mcd. Hammon and his colleagues from children with hemorrhagic disease during an epidemic in Manila, Philippines, in 1956. Although thousands of dengue viruses have been isolated from different parts of the world since that time, all fit antigenically into the four serotype classification.

Many early workers suspected that dengue viruses were transmitted by mosquitoes, but actual transmission was first documented by H. Graham in 1903. In 1906, T. L. Bancroft demonstrated transmission by *Aedes aegypti,* later known to be the principal urban mosquito vector of dengue viruses. Subsequent studies in the Philippines, Indonesia, Japan and the Pacific showed that *Aedes albopictus* and *Aedes polynesiensis* were also efficient secondary vectors for dengue viruses.

During and following World War II, *Ae. aegypti* greatly expanded its distribution in Asia, becoming the dominant day-biting mosquito in most Asian cities. A dramatic increase in urbanization in the postwar years, created ideal conditions for increased transmission of urban mosquito-borne diseases. These changes, plus an increased movement of people within and between countries of the region via air-

See also: **Influenza viruses (*Orthomyxoviridae*): General features, Molecular biology, Structure of antigens; Interference; Pathogenesis: Animal viruses; Vesicular stomatitus viruses (*Rhabdoviridae*).**

Further Reading

Barrett ADT and Dimmock NJ (1986) Defective interfering viruses and infection of animals. *Curr. Top. Microbiol. Immunol.* 128: 55.

Perrault J (1981) Origin and replication of defective interfering particles. *Curr. Top. Microbiol. Immunol.* 93: 151.

Roux L, Simon AE and Holland JJ (1991) Effects of defective interfering viruses on virus replication and pathogenesis. In: Shatkin A (ed.) *Advances in Virus Research,* vol. 40, p. 181. New York: Academic Press.

DENGUE VIRUSES *(FLAVIVIRIDAE)*

Duane J Gubler, Division of Vector-Borne Infectious Diseases, Fort Collins, Colorado, USA

History

Dengue fever is a very old disease; the earliest record of a dengue-like illness found to date is in a Chinese encyclopedia of disease symptoms and remedies, first published during the Chin Dynasty (265–420 AD) and formally edited in 610 AD (Tang Dynasty) and again in 992 during the Northern Sung Dynasty. There are reports of epidemics of dengue-like illnesses in the French West Indies in 1635 and in Panama in 1699. By the late 1700s, the disease had a worldwide distribution in the tropics, with epidemics of a clinically compatible disease occurring in 1779 in Batavia (Jakarta), Indonesia and Cairo, Egypt, and in 1780 in Philadelphia, Pennsylvania, USA. From the late 1700s to World War II, repeated epidemics of dengue-like illness occurred in most tropical and subtropical regions of the world at 10- to 30-year intervals. There is no documentation, however, that dengue viruses were responsible for all of these epidemics because diagnosis was based only on clinical reports. Clinical descriptions of some early epidemics were compatible with chikungunya virus infection, which has a transmission cycle similar to dengue. It is likely that epidemic chikungunya did occur, but recent data show that the dengue viruses, not chikungunya, were responsible for the majority of epidemics in the past 50 years.

The virus etiology of dengue fever was not documented until 1943–1944, when Japanese and American scientists simultaneously isolated the viruses from soldiers in the Pacific and Asian theaters during World War II. Albert Sabin isolated dengue viruses from soldiers who became ill in Calcutta, India, New Guinea and Hawaii. The viruses from India, Hawaii and one strain from New Guinea were antigenically similar, whereas three other strains from New Guinea appeared to be different. These viruses were called dengue 1 (DEN-1) and dengue 2 (DEN-2) and designated as prototype viruses (DEN-1, Hawaii, and DEN-2, New Guinea C). The Japanese virus, isolated by Susumu Hotta, was later shown to be DEN-1. Two more serotypes, called dengue 3 (DEN-3) and dengue 4 (DEN-4), were subsequently isolated by William Mcd. Hammon and his colleagues from children with hemorrhagic disease during an epidemic in Manila, Philippines, in 1956. Although thousands of dengue viruses have been isolated from different parts of the world since that time, all fit antigenically into the four serotype classification.

Many early workers suspected that dengue viruses were transmitted by mosquitoes, but actual transmission was first documented by H. Graham in 1903. In 1906, T. L. Bancroft demonstrated transmission by *Aedes aegypti,* later known to be the principal urban mosquito vector of dengue viruses. Subsequent studies in the Philippines, Indonesia, Japan and the Pacific showed that *Aedes albopictus* and *Aedes polynesiensis* were also efficient secondary vectors for dengue viruses.

During and following World War II, *Ae. aegypti* greatly expanded its distribution in Asia, becoming the dominant day-biting mosquito in most Asian cities. A dramatic increase in urbanization in the postwar years, created ideal conditions for increased transmission of urban mosquito-borne diseases. These changes, plus an increased movement of people within and between countries of the region via air-

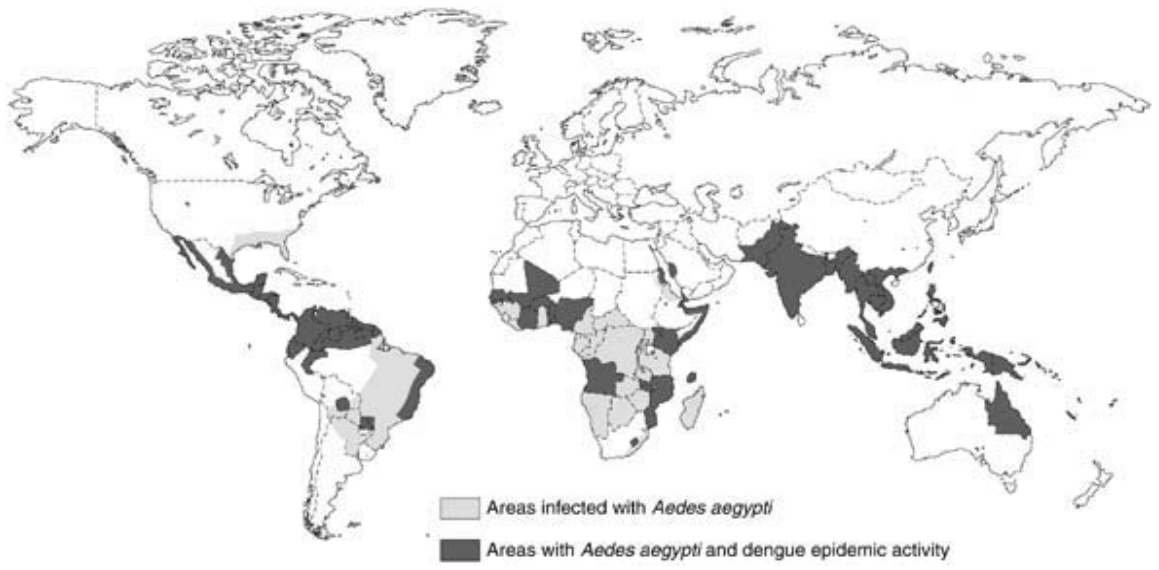


Figure 1 Global distribution of dengue fever and the principal epidemic mosquito vector, *Aedes aegypti*.

plane, resulted in increased movement of dengue viruses between population centers, increased frequency of epidemic activity, the development of hyperendemicity (co-circulation of multiple serotypes) and the emergence of dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) in many countries of southeast Asia during the 1960s. By 1975, DHF/DSS was a leading cause of hospitalization and death among children in the region. During the 1980s and 1990s, epidemic DHF/DSS continued to expand geographically in Asia. DHF/DSS moved into the Pacific islands in the 1970s. In the Americas, where *Ae. aegypti* had been eradicated from many countries to control yellow fever, increased epidemic dengue fever closely followed the reinfestation of countries by the mosquito in the 1970s, 1980s and 1990s. With the development of hyperendemicity, DHF/DSS has emerged as a global public health problem in the past 20 years. In 1997, dengue fever is the most important arbovirus disease of humans with over 2.5 billion people living in areas at risk for dengue in a belt around the tropics of the world (**Fig. 1**). An estimated 100 million cases of dengue fever, and over 500 thousand cases of DHF/DSS occur each year. The average case fatality rate for DHF/DSS is 5%.

Classification

Dengue viruses belong to the family *Flaviviridae*, genus *Flavivirus*. There are four serotypes: DEN-1, DEN-2, DEN-3 and DEN-4. They belong to a larger, heterogeneous group of viruses called arboviruses. This is an ecological classification, which implies that transmission between vertebrate hosts including humans is dependent on hematophagous arthropod vectors.

Dengue viruses, like other flaviviruses, consist of a single-stranded RNA genome surrounded by an icosahedral nucleocapsid. The latter is covered by a lipid envelope, which is apparently derived from the host cell membrane from which the virus buds. The complete virion is about 50 nm in diameter. The mature virion contains three structural proteins as follows: the nucleocapsid core protein (C), a membrane associated protein (M) and the envelope protein (E). Functional domains responsible for virus neutralization, hemagglutination, fusion and interaction with virus receptors are associated with the E protein. Epitope mapping has demonstrated three to four major antigenic sites.

Antigenically, the four dengue viruses make up a unique complex within the genus *Flavivirus*. Although the four dengue serotypes are antigenically distinct, there is evidence that serologic subcomplexes may exist within the group. For example, a close genetic relationship has been demonstrated between DEN-1 and DEN-4 using cDNA hybridization probes. Also, surprisingly, DEN-2 shows a high sequence homology (71%) with Edge Hill virus, an ecologically distinct flavivirus from Australia. There are over 70 antigenically related flaviviruses, including the genus prototype, yellow fever, Japanese encephalitis, Murray Valley encephalitis, St Louis encephalitis, West Nile, Kunjin, Zika and others, which are transmitted by mosquitoes. Another group of flaviviruses are tick-borne and include tick-borne ence-

phalitis, Omsk hemorrhagic fever and Kyasanur Forest disease. A small number of flaviviruses have no known anthropod vector.

Geographic and Seasonal Distribution

Dengue viruses have a worldwide distribution in the tropics (**Fig. 1**). The viruses are endemic in most urban centers of the tropics with transmission occurring year around. Epidemic transmission occurs periodically in most virus-endemic areas, usually at 3- to 5-year intervals. Because surveillance in most endemic countries is poor, cases usually are not reported during interepidemic years, thus misleading tourist and other agencies about the risk of infection. It is well documented, however, that dengue viruses are maintained during interepidemic periods in most tropical areas and, although risk of infection is lower than during epidemic periods, it is still substantial to unsuspecting visitors.

Peak transmission of dengue viruses is usually associated with periods of higher rainfall in most endemic countries. Factors influencing seasonal transmission patterns of dengue viruses are not well understood, but obviously include mosquito density, which may increase during the rainy season, especially in those areas where water level in larval habitats is dependent on rainfall. In areas where water storage containers are not influenced by rainfall, however, other factors such as higher humidity and moderate ambient temperatures associated with the rainy season increase survival of infected mosquitoes, thus increasing the chances of secondary transmission to other persons.

Host Range and Virus Propagation

There are only three known natural hosts for dengue viruses: *Aedes* mosquitoes, humans and lower primates. Viremia in humans may last 2–12 days (average, 4–5 days) with titers ranging from undetectable to over 10^8 mosquito infectious doses 50 (MID_{50}) ml^{-1}. Experimental evidence shows that several species of lower primates (chimpanzees, gibbons and macaques) become infected and develop viremia titers high enough to infect mosquitoes, but do not develop illness. Viremia levels in lower primates are more transient, often lasting only 1–2 days if detectable, with titers seldom reaching 10^6 MID_{50} ml^{-1}.

Dengue viruses are known to cause clinical illness and disease only in humans. Baby mice, which are used for the isolation and assay of many other arboviruses, generally show no signs of illness after intracerebral inoculation with most unpassaged strains of dengue viruses. Experimentally, however, some strains can be adapted to produce illness and death in baby mice.

Only species of the genus *Aedes* appear to be natural mosquito hosts for dengue viruses. Species of the subgenus *Stegomyia* are the most important vectors in terms of human transmission, and include *Ae. (S.) aegypti,* the principal urban vector worldwide, *Ae. (S.) albopictus* (Asia–Americas), *Ae. (S.) scutellaris* spp. (Pacific), *Ae. (S.) africanus* and *Ae. (S.) luteocephalus* (Africa). Species of the subgenus *Finlaya* (Asia) and *Diceromyia* (Africa) appear to be important mosquito hosts involved in dengue forest maintenance cycles. Two other species, *Ae. (Gymnometopa) mediovittatus* (Caribbean) and *Ae. (Protomacleaya)triseriatus* (North America) have been shown to be excellent experimental hosts for dengue viruses.

Low passage or unpassaged dengue viruses can only be propagated with consistent results in laboratory-reared mosquitoes and in mosquito cell lines. Mosquito species most commonly used for *in vivo* propagation include *Ae. aegypti, Ae. albopictus* and *Toxorhynchites* spp., all of which can be reared with ease in the laboratory. Only three mosquito cell lines show high susceptibility to dengue viruses: C6/36 from *Ae. albopictus,* AP-61 from *Ae. pseudoscutellaris* and TRA-284 from *Tx. amboinensis.*

Dengue viruses can also be propagated in baby mice (see above) and in several vertebrate cell lines. These all have lower susceptibility to infection than mosquito cells, however, and dengue viruses must be adapted to each system by serial passage before consistent results can be obtained. Mammalian cell lines commonly used include LLC-MK2 and VERO (monkey kidney), BHK-21 (baby hamster kidney), FRhL (fetal rhesus lung) and PDK (primary dog kidney).

Genetics

Laboratory studies have documented that genetic variants of dengue viruses occur in nature. DEN-3 viruses isolated during epidemics in Puerto Rico in 1963 and in Tahiti in 1965 are antigenically and biologically very similar to each other, but very different from Asian strains of the same serotype. Similar antigenic differences were observed between Caribbean and Asian strains of DEN-4.

Oligonucleotide fingerprinting, restriction enzymes, primer extension sequencing and limited nucleotide sequence comparison using polymerase chain reaction have all been used to study genetic variation among dengue viruses. In general, viruses circulating in the same geographic region during the

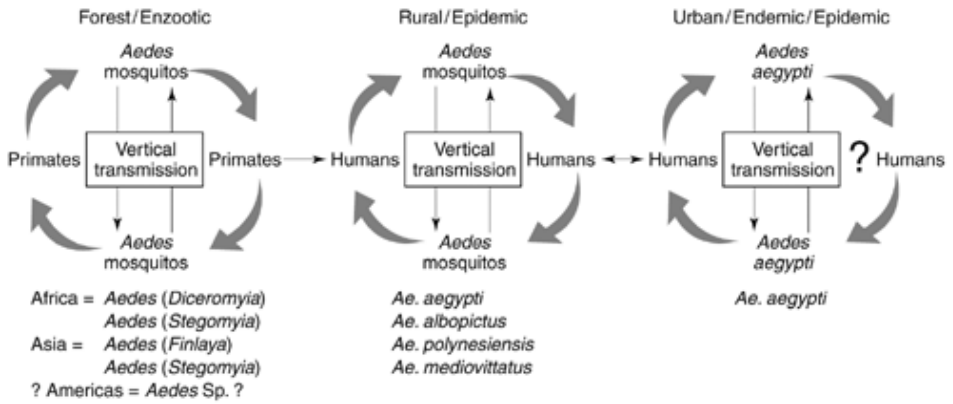


Figure 2 Natural transmission and maintenance cycles of dengue viruses.

same general time frame show genetic homogeneity, while differing from viruses of the same serotype from other regions. Oligonucleotide fingerprint analysis of DEN-2 viruses collected from the same area over a period of over 20 years suggests that the rate of change is very low (1.4% per year).

The number of genetic subtypes identified in each serotype varies with the method used, but more viruses have now been studied by partial nucleotide sequencing. Based on sequencing a 600 bp region of the envelope protein, which correlates well with sequencing the entire envelope protein, there are two distinct genotypes of DEN-1, five of DEN-2, four of DEN-3 and two of DEN-4. Of considerable epidemiologic interest is that there are currently two genotypes of DEN-2 circulating in the American region. One, designated Puerto Rico, has been in the Americas since at least 1952, whereas the second, designated Jamaica, was isolated for the first time from dengue patients in Jamaica during the Cuban epidemic of DHF/DSS in 1981. Analysis by restriction enzymes and primer extension sequencing has shown that the Jamaica virus is nearly identical to strains of DEN-2 isolated in Vietnam in 1987, suggesting that the DEN-2 virus causing the Cuban epidemic was introduced from Vietnam. This conclusion is supported by the fact that many Cubans were working in Vietnam on various aid projects during the late 1970s and early 1980s. The Jamaican genotype has subsequently become the predominant and most widespread strain of DEN-2 virus in the American region. There is no evidence that genetic reassortment or recombination of dengue viruses occur in nature, but this has not been fully studied. In recent years, with increased occurrence of the cocirculation of multiple serotypes in an area (hyperendemicity), there have been increased reports of concurrent infections with two serotypes. This will increase the probability that recombination might occur.

Evolution

The origin of dengue viruses is unknown. Their natural history, however, suggests that they were most likely mosquito viruses prior to becoming adapted to lower primates and humans. Biologically, dengue viruses are highly adapted to their mosquito hosts, being maintained by vertical transmission (from female mosquito to her offspring) in those species responsible for the forest maintenance cycle, with periodic amplification in lower primates (**Fig. 2**). Such forest cycles have been documented in southeast Asia and Africa. At some point in the past, probably with the clearing of the forests and development of human settlements in Asia, these viruses moved out of the jungle and into a rural environment where they were, and still are, transmitted to humans by peridomestic mosquitoes such as *Ae. albopictus*. Migration of people ultimately moved the viruses into the cities of the tropics where they became 'urbanized' and transmitted by the highly domesticated, urban *Ae. aegypti* mosquito.

Because of the rather slow rate of change (genetic drift) of the dengue virus genome, viruses isolated over long periods of time in the same geographic region still show striking homogeneity. The greatest genetic difference between dengue virus strains was observed between DEN-2 isolated from forest mosquitoes in Africa and viruses of the same serotype isolated from humans or mosquitoes in a nearby urban area. This would suggest that there is little gene flow between the forest and urban cycles. On the

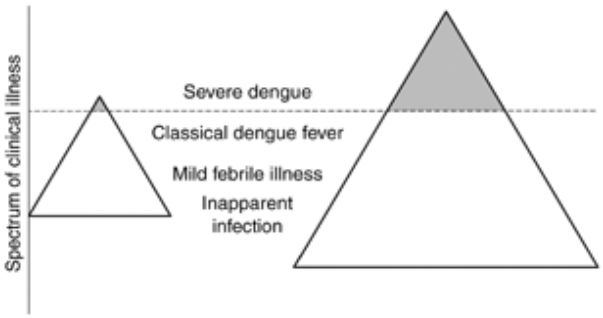


Figure 3 The iceberg concept of dengue/dengue hemorrhagic fever. The severe form of disease represents only the tip that protrudes from the water. As the incidence of infection increases, so too does the severe and fatal form of disease (see also Fig. 4).

other hand, both laboratory and field evidence suggests that significant genetic changes that influence epidemic potential do occur in nature (see below).

Serologic Relationships and Variability

Dengue viruses share common morphology, genomic structure and antigenic determinants with over 70 other flaviviruses. Serologic tests most frequently used to determine antigenic relationships have included the hemagglutination–inhibition (HI), complement fixation (CF) and the plaque reduction neutralization (PRNT) tests. Because all flaviviruses share common antigenic determinants, identification of individual family members using these tests is difficult. The dengue viruses make up one of eight complexes within the family *Flaviviridae*. They share complex-specific antigenic determinants on both structural and nonstructural proteins. Serotypes within the dengue virus complex are most accurately and easily identified with an indirect immunofluorescent antibody (IFA) assay using serotype-specific monoclonal antibodies which react to epitopes on the structural protein. They can also be readily identified using polymerase chain reaction (PCR).

Both antigenic and biologic variation among dengue viruses have been documented. As noted above, DEN-3 viruses isolated in the Caribbean and the South Pacific in the 1960s were found to be antigenically distinct from the prototype and Asian strains of DEN-3 using PRNT. They were also biologically unique in that they did not grow as well as Asian strains in baby mice and mosquitoes. DEN-4 viruses isolated in the Caribbean after the introduction of this serotype into that region in 1981 were antigenically distinct from DEN-4 viruses from Asia.

Field and epidemiologic evidence for natural strain variation among dengue viruses is more circumstantial. When dengue was introduced into the South and Central Pacific islands in 1971 after an absence of over 25 years, epidemics occurred on numerous islands. Marked variation was observed in disease severity, viremia levels and epidemic potential between epidemics. This variation was observed with both DEN-1 and DEN-2 in the Pacific and with DEN-3 in Indonesia. Some DEN virus strains appeared naturally attenuated, causing mild illness with low viremia levels of short duration, whereas others caused explosive epidemics with severe hemorrhagic disease and high viremia levels. Factors that could influence epidemic transmission and disease severity, other than differences in the virus strain, were ruled out as a cause of this variation.

Epidemiology

Dengue viruses exist in nature in three basic maintenance cycles (**Fig. 2**). The primitive forest cycle involves canopy dwelling mosquitoes and lower primates. A rural cycle, primarily in Asia and the Pacific, involves peridomestic mosquitoes (*Ae. albopictus* and *Ae. scutellaris* spp.) and humans. The urban cycle, which is the most important epidemiologically and in public health impact, involves the highly domesticated *Ae. aegypti*, mosquito and humans. The viruses are maintained in most large urban centers of the tropics with epidemics occurring at periodic intervals.

A combination of increased urbanization in the tropics, changing life styles and lack of effective mosquito control has made most tropical cities highly permissive for dengue transmission by *Ae. aegypti*. Increased air travel by humans provides the ideal mechanism for the transport of dengue viruses between population centers. As a result, in the past 20 years there has been a dramatic increase in the movement of dengue viruses within and between regions, resulting in increased epidemic activity, development of hyperendemicity (the cocirculation of multiple serotypes) and the geographic spread and increased incidence of the severe and fatal form of disease, DHF/DSS. Once observed only in southeast Asia, DHF/DSS has spread in epidemic form to west Asia, the Peoples Republic of China, the Pacific islands and the Americas in the past 18 years.

The factors responsible for the emergence and spread of epidemic DHF/DSS are not fully understood. The changing disease pattern described above provides support for both principal hypotheses on the pathogenesis of DHF/DSS, secondary infection and virus virulence. Thus, increased movement of viruses between population centers, increases transmission, and the development of hyperendemicity increases

both the chance of a secondary infection and the chance of a virulent virus emerging via natural selection or being introduced to an area. Increased transmission of multiple dengue serotypes increases the iceberg and thus the probability that severe disease will occur, regardless of whether the underlying cause is due to increased virulence, immune enhancement or, more likely, a combination of both (**Fig. 3**).

Dengue is primarily an urban disease. Most major epidemics of DHF/DSS occur in tropical urban centers where large and crowded human populations live in intimate contact with the principal mosquito vector, *Ae. aegypti*. This mosquito is a highly domesticated, day biting species that lives and breeds in and around the home. High mosquito densities often occur in tropical cities because of water storage practices and domestic trash. Primary larval habitats for *Ae. aegypti* include a variety of domestic water storage containers such as clay jars and pots, metal drums, cement cisterns and many other artificial containers found in the domestic environment that collect and hold rain water. The latter include, but are not limited to flower vases and pots, used automobile tires, buckets, bottles, cans, old machinery, etc.

Transmission and Tissue Tropism

Dengue viruses are only transmitted by the bite of an infective mosquito vector. All four serotypes may cause high levels of viremia in humans ($\geq 10^8$ MID_{50}/ml) that lasts an average of 4–5 days (range, 2–12 days). If a competent mosquito vector takes a blood meal from a person during this viremic phase, virus is ingested with the blood and infects the cells of the mosquito mesenteron. After 8–12 days, depending on ambient temperatures and the virus strain, the virus will disseminate and infect other tissues, including the mosquito salivary glands. When the mosquito takes a subsequent blood meal, virus is injected into the person along with the salivary fluids. Dengue virus infection has no apparent effect on the mosquito, which is infected for life.

Ae. aegypti is a highly competent epidemic vector of dengue viruses. It lives in close association with humans because of its preference to lay eggs in artificial water-holding containers in the domestic environment, and to rest inside houses and feed on humans versus other animals. It has a nearly undetectable bite and is very restless in the sense that the slightest movement will make them interrupt feeding and fly away. It is not uncommon, therefore, to have a single mosquito bite several persons in the same room or general vicinity over a short period of time.

In addition to transmitting the virus to humans or lower primates, the female mosquito may also transmit the virus vertically through the eggs to her offspring. Although the implications of vertical transmission are not fully understood, it is thought to be an important mechanism in the natural maintenance cycles of dengue viruses, especially in rural and forest settings.

The primary site of dengue virus replication after injection into humans by the feeding mosquito is believed to be phagocytic monocytes. Other tissues from which the viruses have been isolated include the liver, lungs, kidneys, lymph nodes, stomach, intestine and brain, but it is not known to what extent the virus replicates in these tissues. Pathological changes similar to those observed in yellow fever, with focal central neurosis, have been observed in the livers of some patients who died of dengue virus infection. There is some evidence that the viruses also replicate in endothelial cells and possibly in bone marrow cells. Although encephalopathy has been documented in dengue infection, evidence to date does not suggest that dengue viruses cross the blood–brain barrier and replicates in the central nervous system.

Pathogenicity

There is still considerable controversy about the pathogenesis of DHF/DSS. Evidence suggests that at least two pathogenetic mechanisms are associated with severe dengue infection. Classical DHF/DSS is characterized by a vascular leak syndrome, which if not corrected, may lead to hypovolemic shock and death. The underlying pathogenetic mechanism for this syndrome is thought to be an immune enhancement phenomenon whereby the infecting virus complexes with nonneutralizing dengue antibody, thus enhancing infection of mononuclear phagocytes which produce a vasoactive mediator responsible for increased vascular permeability. Loss of plasma from the vascular compartment may be mild and transient or severe and prolonged, the latter resulting in severe shock and death. Although classical DSS is most commonly associated with secondary dengue infections, it has also been documented in primary infections, which suggests that subneutralizing levels of homologous antibody or other substances may also cause immune enhancement.

In vitro studies have shown that not all dengue viruses can be enhanced and that there are qualitative differences in the enhancing ability of dengue antibody. This raises the question of whether dengue virus strains vary in their pathogenicity and, if so, how this influences the immune enhancement hypothesis. Because an animal model is not available, no good data exist that demonstrate variation in virulence among dengue viruses. However, an accumulat-

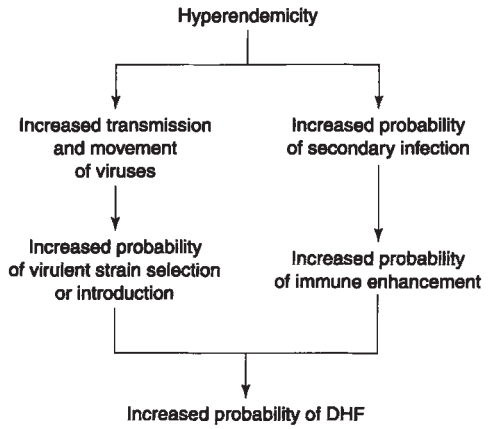


Figure 4 The cocirculation of multiple virus serotypes in a community (hyperendemicity) is the most important risk factor for the occurrence of dengue hemorrhagic fever and is compatible with the two principal hypotheses of pathogenesis, immune enhancement and virus strain variation.

ing mass of both experimental and field data suggest that dengue viruses, like most other animal viruses, do vary in their pathogenicity and their epidemic potential. When DEN-1 and DEN-2 viruses were introduced into the Pacific in the early 1970s after an absence of over 25 years, some islands experienced explosive epidemics with severe and fatal hemorrhagic disease whereas others with similar ecology experienced only low-level, sporadic and even silent transmission with mild illness. Virus strain variation was the only logical explanation for these differences. Recent laboratory evidence suggests that a major Cuban epidemic of DHF/DSS in 1981, the first of its kind in the American Region, was caused by a DEN-2 virus strain introduced from Vietnam, which was genetically distinct from the original American DEN-2 virus. This epidemic is often cited to support both immune enhancement and virus virulence hypotheses, which are not mutually exclusive. The most constant feature associated with the emergence of DHF/DSS in an area is the development of hyperendemicity (the cocirculation of multiple virus serotypes in the same community). This increases the probability of secondary infection, which has been shown to be associated with DHF/DSS, but hyperendemicity is also associated with increased movement of viruses between population centers, which increases the probability of introduction or of genetic selection of virus strains that have greater epidemic potential or virulence (**Fig. 4**).

Patients infected with dengue viruses who do not have classical DHF/DSS, may experience severe and uncontrolled bleeding, usually from the upper gastrointestinal tract. This severe hemorrhagic disease is often more difficult to manage than classical DHF/DSS. The underlying pathogenetic mechanism for this type of bleeding is clearly different from that of the vascular leak syndrome and involves disseminated intravascular coagulation and thrombocytopenia.

A third type of severe and fatal dengue infection, which may or may not involve overt hemorrhagic disease, is encephalopathy. Although many of these latter patients present clinically as viral encephalitis, conclusive evidence that dengue viruses infect the central nervous system has not yet been found. Available data suggest that neurologic symptoms may be secondary to cerebral hemorrhage, edema or other indirect effects of dengue virus infection.

Clinical Features of Infection

Dengue infection causes a spectrum of illness in humans ranging from clinically inapparent to severe and fatal hemorrhagic disease with the latter representing only the tip of the iceberg (**Fig. 3**). The incubation period may be as short as 3 days and as long as 14 days, but most often is 4–7 days. The majority of patients present with mild, nonspecific febrile illness or with classical dengue fever. The latter is generally observed in older children and adults, and is characterized by sudden onset of fever, frontal headache, retroocular pain and myalgias. Rash, joint pains, nausea and vomiting and lymphadenopathy are common. The acute illness, which lasts for 3–7 days, is usually benign and self-limiting, but it can be very debilitating, and convalescence may be prolonged for several weeks.

The hemorrhagic form of disease, DHF/DSS, is most commonly observed in children under the age of 15 years, but it also occurs in adults. It is characterized by acute onset of fever and a variety of nonspecific signs and symptoms that may last 2–7 days. During this stage of illness, DHF/DSS is difficult to distinguish from any number of other viral, bacterial and protozoal infections. In children, upper respiratory symptoms caused by concurrent infection with other viruses or bacteria are not uncommon. The differential diagnosis should include other hemorrhagic fevers, hepatitis, leptospirosis, typhoid, malaria, measles, influenza, etc.

The critical stage in DHF/DSS occurs when the fever subsides to or below normal. At that time, the patient's condition may deteriorate rapidly with signs of circulatory failure, neurologic manifestations, shock and death if proper management is not implemented. Skin hemorrhages such as petechiae, easy bruising, bleeding at the sites of venepuncture and purpura/ecchymoses are the most common hemorrhagic manifestations; gastrointestinal (GI) hemor-

Table 1 World Health Organization classification of dengue hemorrhagic fever

Grade I	Fever accompanied by nonspecific constitutional symptoms, with a positive tourniquet test and/or scattered petechiae as the only hemorrhagic manifestation
Grade II	The same as grade I, but with spontaneous hemorrhagic manifestations
Grade III	Circulatory failure manifested by rapid, weak pulse, narrowing of pulse pressure (20 mmHg or less), or hypotension
Grade IV	Profound shock with undetectable pulse and blood pressure

Source: Anon (1986) *Dengue Hemorrhagic Fever: Diagnosis, Treatment and Control.* Geneva: World Health Organization.

rhage may also occur, usually after, but in some cases before, onset of shock.

The World Health Organization (WHO) has defined strict criteria for diagnosis of DHF/DSS, with four major clinical manifestations: high fever, hemorrhagic manifestations, hepatomegaly and circulatory failure. WHO has classified DHF/DSS into four grades according to severity of illness: grades I and II represent the milder form of DHF and grades III and IV represent the more severe form, DSS (**Table 1**). Thrombocytopenia and hemoconcentration are constant features. However, there is some disagreement with the WHO case definition in that some patients may present with severe and uncontrollable upper GI bleeding with shock and death in the absence of hemoconcentration or other evidence of the vascular leak syndrome. These patients by the WHO criteria, cannot be categorized as having DHF/DSS. Additionally, hepatomegaly may not be a constant feature in all epidemics of DHF/DSS.

Dengue virus infection is associated with a variety of neurologic disorders, including headache, dizziness, hysteria and depression. In addition, some patients present with clinical symptoms of viral encephalitis, but as noted above, there is no conclusive evidence that actual CNS infection occurs.

Treatment for DHF/DSS is symptomatic, and the prognosis of the disease depends on early recognition, initiation of corrective fluid replacement and management of shock. Definitive diagnosis can only be made in the laboratory by serologic and/or virologic methods.

Pathology and Histopathology

The pathology of dengue virus infection is not well understood because systematic post-mortem studies have not been done on patients representing all types of clinical expression. The major pathophysiologic abnormality in classical DHF/DSS is an increase in vascular permeability which leads to leakage of plasma. Patients may have serous effusion in the pleural and abdominal cavities and a variable amount of hemorrhaging in most major organs. Studies have not revealed destructive inflammatory vascular lesions, but some swelling and occasional necrosis have been observed in endothelial cells, as well as some perivascular edema.

Limited studies on patients with a fatal outcome have demonstrated focal necrosis of the hepatic cells, Councilman bodies and hyaline necrosis of Kupffer cells in the liver. Changes in the kidney are suggestive of an immune-complex type of glomerulonephritis. There is depression of bone marrow elements which improve when the patient becomes afebrile. Biopsy studies of the skin rash have demonstrated perivascular edema with infiltration of lymphocytes and monocytes.

Immune Response

Persons infected with dengue viruses produce specific immunoglobulin (Ig)M and IgG antibodies, both of which appear in 5–7 days after onset of illness in primary infections. The highest titers of IgM antibody are produced in primary dengue infections, but it is also produced in secondary and tertiary infections. IgM antibody is transient and disappears in 30–90 days after onset of illness in primary infections and after shorter periods in secondary and tertiary infections. IgG antibody, by contrast, persists for at least 50 years and probably for the life of the patient. In persons experiencing their first dengue or flavivirus infection, peak IgG titers are reached 14–21 days after onset of illness and seldom exceed 1:640 to 1:1280, although there are exceptions. In secondary infections, on the other hand, there is an immediate anamnestic IgG immune response to dengue complex- and/or flavivirus-specific antigenic determinants. In these patients, IgG antibody titers may exceed 1:20 480. Both IgM and IgG antibodies neutralize dengue viruses, and infection provides life-long immunity to that specific dengue virus serotype.

Both IgM and IgG dengue antibodies crossreact with other flavivirus antigens, including yellow fever, Japanese encephalitis and St Louis encephalitis viruses. Crossing with viruses in the dengue complex is more extensive than with other flaviviruses, and

crossreactions make interpretation of serologic results difficult. In patients with second and third flavivirus infections, original antigenic sin reactions are not uncommon. In geographic areas where several flaviviruses are endemic, therefore, definitive laboratory diagnosis can only be made by virus isolation, and in patients with primary infection, by PRNT. Normally, a combination of laboratory (serologic and virologic) clinical and epidemiologic data are used to make a diagnosis of dengue and other flavivirus infection.

Because IgG antibody persists for many years, its presence in a single serum sample is not diagnostic unless it occurs at high titer ($\geq$1:1280 by HI and PRNT, $\geq$1:256 by CF or $\geq$1:163 840 by IgG ELISA), which is considered presumptive evidence of a recent infection. Lower IgG titers simply indicate that the person has had a previous infection at some time in the past. Paired serum samples are thus required to demonstrate a fourfold or greater rise in specific IgG antibody. The presence of detectable IgM antibody in a single serum sample, however, is considered to be diagnostic because this isotype does not persist for long periods. The diagnosis is considered presumptive, however, because IgM antibody does persist for up to 90 or more days.

There is some evidence that cell-mediated immunity may also play a role in terminating dengue infection. Thus, T-cell clones that kill dengue virus-infected cells have been identified.

Prevention and Control of Dengue

The options available for prevention and control of DEN/DHF are limited. Although currently not available, considerable progress has been made in recent years in development of a vaccine for dengue/DHF. Effective vaccination to prevent DHF will require a tetravalent, live attenuated vaccine. Promising candidate attenuated vaccine viruses have been developed and have been evaluated in phase I and II trials in Thailand. A commercialization contract has been signed and the tetravalent vaccine formulation is currently undergoing repeat phase I and II trials.

Promising progress has also been made on developing second-generation, recombinant dengue vaccines by using cDNA infectious clone technology. An infectious clone of the DEN-2, PDK-53 vaccine candidate virus has been constructed, and work is currently in progress to construct chimeric viruses by inserting the capsid, premembrane (PrM) and envelope genes of DEN-1, DEN-3 and DEN-4 into the DEN-2 PDK-53 backbone. These recombinants, through genetic manipulation, may be made to grow better, be more immunogenic and safer.

The development of other new technology such as DNA vaccines is in its infancy. Despite the promising progress, it is unlikely that an effective, safe and economical dengue vaccine will be available in the near future. It will likely be 10 or more years before an effective, safe, economical vaccine is commercially available. Currently, the only way to prevent dengue infection is to control the mosquito vector that transmits the virus.

Unfortunately, our ability to control *Ae. aegypti* is limited. For over 25 years, the recommended method of control was the use of ultra low volume (ULV) application of insecticides to kill adult mosquitoes. Field trials in Puerto Rico, Jamaica and Venezuela, however, showed that this method was not effective in significantly reducing natural mosquito populations for any length of time. This supports epidemiologic observations that ULV has little or no impact on epidemic transmission of dengue viruses.

The only truly effective method of controlling *Ae. aegypti* is source reduction, that is to eliminate or control the larval habitats where the mosquitoes lay their eggs. Most important larval habitats are found in the domestic environment, where most transmission occurs. To have sustainability of prevention and control programs, some responsibility for mosquito control must be transferred from government to citizen homeowners. Mosquito control programs, therefore, must be community-based and integrated. Persons living in *Ae. aegypti* infested communities must be educated to accept responsibility for their own health destiny by helping government agencies control the vector mosquitoes, and thus prevent epidemic dengue/DHF/DSS.

Countries with endemic dengue should develop active, laboratory-based surveillance systems that can provide some degree of epidemic prediction. Finally, prevention of excess mortality associated with DHF/DSS can be achieved by educating physicians in endemic areas on clinical diagnosis and management of DHF/DSS. As demonstrated in countries such as Thailand, early recognition and proper management are the key to keeping DHF/DSS fatality rates low.

Future

Continued population growth and urbanization of the tropics, changing lifestyles, increased air travel and lack of effective mosquito control have been the most important factors responsible for the dramatic increased incidence and geographic expansion of DHF/DSS in the 1980s and 1990s. DEN/DHF/DSS has become a global public health problem in the tropics and it is anticipated that this trend will continue unless something is done to reverse it. More

effective integrated prevention and control strategies must be developed and implemented worldwide in the tropics. Ultimately, development of an economical tetravalent vaccine holds the greatest promise for prevention and control.

See also: **Diagnostic techniques: Detection of viral antigens, nucleic acids and specific antibodies, isolation and identification by culture and microscopy; Encephalitis viruses (*Flaviviridae*): Encephalitis viruses and related viruses causing hemorrhagic disease, Tick-borne encephalitis and Wesselsbron viruses; Epidemiology of viral diseases; Immune response: General features; Yellow fever virus (*Flaviviridae*).**

Further Reading

Anonymous (1986) *Dengue Haemorrhagic Fever: Diagnosis, Treatment and Control*, 58 pp. Geneva: World Health Organization.

Gubler DJ (1988) Dengue. In: Monath TP (ed.) *The Arboviruses: Epidemiology and Ecology*, vol. 2, p. 223. Boca Raton: CRC Press.

Gubler DJ (1989) *Aedes aegypti* and *Aedes aegypti*-borne disease control in the 1990s: top down or bottom up. *Am. J. Trop. Med. Hyg.* 40: 571.

Gubler DJ and Kuno G (eds) (1997) *Dengue and Dengue Hemorrhagic Fever*. Wallingford UK: CAB International.

Halstead SB (1990) Dengue and dengue hemorrhagic fever. *Curr. Opin Infect. Dis.* 3: 434.

Henchal EA and Putnak JR (1990) The dengue viruses. *Clin. Microbiol. Rev.* 376.

PAHO (1994) *Dengue and Dengue Hemorrhagic Fever in the Americas: Guidelines for Prevention and Control*, p. 1. Scientific Publ no. 548. Washington, DC.

Thongcharoen P (ed.) (1993) *Monograph on Dengue/Dengue Hemorrhagic Fever*, p. 1. Reg. Pub. SEARO no. 22. New Delhi, India: World Health Organization.

DENSONUCLEOSIS VIRUSES (*PARVOVIRIDAE*)

Jacov Tal, Department of Virology, Ben-Gurion University of the Negev, Beer-Sheva, Israel

Introduction

Densonucleosis viruses (DNVs) belong to the subfamily *Densovirinae* within the *Parvoviridae* family. The other subfamily, the *Parvovirinae*, includes parvoviruses that infect a wide range of vertebrates, including humans. The DNV name (commonly shortened to densoviruses) is derived from the characteristic appearance of densely stained nuclei in the infected cells. The first DNV was isolated in 1964 from larvae of the greater wax moth *Galleria mellonella*. Over 25 additional DNVs have been isolated since from a variety of Lepidoptera, Diptera, Orthoptera and Odonata.

The criteria for the classification of densoviruses as parvoviruses are the physicochemical properties of the virion and the single-stranded DNA (ssDNA) genome with terminal palindromic structures. *Densovirinae*, however, constitute a highly diverse group of viruses with respect to genome structure and transcriptional patterns. However, the continued discovery of new DNVs with different properties dictates periodic updating of the taxonomy of these viruses.

Virion Composition and Genome Structure and Expression

The DNV virion is icosahedral, 19–24 nm in diameter, and composed of 12 capsomers. Typically, three density classes of 1.32, 1.40 and 1.44 g ml^{-1} are obtained in aqueous CsCl. The 1.32 density class contains 'empty' particles which are devoid of viral DNA and are not infectious. The other two density classes are both DNA-containing, infectious particles, having similar physicochemical properties. The DNV virion is composed of four structural proteins (VP1–VP4) with molecular weights of 76 000–110 000, 64 000–72 000, 52 000–62 000 and 42 000–49 000, respectively. The viral genome is a single-stranded DNA molecule of negative orientation. The 3′ and 5′ ends of the viral DNA can fold to form terminal hairpin structures which are utilized for self-primed DNA replication by the host DNA replication machinery, but these terminal sequences vary in size and in their internal architecture.

The current classification of members within the subfamily *Densovirinae* of the *Parvoviridae* family is

effective integrated prevention and control strategies must be developed and implemented worldwide in the tropics. Ultimately, development of an economical tetravalent vaccine holds the greatest promise for prevention and control.

See also: **Diagnostic techniques: Detection of viral antigens, nucleic acids and specific antibodies, isolation and identification by culture and microscopy; Encephalitis viruses (*Flaviviridae*): Encephalitis viruses and related viruses causing hemorrhagic disease, Tick-borne encephalitis and Wesselsbron viruses; Epidemiology of viral diseases; Immune response: General features; Yellow fever virus (*Flaviviridae*).**

Further Reading

Anonymous (1986) *Dengue Haemorrhagic Fever: Diagnosis, Treatment and Control*, 58 pp. Geneva: World Health Organization.

Gubler DJ (1988) Dengue. In: Monath TP (ed.) *The Arboviruses: Epidemiology and Ecology*, vol. 2, p. 223. Boca Raton: CRC Press.

Gubler DJ (1989) *Aedes aegypti* and *Aedes aegypti*-borne disease control in the 1990s: top down or bottom up. *Am. J. Trop. Med. Hyg.* 40: 571.

Gubler DJ and Kuno G (eds) (1997) *Dengue and Dengue Hemorrhagic Fever*. Wallingford UK: CAB International.

Halstead SB (1990) Dengue and dengue hemorrhagic fever. *Curr. Opin Infect. Dis.* 3: 434.

Henchal EA and Putnak JR (1990) The dengue viruses. *Clin. Microbiol. Rev.* 376.

PAHO (1994) *Dengue and Dengue Hemorrhagic Fever in the Americas: Guidelines for Prevention and Control*, p. 1. Scientific Publ no. 548. Washington, DC.

Thongcharoen P (ed.) (1993) *Monograph on Dengue/Dengue Hemorrhagic Fever*, p. 1. Reg. Pub. SEARO no. 22. New Delhi, India: World Health Organization.

DENSONUCLEOSIS VIRUSES (*PARVOVIRIDAE*)

Jacov Tal, Department of Virology, Ben-Gurion University of the Negev, Beer-Sheva, Israel

Introduction

Densonucleosis viruses (DNVs) belong to the subfamily *Densovirinae* within the *Parvoviridae* family. The other subfamily, the *Parvovirinae*, includes parvoviruses that infect a wide range of vertebrates, including humans. The DNV name (commonly shortened to densoviruses) is derived from the characteristic appearance of densely stained nuclei in the infected cells. The first DNV was isolated in 1964 from larvae of the greater wax moth *Galleria mellonella*. Over 25 additional DNVs have been isolated since from a variety of Lepidoptera, Diptera, Orthoptera and Odonata.

The criteria for the classification of densoviruses as parvoviruses are the physicochemical properties of the virion and the single-stranded DNA (ssDNA) genome with terminal palindromic structures. *Densovirinae*, however, constitute a highly diverse group of viruses with respect to genome structure and transcriptional patterns. However, the continued discovery of new DNVs with different properties dictates periodic updating of the taxonomy of these viruses.

Virion Composition and Genome Structure and Expression

The DNV virion is icosahedral, 19–24 nm in diameter, and composed of 12 capsomers. Typically, three density classes of 1.32, 1.40 and 1.44 g ml^{-1} are obtained in aqueous CsCl. The 1.32 density class contains 'empty' particles which are devoid of viral DNA and are not infectious. The other two density classes are both DNA-containing, infectious particles, having similar physicochemical properties. The DNV virion is composed of four structural proteins (VP1–VP4) with molecular weights of 76 000–110 000, 64 000–72 000, 52 000–62 000 and 42 000–49 000, respectively. The viral genome is a single-stranded DNA molecule of negative orientation. The 3′ and 5′ ends of the viral DNA can fold to form terminal hairpin structures which are utilized for self-primed DNA replication by the host DNA replication machinery, but these terminal sequences vary in size and in their internal architecture.

The current classification of members within the subfamily *Densovirinae* of the *Parvoviridae* family is

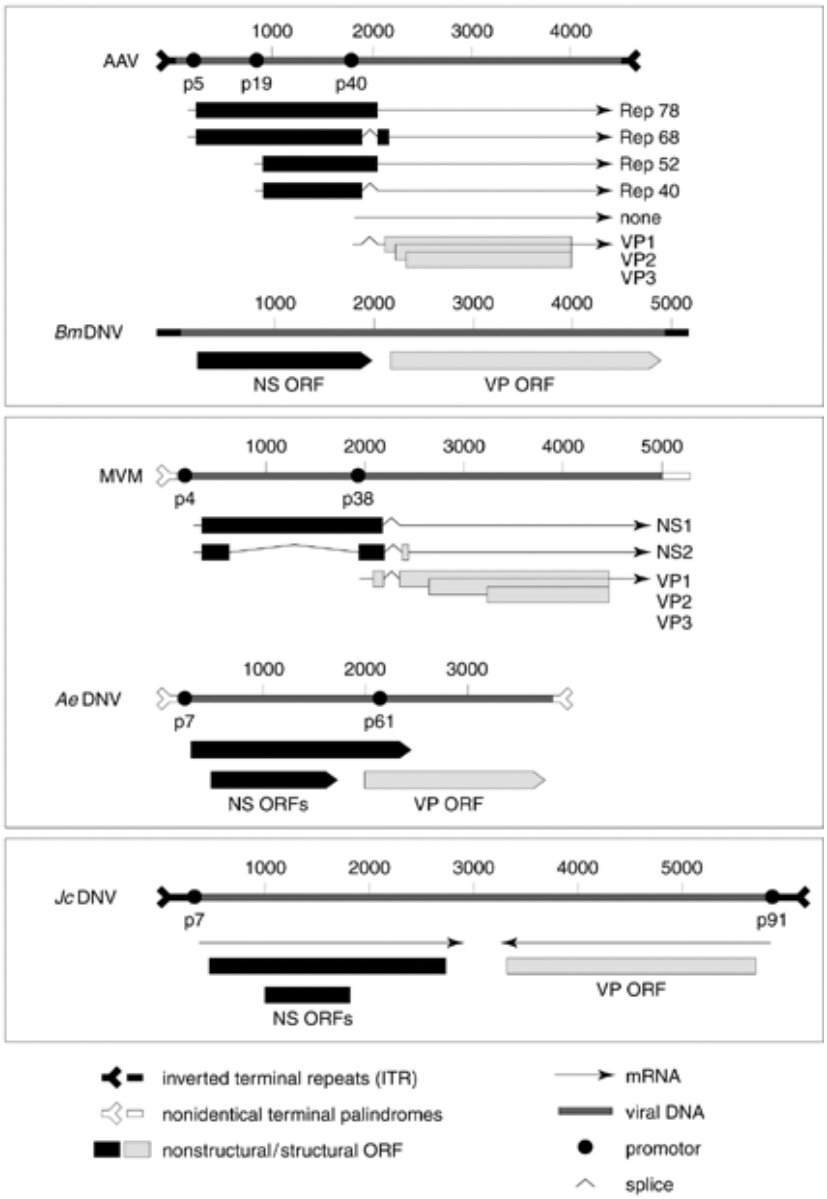


Figure 1 Organization of densovirus genomes compared with selected vertebrate parvoviruses. See text for details.

based primarily on their expression strategies. Genus *Iteravirus* [type species *Bm*DNV-1 (the Ina isolate)] bear the highest resemblance to the AAV (*Dependovirus* in the subfamily *Parvovirinae*). Like AAV, the 5048 nucleotide (nt) genome contains an inverted terminal repeat (ITR) on either end, the first 153 bases of which are palindromic, but the terminal T-shaped conformation, which is characteristic of AAV, is missing. The ITRs flank the coding sequences of the virus, all of which are clustered within the same strand, organized in two ORFs. Another similarity to AAV is that both encapsidate (+) and (−) DNA strands in separate virion particles in equal amounts (**Fig. 1**).

Genus *Brevidensovirus* (type species *Ae*DNV) bears resemblance to the vertebrate, autonomously replicating parvoviruses (genus *Parvovirus*) such as the minute virus of mice (MVM). Their DNA is the smallest among the parvoviruses: 4009 nt in *Ae*DNV and 4176 nt in *Aa*DNV). The hairpin structures on both ends differ in length (146 and 164 nt in *Ae*DNV; 134 and 182 nt in *Aa*DNV, at the 3′ and 5′ ends, respectively) and contain predominantly different nucleotide sequences, as in most autonomously replicating parvoviruses. Both termini can assume a T-shaped structure. Similar to autonomously replicating parvoviruses, the entire coding capacity of the viral DNA, located on one DNA strand, contains

partially overlapping ORFs, and the DNA (−) and (+) strands are encapsidated into separate virus particles. The encapsidation of the (−) strand is preferred and thus (−) strand-containing virions constitute about 90% of the progeny virions.

Genus *Densovirus* (type species *Gm*DNV and *Jc*DNV) form a third group with properties unique to invertebrate parvoviruses. They are characterized by long genomic DNAs (about 6 kb) and a considerably long ITR (440 nt) which, unlike AAV and *Bm*DNV, are all engaged in the terminal hairpin structure. They are the only group of parvoviruses in which both DNA strands encode viral proteins. Two TATA boxes are located within the ITRs at both ends at the junctions with the unique sequence (map positions 9 and 93), and a number of consensus sequences for polyadenylation are clustered in the middle of the genome, between the two ORFs. Both transcripts are polyadenylated and unspliced, approximately 2.5 kb in length. The right ORF encodes four viral structural proteins, and the left ORF encodes at least three nonstructural proteins: a prominent 30 kDa and two minor ones 55 kDa and 60 kDa in size. Hence, the basic organization of the right and left ORFs which encode structural and nonstructural proteins, respectively, is conserved albeit on separate strands.

The wide diversity of *Densovirinae* is only partially known to date and new genomic structures and expression mechanisms are being revealed as new DNVs are discovered. Recently, a new taxonomy has been proposed for DNVs with the suggested names *Densovirus, Brevidensovirus* and *Iteravirus* for genus A, B and C, respectively. A fourth genus, *Bidensovirus*, has also been proposed, based on the molecular structure of *Bm*DNV-2 shown to have a bipartite genome.

As in vertebrate parvoviruses, the cumulative size of these proteins exceeds the coding capacity of the viral DNA. To express all their proteins, vertebrate parvoviruses are therefore making use of a variety of mechanisms including multiple promoters, alternative splicing, the use of unusual initiation codon and proteolytic cleavage. To expand the use of their genomes, some DNVs (e.g. *Gm*DNV, *Jc*DNV) were so far shown to utilize multiple internal initiations of protein synthesis within their nonstructural and structural mRNA molecules. The peptide mapping of *Gm*DNV proteins has demonstrated that all four structural proteins contain extensive sequence homologies.

Host Range and Viral Propagation

The most extensively studied DNVs are those which infect the greater wax moth, *Galleria mellonella* (*Gm*DNV), the Buckeye caterpillar *Junonia coenia* (*Jc*DNV), the silkworm, *Bombyx mori* (*Bm*DNV), and the mosquitoes *Aedes aegypti* (*Ae*DNV) and *Aedes albopictus* (*Aa*DNV). Other DNVs include isolates from the cutworm, *Euxoa auxiliaris*, the cricket, *Acheta domestica*, the sugarcane pest, *Diatraea saccharalis*, the lepidopteran pest of palm, *Sibine fusca*, the black fly, *Similium vittatum*, the smoky-brown cockroach, *Periplaneta fuliginosa*, the Swedish dragonfly, *Leucorrhinia dubia*, the butterfly, *Agraulis vanillae*, the cabbage worm, *Pieris rapae*, the soybean looper, *Pseudoplusia includens* and the crab, *Carcinus mediterraneus*.

Some DNV have a wide host range whereas others exhibit species specificity so narrow as to infect one host only. Thus, *Jc*DNV infects a wide spectrum of insect larvae including *Spodoptera littoralis, B. mori*, but not *G. mellonella*, and the *Ae*DNV is infectious to a number of *Aedes* and *Culex* mosquito species. In contrast, the host range of *Gm*DNV (which is closely related to *Jc*DNV by serological and nucleotide sequence homology criteria) is confined to *G. mellonella*. The two viruses also vary widely in their tissue specificities and histopathological effects in their respective hosts. Infectivity of all three isolates of *Bm*DNV (the Japanese isolates *Bm*DNV-1 and *Bm*DNV-2, and the China isolate *Bm*DNV-3) is restricted to silkworm strains, but most silkworm strains are susceptible to one of the three isolates only. Genetic studies revealed that the resistance to *Bm*DNV-1 and BmDNV-2 is controlled by a recessive gene which is not sex linked, hence strains homozygous strains for the resistance gene are the preferred ones for silkworm rearing.

A major impediment to DNV research is the absence of a tissue culture host system for most of these viruses (although there are some exceptions: *Jc*DNV, for example, can grow in two *Spodoptera* cell lines following virus infection and also after transfection with an infectious DNA clone). As a result, the cultivation of the virus is mostly done *in vivo*; genetics studies are made complicated, and *in vitro* infectivity assays cannot be established. At present, there are only partial solutions to this technical problem. First, it was shown that following the injection of viral DNA in the form of calcium phosphate or DEAE dextran precipitates, replication and expression take place. Second, when tissue cultures from a variety of insect sources (e.g. *S. littoralis, Trichoplusia ni* and *Galleria*) are infected with *Gm*DNV, viral DNA replication can be readily demonstrated. Hence, the nonpermissiveness of insect cell lines is manifested at later stages in the virus replication cycle. In contrast, recent, extensive studies have shown conclusively that *Gm*DNV is unable to

replicate in vertebrate cell lines and viral DNA replication was not detected in mouse, hamster and human cell lines, even after infection with 10^4 virus input in excess of that used to demonstrate DNA replication in insect cell lines.

DNV Disease and Ecology

There have been a number of reports of DNV diseases of economically important insects in Japan and the Peoples' Republic of China. A disease of the silkworm *B. mori* in the vicinity of Ina City, Japan, in 1968 has led to the discovery of *Bm*DNV. At least two other isolates of DNV from diseased silkworms were later discovered in Japan and in China. Epizootic spread of DNV was observed in Japan in silkworm sericulture farms and in mulberry pyralids in mulberry plantations. The virus isolated from the mulberry pyralid was serologically indistinguishable from *Bm*DNV-1 and was infectious to *Bm*DNV-1-susceptible silkworm strains, suggesting that they are either identical or have a common origin. DNV antigen was detected in the dust collected from several silkworm-rearing farms in Japan, in which *Bm*DNV-resistant silkworm strains are being used. These findings are interpreted to suggest that enzootic distribution of *Bm*DNV is widespread, and probably demonstrate that epizootics are prevented by the use of nonsusceptible silkworm strains.

Some evidence suggests that immunity to infection may play a role in the spread of disease by DNVs. Primarily, extracts from uninfected *Galleria* larvae were found to contain substances with affinity to, or associated with *Gm*DNV. There is no evidence for acquired immunity.

Although some DNVs inflict economical damage, others are considered as potential biological control agents. Some success has been reported using the virus as an insecticide. In Columbia, extracts of DNV-infected larvae were used to control the disease of palm trees caused by *Sibine fusca*, and in the Ivory Coast DNV has been highly successful in the eradication of *Casphalia extranea*, a pest of oil palm and coconut trees. The advantages of DNV as pest control agents are the high resistance of the virions to environmental conditions such as heat and organic solvents and their high infectivity ratios to susceptible insects. The major disadvantage is that the extreme sensitivity of these ssDNA-containing, nonenveloped virions to UV radiation imposes limitations on their use as insecticides.

Densoviruses differ in their pathology and tissue specificity; some (e.g. *Gm*, *Jc*DNVs) can cause an extensive viremia in most or all tissues, whereas the replication of others, such as *Sibine* DNV, is restricted primarily to the midgut. *Bm*DNV multiplies only in the nuclei of the columnar cells of the midgut epithelium columnar cells.

DNV-caused disease is lethal, and is characterized by the accumulation of DNV particles in the nuclei of infected cells. The nuclei degenerate gradually and vacuoles and inclusion bodies appear in the cytoplasm. In the nucleus, the virus forms multilayer crystalline-like structures which occupy the majority of the nucleoplasm volume. The infected larvae become anorexic, sluggish and flaccid, and are progressively paralyzed. Death occurs within 5–10 days, depending on the larval stage, multiplicity of infection and environmental conditions.

DNV histopathology can assume many forms. In *Ae*DNV infected *Aedes aegypti* larvae, the nuclei of the infected cells (hypodermis, imaginal disk, endocrine glands, trachea, hypodermis, etc.) become double in size of those of normal cells and intranuclear structures are disrupted. In *Gm*DNV infection the nuclei of the midgut cells become hypertrophied and eosinophilic, and dense bodies accumulate in the nucleoplasm. *S. fusca* DNV produces distinct pathological changes: the digestive tracts of the infected larvae become thickened, opaque and whitish.

DNV infections of last instar larvae often result in inhibition of pupation. Infection at lower concentrations results in partial metamorphosis, but larval tissues in the pupae are affected. Silk production and cocoon spin are also inhibited.

DNV Gene Vectors

Mammalian parvoviruses, especially AAV, are being studied extensively as transducing and expression vectors. The growing understanding of DNV molecular biology is accompanied by similar attempts to exploit these viruses as vectors for the expression of foreign genes in larvae. Success has been reported in some cases, especially with the lepidopteran *Jc*DNV and the mosquito DNVs. However, the possible application of these vectors to other insects is not clear since these studies were so far confined to their own natural hosts. Both promoters were shown to be capable of driving the expression of heterologous genes such as β-galactosidase and chloramphenicol acetyltransferase, and of allowing the selection of G418 resistant cells using neo^R gene. Success has also been reported in the construction of infectious clones and in packaging them into infectious particles. DNVs are similar to vertebrate parvoviruses in that their major nonstructural protein, NS1, is essential for viral DNA replication and for transactivating transcription from the structural promoter. Finally,

*Jc*DNV has been reported to integrate into the DNA of *Spodoptera* cell lines.

See also: **Parvoviruses (*Parvoviridae*): Cats, dogs and mink, Molecular biology, Rodents, pigs, cattle and waterfowl, General features; Vectors: Animal viruses, Plant viruses.**

Further Reading

Tijssen P and Bergoin M (1995) Densonucleosis viruses constitute an increasingly diversified subfamily among the parvoviruses. *Semin. Virol.* 6: 347.

Corsini J, Afanasiev B, Maxwell IH and Carlson JO (1996) Autonomous parvovirus and densovirus gene vectors. *Adv. Virus Res.* 47: 303.

DIAGNOSTIC TECHNIQUES

Contents

Detection of viral antigens, nucleic acids and specific antibodies

Aloys CM Kroes and **Linda FF Kox**, Department of Virology, Leiden University Medical Center, Leiden, The Netherlands

Introduction

Today, *in vitro* virus isolation techniques are still widely used and these constitute perhaps the most convincing diagnostic method available. The identification of complete infectious viruses in clinical specimens is considered the gold standard of diagnostic virology; however, other approaches are becoming increasingly important, for several reasons. First, the maintenance of tissue cultures is labor intensive and time consuming. Furthermore, even with some technical improvements, diagnosis based on virus isolation is relatively slow, taking one to several days. But most important in this respect is the simple fact that many important viruses cannot be cultured *in vitro* at all, e.g. many gastroenteritis viruses, hepatitis viruses and papilloma viruses. This has stimulated diagnostic approaches aimed not at isolation of the virus but just at the identification of viral proteins, of antibodies in the host against viral antigens, or of viral nucleic acids. The techniques employed for these purposes are developing rapidly, leading to impressive increases in sensitivity and also to reduced demands for time and cost. Although these approaches have in common the fact that they do not formally demonstrate the presence of biologically active, complete virus, they are of great practical importance in diagnostic virology. It is to be expected that the increasing demand for rapid and efficient large-scale testing in virology will continue to stimulate the use of these techniques. In particular, the detection of viral nucleic acids, which benefits most fully from biotechnological advances, will probably be the primary approach in diagnostic virology of the twenty-first century.

Viral Antigens

Immunological techniques are usually employed to demonstrate the presence of a viral protein in a clinical specimen; therefore, this viral protein is then referred to as a viral antigen, recognized by the specific antibodies used in the test. Assays of this kind have benefited greatly from the improved availability of antibodies of any desired specificity, by the continuous stable *in vitro* production of monoclonal antibodies, which can recognize single epitopes of viral proteins. This has led to increased specificity and sensitivity of this category of tests.

The principal limitation of this approach is the concentration of viral antigens in the specimens. Antigens are not always present in sufficient concentrations for even the most sensitive assays, and there is no additional technique available to increase this target concentration, in contrast to the detection of viral nucleic acids. The signal obtained after the binding of an antigen can be detected with increasing sensitivity, although still not comparable to the thresholds of culture and viral nucleic acid amplification.

The main advantage of antigen detection is its speed, often producing results after one simple

*Jc*DNV has been reported to integrate into the DNA of *Spodoptera* cell lines.

See also: **Parvoviruses (*Parvoviridae*): Cats, dogs and mink, Molecular biology, Rodents, pigs, cattle and waterfowl, General features; Vectors: Animal viruses, Plant viruses.**

Further Reading

Tijssen P and Bergoin M (1995) Densonucleosis viruses constitute an increasingly diversified subfamily among the parvoviruses. *Semin. Virol.* 6: 347.

Corsini J, Afanasiev B, Maxwell IH and Carlson JO (1996) Autonomous parvovirus and densovirus gene vectors. *Adv. Virus Res.* 47: 303.

DIAGNOSTIC TECHNIQUES

Contents

Detection of viral antigens, nucleic acids and specific antibodies

Aloys CM Kroes and **Linda FF Kox**, Department of Virology, Leiden University Medical Center, Leiden, The Netherlands

Introduction

Today, *in vitro* virus isolation techniques are still widely used and these constitute perhaps the most convincing diagnostic method available. The identification of complete infectious viruses in clinical specimens is considered the gold standard of diagnostic virology; however, other approaches are becoming increasingly important, for several reasons. First, the maintenance of tissue cultures is labor intensive and time consuming. Furthermore, even with some technical improvements, diagnosis based on virus isolation is relatively slow, taking one to several days. But most important in this respect is the simple fact that many important viruses cannot be cultured *in vitro* at all, e.g. many gastroenteritis viruses, hepatitis viruses and papilloma viruses. This has stimulated diagnostic approaches aimed not at isolation of the virus but just at the identification of viral proteins, of antibodies in the host against viral antigens, or of viral nucleic acids. The techniques employed for these purposes are developing rapidly, leading to impressive increases in sensitivity and also to reduced demands for time and cost. Although these approaches have in common the fact that they do not formally demonstrate the presence of biologically active, complete virus, they are of great practical importance in diagnostic virology. It is to be expected that the increasing demand for rapid and efficient large-scale testing in virology will continue to stimulate the use of these techniques. In particular, the detection of viral nucleic acids, which benefits most fully from biotechnological advances, will probably be the primary approach in diagnostic virology of the twenty-first century.

Viral Antigens

Immunological techniques are usually employed to demonstrate the presence of a viral protein in a clinical specimen; therefore, this viral protein is then referred to as a viral antigen, recognized by the specific antibodies used in the test. Assays of this kind have benefited greatly from the improved availability of antibodies of any desired specificity, by the continuous stable *in vitro* production of monoclonal antibodies, which can recognize single epitopes of viral proteins. This has led to increased specificity and sensitivity of this category of tests.

The principal limitation of this approach is the concentration of viral antigens in the specimens. Antigens are not always present in sufficient concentrations for even the most sensitive assays, and there is no additional technique available to increase this target concentration, in contrast to the detection of viral nucleic acids. The signal obtained after the binding of an antigen can be detected with increasing sensitivity, although still not comparable to the thresholds of culture and viral nucleic acid amplification.

The main advantage of antigen detection is its speed, often producing results after one simple

reaction. This had led to widespread use of antigen detection in several areas of diagnostic virology, to trace those infections leading to the release of relatively large amounts of viral antigens, in particular when a rapid diagnosis is important.

In addition, it is possible to detect viral antigens in infections in which viral culture is difficult or impossible.

The following techniques are in use for the detection of viral antigens in clinical specimens.

Immunofluorescence If specific antibodies against viral antigens are used, and are conjugated with fluorochrome dyes (usually fluorescein isothiocyanate or FITC), this is termed direct immunofluorescence. If an unconjugated antiviral antibody is used, a second incubation is necessary with an appropriate fluorochrome-labeled anti-immunoglobulin, which is known as indirect immunofluorescence. After the incubations, the clinical specimen is examined microscopically in ultraviolet light and typical staining patterns of virally infected cells can be observed if the test is positive.

This approach is feasible when virally infected cells can be easily obtained. It has been particularly successful for rapid diagnosis of respiratory infections, as caused by respiratory syncytial virus (RSV) and others. The same principle is also used extensively to detect viral antigens in virus isolation procedures.

Immunoenzyme staining This approach is identical to immunofluorescence, except for the label used, which is an enzyme, usually peroxidase or phosphatase. Local color change is observed microscopically if a suitable substrate is added after the antibody incubation. The technique is widely used to demonstrate viral antigens in tissue sections but has many applications. It is used to demonstrate cytomegalovirus antigen in peripheral blood cells, enabling a rapid diagnosis of active infections without viral culture.

Enzyme immunoassays (EIA, or ELISA) The viral antigen again is recognized by a specific antibody, either directly or indirectly through labeling with an enzyme. The reaction catalyzed by the enzyme is usually the production of colored dye, which then is detected by photometry. Recently, the same principle has been applied for several other enzyme-mediated conversions, leading to a product detectable by chemiluminescence or fluorescence. To allow the separation of antibody bound to the antigen from unbound labeled antibody, a 'solid phase' is essential in this type of immunoassay, which means a surface to which a specific antibody is stably bound and immobilized during subsequent washing steps. Many viral antigens are detected with great sensitivity by these techniques. Routine testing for hepatitis B virus (HBV) infection is generally done by EIA detection of the viral surface antigen (HBsAg) in serum. EIA techniques are also used for the detection of gastroenteritis viruses in feces and for respiratory viruses in nasal or throat washings. Some commercial versions are easily carried out in handheld devices with visual detection of color production if a viral antigen is present in the specimen. But, in contrast, EIAs are also widely employed in large-scale fully automated analyzers.

Radioimmunoassay (RIA) The antiviral antibody in this technique is labeled with a radioactive isotope, which can be detected by gamma counters or liquid scintillation counters, depending on the nature of the emission. The traditional advantage of this technique was always its sensitivity. Nowadays, the EIA-based methods are not much different in this respect, and in other respects the RIA technique has a number of important disadvantages, mainly the limited shelf-life of many radiolabels and the extensive regulations for safe handling and waste disposal, which make this technique less suitable for routine viral diagnosis.

Particle agglutination Particles which are coated with antiviral antibodies can produce macroscopic agglutination if mixed with a specimen containing viral antigens. Although this is an elegant technique with a minimal requirement for technical support, its applications are limited owing to its low sensitivity. Erythrocytes, latex, gelatin and other particles can be used. A classical example has been the field test for hepatitis B surface antigen. Nowadays, this technique is still used frequently to detect viruses causing gastroenteritis, mainly rotavirus, as these are excreted in feces in high concentrations.

Specific Antibodies

The detection of antibodies in the host, to demonstrate a viral infection, is extremely successful because of its many practical advantages. These include: the nature of the clinical specimen, which is just a sample of venous blood for all tests; and the simplicity of the techniques involved and therefore the speed and low cost of the procedures. Immunoglobulins are generally produced in high concentrations, easily detectable by several techniques. Antibody testing can also be utilized for most viruses which cannot be recovered by virus culture. With all these benefits in mind, it should be emphasized that the detection of specific

antiviral antibodies in the host, as a diagnostic approach, also has several important weaknesses. The disadvantages of antibody detection for viral diagnosis (sometimes shortly referred to as 'serology') include the following:

- Antibody production by the host inevitably requires time, to allow for a primary or secondary immune response. This precludes a diagnosis in the acute phase of some infections, although other infections at the time of clinical presentation may have given rise to an antibody response.
- The antibody response requires a properly functioning immune system in the host; however, several infections do occur preferentially in patients with decreased immunity (immunocompromised hosts). In these cases, a diagnosis based on antibody detection can be either impossible or, at best, will be delayed. This category of patients is by no means rare in modern medicine (transplant recipients, acquired immune deficiency syndrome (AIDS), tumor chemotherapy, etc.).
- Antibody detection does not discriminate between past and current infections without special precautions. For any infection that is not extremely rare in the population studied, to link the presence of antibodies to an actual clinical problem requires either the demonstration of a rise in antibody titers in the course of the disease or the demonstration of the usually short-lived IgM class of antibody against the virus. Although neither procedure provides absolute proof of a viral infection in the period involved, positive results can be considered strong support for this assumption.
- Special problems are encountered in the diagnosis of congenital infections, as the fetal antibody response may be lacking, transient or masked by the maternal response.

To resolve many of the problems listed above, one should resort to diagnostic techniques demonstrating the virus or viral components directly. Essentially, antibody detection should be considered only indirect proof of the presence or absence of a virus.

All techniques aimed at detection of antiviral antibodies rely heavily on the availability of specific antigens. While in the past these were often obtained from viral cultures or even from infected patients, nowadays recombinant DNA techniques are rapidly becoming the dominant method of producing viral antigens. Increasingly, naturally occurring antigens are even replaced by carefully selected synthetic peptides containing single epitopes of which specificity and immunological recognition have been demonstrated. These developments have greatly improved many serological tests in terms of sensitivity and specificity.

Table 1 Principal techniques for the detection of viral antigens and antiviral antibodies

Immunofluorescence	Antigen and antibody
Immunoenzyme staining	Antigen
Enzyme immunoassay	Antigen and antibody
Radioimmunoassay	Antigen and antibody
Particle agglutination	Antigen and antibody
Neutralization	Antibody
Hemagglutination inhibition	Antibody
Complement fixation	Antibody

Antibodies against viral antigens can be demonstrated by various methods, several of these being similar to those applied to antigen detection as discussed earlier (**Table 1**). The following techniques are relevant in clinical and veterinary medicine.

Neutralization This technique, like the next one, constitutes a classical virological approach to find antiviral antibodies but is infrequently used in routine practice. Serum or preferentially paired serum samples are used to inhibit ('neutralize') the culture of a specific virus. The neutralizing capacity is considered an important biological function of antibodies, but its detection requires culturing the virus involved. It is cumbersome and not applicable to many viruses.

Hemagglutination inhibition Certain viruses cause hemagglutination if a suspension of cultured virus is incubated with erythrocytes. The presence of antiviral antibodies can prevent this macroscopically visible effect. Classically, it is used to detect type-specific antibodies against influenza viruses. Apart from this application, the technique is rarely used.

Complement fixation One of the typical biological effects of antigen–antibody complexes is the binding of complement. Complement is a system of serum enzymes, which on activation may lead to cell lysis. If complement is bound by a complex, in a hemolytic system which relies on the presence of a critical concentration of complement, hemolysis will be suppressed.

In this way, specific antibody is detected if after the incubation phase with a suitable antigen no hemolysis is observed. This technique also allows macroscopical reading of results. It requires technical experience and has low sensitivity. Its main advantage is that all (sub)classes of immunoglobulin able to bind complement are detected simultaneously. It is still widely used in tests for antibodies against respiratory viruses.

Immunofluorescence Antibodies against a virus are detected by incubation of serum with cells expressing viral antigens. These cells are usually obtained from standard viral cultures. Alternatively, cells can be made to express certain antigens by recombinant DNA techniques. In a second incubation step, the bound antiviral antibodies are visualized microscopically by a FITC-labeled anti-immunoglobulin. Appropriate controls should demonstrate a typical staining pattern. The technique is relatively time consuming and the reading of results is subjective to some extent. It is widely used to detect antibodies against viruses that are easily cultured, as the slides for these tests can then be prepared in the laboratory.

Enzyme immunoassays (EIA, or ELISA) The binding of antiviral antibody to a prepared antigen is recognized by the reaction of an enzyme-linked anti-immunoglobulin. As described for the antigen detection by EIA, a solid phase is required to immobilize the antigen–antibody complex. Several technical variations are in use. One of the most important is particularly suitable for detection of IgM antibodies: the capture technique, which binds all IgM to the solid phase by using anti-IgM antibody. Afterwards, viral antigen is added, detected by labeling this protein or by a labeled antibody in a subsequent step. EIA testing for viral antibodies is the most widely used technique and is employed in many different ways, from handheld visually readable tests to sophisticated analyzers. Improvements include kinetic measurements instead of end-point reactions, the use of luminescent or fluorescent reaction products and the increase of solid-phase surfaces, for example by using microparticles.

Radioimmunoassay (RIA) Like its application in the detection of viral antigens, the principle of using antibodies or antigens labeled with a radioactive isotope can provide very sensitive assays but its practical applications are increasingly limited by the typical disadvantages, which were discussed before.

Particle agglutination Particles coated with viral antigens are used, which will produce agglutination if antiviral antibody is present. Sensitivity is limited and the tests are used exclusively in some rapid test procedures, usually with single specimens.

Immunoblot techniques These constitute an important approach in antibody diagnosis which is used extensively when tests with maximal specificity are required. Essentially, a number of viral antigens derived from the same virus are separated and transferred to an immobile matrix. The antibody reactivity of a specimen against each of the antigens is visualized by a secondary incubation with enzyme-labelled anti-immunoglobulin. The observed patterns can provide a high degree of confidence that a response is directed at the virus. Originally, antigens were prepared from lysates of viral cultures; recombinant proteins or synthetic peptides are now often used. The test is frequently used for confirmation of screening test results by EIA, routinely for human immunodeficiency virus (HIV) and hepatitis C virus (HCV), for example.

Typical pitfalls in diagnostic use of antibody detection include:

- cross-reacting antibodies directed against related viruses;
- false-positive IgM assays due to the presence of rheumatoid factor (RF) in samples, an autoreactive IgM antibody binding to IgG, which can produce a positive signal with any labelled anti-IgM if IgG is present;
- false-negative IgM assays, due to the competing presence of IgG.

The described IgM-capture technique can resolve the last problems, otherwise IgG should be selectively removed before IgM detection.

Nucleic Acids

An overview is presented of techniques currently in use for detection and identification of viral nucleic acids in routine medical or veterinary laboratories, with the exception of *in situ* techniques. Since only in a few cases clinical specimens contain sufficient virus to allow identification of their genomes directly by physical methods (e.g. gel electrophoresis of feces samples containing rotavirus), these tests are usually based on hybridization or amplification reactions.

Hybridization

Nucleic acids are able to form a double-stranded structure with a complementary nucleotide sequence and this reaction is highly specific as a result of the base pairing between nucleic acid molecules.

By using a probe, a DNA or RNA molecule with a complementary sequence to the genome to be detected, the presence of viral DNA or RNA can be demonstrated by hybrid formation. This hybrid formation or hybridization is detected by a suitable label which is incorporated in the probe: radioisotopes, enzymes or fluorochromes.

The use of non-isotopic labeled probes is preferred in diagnostic laboratories. In addition, a number of indirect detection systems have been developed, for

Table 2 Detection of nucleic acid by amplification: an overview

Target amplification
PCR, polymerase chain reaction (Roche)
TAS, transcription-based amplification systems
NASBA, nucleic acid sequence based amplification (Organon Teknika)
3SR, self-sustaining sequence replication
TMA, transcription-mediated amplification (Gen-Probe)
SDA, strand displacement amplification (Becton-Dickinson)
Probe amplification
LCR, ligase chain reaction (Abbott)
QBR, Q-β replicase (Gene-Trak)
Signal amplification
bDNA, branched DNA (Chiron)

Current license-holder in parantheses.

example probes labeled with biotin, detected with enzyme-labeled avidin or streptavidin, and probes labeled with digoxigenin, detected with an enzyme-labeled antibody. Another recently developed indirect detection system employs enzyme-labeled antibodies directed against DNA:RNA hybrids. In all these cases, final detection takes place by colored or chemiluminescent products.

Viral nucleic acids can be detected by hybridization in a wide range of clinical specimens like blood, cerebrospinal fluid, urine, bronchoalveolar lavage and others. Before the identification with a probe is possible, viral nucleic acids have to be isolated from the specimens and separated to single strands if the genome is double stranded. Hybridizations can be performed as solid-phase assays or in solution. In solid-phase hybridizations nucleic acids are fixed in a single-stranded form on a nitrocellulose or nylon filter. In solution-phase hybridizations both the nucleic acid and the probe are free to interact in the reaction mixture. Specific procedures to enhance the hybridization signals can be employed (see also below).

Amplification

Although hybridization assays are able to detect minuscule amounts of DNA and RNA, the sensitivity is too limited for many applications. Amplification of nucleic acids and amplification of hybridization signals have realized a considerable increase in sensitivity. In recent years, much progress has been made in the development of several amplification techniques for the detection of specific viral nucleic acids.

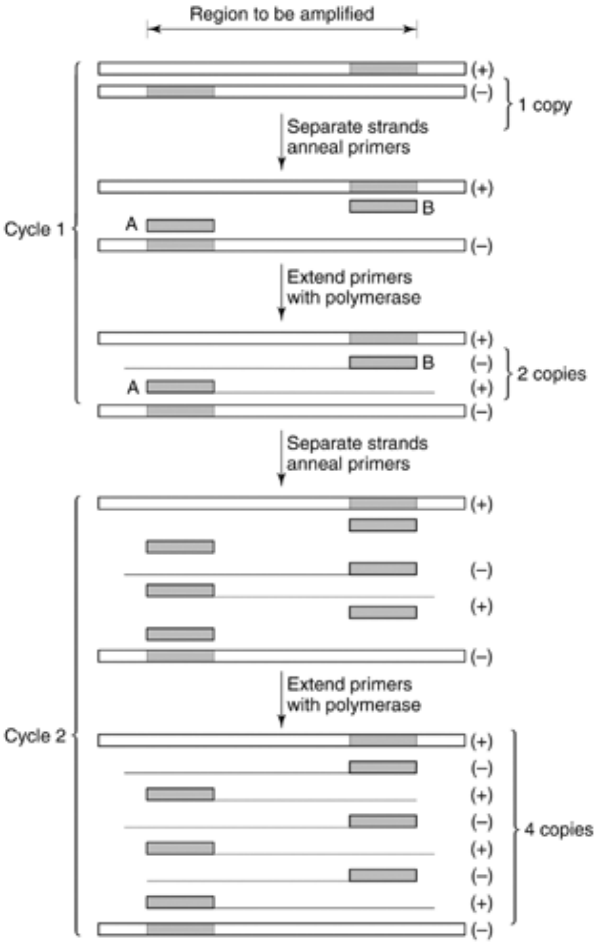


Figure 1 PCR. (+), Sense or coding strand; (−) antisense or noncoding strand; hatched (A) bar, sense primer; stippled (B) bar, antisense primer; empty bar, DNA.

The first nucleic acid amplification technique to be used was the polymerase chain reaction (PCR). Several other methods are currently in use for the detection of specific viral nucleic acids, as summarized in **Table 2.** The transcription-based amplification systems (TAS) include nucleic acid sequence-based amplification (NASBA), self-sustaining sequence replication (3SR) and transcription-mediated amplification (TMA). Other amplification strategies are: ligase chain reaction (LCR), strand displacement amplification (SDA), branched DNA (bDNA) signal amplification and Q-β replicase (QBR). These techniques can be subdivided in target, probe and signal amplification techniques, as indicated.

Target amplification *PCR* With the PCR technique (**Fig. 1**), target DNA sequences are exponentially replicated in repeated cycles of DNA synthesis driven by specific primers (short stretches of nucleotides, complementary to a DNA strand). At a high

temperature (94°C), the double-stranded viral target DNA is separated to single strands. Subsequently the primers are hybridized at a temperature of 40–60°C to both single strands of the target DNA and the primers are extended by the action of a heat-stable DNA polymerase (*Thermus aquaticus (Taq)* DNA polymerase, *Thermus thermophilus (Tth)* DNA polymerase) at a temperature of 72°C, resulting in the synthesis of new complementary strands. These three incubation steps, denaturation, annealing and extension, give a doubling of the target DNA and the new strands serve as a template in the next cycle. By repeating the cycle several (20–40) times, a large amount of viral DNA with fixed ends, determined by the distance of the two primers on the target DNA, is generated. If viral RNA is the target it is first converted into copy DNA (cDNA) by the action of the enzyme reverse transcriptase (RT) before the amplification reaction. This modification is known as RT-PCR.

The specificity of the PCR is dependent on the nucleotide sequence of the primers, which has to be virus specific, and the temperature and salt conditions used during the incubations. Both specificity and sensitivity of the PCR can be increased by performing a nested PCR: a second round of amplification, using a second pair of primers that is specific for the internal sequence amplified by the first primer pair. The strength of PCR is that it can be applied to relatively crude samples containing only small numbers of viral fragments. Using PCR it is possible to amplify any target DNA or RNA molecule to a detectable level. The amplified DNA can be detected by electrophoresis in ethidium bromide-stained agarose gels. However, this only provides information on the size of the PCR product. For diagnostic purposes, further confirmation of the result is usually required. This can be done by subsequent hybridization with a specific probe. When a suitable restriction site is present, the PCR product can be cut in defined fragments. PCR products can also be analyzed by sequencing, but this approach is not practical for routine diagnosis.

TAS The transcription-based methods NASBA, 3SR and TMA are very similar. NASBA is a continuous, isothermal process, which can amplify RNA (**Fig. 2**). Target RNA is first converted into cDNA by RT using a virus-specific primer, containing a promoter sequence that is recognized by T7 RNA polymerase (P1). After removal of the RNA from the DNA:RNA hybrid by RNase H and synthesis of the complementary DNA strand with a second virus-specific primer (P2), about 1000 RNA copies from the DNA template are transcribed by incubation with T7 RNA polymerase. Each RNA copy now serves again as a template for a new round of amplification. 3SR differs from NASBA in that both primers contain T7 promoter sequences. In TMA, only an RT and an RNA polymerase are used. These methods are increasingly employed, mainly in complete commercial systems.

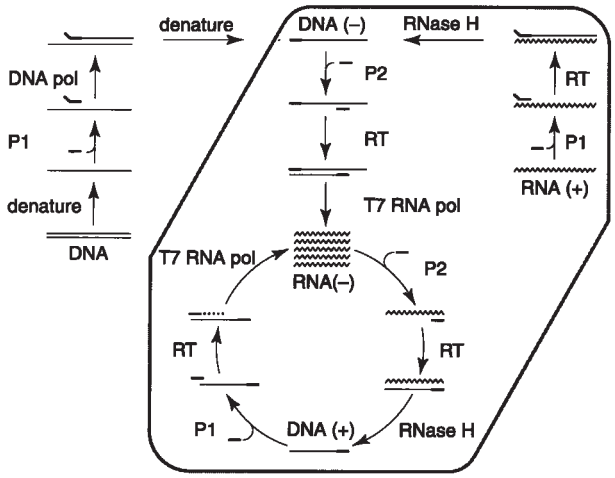


Figure 2 NASBA. RT, reverse transcriptase; P1, antisense primer; P2, antisense primer; pol, polymerase; (+), sense or coding strand; (–) antisense or noncoding strand; bold lines, primers; light lines, DNA; wavy lines, RNA; dotted line, incomplete DNA strand.

SDA This is an isothermal method of amplifying target DNA which requires two enzymes, a DNA polymerase and a restriction enzyme. It uses two primer sets, one to define the target sequence and a second to introduce a restriction site. Extending upstream and downstream primers displace each other from the template on both strands simultaneously. The first SDA assays used the exonuclease deficient (exo$^-$) Klenow fragment of *Escherichia coli* DNA polymerase and *HincIII* as restriction enzyme. It produces a 10^8-fold amplification during a single 2 h incubation at 40°C. The second generation SDA assays use exo$^-$ *Bst* or exo$^-$ *Bca* DNA polymerase and *Bso*BI and produces a 10^{10}-fold amplification in 15 min at 50–60°C.

Probe amplification *LCR* This method utilizes four oligonucleotide primers (instead of two used in PCR) and a thermostable DNA ligase to ligate the contiguous primers and cycles between the denaturation temperature (94°C) and the ligation temperature (72°C). A variation of LCR called gapped LCR employs *Taq* polymerase to fill in one nucleotide prior to the ligation of adjacent primers.

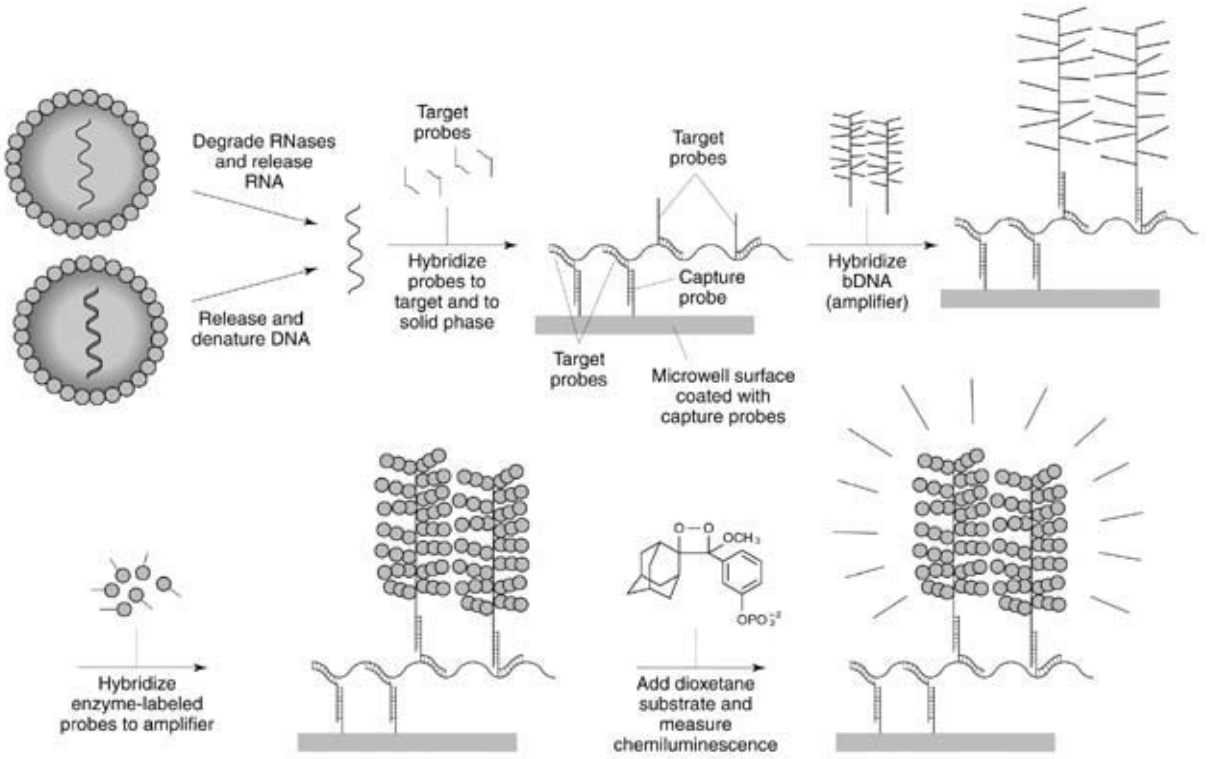


Figure 3 bDNA signal amplification.

QBR This amplification is named for the enzyme carrying out amplification, Q-β replicase, a RNA-directed RNA polymerase produced by the Q-β bacteriophage. The enzyme recognizes and produces copies of a 'reporter' RNA molecule, midivariant 1 (MDV-1) which can incorporate a short foreign RNA probe. The presence of target DNA leads to hybrid formation with subsequent activation of the Q-β replicase, generating many new RNA molecules. The RNA molecules serve as probes in the following rounds. So far, this system has not been extensively used.

Signal amplification *bDNA signal amplification* This is a sandwich hybridization assay performed in the wells of a microtiter plate in which lysis, hybridization, capture and signal amplification occur (**Fig. 3**). A mixture of capture probes and target probes hybridizes to highly conserved regions of the nucleic acid, thereby capturing the nucleic acid molecules on to the surface of a microtiter plate. After hybridizing the target to synthetic bDNA molecules added to the well, multiple copies of an alkaline phosphatase-linked synthetic probe hybridize to the immobilized complex, thereby amplifying the target signal. Detection is achieved by incubating the complex with a chemiluminescent substrate and measuring light emission, which is proportional to the concentration of target nucleic acid in the specimen.

Potential problems

Although amplification assays are rapidly becoming 'routine' tests in diagnostic laboratories there are some pitfalls to be aware of. A summary of these will focus on PCR, as it is the most widely used amplification technique.

A major problem with diagnostic assays based on amplification is the occurrence of false-positive results due to nucleic acid contamination. This can be either viral nucleic acid or products from previous amplifications (amplicons). The amplicons are particularly likely to cause contamination because of the large number of molecules produced. To perform different steps of the PCR procedures at different locations is a first step in the prevention of contamination. In addition, amplicon contamination can be prevented by using uracil DNA glycosylase (UDG) together with dUTP, instead of dTTP. In the incubation step prior to amplification, UDG is allowed to excise uracil from any contaminating dU-containing PCR product. At the high cycling temperatures during amplification the UDG is inactivated and any possible contaminants are

broken into small fragments. An alternative to this pre-PCR enzymatic amplicon degradation method is the post-PCR photochemical method. In this method high concentrations of isopsoralen inactivate the amplicons. This method has the disadvantage that an excess of isopsoralen can inactivate the DNA polymerase. Furthermore isopsoralens are mutagenic.

Another problem with the diagnostic use of PCR can be the occurrence of false-negative results due to inhibition of the *Taq* DNA polymerase. Most inhibitors are poorly characterized and occur rather unpredictably in clinical materials. Inhibition can be detected by testing specimens in duplicate, with and without exogenously added DNA or RNA (or virus) that can be amplified with the same or different primers. This external inhibition control increases the number of PCR tests necessary for each sample. Therefore internal controls, included in the same reaction, are preferred, although these require a careful design.

The extraction of nucleic acid from the clinical samples is a critical process. Different procedures are needed depending on the nature of the sample and it is important to identify the optimal procedure for a specific assay. Inhibitors of the PCR must be removed effectively while minimizing the loss of nucleic acid.

Amplification procedures demand strict quality control of the reaction mixtures, standardization of the procedures and a dependable thermocycler. The use of multiple positive and negative controls to monitor each step of the assay is mandatory for diagnostic purposes. With this approach, amplification assays are reliable diagnostic tests and can be employed in any routine diagnostic laboratory. The commercial availability of complete test systems theoretically could make quality control easier. However, several studies have shown that the use of commercial test systems is not a guarantee for reliable results. All nucleic acid amplification methods, as a category of diagnostic tests, require strict quality control measures.

Significance in diagnostic virology

Nucleic acid amplification techniques will be increasingly used for the diagnosis of viral infections, as they combine sensitivity, specificity and speed and are universally applicable. Amplification techniques are therefore well suited for the detection of viruses that cannot be recovered by culture. This has been effective for detection of hepatitis C virus (HCV), human parvovirus B19, papilloma viruses and many others. In addition to detecting this category of viruses, molecular methods can be used in quantitative assays to determine viral concentrations ('viral load') in several chronic infections, such as those of HIV, HCV, HBV and cytomegalovirus. These quantitative assays are particularly important to monitor the response to new antiviral drug therapies.

See also: **Hepadnaviruses (*Hepadnaviridae*): Hepatitis B Virus: General features; Hepatitis C virus (*Flaviviridae*); Rotaviruses (*Reoviridae*): General features; Influenza viruses (*Orthomyxoviridae*): General features; Immune response: General features; Antivirals; Diagnostic techniques: Isolation and identification by culture and microscopy.**

Further Reading

Becker Y and Darai G (eds) (1992) *Diagnosis of Human Viruses by Polymerase Chain Reaction Technology.* Berlin: Springer.

James K (1990) Immunoserology of infectious diseases. Clin. Microbiol. Rev. 3: 132.

Lennette EH (ed.) (1991) *Laboratory Diagnosis of Viral Infections*, 2nd edn. New York: Dekker.

Persing DH, Smith TF, Tenover FC and White TJ (eds) (1993) *Diagnostic Molecular Microbiology: Principles and Applications.* Rochester, MN: Mayo Foundation.

Wiedbrauk DL and Farkas DH (eds) (1995) *Molecular Methods for Virus Detection.* San Diego: Academic Press.

Isolation and Identification by Culture and Microscopy

Marie L Landry and **GD Hsiung**, Virology Reference Laboratory, VA Connecticut Healthcare System, West Haven, Connecticut, USA; and Department of Laboratory Medicine, Yale University School of Medicine, New Haven, Connecticut, USA

Introduction

Methods commonly used to test for viruses vary greatly from laboratory to laboratory. These variations depend not only on the availability of resources and equipment of each setting, but also on the availability of trained personnel.

In general, the isolation of a viral agent is the most accurate method for diagnosis of a virus infection and is still considered the gold standard, with a few exceptions. Since viruses only replicate in living cells, virus isolation requires a constant supply of cell cultures or laboratory animals, as well as labor-intensive procedures. This has been an impetus for the development of methods for direct detection of viral

broken into small fragments. An alternative to this pre-PCR enzymatic amplicon degradation method is the post-PCR photochemical method. In this method high concentrations of isopsoralen inactivate the amplicons. This method has the disadvantage that an excess of isopsoralen can inactivate the DNA polymerase. Furthermore isopsoralens are mutagenic.

Another problem with the diagnostic use of PCR can be the occurrence of false-negative results due to inhibition of the *Taq* DNA polymerase. Most inhibitors are poorly characterized and occur rather unpredictably in clinical materials. Inhibition can be detected by testing specimens in duplicate, with and without exogenously added DNA or RNA (or virus) that can be amplified with the same or different primers. This external inhibition control increases the number of PCR tests necessary for each sample. Therefore internal controls, included in the same reaction, are preferred, although these require a careful design.

The extraction of nucleic acid from the clinical samples is a critical process. Different procedures are needed depending on the nature of the sample and it is important to identify the optimal procedure for a specific assay. Inhibitors of the PCR must be removed effectively while minimizing the loss of nucleic acid.

Amplification procedures demand strict quality control of the reaction mixtures, standardization of the procedures and a dependable thermocycler. The use of multiple positive and negative controls to monitor each step of the assay is mandatory for diagnostic purposes. With this approach, amplification assays are reliable diagnostic tests and can be employed in any routine diagnostic laboratory. The commercial availability of complete test systems theoretically could make quality control easier. However, several studies have shown that the use of commercial test systems is not a guarantee for reliable results. All nucleic acid amplification methods, as a category of diagnostic tests, require strict quality control measures.

Significance in diagnostic virology

Nucleic acid amplification techniques will be increasingly used for the diagnosis of viral infections, as they combine sensitivity, specificity and speed and are universally applicable. Amplification techniques are therefore well suited for the detection of viruses that cannot be recovered by culture. This has been effective for detection of hepatitis C virus (HCV), human parvovirus B19, papilloma viruses and many others. In addition to detecting this category of viruses, molecular methods can be used in quantitative assays to determine viral concentrations ('viral load') in several chronic infections, such as those of HIV, HCV, HBV and cytomegalovirus. These quantitative assays are particularly important to monitor the response to new antiviral drug therapies.

See also: **Hepadnaviruses (*Hepadnaviridae*): Hepatitis B Virus: General features; Hepatitis C virus (*Flaviviridae*); Rotaviruses (*Reoviridae*): General features; Influenza viruses (*Orthomyxoviridae*): General features; Immune response: General features; Antivirals; Diagnostic techniques: Isolation and identification by culture and microscopy.**

Further Reading

Becker Y and Darai G (eds) (1992) *Diagnosis of Human Viruses by Polymerase Chain Reaction Technology.* Berlin: Springer.

James K (1990) Immunoserology of infectious diseases. Clin. Microbiol. Rev. 3: 132.

Lennette EH (ed.) (1991) *Laboratory Diagnosis of Viral Infections*, 2nd edn. New York: Dekker.

Persing DH, Smith TF, Tenover FC and White TJ (eds) (1993) *Diagnostic Molecular Microbiology: Principles and Applications.* Rochester, MN: Mayo Foundation.

Wiedbrauk DL and Farkas DH (eds) (1995) *Molecular Methods for Virus Detection.* San Diego: Academic Press.

Isolation and Identification by Culture and Microscopy

Marie L Landry and **GD Hsiung**, Virology Reference Laboratory, VA Connecticut Healthcare System, West Haven, Connecticut, USA; and Department of Laboratory Medicine, Yale University School of Medicine, New Haven, Connecticut, USA

Introduction

Methods commonly used to test for viruses vary greatly from laboratory to laboratory. These variations depend not only on the availability of resources and equipment of each setting, but also on the availability of trained personnel.

In general, the isolation of a viral agent is the most accurate method for diagnosis of a virus infection and is still considered the gold standard, with a few exceptions. Since viruses only replicate in living cells, virus isolation requires a constant supply of cell cultures or laboratory animals, as well as labor-intensive procedures. This has been an impetus for the development of methods for direct detection of viral

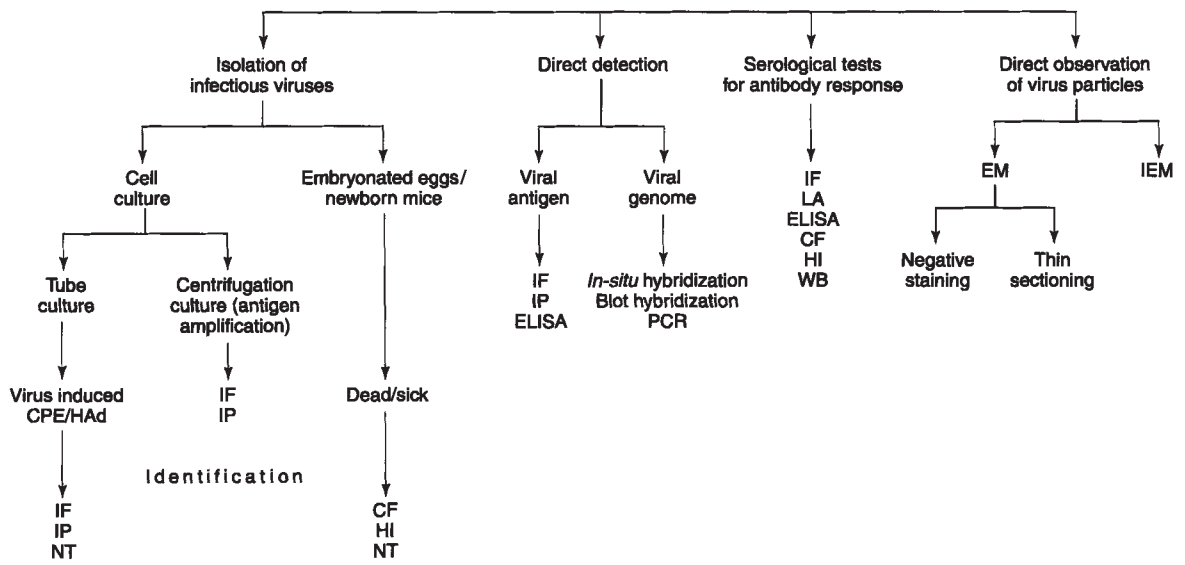


Figure 1 Flow chart of diagnostic tests for viruses. CPE, cytopathic effect; CF, complement fixation test; HAd, hemadsorption test; HI, hemagglutination–inhibition test; EM, electron microscopy; IEM, immunoelectron microscopy; ELISA, enzyme-linked immunosorbent assay; LA, latex agglutination test; IF, immunofluorescent staining; IP, immunoperoxidase staining; NT, neutralization test; PCR, polymerase chain reaction; WB, Western immunoblot.

antigens and/or viral products. In addition, recent advances in the development of molecular biologic techniques have been applied to the detection of viral genomes with a sensitivity that approaches that of culture. The four basic methods are illustrated in the flow chart in **Fig. 1**. This chapter discusses the isolation of animal viruses in cell culture and their identification by electron and light microscopy. Techniques for detecting viral antigens, specific antibodies and viral nucleic acids are discussed in the preceding chapter (*see also* **Diagnostic Techniques: Detection of Viral Antigens, Nucleic Acids and Specific Antibodies**).

Primary Virus Isolation and Identification

Viruses are obligate intracellular parasites. Thus, isolation of a virus requires inoculation of either cell culture, suckling mice, embryonated eggs or other host systems. The host system selected depends on the virus sought. When attempting to isolate virus from a clinical specimen and the identity of the virus is uncertain, the use of multiple systems may be necessary for maximum sensitivity.

Use of cell culture monolayers for virus isolation

Isolation of virus in cell culture monolayers is the most convenient method available and is the sole culture system used in most diagnostic laboratories. A variety of cell cultures in flasks or roller tubes are available from commercial suppliers or can be prepared in the laboratory. Cell cultures are categorized as either primary, passaged or continuous. Primary cells, such as monkey kidney or rabbit kidney, are obtained directly from a tissue or organ and cannot be serially passaged. Embryonic tissues such as human embryonic fibroblasts can be passaged a finite number of times. Continuous cell lines, such as HEp-2 cells, are transformed cells and can be passaged indefinitely. Representative primary cultures and passaged cell lines are essential in a diagnostic laboratory to isolate a broad spectrum of viruses.

Conventional methods

Cytopathic effects (CPE) in tube culture For conventional virus isolation, specimens are inoculated into susceptible cell cultures in roller tubes which are incubated at 35–37°C in either stationary racks or roller drums. For many viruses, rotation of the tubes in a roller drum provides for more rapid onset and easier recognition of cytopathic effects (CPE). The cell cultures are examined serially under the light microscope for the development of viral-induced CPE, which is often characteristic for different virus groups (**Fig. 2**). For example, clusters of swollen cells, diffuse rounding, syncytium formation, or foci of refractile cells may be observed. The characteristics of the cellular changes, the rapidity of both onset and

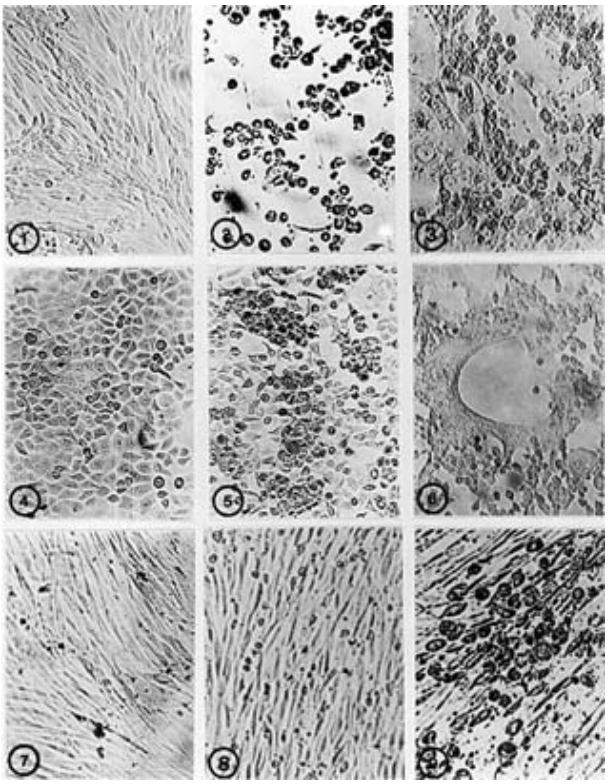


Figure 2 Examples of characteristic cytopathic effect of different viruses. 1, Uninfected rhesus monkey kidney cells (RhMK); 2, poliovirus induced CPE in RhMK; 3, influenza B virus induced CPE in RhMK cells; 4, uninfected human carcinoma cell line (Hep-2); 5, adenovirus induced CPE in Hep-2 cells; 6, respiratory syncytial virus induced CPE in Hep-2 cells; 7, uninfected human diploid fibroblast (HDF); 8, rhinovirus induced CPE in HDF cells; 9, cytomegalovirus induced CPE in HDF cells. (Reprinted with permission from Landry and Hsiung (1992) in Spector S and Lancz GJ (eds) *Clinical Virology Manual*, 2nd edn. Norwalk, CT: Appleton and Lange.)

progression of CPE, and the cell culture exhibiting the changes all lead to a presumptive diagnosis of the causative virus.

Differential cell susceptibility Although a virus may be able to infect and replicate in several cell systems, some cell cultures will be more sensitive than others. When the virus concentration in the inoculum is high, the differences in sensitivity will not be apparent. However, when the concentration is low, less-sensitive cell systems may give false negative results or the onset of CPE may be delayed.

Hemadsorption For some viruses, such as influenza or parainfluenza viruses, cellular changes may not be evident. To detect the presence of these viruses, the hemadsorption test is commonly used. Influenza and parainfluenza viruses express a viral hemagglutinin on the surface of infected cells. By the hemadsorption test, the culture medium is removed and replaced with a 0.5% dilute solution of guinea-pig red blood cells. After an incubation of 20–30 min either at room temperature or 4°C, the monolayer is washed to remove the red cells. If a viral hemagglutinin is present in the cells, the red blood cells will adhere in a specific pattern. If the culture is placed at 37°C, the viral neuraminidase will cleave the hemagglutinin and the red cells can be removed.

Plaque formation Viruses may induce plaque formation when infected cell monolayers are overlaid with a semisolid medium. The virus infection spreads from cell to cell resulting in a localized plaque. After an appropriate incubation period, the overlay medium is removed and the monolayers are fixed and stained with crystal violet. Uninfected cells take up the dye. Areas of cell destruction represent viral plaques and are enumerated as plaque forming units (PFU). Plaque formation is useful to identify a virus, determine the infectivity titer of a virus stock, or perform antiviral susceptibility testing.

Centrifugation culture in shell vials

Detection of CPE in conventional cell cultures may require one day for viruses such as herpes simplex virus (HSV) or 3–4 weeks for other viruses, such as cytomegalovirus (CMV). As antiviral therapy has become available, obtaining a rapid diagnosis has assumed increasing importance. For many years it has been recognized that centrifugation of viruses and chlamydia onto cell monolayers enhances infectivity. In fact, the standard method for isolating chlamydia has been centrifugation culture in shell vials followed by incubation for 24–48 h then staining for inclusion bodies in infected cells. This methodology has been applied to the diagnosis of CMV with significant enhancement of both the speed and sensitivity of virus detection. Samples are inoculated into shell vials containing coverslips with cell monolayers. Following low-speed centrifugation for 30–60 min, shell vial cultures are incubated at 37°C for 24–48 h. Then monolayers are fixed and stained with a fluorescein or peroxidase labeled monoclonal antibody to CMV early antigens, which are expressed in the nuclei of infected cells (**Fig. 3**). Thus viral antigens can be detected in infected cells as early as 16 h postinoculation, whereas days to weeks may be required before viral CPE can be observed by light microscopy. This technique can also be used for the rapid detection of other viruses that replicate in cell culture for which a specific antibody is available. Its greatest benefit is for

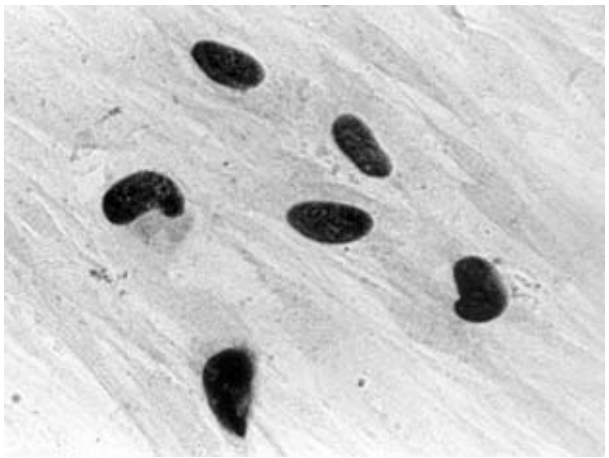


Figure 3 Shell vial centrifugation culture: 24 h after specimen inoculation, CMV immediate early antigen can be detected in infected HDF nuclei (immunoperoxidase staining method).

those viruses with a long replication cycle for which monoclonal antibody to early antigens is available. For maximum sensitivity, conventional cultures should be performed in parallel with centrifugation cultures.

Use of genetically modified cell lines

The use of genetically modified cell lines is an emerging technology with great potential for the diagnostic laboratory. By this method, genetic elements derived from viral, bacterial or cellular sources are stably introduced into a cell. When a particular virus subsequently enters the cell, a specific event in the viral replication cycle triggers the production of an easily measurable enzyme. This approach has been given the acronym enzyme-linked inducible system (ELVIS) and has been shown to be feasible for both DNA and RNA viruses, although different strategies are necessary for enzyme induction. In contrast to CPE, ELVIS can be read by an untrained observer and the earliest stages of infection reliably detected.

However, both the cell line and the reporter gene chosen can have significant effects on the sensitivity and specificity of the system. *Escherichia coli* β-galactosidase is a commonly used reporter gene, but many cell types have high background due to endogenous enzyme activity. Firefly luciferase may offer greater sensitivity since it is absent in mammalian cell lines.

Recently, a baby hamster kidney cell line (BHK ICP6Laz-5) has been genetically modified to contain an HSV-inducible promoter gene linked to an *E. coli* *Lac Z* reporter gene. When these cells are infected with HSV, the β-galactosidase gene is expressed, leading to intracellular accumulation of the enzyme. HSV-infected cells then stain blue in a simple histochemical assay. The ELVIS method for detection of HSV appears to be simple, sensitive and rapid, and is an attractive assay especially for laboratories processing large numbers of specimens. However, ELVIS remains somewhat less sensitive than the most sensitive of conventional cell culture systems when specimens contain only a few infectious HSV particles.

ELVIS culture methodology is amenable to automation with some advantages over standard immunological methods, such as ELISA: it can provide more sensitive results and can detect infectious virus. Lastly, genetically modified cell lines can also facilitate antiviral susceptibility testing.

Use of lymphocyte cultures

Although not available in routine diagnostic laboratories, human lymphocyte cell cultures are used in the research laboratory to isolate several human herpesviruses, Epstein–Barr virus (EBV), human herpes virus type 6 (HHV-6) and type 7 (HHV-7) and retroviruses, human immunodeficiency viruses (HIV) 1 and 2 and human T cell leukemia viruses (HTLV) I and II. Human leukocytes for cocultivation can be obtained from seronegative donors or from cord blood of uninfected newborn infants.

EBV is able to transform uninfected human leukocytes into continuous cell lines and EBV nuclear antigen can be detected in the nuclei of transformed cells. Throat washings are filtered, added to leukocytes and placed on placental fibroblast feeder layers. Transformation by EBV is heralded by a sudden increase in cell numbers, cell clumping and ability to be subcultured indefinitely. Unfortunately transformation usually requires 30–90 days of incubation.

HTLV I and II, HHV-6 and HHV-7 can be isolated by cocultivating samples with cord blood lymphocytes stimulated with phytohemagglutinin or interleukin 2 (IL-2). Identification is by polymerase chain reaction (PCR) or monoclonal antibodies, as well as by reverse transcriptase assay of supernates for HTLV.

Since HIV lyses CD4 lymphocytes, successful isolation necessitates repeated additions of fresh stimulated, uninfected peripheral blood lymphocytes to cultures. Virus growth is detected by periodically assaying supernatant fluid for reverse transcriptase or viral antigen, or by electron microscopy (EM). Continous mature T cell lines from leukemic patients are useful in propagating HIV laboratory strains but

are not as sensitive for primary isolation of wild-type viruses.

Use of embryonated eggs and infant mice in selected instances

Although most diagnostic laboratories confine their virus isolation techniques to monolayer cell culture, certain virus isolates may be missed without the inoculation of embryonated hens' eggs, in particular some influenza type A strains (e.g. H2N2). Suckling mice remain the universal host for group A coxsackieviruses and toga- and flaviviruses, although a number of virus types can be isolated in specialized cell cultures. Mice are also useful for isolation of rabies and are essential for the recovery of hantaviruses which can be slow to adapt to cell cultures.

Embryonated eggs can be inoculated via the amniotic or allantoic cavities or via the chorioallantoic membrane. For the isolation of influenza, the amniotic and allantoic cavities of 10–13-day-old embryos are inoculated; for mumps, 7–8-day-old embryos are used. Inoculated eggs are incubated 2–4 days for influenza and 5–7 days for mumps. At that time, eggs are chilled for 2–4 h, then amniotic and allantoic fluids are harvested separately under sterile conditions. The fluids are then assayed for the presence of viral hemagglutinin by mixing an aliquot of the fluids with a 0.5% suspension of guinea-pig red blood cells. Virus isolates can be passaged by egg inoculation and can be identified by hemagglutination inhibition with specific antisera.

To inoculate the chorioallantoic membrane (CAM) of hens' eggs (9–12 days old), an artificial air sac is first induced. Then an inoculum is dropped onto the membrane through a hole in the egg shell. The opening is sealed and the egg incubated for 2–3 days, at which time the membrane is harvested and placed in a Petri dish containing saline to facilitate counting of virus-induced pocks. Though rarely performed today, pock formation can still be useful for the biologic characterization of poxvirus isolates.

Most group A coxsackieviruses and many toga- and flaviviruses are best isolated in the newborn mouse. For optimal sensitivity, when these viruses are sought, newborn mice, 24–48 h old, should be inoculated intracerebrally and/or intraperitoneally. For coxsackie A viruses, a characteristic hindlimb paralysis develops in the mice. Mice should be checked twice daily for signs of illness, paralysis or death. The mouse brain or skeletal muscle (coxsackie viruses) should then be harvested, a 20% suspension prepared and inoculated into appropriate cell cultures and/or mice for further study and identification.

Virus Identification

When a virus isolate is obtained in monolayer cultures, a presumptive identification is made based on the type of cell culture exhibiting cytopathic effects, the characteristic CPE observed, and the specimen source. A final identification can be made by the use of virus-specific antiserum to detect viral antigens or to inhibit virus-induced effects in infected cultures. The three tests below are commonly used for virus identification, however other tests such as complement fixation (CF), hemadsorption–inhibition and hemagglutination–inhibition can also be used.

Immunofluorescence test

Immunofluorescence (IF) techniques include the direct fluorescent antibody (DFA) procedure and the indirect fluorescent antibody (IFA) procedure. These tests have long been used in the diagnosis of viral diseases. By this technique, specific antibody is tagged with a fluorescent dye, allowed to react with the viral antigen in infected cells and after an incubation period, the antigen–antibody complex is visualized using a microscope with an ultraviolet light source. With DFA, the virus specific antibody is conjugated with a fluorescent dye, whereas with IFA specific antibody is not labeled. Rather, a second antibody, directed against the animal species in which the virus-specific antibody was prepared, is fluorescein-labeled. The direct method is quicker and simpler, whereas the indirect method requires only one conjugate for the detection of many antigen–antibody reactions, provided all antisera are made in a single animal species.

In the past, only polyclonal antiserum made from animals was available and variation in quality was problematic. At present, a variety of high quality monoclonal antibodies are available commercially and as a result, the use of IF techniques to identify viruses has become routine in many laboratories. For herpes simplex, influenza and parainfluenza viruses, type-specific monoclonal antibodies are also available. Once CPE or hemadsorption is detected and involves 50% or more of the infected cell monolayer, the cells are removed, centrifuged, resuspended in a small volume of buffer and dropped onto welled-slides. The cells are fixed in acetone, then stained with specific antibody. The fluorescent pattern seen in infected cells will vary with the virus, the cell type and the antibody used. Appropriate positive and negative controls should be tested with each assay.

Immunoperoxidase test

Immunoperoxidase (IP) techniques follow the same principles as IF, however, the conjugate is an enzyme, such as horseradish peroxidase. In the direct method,

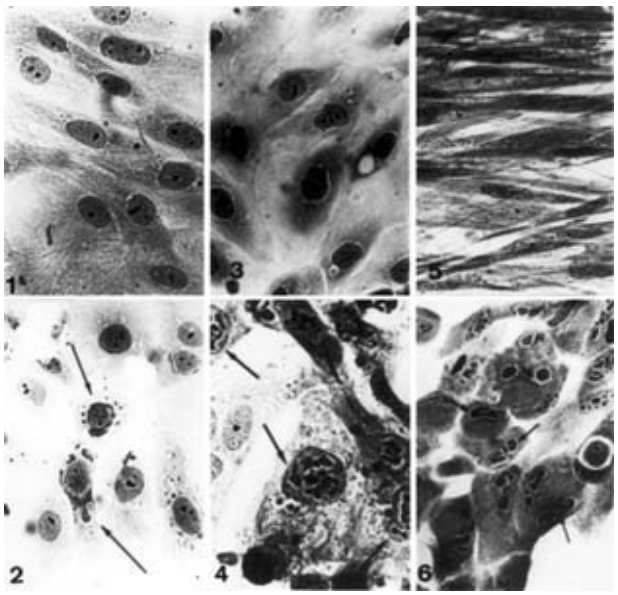


Figure 4 Examples of virus-induced inclusions (arrows) by different viruses (H&E stained preparations) 40 × objectives. 1, Uninfected rhesus monkey kidney cells; 2, intracytoplasmic inclusions induced by reovirus type 1; 3, uninfected human embryonic kidney cells; 4, intranuclear inclusions induced by adenovirus type 2; 5, uninfected human diploid fibroblasts; 6, intranuclear inclusions induced by CMV. Arrows indicate inclusions.

the virus specific antibody is enzyme-labeled, whereas in the indirect method, an anti-animal species antibody is labeled. The presence of the enzyme conjugate bound to the virus-infected cells is detected by adding a substrate, such as diaminobenzidine or aminoethylcarbazole, then oxidizing it in the presence of hydrogen peroxide which results in a reddish-brown color. Although IP staining requires an additional step (the addition of the substrate), it has several advantages over IF. The reaction is read using a light microscope or even the naked eye. The slides are permanent, the reagents are more stable, there are fewer nonspecific reactions and IP has been more successful than IF on processed tissue sections.

Neutralization test

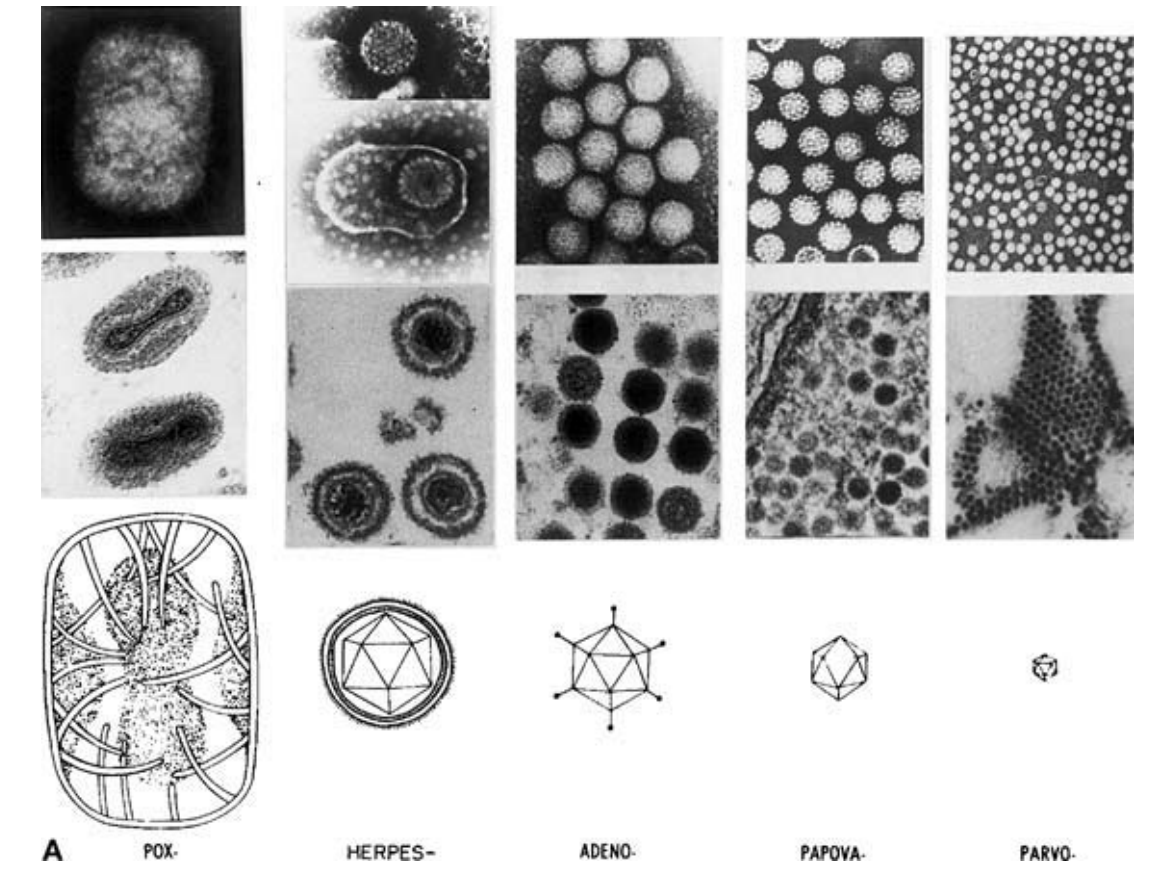
The neutralization test (NT) is arguably the most sensitive and accurate method available to identify a virus isolate. However, it is also very time consuming and labor-intensive. By this technique, virus infectivity is neutralized by the incubation of the virus isolate with specific antiserum, followed by the inoculation of the virus–serum mixture into sensitive cell cultures. The NT is used for the type-specific identification of enteroviruses and rhinoviruses. Unfortunately, these virus groups have no common antigen against which to prepare a monoclonal antibody. The NT is also used for type-specific identification of adenoviruses. For adenovirus-group identification, a monoclonal antibody to the common hexon antigen is available for use in IF and IP assays.

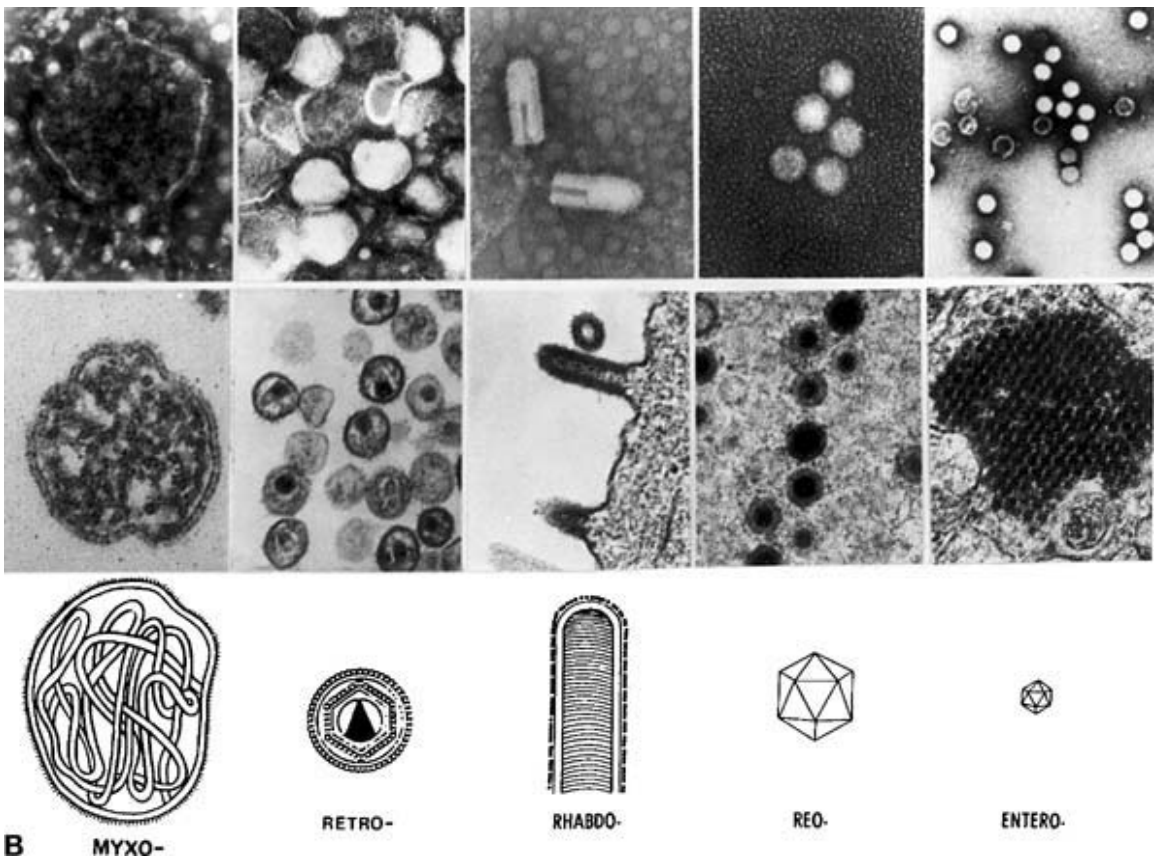
In order to perform the NT, a virus stock must be prepared and the infectivity titer determined. Then a challenge dose of approximately 100 PFU or tissue culture infectious dose ($TCID_{50}$) is prepared. An aliquot of the virus at the selected titer is allowed to react with 20 units of specific antiserum in a test tube for 1–2 h. The virus–antiserum mixture is then inoculated into susceptible cell cultures and observed for virus-induced CPE or plaque formation. Virus mixed with sterile buffer is inoculated as a positive control. Complete inhibition of virus-induced changes by 20 units of antiserum is considered a positive neutralization test.

Direct Observation of Virus-Induced Changes by Light Microscopy

Microscopic examination of fixed and stained cells is a technique that can be used as an additional aid in the diagnostic laboratory. When cultured cells are stained and mounted, these preparations are a distinct aid in recognizing and identifying viruses and can be filed for reference and become part of the permanent record. The most commonly used staining technique is with hematoxylin and eosin (H&E), a technique which every pathology laboratory uses routinely. Zenker's fixing solution is particularly useful for the demonstration of virus-induced intranuclear inclusions and preserves sharp details of virus-infected cells (**Fig. 4**). For determination of DNA viruses by the Feulgen reaction or RNA viruses by the acridine orange stain, infected cells are fixed in freshly prepared Carnoy's fixing fluid followed by Schiff's reagent for DNA viruses or acridine orange for RNA viruses. Good reagents and very meticulous procedures are required.

Figure 5 Relative size and shape of representative (**A**) DNA- and (**B**) RNA-containing viruses as revealed in negatively stained preparations (top row), in thin-sectioned cells (middle row), and as compared with schematic diagrams (bottom row). Magnification ×50 000. (Reprinted with permission from *Hsiung's Diagnostic Virology* (1994), 4th edn, pp. 106, 107. New Haven: Yale University Press.)


A
POX-
HERPES-
ADENO-
PAPOVA-
PARVO-


B
MYXO-
RETRO-
RHABDO-
REO-
ENTERO-

Direct Observation of Virus Particles by Electron Microscopy

The recognition of virus particles in clinical specimens by means of electron microscopy (EM) has been made for years. However, specimen preparation is a time-consuming and tedious task. In recent years, improved technology has simplified many of the procedures and reduced the time needed for processing specimens.

Essentially, there are two basic techniques available for routine use: negative staining of virus particles (**Fig. 5**, top row) and thin sectioning of virus infected cells (**Fig. 5**, middle row). Negative staining of clinical materials provides the simplest and most rapid method for detection and recognition of virus particles. Thin sectioning, although less rapid, provides a more reliable diagnosis, since the examination of virus infected cells reveals the site of viral replication. In the following sections, the two basic techniques for preparing samples for electron microscopy are briefly described.

Negative staining

The negative staining technique for visualization of virus particles was introduced by Horne and Wildy in 1959. They described how the use of heavy salts may enhance the contrasts in virus particle images. The procedure is simple and needs no special equipment. Generally, viruses in aqueous suspension are mixed with an equal volume of heavy metal salt solution (2–4%), such as phosphotungstic acid (PTA) or 0.5% uranyl acetate. The mixture is then placed on a Formvar or collodion-coated electron microscope grid. Excess fluid is blotted away and the specimen is then air dried. The grid containing the mixture is then examined under an electron microscope. The virus particles are surrounded by heavy metal atoms and are revealed against a dark background, the so-called negative stain because the electron beam can pass through the low electron density of the virus but not through the metallic background.

However, most clinical specimens contain small numbers of virus particles; therefore, additional procedures have been developed for concentration of virus particles and are commonly used in diagnostic laboratories.

In order to facilitate recognition of the relatively small number of virus particles in the specimen, immunoelectron microscopy (IEM) has been used for enhancing concentration of virus particles and for rapid serodiagnosis of virus infection. IEM has been widely used for recognizing rotaviruses and Norwalk agent in stool samples of patients with gastroenteritis and to detect hepatitis B virus antigen in patient's serum.

Thin sectioning

The thin-sectioning method is used to prepare fixed and embedded tissues or cells for electron microscopy. It is, however, more time-consuming and requires personnel with special skills. Examining well-preserved virus-infected cells offers the advantage of direct observation of virus–cell interaction, which in turn reveals the site of virus replication and maturation in the host cell, thus aiding identification of unknown viruses. The conventional procedures consist of primary fixation (with glutaraldehyde or paraformaldehyde), postfixation (osmium tetroxide), *en bloc* staining with uranyl acetate (optional), dehydration (ethanol or acetone), infiltration (propylene oxide or other solvents), embedding (epoxy resin or other embedding media), thin-sectioning and staining. The choice of reagents for fixing and embedding varies from laboratory to laboratory and usually depends on experience.

The various virus morphologies as seen in negative stain or in thin-sectioning with their respective schematic diagrams are illustrated in **Fig. 5**.

Future Perspectives

Over the past decade, significant changes have occurred in diagnostic virology. A plethora of high quality reagents, including monoclonal antibodies, antigen and antibody detection kits, and nucleic acid probes and amplification kits, have become commercially available. The demand for rapid and accurate viral diagnostic testing continues to escalate due to the increasing recognition of serious viral infections and the need to intervene with antiviral therapy.

A variety of test methods are now available, each with advantages and disadvantages. Rapid antigen, nucleic acid and antibody detection assays have provided rapid results and had a favorable impact on patient care. However, only the suspected viral agent is tested for in these assays, and they tend to be less sensitive than isolation in cell culture. With the availability of genetically modified cell lines, new avenues for virus detection are available. Virus isolation is more open minded, since unsuspected viruses, as well as multiple viruses, may be detected. At present, a number of viruses are still not cultivable by methods used in a routine diagnostic laboratory. Thus, for optimal results, a combination of rapid and conventional methods is often required.

See also: **Diagnostic techniques: Detection of viral antigens, nucleic acids and specific antibodies.**

Further Reading

Evans AS and Kaslow RA (eds) (1997) *Viral Infections of Humans*, 4th edn. New York: Plenum Medical Book Company.

Fields BN, Knipe DM and Howley PM (eds) (1996) *Fields Virology*, 3rd edn, Philadelphia: Lippincott–Raven.

Hsiung GD, Fong CKY and Landry ML (1994) *Hsiung's Diagnostic Virology*, 4th edn. New Haven: Yale University Press.

Murray PR, Baron EJ, Pfaller MA, Tenover FC and Yolken RH (eds) (1995) *Manual of Clinical Microbiology*, 6th edn. Washington, DC: American Society of Microbiology.

Olivo PD. (1996) Transgenic cell lines for detection of animal viruses. *Clin. Microbiol. Rev.* 9: 321.

Schmidt NJ and Emmons RW (eds) (1989) *Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections*, 6th edn. Washington, DC: American Public Health Association.

DIANTHOVIRUSES (*TOMBUSVIRIDAE*)

Steven A Lommel, Department of Plant Pathology, North Carolina State University, Raleigh, North Carolina, USA

History

Carnation ring spot virus (CRSV) was first isolated and described as a disease of carnations in 1951. Red clover necrotic mosaic virus (RCNMV) was first described in red clover and alfalfa in 1967. Sweet clover necrotic mosaic virus (SCNMV) was first defined as a new *Dianthovirus* in 1986. The International Committee on the Taxonomy of Viruses formed the genus *Dianthovirus* for *Dianthus* the generic name for carnation (*Dianthus caryophyllus*) the major host for the type member of the species, CRSV.

Geographic and Seasonal Distribution

Dianthoviruses, with the possible exception of furcraea nectrotic streak virus (FNSV), which appears to be tropical in range, are widespread throughout the temperate regions of the world. CRSV has been identified wherever carnations are grown on a large scale. The worldwide distribution of CRSV is most likely due to the practice of distributing vegetative propagules within the floral industry. Quite separately, CRSV is also established in stone fruit orchards and grape-growing regions of Eastern and Central Europe. RCNMV has been identified in temperate forage legume growing regions of Canada, Australia, New Zealand, England, and Northern and Central Europe. SCNMV has been found only in Alberta, Canada.

Taxonomy and Classification

The *Dianthovirus* genus in the *Tombusviridae* family is defined by those plant-infecting viruses which have 32–35 nm icosahedral virions composed of 180 copies of a 37 kDa capsid protein and two nonhomologous positive polarity single-stranded RNA species of 4 and 1.5 kb. The key taxonomic feature which distinguishes members of this genus from other genera within the *Tombusviridae* is the segmented nature of their single-stranded RNA genomes. Based on significant viral replicase and capsid protein amino acid sequence similarity, the *Dianthovirus* genus has been placed within the recently established family *Tombusviridae*. The *Dianthovirus* genus is currently composed of three definitive members; carnation ring spot virus (CRSV), the type member; red clover necrotic mosaic virus (RCNMV); and sweet clover necrotic mosaic virus (SCNMV); and one potential member, furcraea necrotic streak virus (FNSV). For each definitive member, several biological and serological strains have been identified.

Properties of Virion

Electron microscopic examination of dianthoviruses show that they are 32–35 nm icosahedral virus particles with a $T=3$ symmetry. The particles have distinctively granular surfaces similar to members of the *Tombusvirus* and *Carmovirus* genera. The virion is composed of 180 copies of the 37 kDa capsid protein. This forms the protein shell that packages the 4 kb and 1.5 kb genomic single stranded RNAs. X-ray crystallographic studies have not been performed on any dianthoviruses. However, several structural features can be inferred as a result of amino acid sequence similarity between RCNMV and both turnip crinkle carmovirus and tomato bushy stunt tombusvirus (TBSV) (**Table 1**), whose structures have

Further Reading

Evans AS and Kaslow RA (eds) (1997) *Viral Infections of Humans*, 4th edn. New York: Plenum Medical Book Company.

Fields BN, Knipe DM and Howley PM (eds) (1996) *Fields Virology*, 3rd edn, Philadelphia: Lippincott–Raven.

Hsiung GD, Fong CKY and Landry ML (1994) *Hsiung's Diagnostic Virology*, 4th edn. New Haven: Yale University Press.

Murray PR, Baron EJ, Pfaller MA, Tenover FC and Yolken RH (eds) (1995) *Manual of Clinical Microbiology*, 6th edn. Washington, DC: American Society of Microbiology.

Olivo PD. (1996) Transgenic cell lines for detection of animal viruses. *Clin. Microbiol. Rev.* 9: 321.

Schmidt NJ and Emmons RW (eds) (1989) *Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections*, 6th edn. Washington, DC: American Public Health Association.

DIANTHOVIRUSES (*TOMBUSVIRIDAE*)

Steven A Lommel, Department of Plant Pathology, North Carolina State University, Raleigh, North Carolina, USA

History

Carnation ring spot virus (CRSV) was first isolated and described as a disease of carnations in 1951. Red clover necrotic mosaic virus (RCNMV) was first described in red clover and alfalfa in 1967. Sweet clover necrotic mosaic virus (SCNMV) was first defined as a new *Dianthovirus* in 1986. The International Committee on the Taxonomy of Viruses formed the genus *Dianthovirus* for *Dianthus* the generic name for carnation (*Dianthus caryophyllus*) the major host for the type member of the species, CRSV.

Geographic and Seasonal Distribution

Dianthoviruses, with the possible exception of furcraea nectrotic streak virus (FNSV), which appears to be tropical in range, are widespread throughout the temperate regions of the world. CRSV has been identified wherever carnations are grown on a large scale. The worldwide distribution of CRSV is most likely due to the practice of distributing vegetative propagules within the floral industry. Quite separately, CRSV is also established in stone fruit orchards and grape-growing regions of Eastern and Central Europe. RCNMV has been identified in temperate forage legume growing regions of Canada, Australia, New Zealand, England, and Northern and Central Europe. SCNMV has been found only in Alberta, Canada.

Taxonomy and Classification

The *Dianthovirus* genus in the *Tombusviridae* family is defined by those plant-infecting viruses which have 32–35 nm icosahedral virions composed of 180 copies of a 37 kDa capsid protein and two nonhomologous positive polarity single-stranded RNA species of 4 and 1.5 kb. The key taxonomic feature which distinguishes members of this genus from other genera within the *Tombusviridae* is the segmented nature of their single-stranded RNA genomes. Based on significant viral replicase and capsid protein amino acid sequence similarity, the *Dianthovirus* genus has been placed within the recently established family *Tombusviridae*. The *Dianthovirus* genus is currently composed of three definitive members; carnation ring spot virus (CRSV), the type member; red clover necrotic mosaic virus (RCNMV); and sweet clover necrotic mosaic virus (SCNMV); and one potential member, furcraea necrotic streak virus (FNSV). For each definitive member, several biological and serological strains have been identified.

Properties of Virion

Electron microscopic examination of dianthoviruses show that they are 32–35 nm icosahedral virus particles with a $T=3$ symmetry. The particles have distinctively granular surfaces similar to members of the *Tombusvirus* and *Carmovirus* genera. The virion is composed of 180 copies of the 37 kDa capsid protein. This forms the protein shell that packages the 4 kb and 1.5 kb genomic single stranded RNAs. X-ray crystallographic studies have not been performed on any dianthoviruses. However, several structural features can be inferred as a result of amino acid sequence similarity between RCNMV and both turnip crinkle carmovirus and tomato bushy stunt tombusvirus (TBSV) (**Table 1**), whose structures have

Table 1 Percentage amino acid sequence identity among *Dianthovirus* species and TBSV capsid proteins

	CRSV	*RCNMV*	*SCNMV*	*TBSV*
CRSV	—	46	54	34
RCNMV	46	—	84	36
SCNMV	54	84	—	35
TBSV	34	36	35	—

been solved at 3.2 Å resolution. The RCNMV capsid protein is organized into four domains. The N-terminal RNA binding (R) domain, which is basic in nature and extends into the interior of the virion, is truncated relative to the R domains of the carmo- and tombusviruses. The R domain is connected to the shell domain (S) by a short intervening arm (a) (**Fig. 1**). The 160 residue S domain is highly conserved among members of the diantho-, carmo- and tombusviruses, suggesting that the dianthovirus virion shell is composed of the conserved 'jellyroll β barrel' structure found in all spherical viruses of eukaryotes. In addition, clusters of conserved negatively charged residues, including an invariant aspartic acid residue within the S domain, constitute a Ca^{2+} binding site which may be associated with structural integrity and virion assembly. The internal S domain is linked to the C-terminal protruding domain (P), which projects outward from the surface of the virion. The P domain most likely gives dianthoviruses their distinctive granular appearance in the electron microscope. The putative RCNMV P domain possesses only moderate amino acid sequence conservation with the P domains of the tombus- and carmoviruses.

Several different strains of CRSV are distinguished by distinctive aggregation properties. CRSV-A assembles into clusters of 12 particles assuming an

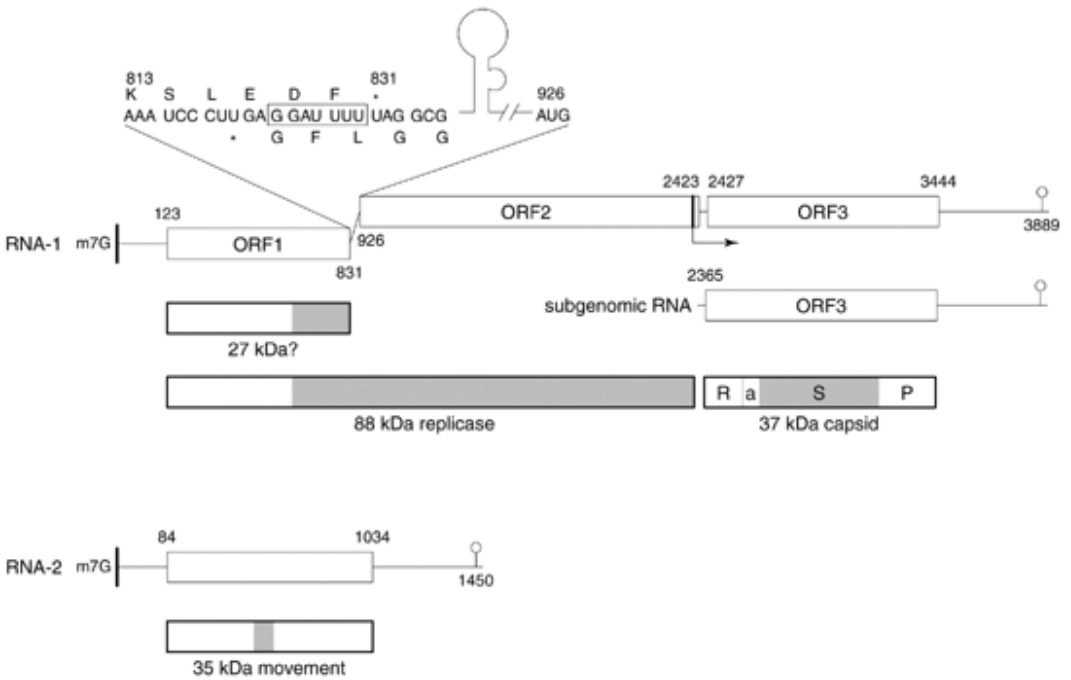


Figure 1 Schematic of the genome organization of RCNMV, illustration of sequences predicted to be involved in replication and gene expression, and identification of conserved protein domains. RCNMV RNA-1 and RNA-2 are depicted as solid lines with ORFs identified as open rectangles. Numbers above or below the borders of the ORFs identify initiation and termination codons defining the ORF. ORFs are labeled with respect to the size of the polypeptide they encode. Solid vertical bars at the 5′ end of RNA-1, RNA-2 and near the C-terminus of the p57 ORF identify a 13 nucleotide conserved sequence. Right angle arrow identifies the location of the beginning of the capsid protein subgenomic RNA. The stem–loop at the 3′ end of both viral RNAs symbolizes the 3′ terminal 27 homologous nucleotides capable of forming a stable stem–loop structure. Above RNA-1 is an expanded region of sequence between RNA-1 nucleotides 813 and 926. The heptanucleotide sequence facilitating ribosomal frameshifting immediately preceding the p27 ORF amber termination codon is boxed. Following this sequence is a schematic of a predicted stable stem–loop structure that may be involved in ribosomal frameshift expression of the 88 kDa fusion polypeptide. Shaded rectangles below the RNAs represent viral encoded polypeptides identified *in vivo*. Shaded areas within RNA-1-encoded polypeptides identify domains with significant amino acid sequence similarity to proteins from the carmo- and tombusviruses. The shaded region in the RNA-2 encoded 35 kDa polypeptide identifies a domain conserved between the *Bromoviridae* movement proteins. The R (RNA binding), a (arm), S (shell) and P (protruding) domains of the RCNMV capsid protein are indicated. Polypeptide functions are stated below the protein, if known.

icosahedral symmetry. This 12 particle unit can form higher order aggregates. CRSV-N and -R strains form alternative two-dimensional net-like aggregates. These aggregation properties appear to be unique to CRSV and have not been observed for the other dianthoviruses.

Properties of Genome

The structure of the dianthovirus genome is based on the complete nucleotide sequence of CRSV, RCNMV and SCNMV. The dianthovirus genome is composed of two, essentially nonhomologous, positive polarity, single-stranded RNAs of approximately 4 kb (RNA-1) and 1.5 kb (RNA-2). The genomic RNAs are infectious as naked nucleic acids. Each RCNMV genomic RNA is capped at the 5′ terminus with m^7GpppA and is not 3′-terminally polyadenylated. Only the 5′ terminal six nucleotides and 3′ terminal 27 nucleotides are identical between RNA-1 and RNA-2. The identical 3′ 27 nucleotides are capable of forming a stem–loop structure which may be involved in viral replicase recognition (**Fig. 1**). RNA-1 contains three open reading frames (ORFs). The first ORF (p27) initiates at the first methionine codon, 123 nucleotides from the 5′ terminus. The p27 ORF is capable of encoding a 27 kDa polypeptide. An internal ORF (p57) could encode a 57 kDa polypeptide. The most 3′ terminal ORF encodes the 37 kDa capsid protein, followed by a 445 nucleotide 3′ terminal noncoding sequence. RNA-2 is monocistronic, containing a single ORF encoding a 35 kDa polypeptide. RNA-1 directs the synthesis of 27, 57, 88 and 37 kDa polypeptides *in vitro*. Only the 88 kDa polypeptide, which is made in small quantities relative to other products, does not correlate in size to one of the three RNA-1 ORFs. The p88 polypeptide is a fusion protein which is serologically related to both the p27 and p57 polypeptides. Immediately preceding the p27 ORF termination codon, a seven-nucleotide element has been identified which facilitates ribosomal frameshifting in accordance with the simultaneous slippage model (**Fig. 1**). Following the frameshift heptanucleotide, a stable stem–loop structure is predicted. These structural features have been shown to facilitate ribosomal frameshift expression of *gag-pol* fusion proteins in retrovirus systems. Mutation of the frameshift element affects the expression of p88 synthesis *in vitro*. Together, this information suggests that p88 is a fusion protein resulting from translational readthrough of the p27 ORF into the p57 ORF by a ribosomal frameshifting mechanism. The 57 kDa polypeptide has not been observed *in vivo* and the independent production of this protein may well be an *in vitro* translation artifact.

Physical Properties

The icosahedral *Dianthovirus* virions sediment as a single species with a sedimentation coefficient ($s_{20,w}$) of 132 S and have an equilibrium density of 1.366 g cm^{-3} in CsCl. The molecular weight of a virion, assuming that each particle possesses one copy of RNA-1 and RNA-2 is 8.2 MDa. It is not known whether a virion contains one copy of both genomic segments or whether a virion contains one copy of RNA-1 and another contains three copies of RNA-2. Dianthovirus particles are stabilized by RNA–protein interactions and pH-dependent protein–protein interactions. Dianthoviruses are generally quite stable with RCNMV being more stable than CRSV. Particles slowly swell when pH is increased from 5.0 to 7.5. EDTA also induces particle swelling whereas Mg^{2+} and Ca^{2+} ions prevent particle swelling. CRSV particles have an isoelectric point of pH 5.1. RCNMV strains A, B and C have isoelectric points of pH 5.0, 4.8 and 4.6, respectively.

Replication

Little is known regarding the specifics of dianthovirus replication. Cytological studies suggest that replication occurs in the cytoplasm of infected cells, although mature virions have been reported in the nucleus. Dianthoviruses replicate by producing a negative sense complementary copy of the viral RNA. The viral replicase most likely recognizes the 3′ terminal stem–loop structure on the viral sense RNA-1 and -2, enabling it to initiate synthesis of the full length complementary strand. After completion of complementary strand synthesis, the viral replicase then recognizes the conserved 3′ termini as an initiation site for the production of progeny genomic RNA-1 and -2. This replication strategy dictates that prior to replication, viral replicase must be expressed from the original infecting genomic RNA-1. Consistent with this assumption, the putative replicase ORFs are located at the 5′ terminus of RNA-1 making them easily accessible to translation. A template-bound RNA replicase has been isolated from RCNMV-infected tissue that upon digestion with micrococcal nuclease became template-dependent. The replicase complex was composed of the RCNMV 27 kDa and 88 kDa polypeptides in addition to several proteins thought to be of host origin. The RNA-1 3′ terminal capsid protein ORF is expressed *in vivo* from a 1.4 kb subgenomic RNA. Presumably the viral replicase recognizes an internal sequence con-

taining a 13 nucleotide element upstream of the capsid protein ORF initiation codon. This sequence element is identical to the 3′ terminus of the full length complementary strand. Once bound, the replicase synthesizes a positive sense copy of the capsid protein subgenomic RNA.

Host Range and Virus Propagation

Dianthoviruses have moderately broad natural and experimental host ranges. CRSV was originally isolated from commercial carnations, but has since been found to infect a wide range of orchard trees, including plum, pear, apple and sour cherry, and also grapevines. CRSV has also been identified in several weed species growing within infected fruit orchards. RCNMV has been isolated from naturally infected alfalfa and red, sweet and white clovers. SCNMV has been identified in sweet clover and alfalfa, as well as in various leguminous weeds. The experimental host range of the dianthoviruses is much broader than that found in nature. The dianthoviruses are easily mechanically transmissible to a wide range of herbaceous species. These viruses can systemically infect a number of members of the *Solanaceae, Leguminosae, Cucurbitaceae* and the *Compositae*. Dianthoviruses infect an even larger number of plants locally (nonsystemically).

CRSV infection in carnations results in diagnostic ring spots, mottling and leaf and flower distortions. The disease symptoms are enhanced when carnations are co-infected with carnation mottle carmovirus. On experimental systemic hosts, CRSV causes concentric ring-spots with necrotic centers on the inoculated leaves and mosaics, necrotic flecks and often veinal necrosis on the systemically infected leaves. RCNMV and SCNMV infections result in necrotic mosaics, and necrotic and chlorotic spots on the inoculated and systemic leaves of naturally infected clover species. Systemically infected leaves of *Nicotiana* and legume species exhibit necrotic mosaics which result in leaf deformation and plant stunting. In general, dianthovirus infections do not kill the host plants, however, necrosis and symptom severity can become quite severe at sustained temperatures between 15 and 20°C versus temperatures above 20°C. In nature, symptoms generally disappear and plants recover when the average mean temperature rises above 20°C for an extended period of time.

Genetics

RCNMV RNA-1 can replicate in plant protoplasts in the absence of RNA-2. This fact allows for the assignment of replication and encapsulation functions to RNA-1 and cell-to-cell movement function to RNA-2. The RNA-1 encoded 88 kDa polypeptide contains the conserved glycine-aspartate-aspartate (GDD) motif present in all RNA-dependent RNA polymerases. In addition, extensive amino acid sequence similarity exists between the RCNMV 27 kDa and 88 kDa polypeptides and the replicases encoded by all of the species within the *Tombusviridae* (**Table 2**). Deletion mutagenesis has shown that RNA-1 is capable of replication in the absence of both the capsid protein gene and RNA-2. From this information it is concluded that the RCNMV RNA-1-encoded 27 and 88 kDa polypeptides are required and sufficient for viral replication. Interestingly, unlike many viral replicase proteins, those encoded by the dianthoviruses and other viruses in the *Tombusviridae* do not contain an identifiable helicase motif.

Table 2 Percentage amino acid sequence identity among *Dianthovirus* species and TBSV replicase in both the pre- and post-read through domains

	CRSV	*RCNMV*	*SCNMV*	*TBSV*
CRSV	—	65/81	62/76	19/45
RCNMV	65/81	—	91/93	19/45
SCNMV	62/76	91/93	—	18/46
TBSV	19/45	19/45	18/46	—

The 37 kDa polypeptide encoded by the RCNMV RNA-1 3′ proximal ORF is the viral capsid protein. Mutagenesis of this ORF revealed that C-terminal amino acid sequence deletions completely destabilize the protein, such that neither virion formation nor truncated forms of the capsid protein can be detected *in vivo*. These same C-terminal, as well as other internal capsid protein deletions prevent rapid systemic infection from occurring in all hosts, with the exception of *Nicotiana benthamiana* at low temperatures. However, as many as 16 amino acids can be deleted from the N-terminus of the RCNMV capsid protein with no effect on polypeptide stability, virion formation, or ability to rapidly systemically infect host plants.

RCNMV infections, in the absence of RNA-2 or with deletion mutations within the RNA-2 p35 ORF, are restricted to the initially inoculated cell and are incapable of moving to adjacent cells. Consequently, it was concluded that the 35 kDa polypeptide is the viral cell-to-cell movement protein which facilitates movement of the virus from infected to neighboring healthy cells through the plasmodesmata. The RCNMV capsid protein is not necessary for cell-to-cell movement, but is required for rapid systemic

Table 3 Percentage amino acid sequence identity among *Dianthovirus* species movement proteins

	CRSV	*RCNMV-Aus*	*RCNMV-TpM*	*SCNMV-38*	*SCNMV-59*
CRSV	—	64	60	63	62
RCNMV-Aus	64	—	78	79	77
RCNMV-TpM-34	60	78	—	94	93
SCNMV-38	63	79	94	—	92
SCNMV-59	62	77	93	92	—

infection through the vascular tissue. The RCNMV and CRSV movement proteins contain movement protein motifs conserved among species within the RNA plant virus *Bromoviridae* family (**Fig. 1**)(**Table 3**).

Pseudorecombination studies between various strains, as well as distinct members within the *Dianthovirus* genus, further support the assignment of cell-to-cell movement function to RNA-2. In addition, RNA-2 controls lesion morphology and the ability to systemically invade cowpea. RNA-1 contains host range specificity, serological specificity, and symptom expression determinants.

Serologic Relationships and Variability

The dianthoviruses, like most spherical RNA plant viruses, are good immunogens. Studies using polyclonal antisera have identified three serologically distinct strains of CRSV (A, N and R), four strains of RCNMV (A, B, C and D), and two SCNMV strains (38 and 59). Clover primary leaf necrosis virus was once considered to be a distinct dianthovirus, but upon immunological analysis, has now been determined to be a previously recognized RCNMV serotype. The various SCNMV and RCNMV serotypes weakly crossreact with one another using polyclonal antibodies. In addition, RCNMV polyclonal antibodies react with FNSV virions. No cross-reactivity has been observed between CRSV and RCNMV or SCNMV with polyclonal antibodies. However, a single monoclonal antibody to SCNMV does react with CRSV and RCNMV. This monoclonal antibody most likely recognizes a highly conserved epitope in the capsid protein shell domain.

Epidemiology

CRSV is an established and persistent disease problem in commercial carnation facilities. The virus has diminished as a major problem in recent years due to vigilant and effective control measures. Infected nuclear block plants, from which production cuttings are derived, serve as sources of virus inoculum in commercial glasshouses. Rouging, selection, indexing and meristem tip culture are all effectively employed to insure that these stock plants are free of all viruses, including CRSV. CRSV in carnations spreads by careless propagation and leaf and root contact. In orchard crops, it has been observed that nematode infestation increases the incidence of CRSV. Cultural practices and chemicals which control nematode populations control the spread of CRSV in orchard crops. Little information exists regarding the epidemiology and control of RCNMV and SCNMV.

Transmission and Tissue Tropism

Dianthoviruses are not transmitted through seed, nor by insects or soil-inhabiting fungi. However, confusion exists as to whether dianthoviruses can be transmitted from plant to plant by nematodes. Early reports suggested that CRSV could be transmitted by several species of nematodes. These data tended to confirm observations that CRSV infections were more widely spread in orchards and vineyards when the soil was infested with known virus-vectoring nematodes. However, more recent reports suggest that CRSV, RCNMV and SCNMV particles are released directly from infected roots into the soil. Plant-to-plant transmission can then occur passively through the soil in the absence of a biological vector. Presumably, infection occurs through microscopic sized root wounds. Nematode and soil fungus colonization of roots tends to increase the possibility of soil transmission of dianthoviruses by generating virus entry sites, but transmission is not absolutely dependent on this. Thus, dianthoviruses require no biological vector for soil transmission. Dianthoviruses are readily mechanically transmissible. In nature, dianthoviruses are transmitted from plant-to-plant by physical contact or by contaminated soil. In addition, CRSV spread in carnations is most likely due to vegetative propagation.

RCNMV infects most cell and tissue types in systemic hosts. RCNMV also appears to infect most,

if not all tissues of the roots of systemic hosts. In the systemic host *N. benthamiana* the virus titer in root tissue is twice that of foliar tissues. In hosts of RCNMV that are capable or supporting only a localized infection (*Nicotiana tabacum,* for example), the virus is limited to the epidermal, mesophyll, bundle sheath and phloem parenchyma cells. The infection is incapable of infecting either the companion cells or sieve element.

Pathogenicity

CRSV is an economically significant pathogen of commercial carnations. Extensive indexing and eradication programs are employed to eliminate CRSV from commercial carnation production facilities. Carnations infected with CRSV alone, or in combination with several other viruses, yield low quality unmarketable flowers. In Eastern and Central Europe, CRSV infects a wide range of orchard and vine crops. However, the role of CRSV in economically important diseases of these crops has not been fully established. Diseased orchard trees in addition to having low titers of CRSV are always infected with one or more other viruses. Typically, RCNMV and SCNMV infections of forage legumes result in rather mild diseases which have limited impact on yield. However, infection by either RCNMV or SCNMV can result in economic losses when temperatures remain unusually cool for an extended period of time.

Pathology and Histopathology

Dianthovirus-infected cells contain large amorphous inclusions which can be readily observed with a light microscope after staining. Electron microscopic studies show that these inclusions are composed of large aggregates of virus particles within the cytoplasm. Inclusions form as intracellular virus titer increases and become readily observable when the virus titer reaches a maximum, approximately one week after inoculation. CRSV infections in cowpea result in crystalline arrays composed of virions in the cytoplasm. The nuclei also contain aggregates of virus particles, in addition to tubular inclusions, which frequently have virus particles apparent on the surface. In carnation, spherical inclusion bodies are also observed. Inflated and electron transparent mitochondria, clusters of proliferated endoplasmic reticulum with dilated cisternae are apparent in cells with mature infections. CRSV particles are commonly observed scattered throughout the cytoplasm of mesophyll cells, with necrotic cells having massive aggregates of virions. RCNMV induces pronounced chloroplast vasiculation. FNSV particles are detected in the cytoplasm of both leaf and root cells and are sometimes associated with electron-dense tubular inclusions.

Prevention and Control

CRSV is the most pathologically relevant species within the *Dianthovirus* genus. The most effective control is sanitation during plant propagation. In vegetatively propagated hosts such as carnation and fruit trees, eradication of CRSV in plant shoots or meristems by chemo- and thermotherapy is routinely accomplished to produce certified virus free nuclear stocks which are then commercially propagated under a strict sanitation regime. Production from virus-free nuclear stocks coupled with constant monitoring for CRSV infection by ELISA has ensured the production of CRSV-free commercial carnations.

Control of RCNMV is more problematic. RCNMV is not seed transmitted and legume seedlings probably are infected by plant-to-plant contact or by direct soil transmission. Little if any breeding for resistance to RCNMV has been performed, consequently most commercial cultivars of red clover are fully susceptible to the virus.

Evolution

The following hypotheses on the evolution of the *Dianthovirus* genus are based exclusively on analysis of the extent of similarity of the amino acid sequence of the various viral gene products. The replicases of all known and sequenced positive strand RNA viruses have been placed into three distinct phylogenetic supergroups. The dianthovirus replicase belongs in supergroup II along with the tombus-, carmo-, necro-, a subset of the luteoviruses, pestiviruses, hepatitis C virus, flaviviruses and the positive strand RNA coliphages (notably Qβ) and the related yeast dsRNA elements. It is generally assumed that the replicase from all of these viruses evolved from a common progenitor and was subsequently dispersed by modular evolution. The dianthovirus capsid protein fits into a rather large group of icosahedral-shaped viruses whose capsid protein(s) possess a conserved structural element termed the 'jelly roll' confirmation. It is suspected that this type of capsid protein has evolved only once. All genera within the *Tombusviridae* have capsid proteins that fit into this group. *Dianthovirus* capsid proteins possess an additional structural feature termed the protruding domain. Tombus- and carmoviruses share this feature. The movement protein gene is unique to plant viruses (**Table 3**). It is hypothesized that this gene was

recruited by the virus from the plant as viruses evolved to adapt to plant hosts.

Future

Since a putative dianthovirus (FNSV) has recently been identified from the tropics, it is possible that additional species within the *Dianthovirus* genus exist and will be discovered. However, with the possible exception of the tropical regions of the world, it is unlikely that any members of this genus will become a major disease problem. RCNMV is clearly the best studied member of the genus. RCNMV is being used as a model system in several research laboratories to study plant–virus interactions, movement, and replication. RCNMV is a convenient model system because of the extreme simplicity of the genome, it does not require a biological vector for infection, and clones from which infectious RNA transcribed are derived are available.

See also: **Carmoviruses (*Tombusviridae*); Necroviruses (*Tombusviridae*); Plant virus disease – economic aspects; Tombusviruses.**

Further Reading

Bates HJ, Farjah M, Osman TAM and Buck KW (1995) Isolation and characterization of an RNA-dependent RNA polymerase from *Nicotiana clevelandii* plants infected with red clover necrotic mosaic dianthovirus. *J. Gen. Virol.* **76:** 1483.

Hamilton RI and Tremaine JH (1996) Dianthoviruses: properties, molecular biology, ecology, and control. In: Harrison BD and Murant AF (eds) *The Plant Viruses*, vol. 5, *Polyhedral Virions and Bipartite RNA Genomes*, pp. 251–282. New York: Plenum Press.

Hiruki C (1987) The Dianthoviruses: a distinct group of isometric plant viruses with bipartite genome, *Adv. Virus Res.* **33:** 257.

Lommel SA, Weston-Fina M, Xiong Z and Lomonossoff GP (1988) The nucleotide sequence of red clover necrotic mosaic virus RNA-2. *Nucleic Acids Res.* **16:** 8587.

Osman TAM and Buck KW (1987) Replication of red clover necrotic mosaic virus RNA in cowpea: RNA-1 replicates independently of RNA-2. *J. Gen. Virol.* **68:** 289.

Xiong Z and Lommel SA (1989) The complete nucleotide sequence and genome organization of red clover necrotic mosaic virus. *Virology* **171:** 543.

Drosophila C Virus

see **Picornaviruses – Insect**

Drosophila Melanogaster Gypsy Virus: (*Metaviridae*)

see **Retroviruses of Drosophila: The Gypsy Paradigm**

E

Eastern equine encephalitis virus *see* **Equine encephalitis viruses**

Ebola virus *see* **Marburg and Ebola Viruses**

ECHOVIRUSES (*PICORNAVIRIDAE*)

Helena Kopecka, CNRS, Institut Pasteur, Paris, France

History

Echoviruses (echo for *e*nteric *c*ytopathogenic *h*uman *o*rphan) were defined initially in 1955 as a group of nonpoliomyelitis viruses that are nonpathogenic for newborn mice. They were thus distinguished from coxsackieviruses, another subgroup of enteroviruses pathogenic for newborn mice. Although the echoviruses are the most frequently isolated enteroviruses, they remain the least well known and characterized subgroup.

Taxonomy and Classification

Echoviruses are members of the *Enterovirus* genus of the *Picornaviridae* family. Enterovirus classification relies mainly on neutralization assays using specific antibodies raised against the prototype strains. The subgroup of echoviruses consists of 31 types: types 1–9, 11–27, 29–33. On the basis of recent evidence from cell culture and from molecular organization, echovirus types 22 and 23 constitute a new enterovirus genus, *Parechovirus*.

Geographic and Seasonal Distribution

Echoviruses circulate worldwide in all geographical areas. They are isolated primarily during the summer and early autumn months, usually as cyclic epidemics over comparatively short periods of time in temperate climates. In tropical and semitropical areas circulation tends to be endemic, produced throughout the year with longer periods of prevalence, often associated with the rainy season.

Host Range and Virus Propagation

The important primary determinants of virus cell tropism and host range are cell surface molecules that act as receptors. The subunits α_2 and β_1 of the integrin VLA-2, the adhesion molecule, have been identified as receptors for echovirus types 1 and 8 (**Fig. 1**). Human decay accelerating factor (DAF), or CD55, was shown to be a cellular receptor for echoviruses types 7, 13, 21, 29 and 33.

Echovirus multiplication *in vivo* is limited to primates, although certain strains can infect mice and eventually induce paralysis in suckling mice (e.g. echovirus type 9, strain Barty). Two cases of endomyocarditis with histological lesions were observed in newborn mice inoculated with echovirus type 25.

The *in vitro* propagation of echoviruses in cell culture gives apparent cytopathic effect (with exception of types 22 and 23). Most echoviruses shut off host cell protein synthesis after infection. Primary monkey and human cells and primate tissue culture are the most appropriate systems for echovirus isolation. These include Buffalo green monkey kidney (BGM), rhesus monkey kidney (MK) cells or Vero cells, human embryonic kidney (HEK) cells, human diploid fibroblasts (MRC-5), human amniotic cells or human rhabdosarcoma (RD) cells. Other continuous

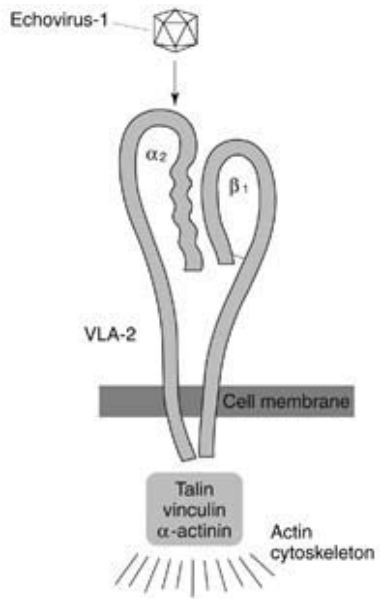


Figure 1 Echovirus types 1 and 8 receptor – the integrin VLA-2. (Reproduced with permission from Bergelson *et al* (1993).)

cell lines (HeLa, KB, HEp-2) are usually less sensitive, although different cells vary in their susceptibility to different echoviruses. Thus, the use of multiple cell lines increases yield and enhances the chance and speed of virus isolation.

Genetics

Echoviruses are typical members of the *Enterovirus* genus and share many of its characteristics. Echoviruses are small nonenveloped viruses; their capsid is formed from 60 copies of four nonglycosylated proteins, organized in an icosahedron (VP1, VP2, VP3 and the relatively unstructured and wholly internal protein VP4). The genome is a monocistronic single-stranded RNA molecule of plus polarity, about 7500 nucleotides long, covalently linked on the 5′ extremity to a small, virus-encoded protein (VPg). It has a long untranslated sequence in the 5′ region with highly conserved structures important for replication and translation initiation. A single large open reading frame encodes a polyprotein of about 250 kDa, proteolytically processed by virus-encoded proteases to yield structural and nonstructural proteins. On the 3′ end is located a short (70–100 nucleotides long) untranslated region (UTR), forming a pseudoknot structure. The 3′ terminal is polyadenylated; the mean length of the poly(A) tail is 60 nucleotides and it plays a role in RNA stability and infectivity of the virus.

The genome sequences of several echoviruses have been determined (types 6, 9, 11, 12, 22 and 23). Sequence comparisons with other enteroviruses revealed a strong overall amino acid identity of echovirus type 9 to types 11 and 12. The echovirus

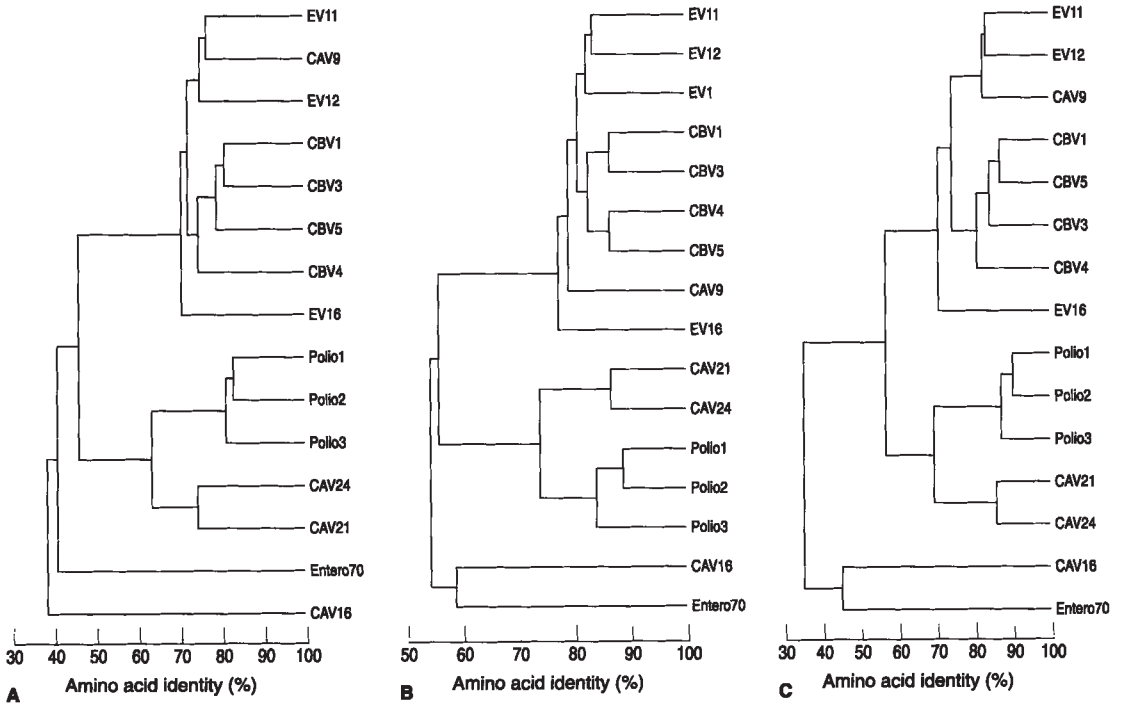


Figure 2 Dendrograms generated using sequences of the major capsid proteins. (A) VP1; (B) VP2; (C) VP3. (Reproduced with permission from Huttunen et al (1996).)

type 11 also showed a remarkable similarity with coxsackievirus A9. These viruses have insertion sequences at the C-terminus of VP1 polypeptide. The organization of the 5′ untranslated region resembles that of other enteroviruses, but also contains a long poly(U) stretch not seen in any other enteroviruses sequenced to date.

Echovirus types 22 and 23 have particular structural, genomic and biological properties when compared with other echoviruses. Their major capsid protein (VP0) is an uncleaved form in the mature virion. In addition, an N-terminal basic extension was determined in VP3. In contrast to the overall low degree of sequence identity with other enteroviruses, a number of sequences in the 5′ untranslated region are shared with the cardiovirus encephalomyocarditis virus. Moreover the incomplete cytopathic effect in cell culture and the absence of shutoff of host cell protein synthesis in infected cells, properties distinctive in both molecular and biological terms, justify their classification in a new genus in the *Picornaviridae* family.

The study of genomic relations in the *Enterovirus* genus has shown that echoviruses are closely related to the coxsackie B virus group (**Fig. 2**).

Evolution

Most echovirus infections of a single serotype contain a heterologous population of viruses that constitutes a quasi-species.

Echoviruses, like other enteroviruses, demonstrate reasonable genome stability in some situations, but can evolve extensively upon replication in the human gut, as is reflected in their rapid evolution during outbreaks in human host populations. They may well be the most variable enteroviruses, with 31 different types discriminated with polyclonal sera. Echoviruses, represented by a very heterologous population of viruses, allow great adaptability and rapid evolution of their genomes. The rate of evolution of continuously replicating RNA viruses is 10^{-3}–10^{-4} substitutions per site per year. In addition to stepwise evolution due to the accumulation of point mutations, RNA recombination between different viruses may also contribute to variation; this event has been demonstrated for polioviruses, but has not yet been described for echoviruses.

Serologic Relationships and Variability

Identification of echovirus serotypes is based on the profile of seroneutralization with monospecific sera raised against the prototype virus, usually the first isolate. There is a general problem of antigenic variation in enteroviruses. It seems that the impact of the variations in echoviruses is greater than in other enteroviruses. One possible explanation may be that echoviruses occur widely in human populations and persist in nature, although their presence is probably underestimated because they give rise to a wide range of clinical symptoms and the majority of infections are asymptomatic. The major problem encountered with human sera often relates to their crossreactivity with other serotypes. Because of the antigenic variation observed within the serotype, the selection of one prototype strain for the production of antiserum for diagnostic purposes is unsatisfactory. It is clear that the panel of prototype antisera is not broad enough antigenically for virus diagnosis by this method. This statement is supported by the observation that many antigenic variants of the same serotype are frequently isolated from clinical specimens; about 10% escape identification using sera raised against the prototype strain. Antigenic variants of echoviruses were isolated rapidly after description of prototype strains; the intratypic variations were observed in echovirus type 1 and type 8 at the same time as the antigenic relations between these two types and also have been documented with echovirus type 4, 12 and 29. Similar antigenic relations were observed between echovirus type 6 and 30. Despite antigenic differences, the prototype strains and the variants of echovirus types 4, 9, 18, 19 and 25 also present variations in their structural capsid proteins VP1, VP2 and VP3. Considerable biological and antigenic variations of natural isolates of echovirus type 25 were observed when compared with the prototype strain JV-4. Antigenic heterogeneity of echovirus type 30 was used to divide these variants in different, but overlapping, antigenic subgroups. The genomic variations in this serotype have been located in the 5′ UTR and in the VP2 and VP4 coding sequence.

Epidemiology

Outbreaks of infection with echoviruses occur frequently in widely separated areas of the world, in various climates, conditions and at different time intervals. Global figures from 1975 to 1983 published by the World Health Organization showed prevalence of 64% for echoviruses in a total of nearly 60000 nonpolio enterovirus reports. Echoviruses clearly continue to be an important cause of significant morbidity and mortality worldwide. The prevalent serotypes have changed since 1983; several outbreaks of echovirus type 30 spread in the United States, in Canada and in Japan between 1990 and 1992. The outbreak of echovirus type 3 in China in 1985, which was never isolated in this country, affected 20% of the

population in the Guizhon province, with a case fatality of 12%. The retrospective epidemiological studies of echovirus type 22 infections from 1966 to 1990 revealed a peak in 1986, with the predominance in infants under 1 year old. In Jamaica in 1986 there was an outbreak of echovirus 22, with several cases of flaccid paralysis, two deaths and a case of aseptic meningitis. Why particular serotypes appear and disappear and the factors dictating endemic versus epidemic behavior are not well understood. One theory is that some epidemic strains, such as echovirus 9, may spread rapidly and exhaust susceptible individuals in the population, whereas those strains appearing endemically over several years may be less contagious.

The study of enteroviral epidemiology has been enhanced recently by the application of molecular genetics methods, such as two-dimensional oligonucleotide gel electrophoresis of viral RNA, hybridization with specific subgenomic probes, amplification and identification of defined RNA sequences using reverse transcription and polymerase chain reaction and nucleotide sequencing. These methods have been used to trace the routes of spread and to determine the degree of relatedness among epidemiologically distinct isolates. The nucleotide sequences in the 5′ UTR of 60 isolates of echovirus type 30 from the recent outbreak of meningitis in Canada compared to prototype strains showed a difference of 11–15%, whereas all isolates originated from seven different provinces shared 99% or greater sequence identity. These approaches can be utilized to rapidly identify enteroviral strains associated with particular outbreaks and distinguish them from other strains and serotypes.

Transmission and Tissue Tropism

Humans are the only known reservoir for echoviruses and therefore close human contact appears to be the primary route of spread. The limited replication of echoviruses takes place in the oropharynx, followed by transit through the stomach and implantation in the lower intestinal tract, where they undergo more extensive multiplication. Virus can be recovered from the oropharynx and intestine of individuals with apparent or inapparent infection. Viruses are generally shed for periods ranging from one to many months in stools; their concentration is highest early in infection. In rare cases of persistent infection, viruses can be shed for several years.

Besides fecal–oral transmission, other routes to be considered are water, food, inoculation, blood and vertical transmission from mother to fetus. Waterborne transmission can be considered as an extension of fecal–oral transmission in which the intermediate vector is water. Indeed the viruses can survive for prolonged periods in favorable environmental conditions (low temperature, neutral pH, presence of organic matter). Echoviruses can be found in surface and ground waters, and in swimming pools, even after chlorination. They can be isolated from raw or partly cooked mollusks and crustacea and their overlying waters. Shellfish rapidly concentrate many viruses, including enteroviruses, which may survive for several weeks, and their consumption may be the route of infection. Soil and crops also provide conditions favorable to echovirus transmission.

Although most neonatal echovirus infections are acquired directly from the mother, some infections are transmitted nosocomially; this type of transmission, typical in newborn nurseries, has been well documented. The incidence and severity of perinatally acquired infections from an infected mother reflect the occurrence of infection, usually in the enterovirus season. Clusters of vertically transmitted neonatal infection have been recorded during community outbreaks. Airborne transmission is probably not significant in echovirus infections.

Echoviruses, like other enteroviruses, multiply throughout the alimentary tract, the primary site of colonization. The virus is translocated from the oropharynx and from the intestine to other organs. Enteroviral replication in ileal lymphoid tissue is detectable 1–3 days after ingestion of virus. After multiplication in submucosal lymphatic tissues, the viruses pass to regional lymph nodes and give rise to a transitional minor viremia. The major viremia results in dissemination to target organs, such as meninges, heart and skin. In these tissues, necrosis and inflammatory lesions are observed, whereas histopathologic lesions are generally not seen in the gut and lymphoid tissues during the earlier replicative events.

Pathogenicity

Most enterovirus infections are characterized by subclinical manifestations; however, they can also cause a wide spectrum of more or less several clinical diseases, including aseptic meningitis, encephalitis, flaccid motor paralysis, exanthema, herpangina, myocarditis, pleurodynia, hepatitis, upper respiratory symptoms, etc.

The genetic heterogeneity of the virus population and selective pressure can lead to the evolution of variants with altered pathogenic properties such as virulence, tissue specificity and host range, which may increase virulence and cause syndromes not ordinarily associated with the prototype virus. Alternatively, an attenuated virus variant can emerge from the adapted

Table 1 Clinical spectrum of infections with echoviruses[a]

Asymptomatic infection	Most echoviruses
Aseptic meningitis	All types (except 24, 26, 29, 32)
Encephalitis	Types 2, 3, 4, 6, 7, 9, 11, 14, 17, 18, 19, 25
Paralysis	Types 2, 4, 6, 7, 9, 11, 14, 16, 30
Exanthema	Especially types 9 and 16 but also several other types
Myopericarditis	Types 9, 22
Generalized disease of newborn	Types 4, 6, 7, 9, 11, 12, 14, 19, 21, 25, 31
Neonatal diarrhea	Types 11, 14, 18
Diarrhea	Several types
Hemolytic-uremic syndrome	Type 22
Myositis	Types 9, 11
Guillain–Barré syndrome	Types 6, 22
Chronic meningoencephalitis in agammaglobulinemic patients	Types 2, 3, 5, 9, 11, 19, 24, 25, 30, 33
Infectious lymphocytosis	Type 25

[a] According to Modlin.

subpopulation. Attenuated variants of echovirus type 2 and type 12 have been isolated. Echovirus type 6 can cause persistent infection *in vitro* and *in vivo*. In immunodepressed individuals with congenital agammaglobulinemia, echovirus types 5, 11 and 25 were detected in the central nervous system.

Most echoviruses lack the property of mouse pathogenicity. However, different strains can produce variants that exibit paralytogenic activity in newborn mice. The analysed sequences of the nonpathogenic prototype strain Hill and the mouse virulent strain Barty of the echovirus type 9 differ, particularly in an insertion coding for an RGD motif at the C-terminus of the capsid protein VP1 in the genome of the strain Barty. It is suggested that this motif is an important determinant for pathogenicity of newborn mice.

Clinical Features and Infection

The majority of echovirus infections are asymptomatic or result in undifferentiated febrile illness. When disease occurs, the spectrum and severity of clinical manifestations vary with the age and immune status of the host and with the serotype and even the intratypic viral strain. While a variety of distinct clinical syndromes are caused by infection with echoviruses some manifestations are associated with many echovirus serotypes (**Table 1**).

Echoviruses together with coxsackieviruses cause more than 90% of cases of aseptic meningitis. Infants under 3 months of age have the highest rates of recognized aseptic meningitis. Pharyngitis and other symptoms of upper respiratory tract infections are often present during the meningitis. Symptoms of encephalitis sometimes complicate the course of aseptic meningitis. In perinatally acquired echovirus infection, encephalitis may be one manifestation of generalized viral infection. Most patients with echovirus encephalitis beyond the neonatal period recover fully; however, one fatal case of echovirus type 9 encephalitis has been reported. Sporadic cases of flaccid motor paralysis have been associated with several echovirus types, most often types 2, 6, 7, 9, 11 and 16. The disease is generally milder than poliomyelitis; paralysis is not usually permanent. The Guillain–Barré syndrome has been reported in a small number of patients infected with echovirus types 6 and 22.

Echoviruses cause a variety of exanthems; they cause little morbidity, but are important sentinels of the prevalence of echoviruses in the community. Summer outbreaks of herpangina have been associated with infection by echovirus types 3, 6, 9, 16, 17, 25 and 30, but other types have been also isolated from persons with herpangina. Echovirus types 1, 6, 9, 16 and 19 are sometimes implicated in pleurodynia. During the summer, disease of the upper respiratory tract is often associated with echovirus type 11 infection.

Direct evidence linking echovirus types 9 and 22 with myopericarditis has been demonstrated by the presence of the infectious viruses or viral antigens in myocardium or pericardial fluid. There is less substantive evidence for echovirus types 1–4, 6–8, 11, 14, 19, 25 and 30; these serotypes have been recovered from noncardiac sources during the episode of acute myopericarditis.

Human neonates are uniquely susceptible to echovirus and coxsackievirus diseases. Echovirus 11 is most frequently associated with overwhelming, systemic neonatal infection. Generalized enterovirus disease in the newborn usually occurs with myo-

carditis or fulminant hepatitis. The serotype 11 is responsible for most cases of fulminant hepatitis but other serotypes (4, 6, 7, 9, 12, 14, 19, 21 and 31) are also responsible for severe hepatitis in neonates. There are several reports of cases of fatal pneumonia occurring in the first few days of life caused by echovirus types 6, 9 and 11. Most cases of persistent, sometimes fatal, infection of the central nervous system in children with hereditary or acquired agammaglobulinemia are mainly associated with echovirus type 11, but other types have also been detected in these patients.

Pathology and Histopathology

Echoviruses have found clinical expression mainly in injury to the central nervous system; occasionally peripheral neuropathies, hepatoneuronal dysfunction, orchitis, pleurodynia or carditis may intervene during infections with several serotypes.

Neonatal myocarditis, which is usually accompanied by encephalitis, is mostly the manifestation of coxsackie B virus infection, and less commonly echovirus 11 infection. Infants dying of myocarditis have enlarged, dilated hearts, extensive myonecrosis and a variable degree of cardiac inflammation. Lymphocytic infiltration of the brain, meninges, lungs, liver, pancreas and adrenal glands may also be found.

Neonatal echovirus encephalitis with white matter sclerosis has been documented. A fatal case of echovirus type 7 infection in a neonate, which was probably acquired from the symptomatic mother, exhibited extensive hemorrhagic necrosis of brain, liver, adrenal glands and kidneys and disseminated intravascular coagulation.

Clinical and pathological findings in a fatal case of disseminated echovirus infection complicating bone marrow transplantation, in which the patient developed noncardiogenic pulmonary edema, has been reported. Viral cultures of the pericardiocentesis were positive for echovirus type 11, and the virus was isolated post mortem from the brain, cerebrospinal fluid, spleen and heart. The virus isolated was probably a variant of echovirus type 11, according to the neutralization titers of the variant versus prototype virus.

Fulminant hepatitis presents with hypotension, profuse hemorrhage and multiple organ failure; the major pathologic findings for infants dying of hepatitis are massive hepatic necrosis and extensive hemorrhage into the cerebral ventricles, renal medullae and interstitial spaces of solid organs. Inflammation is commonly found in the liver and adrenal glands.

Several cases of spontaneous abortion and stillbirth associated with echovirus types 11, 17, 19, 27, and 33 infection have been reported.

Acute renal failure due to rhabdomyolisis has been associated with echovirus type 9 infection.

Echovirus type 4, classically associated with acute lytic infection, appears to have the potential to persist in β cells or perinsular tissue, causing autoimmunity and impairment of insulin secretion without massive β cell lysis.

Echoviruses may be at the origin of symptoms mimicking bulbospinal poliomeningitis. Bilateral limb paralysis in children, and in one adult, was associated with echovirus types 4, 9, 14 and 30.

Immune Response

Neutralizing type-specific humoral antibodies appear as a consequence of infection with echoviruses; they develop shortly after infection and persist for many months or years, depending upon the severity of infection. Immunity to infection is type specific and disease relates not only to the presence, but also to the concentration, of specific humoral antibodies.

In the serum, both IgM and IgG humoral antibodies are produced as early as 1–3 days after echovirus infection. IgM antibodies predominate during the first month and gradually disappear; neutralizing IgG antibodies in serum persist for life after natural infection with these viruses. There are indications that macrophage function may also be a critical component in the immune response to enterovirus infection. Antibody-mediated immune mechanisms operate both in the alimentary tract to prevent the implantation of the virus and in blood to prevent dissemination to the target organs. While antibodies, either passively or actively acquired, may modulate the course of infection, they may not prevent infection. Serologic cross-relationship between echoviruses or other members of the human enteroviruses may be a problem in the control of echovirus infection.

Echovirus infections are high risk for persons with deficiencies in their humoral or cell-mediated immunity. A number of persistent or fatal infections of immunodeficient persons by echoviruses have been reported. In most of the patients the main deficit was in the B cell functions associated with humoral immunity. The majority of the subjects were unable to eradicate the virus from their central nervous system.

Prevention and Control

Echoviruses are the most frequently represented enteroviruses in severe or less severe pathologies of

young children. Despite the frequency and severity of their infection, these viruses are the least understood of the picornaviruses. The only enterovirus vaccines available at present are those against the three poliovirus serotypes. Their application for almost 40 years has resulted in the goal of eradication of poliomyelitis in the world becoming a reality. However, there are no vaccines against other enteroviruses. In view of the large number of different serotypes of echoviruses, it is difficult to design vaccines against them all. A better understanding of their genomic organization and function, as well as the mechanisms of their rapid evolution and variability, would be a great help in the design of antiviral components, where the essential functions of the virus would be a target. The studies of cell receptors are of great importance for the understanding of the interactions of a virus with its host cell, and thus the possible inhibition of virus–cell interaction. A better understanding of the localization and structural configuration of epitopes is necessary for the development of recombinant vaccines. The application of rapid, sensitive and specific diagnostic molecular biology tools for echovirus detection in clinical specimens and in environmental samples is important for epidemiological control. Surveillance of newly isolated strains is also of great importance, as viruses with new pathologies, such as neurotropism and cardiotropism, may emerge. The study of the resistance of echoviruses in the environment in general, and in the clinical environment in particular, is important for epidemiology and for the risk of severe pathologies in immunodepressed persons. The coming years will surely lead to new and important information regarding the potential of the echoviruses to trigger autoimmunity and/or cause chronic infection.

Eradication of poliomyelitis by vaccination is an objective of WHO. However, a number of cases of infection-associated paralysis and meningoencephalitis due to enteroviruses other than polioviruses will still exist. The exact diagnosis of such infections is imperative in order to obtain the correct epidemiological data to demonstrate the role of viruses other than polioviruses in these clinical manifestations.

Future Perspectives

A knowledge of genomic composition and organization will simplify the classification of the enterovirus group and may well mean the reclassification of some echoviruses into the different subgroups. More information on the properties, ecological variations and natural history of isolates of echoviruses will shed further light on this important group of enteroviruses. We have to be concerned about the future occupation by other enteroviruses, and mainly by echoviruses, of the ecological niches liberated by the polioviruses after their eradication.

See also: **Coxsackieviruses (*Picornaviridae*); Enteric viruses; Enteroviruses (*Picornaviridae*): Animal and related viruses, Human enteroviruses (serotypes 68–71); History of virology: Bacteriophages, General, Polio, coxsackie, echo and other enteroviruses; Persistent viral infection; Polioviruses (*Picornaviridae*): General features, Molecular biology.**

Further Reading

Bergelson JM, Shepley MP, Chan M, Hemler ME and Findberg RW (1993) Identification of the integrin VLA-2 as a receptor for echovirus 1. *Science*, 255: 1718.

Bergelson JM, Chan M, Solomon N *et al* (1994) Decay-accelerating factor (CD55), a glycosylphosphatidylinositol-anchored complement regulatory protein, is a receptor for several echoviruses. *Proc. Natl Acad. Sci USA* 91: 6245.

Huttunen P, Santti J, Pulli T and Hyypiä T (1996) The major echovirus group is genetically coherent and related to coxsackie B viruses. *J. Gen. Virol.* 77: 715.

Melnick JL (1990) Enteroviruses: polioviruses, coxsackieviruses, echoviruses and newer enteroviruses. In: Fields BN *et al* (eds) *Virology*, p. 549. New York: Raven Press.

Modlin JF (1990) Coxsackieviruses, echoviruses, and newer enteroviruses. In: Mandell GL, Douglas RG, Jr and Bennet JE (eds) *Principles and Practice of Infectious Diseases*, p. 1620. Edinburgh: Churchill Livingstone.

Rotbart HA (ed.) (1995) *Human enterovirus infections*. Washington, DC: ASM Press.

Ectromelia virus *see* **Mousepox and Rabbitpox**

EMERGING AND RE-EMERGING VIRUS DISEASES

Frederick A Murphy, School of Veterinary Medicine, University of California Davis, Davis, California, USA

Introduction

Many elements can contribute to the emergence of a new viral disease; these include *viral determinants* (such as mutation, recombination, natural selection, evolution), *natural determinants* (such as ecologic and environmental determinants) and *determinants pertaining to human activity* (such as behavioral, societal, transport, commercial and medical care activities). In general, there is no way to predict when or where the next important new viral pathogen will emerge; nor is there any way to predict the ultimate importance of a virus as it first emerges. Given this reality, initial investigation at the first sign of the emergence of a new viral disease must focus on characteristics such as mortality, severity of disease, transmissibility and remote spread, all of which are important predictors of epidemic potential and societal threat. Clinical observations, pathologic examinations and preliminary identification and characterization of the virus often provide early clues because new, emerging viruses often resemble their closest genetic relatives in regard to their epidemiologic and pathogenic characteristics. The elements of disease control and prevention programs include surveillance, diagnostics and communications, linked to a professional response network. This network, actually a web of many national, state and local networks with only modest international links, must be flexible; capable, for example, in one instance of emphasizing local resources as the basis for actions, and in the next of emphasizing global epidemic aid. Given the nature and magnitude of the threats represented by the many new and emerging viral diseases, the importance of all the elements of the global network seems clear.

The Emergence of New Viral Diseases is Inevitable yet Unpredictable

New or previously unrecognized viruses are constantly being identified. New diseases emerge as the cause of geographically limited curiosities, as the cause of intermittent disease outbreaks and as the cause of new epidemics. No one would have predicted the emergence a new rodent-borne hantavirus as the cause of a severe acute respiratory distress syndrome in the southwestern region of the USA in 1993 and certainly no one would have predicted the epidemic emergence of human immunodeficiency virus (HIV) as the cause of acquired immune deficiency syndrome (AIDS) before the initial description of the disease in the early 1980s.

New viral diseases appear to be emerging with increasing frequency, as suggested by published reports of cases, outbreaks and epidemics, and by the rate of identification of new pathogenic viruses. The list of newly emergent viruses of humans and animals is impressive, indeed, and is seemingly prophetic of more to come in the future (**Tables 1–3**). There are several reasons why emergence of new viral diseases seems to be accelerating. The global human and domestic animal populations have continued to grow inexorably, bringing increasingly larger numbers of people and animals into close contact. There have been successive revolutions in transportation of humans and animals, making it possible to circumnavigate the globe in less than the incubation period of most viral diseases. Ecological changes brought about by human activity are occurring at a rapidly accelerating rate.

When a new viral disease is suspected, it must be characterized (by the work of clinicians and pathologists), and assessed in regard to its impact on populations at risk (by the work of epidemiologists). When a new virus is suspected, it must be isolated, identified and characterized and methods must be developed for diagnosis and field investigation. All these activities must be integrated to construct a comprehensive view of the immediate problem at hand. Today, this kind of integrated investigative problem-solving activity is usually thought to be the sole responsibility of governmental agencies, but in fact, many professionals from throughout the human and animal health sectors have played central roles in recent episodes. As it turns out, assessing risk and guiding intervention involve quite diverse activities; for example, in some cases complex field studies of the incidence of infection in the general population or in a selected subpopulation are necessary to determine risk factors for infection, mode of transmission, targets for intervention, etc., while in other cases, studies of the pathogenetic mechanisms underpinning the clinical presentation in the individual human or animal patient might hold the key. Today, in nearly every instance, important clues lie in characterizing the molecular structure and replication strategy of the virus and the cellular pathobiology of the infection.

Table 1 Some of the most important emerging and re-emerging human viruses

- *Crimean-Congo hemorrhagic fever virus** (tick-borne; severe human disease with 10% mortality; widespread across Africa, Middle East and Asia)
- *Dengue viruses** (mosquito-borne; the cause of millions of cases of febrile disease in the tropics and dengue hemorrhagic fever, a life-threatening disease, especially in children)
- *Ebola** *and Marburg** *viruses* (natural reservoirs unknown, the cause of the most lethal hemorrhagic fevers known)
- *Guanarito virus** (rodent-borne; the cause of Venezuelan hemorrhagic fever)
- *Hantaviruses** (rodent-borne; the cause of important rodent-borne hemorrhagic fever in Asia and Europe and severe, often fatal acute respiratory distress syndrome in the USA and elsewhere in the western hemisphere)
- *Hepatitis C virus* (the cause of much severe chronic liver disease worldwide)
- *Hepatitis E virus* (the cause of epidemic hepatitis, especially in Asia and along the USA/Mexico border; the infection has a high mortality rate in pregnant women)
- *Human herpesviruses 6 and 7* (the cause of febrile disease in children)
- *Human immunodeficiency viruses, HIV-1 and HIV-2* (the cause of AIDS, still emerging in many parts of the world)
- *Human papillomaviruses* (over 70 viruses, some associated with cervical, esophageal and rectal cancers)
- *Human T-lymphotropic viruses, HTLV-1 and HTLV-2* (the cause of adult leukemia and neurologic disease, especially in the tropics)
- *Influenza viruses* (the cause of thousands of deaths every winter in the elderly; the cause of the single most deadly epidemic ever recorded – the worldwide epidemic of 1918, in which 25–40 million people died)
- *Japanese encephalitis virus* (mosquito-borne; severe, lethal encephalitis; now extending across southeast Asia; great epidemic potential)
- *Junin virus** (rodent-borne; the cause of Argentine hemorrhagic fever)
- *Lassa virus** (rodent-borne; the cause of severe disease in West Africa)
- *Machupo virus** (rodent-borne; the cause of Bolivian hemorrhagic fever)
- *Measles virus* (still the cause of more than 1 million deaths per year in children, mostly in the tropics)
- *New-variant Creutzfeldt–Jakob disease* (prion disease, zoonotic, transmitted to humans from cattle with bovine spongiform encephalopathy)
- *Norwalk and related viruses* (major causes of outbreaks of severe diarrhea)
- *Polioviruses* (still an important problem in some developing countries; targeted by the World Health Organization for worldwide eradication by the year 2002)
- *Rabies virus* (transmitted by the bite of rabid animals; still the cause of 25 000–50 000 human deaths per year, mostly in developing countries)
- *Rift Valley fever virus** (mosquito-borne; the cause of explosive epidemics in Africa)
- *Ross River virus* (mosquito-borne; the cause of epidemic arthritis that has moved across the Pacific region several times)
- *Rotaviruses* (rotavirus enteric disease is the second leading cause of death among infants in the world)
- *Sabiá virus* (rodent-borne; the cause of hemorrhagic fever in Brazil and two laboratory-acquired cases)
- *Venezuelan encephalitis virus* (mosquito-borne; great potential for causing explosive epidemics in humans and horses in Central and South America)
- *Yellow fever virus** (mosquito-borne; one of the most deadly diseases in history)

* The viruses that cause hemorrhagic fevers in humans.

Of course, the immediate goal of such enterprise is to invent and guide prevention and control measures as these become necessary.

Variation and Evolution of Viruses

Variation in the genome of viruses, altering any of many characters that affect their pathogenicity and transmissibility, can increase their potential for emerging incrementally. For this reason, the great breakthroughs in viral molecular and cellular biology have had great value in dealing with emergent viral diseases. All of the many mechanisms that drive viral evolution must be evaluated in trying to predict the future course of a disease; first, there is the high frequency of *viral mutation* during replication, especially with RNA viruses, especially with the greatly expanded number of viral replication cycles that occurs early in an epidemic. Second, there is the opportunity for *recombination or reassortment* of genes that is afforded in the same circumstances. Finally, there is the long-term risk of viral recombination with genetic elements of host cells, the ultimate risk whereby retroviruses acquire new genes.

Classical examples of such viral variation and evolution include the definition many years ago of smallpox variants, variola major (Indian subcontinent and Europe, mortality up to 30%) and variola

Table 2 Some of the most important emerging and re-emerging viruses of animals

- *African horsesickness viruses* (mosquito-borne; a historic problem in southern Africa; now becoming entrenched in the Iberian peninsula; a major threat to horses worldwide)
- *African swine fever virus* (tick-borne and also spread by contact; an extremely pathogenic virus; recently present in Europe and South America)
- *Australian bat lyssavirus* (new rabies-like virus in fruit bats in Australia, one human death)
- *Avian influenza viruses* (spread by wild birds; a major threat to poultry industries globally)
- *Bluetongue viruses* (*Culicoides*-borne; the isolation of several strains in Australia became an important nontariff trade barrier issue)
- *Bovine spongiform encephalopathy prion* (the cause of a major epidemic in cattle in the UK, resulting in major economic loss and trade embargo)
- *Canine parvovirus* (the cause of a pandemic of severe disease in dogs that rapidly swept around the world; a new virus, having mutated from feline panleukopenia virus)
- *Chronic wasting disease of deer and elk prion* (spongiform encephalopathy of unknown source, discovered in captive breeding herds in the USA)
- *Feline immunodeficiency virus* (important cause of AIDS-like disease in cats globally)
- *Foot-and-mouth-disease viruses* (still considered the most dangerous exotic viruses of animals in the world because of their capacity for rapid transmission and great economic loss; still entrenched in Africa and Asia)
- *Australian equine morbillivirus* (new virus causing lethal respiratory disease in horses and humans; reservoir host recently found to be fruit bats)
- *Lelystad virus (porcine respiratory and reproductive disease virus)* (the cause of an important new disease in swine in Europe and the USA)
- *Malignant catarrhal fever virus* (an exotic, lethal herpesvirus of cattle; an important nontariff trade barrier issue throughout the world)
- *Rabbit hemorrhagic disease virus* (being used to control rabbits in Australia, with great success)
- *Rinderpest virus* (still considered very dangerous, with potential for causing great economic loss in Africa and Asia)

minor (South America, mortality about 1%) and naturally occurring attenuated poliovirus variants, some of which inspired the development of Sabin live-attenuated polio vaccines.

There are several other kinds of change that can contribute to the emergence of new viral diseases. A *change in host range* can permit a virus to spread into a new species with devastating consequences (e.g. following minimal mutations a feline parvovirus evolved into canine parvovirus 2, which then caused a global pandemic in dogs). An *increase in virulence* can convert a nonpathogenic virus into an important pathogen (e.g. following minimal mutations temperate endemic Venezuelan equine encephalitis viruses

Table 3 The special case of emerging and re-emerging viruses in endangered/threatened species

- *Callitrichid arenavirus in tamarins (LCM/marmoset hepatitis)* (lethal disease; a major problem in the golden lion-tamarin, an endangered species)
- *Canine distemper virus in the black-footed ferret* (lethal disease; its control is crucial to the survival of this endangered North American species; being controlled by vaccination)
- *Canine parvovirus in the red wolf* (severe disease in endangered wolf pups reintroduced into the Great Smoky Mountains National Park; being controlled by annual vaccination)
- *Eastern equine encephalitis virus in the whooping crane* (lethal disease; its control is crucial to the survival of this endangered North American species; being controlled by annual vaccination)
- *Feline infectious peritonitis virus in the cheetah* (lethal disease; its control is crucial to the survival of this endangered African species)
- *Dolphin, porpoise and phocine (seal) morbilliviruses and related viruses* (newly recognized; a complex of lethal diseases in several species of seals, sea lions, dolphins and porpoises, caused by several related morbilliviruses; control is crucial to the survival of several endangered/threatened species, worldwide)
- *Rabies in endangered free-living wild canid species* (lethal disease; its control is crucial to the survival of several endangered wild canid species)
- *Rinderpest virus in endangered free-living ruminant species* (several wild ungulate species of Africa serve as reservoir hosts of this virus, the source of virus causing epidemics in cattle; control is crucial to the acceptance of cohabitation of domestic and wild animals in large areas of Africa)

have evolved into lethal epidemic pathogens). A *change in antigenic signature* can permit a virus to infect a population already immune to parental strains of the same virus (e.g. human influenza viruses undergo 'drift' and 'shift', in many instances resulting in new epidemic emergence).

In some instances, a new viral disease is the consequence of the appearance of a virus which is truly new to its host. The most dramatic instance of this phenomenon is the global pandemic caused by HIV-1 and HIV-2, each of which evolved in nonhuman primates from viral ancestors resembling simian immunodeficiency viruses. In this instance, the human population had no background immunity nor any resistance derived from long-term genetic selection. Without such natural barriers to dissemination, international spread and a global pandemic followed.

Epidemiologic Considerations in the Emergence of Viral Diseases

Epidemics are classically divided according to their means of spread into two major categories, propagated and common source. *Propagated epidemics* depend upon spread from host to host; they continue to expand as long as each infection gives rise to more than one new infection. *Common source epidemics* occur when a virus is disseminated from a single focus; they usually result from contamination of air, food, water, drugs, medical devices, or the like.

Propagated epidemics

The epidemic potential of newly emerging acute viral diseases varies depending upon the mode of transmission, the immunological and genetic susceptibility of the host population and the size of the population at risk. Epidemic potential is greatest for a virus which is readily transmitted from host to host, particularly via the respiratory route. Conversely, zoonotic agents and arthropod-borne agents are usually limited in their geographic range, although the latter are certainly not limited in their capacity for causing very large epidemics.

Continuing evolution of a virus which is already present in a population can cause what appears to be re-emergence of epidemic disease. For example, the evolution of influenza viruses is accompanied by variances in virulence, with some variants being responsible for greatly increased mortality. The greatest example of this phenomenon is the global influenza pandemic of 1918, in which 25–40 million people died.

On occasion, a virus may emerge in epidemic form simply because of an alteration in the ecology of its host. An example of historic importance is the emergence of epidemic poliomyelitis in the mid-nineteenth century. This was not due to any change in the properties of the virus, as epidemiological studies strongly suggest that the same virus strains could be simultaneously associated with both pathogenic and nonpathogenic infection. Rather, this was due to an improvement in public sanitation and personal hygiene, which led to a delay in the acquisition of enteric virus infection from infancy to childhood. Since infants were protected by maternal antibody, they usually underwent silent immunizing infections, whereas when older children were infected they more often suffered paralytic poliomyelitis.

Common source epidemics

If a virus is introduced from a common source into a large population, disease can emerge on an epidemic scale. A recent example is the ongoing epidemic of bovine spongiform encephalopathy in the UK. In this instance it appears that the scrapie prion of sheep contaminated meat-and-bone meal which was fed to cattle. This resulted in the apparent adaptation of the prion to cattle, and the emergence of a devastating new cattle disease. When the prion associated with bovine spongiform encephalopathy emerged as the cause of new-variant Creutzfeldt–Jakob disease in humans, the ultimate risk of a common source contagion became manifest.

Persistent infections, long incubation periods and emerging diseases

The dynamics of emergence of a new viral disease can vary greatly, depending upon the incubation period, whether the infection is acute or persistent, and whether the resulting disease (and shedding pattern) is acute or chronic. When associated with a short incubation period and acute disease, emergence can be a dramatic event. An example is hantavirus-associated respiratory distress syndrome, which first came to attention in 1993. However, when the emerging agent is associated with a long incubation period, the resulting epidemic may rise over a period of years, its presence obscured by a failure to appreciate its entrenchment in the population. Of course, the example *par excellence* is AIDS: first, the length of its incubation period (as long as 8–10 years) led to underestimates of its potential epidemic potential and case–fatality rate; next, its initial epidemic behavior led to misjudgments of its shedding and transmissibility patterns; finally, misconceptions about its clinical course led to gross underestimates of its burden on the health care systems of developed countries.

New recognition of previously unrecognized diseases

In some instances, a long-existing viral disease may appear to emerge simply as a matter of recognition – the occurrence of a notable outbreak and intensified investigation, the isolation of the virus and the development of diagnostics, etc. New diseases that are clinically unique are more likely to be recognized early than diseases that closely resemble well-established clinical entities, such as hepatitis, diarrhea or encephalitis. One example of 'delayed emergence' due to late recognition is California encephalitis, caused by La Crosse virus. The virus was first isolated in 1964; using an isolate as a source of antigen, retrospective serological surveys showed that the virus has been an important endemic cause of encephalitis in children for many years in midwestern USA.

Nature of Ecological and Zoonotic Factors in Regard to Emerging and Re-emerging Viral Diseases

One set of factors contributing to the emergence of new viruses relates to their capacity to adapt to extremely diverse and changing econiches. One of the most complex sets of adaptive characteristics concerns the transmission of viruses by arthropods. The arthropod-borne viruses are examples *par excellence* where emergence and re-emergence follow upon human environmental manipulation:

- Population movements and the intrusion of humans and domestic animals into new arthropod habitats have resulted in many new emergent disease episodes. The classic example of this was the emergence of yellow fever during the building of the Panama Canal; there are many contemporary examples as well.
- Ecologic factors pertaining to unique environments have contributed to emergent disease episodes. Remote econiches, such as islands, free of particular species of reservoir hosts and vectors, are often particularly vulnerable to an introduced virus. For example, the initial Pacific 'island-hopping' of Ross River virus in the 1980s from its original niche in Australia, caused 'virgin-soil' epidemics of arthritis–myalgia syndrome in Fiji and Samoa – this virus will surely re-emerge in a similar way again.
- Deforestation has been the key to the exposure of farmers and domestic animals to new arthropods. The occurrence of Mayaro virus disease in Brazilian woodcutters as they cleared the Amazonian forest in recent years is a case in point.
- Increased long-distance travel facilitates the carriage of exotic arthropod vectors around the world. The carriage of the Asian mosquito, *Aedes albopictus*, a proven vector for dengue, yellow fever and California encephalitis viruses, into the USA in the water contained in imported used tires represents an unsolved problem of this kind.
- Increased long-distance livestock transportation facilitates the carriage of viruses and arthropods (especially ticks) around the world. The introduction of the tick-borne virus, African swine fever virus, from Africa into Portugal (1957), Spain (1960) and South America (1960s and 1970s) is thought to have occurred in this way – it is just a matter of time until this virus makes further international forays.
- New routings of long-distance bird migrations, brought about by new manmade water impoundments, represent an important yet still untested new risk of introduction of arboviruses into new areas. This may be one key to the movement of Japanese encephalitis virus into new areas of Asia.
- Ecologic factors pertaining to environmental pollution and uncontrolled urbanization are contributing to many new, emergent disease episodes. Arthropod vectors breeding in accumulations of water (tin cans, old tires, etc.) and sewage-laden water are a worldwide problem. Environmental chemical toxicants (herbicides, pesticides, residues) can also affect vector–virus relationships directly or indirectly.
- Ecologic factors pertaining to water usage – that is, increasing irrigation and the expanding reuse of water – are becoming important factors in viral disease emergence. The problem with primitive water and irrigation systems, which are developed without attention to arthropod control, is exemplified in the emergence of Japanese encephalitis in new areas of Southeast Asia.
- Global warming, affecting sea level, estuarine wetlands, swamps and human habitation patterns, may be affecting arthropod vector relationships throughout the tropics; however, specific data are scarce because such long-term trends are involved.

Nature of Behavioral and Societal Factors

There are diverse behavioral, commercial, societal and iatrogenic factors that lead to the emergence of new diseases or new viral disease patterns. In many countries these represent overarching societal health problems:

- Sexually transmitted viral diseases are increasing in incidence in many populations. After widespread personal behavioral changes made in the wake of the global AIDS pandemic, prevention programs based upon public education seem to be burning out.
- Virus transmission associated with daycare has become recognized as the most important factor in community and family spread of many respiratory and enteric viruses. Daycare-acquired infection is also important in the spread of viruses, such as hepatitis A virus, that usually cause only subclinical infection in children but which cause serious disease when transmitted to parents and staff.
- The re-emergence of childhood viral diseases transmitted in the community is often a sentinel of the condition of all public health disease prevention programs. When the incidence of these diseases increases it is usually because of inadequate vaccine coverage.
- Viral diseases associated with agriculture and food processing and distribution systems are increasing in incidence as the sources, scale and technologies involved are driven to change by economic forces. Foods are produced by fewer but larger processors, resulting in increasing numbers of large regional outbreaks of disease. The centralization of the food industry also means that a single contaminated ingredient may appear in many different end-products. The USA now imports 30 billion tons of food a year, including meat, fruit, vegetables and seafoods; such foods, representing an increasing proportion of the diet, often come from developing countries where hygiene and sanitary conditions are less than adequate. In such circumstances, hepatitis A virus has become an important food-borne pathogen.
- Several viral diseases associated with medical care have increased in incidence in recent years, partly because of the wider use of advanced invasive medical technologies. Viral diseases have been associated with immunosuppressive therapy, organ transplantation, dialysis and blood transfusion. Hepatitis B, hepatitis C, human cytomegalovirus and AIDS are notable in these regards, but so are less common diseases such as Crimean–Congo hemorrhagic fever, Lassa fever and Ebola hemorrhagic fever.

Prevention and Control

The world faces immediate and urgent problems in regard to emerging and re-emerging viral diseases. Health systems are being challenged by these diseases, each of which seems to have qualities that defy established conventional prevention and control strategies. Innovative strategies based upon agent-specific epidemiologic and virologic research must address several distinct needs: (1) a global disease surveillance system, (2) a global diagnostics system, (3) a global integral research base, (4) a global communications system, (5) a global technology transfer system, (6) a global emergency response system, (7) a global training program, and (8) a stable global funding base.

International organization is the key to many of these elements. There would be value in designing a single overarching global organization to deal with the emerging and re-emerging viral disease problems of humans and the similar problems of interest in animal agriculture (the many viral diseases of livestock, poultry, fish and shellfish), crop agriculture (the many viral diseases of commercially grown food and fiber plants) and national and international security and law enforcement agencies (the viral diseases involved in biological warfare threats from rogue governments and bioterrorism from the same sources as well as amateur groups).

Further Reading

Garrett L (1994) *The Coming Plague. Newly Emerging Diseases in a World Out of Balance.* New York: Farrar, Straus and Giroux

Henderson DA (1993) Surveillance systems and intergovernmental cooperation. In: Morse SS (ed.) *Emerging Viruses.* New York: Oxford University Press.

Lederberg J, Shope RE and Oaks S (eds) (1992) *Emerging Infections, Microbial Threats to Health in the United States.* Washington, DC: US National Academy of Sciences Press.

Murphy FA (1993) New, emerging and re-emerging viral diseases. *Adv. Virus Res.* 43: 1.

Murphy FA and Nathanson N (eds) (1994) New and emerging viral diseases. *Semin. Virol* 5(2): 85.

Walker DH (ed.) (1992) *Global Viral Diseases. Prevention, Control and Eradication.* Vienna: Springer.

ENCEPHALITIS VIRUSES (*FLAVIVIRIDAE*)

Contents

Encephalitis Viruses and Related Viruses causing Hemorrhagic Disease

James S Porterfield, Barnstaple, Devon, UK

History

'Brain fevers' such as rabies and poliomyelitis have been known since ancient times, and early in this century it was recognized that these two diseases were of viral origin. In the 1930s and 1940s it became clear that a number of other neurotropic viral infections occurring in different parts of the world differed from rabies and poliomyelitis in that they were transmitted by mosquitoes or ticks; these became known as the arthropod-borne encephalitides. Later, when it was found that some diseases not associated with encephalitis were caused by viruses serologically related to those responsible for the arthropod-borne encephalitides, the more general collective term 'the arthropod-borne viruses' or 'arboviruses', was introduced. In the 1950s arboviruses were divided into a number of serological groups, A, B, C and others, and this serological division formed the basis upon which these viruses were later placed within genera and families recognized by the International Committee for the Taxonomy of Viruses, group A arboviruses becoming the genus *Alphavirus* and group B arboviruses the genus *Flavivirus*, both initially within the family *Togaviridae* (but see Taxonomy and Classification, below). Encephalitic alphaviruses are considered in separate entries elsewhere in the Encyclopedia: this entry is concerned with flaviviruses that cause encephalitis and/or hemorrhagic disease (see **Table 1**). A chronological account of the recognition of these viruses and their associated diseases now follows.

Outbreaks of summer encephalitis were recognized in Japan as early as 1873, but no virus was isolated until 1935. Japanese encephalitis virus (JEV) is described in greater detail elsewhere in the Encyclopedia and is not considered further here, except to note that it is another flavivirus.

In the Australian summer of 1917–18 epidemic encephalitis, at first diagnosed as poliomyelitis, affected 134 people, half of them children under 5 years of age; the mortality rate approached 70%. Brain tissue from fatal cases yielded an agent that was passaged through rhesus monkeys, some of which developed a fatal meningoencephalitis, and other animal species were also infected. Unfortunately, this agent was lost before its nature could be established. Lesser outbreaks of what came to be known as Australian X disease followed in the summers of 1922, 1925 and 1926, after which no further cases were recognized until another outbreak of encephalitis struck the Murray and Darling River areas of Victoria and New South Wales in 1951. A virus that became known as Murray Valley encephalitis virus (MVEV) was isolated from a fatal case and was successfully propagated. Other isolates were made from mosquitoes. An examination of sera from survivors of Australian X disease made it very likely that the earlier Australian outbreaks were also due to MVEV.

In the summer of 1932 separate outbreaks of encephalitis were recorded in the USA, at Paris, Illinois, and in far eastern regions of the former USSR. The following summer encephalitis again struck the USA, this time in St Louis and Kansas City. The intracerebral inoculation of rhesus monkeys and albino mice yielded strains of a virus that became known as St Louis Encephalitis virus (SLEV). Subsequent studies showed that the infection was transmitted by mosquitoes.

In 1937 a virus was isolated from blood collected in the West Nile district of Uganda from an African woman suffering from a mild fever; it became known as West Nile virus (WNV). Although antibodies against WNV are commonly found in Africa, association with human disease was not at first apparent. The same virus was later (1951) isolated in Egypt from healthy children and also from wild birds, mosquitoes and from the brain of a horse with encephalitis. An association with encephalitis was demonstrated in elderly human subjects in Israel, and more recently in Europe.

Meanwhile, cases of encephalitis continued to occur in the eastern USSR, and in 1937 Russian spring summer encephalitis virus (RSSEV) was isolated. By 1940 it was established that RSSEV was transmitted by the tick *Ixodes persulcatus*, and that a second, closely related virus could be recovered from *Ixodes ricinus* ticks collected in western regions of the former USSR where it also produced encephalitis;

Table 1 Principal features of the seven flaviviruses

Virus	*First isolated*	*Geographic distribution*	*Host range*	*Principal vector*	*Clinical disease*
Murray Valley encephalitis virus	(1917)[a] 1951	Australia, New Guinea	Birds, humans	Mosquito	Encephalitis
St Louis encephalitis virus	1933	Epidemic USA and Canada, endemic South America	Rodents, humans	Mosquito	Encephalitis
West Nile virus	1937	Africa, Mediterranean, France, Portugal, Cyprus, Romania, Bulgaria, CIS, Asia.	Birds, humans	Mosquito (tick)	Mild fever, rarely rash, arthralgia, encephalitis
Russian spring–summer encephalitis virus	1937	Eastern CIS	Rodents, humans	Ticks	Encephalitis
Central European encephalitis virus	1940	Europe, from Scandinavia in north to Greece in south	Rodents, humans	Ticks	Encephalitis
Omsk HF virus	1947	Central CIS	Muskrats, humans	Ticks	Gastrointestinal symptoms, hemorrhagic fever
Kyasanur Forest disease virus	1957	India (Mysore)	Monkeys, humans	Ticks	Hemorrhagic fever

[a]The agent isolated in 1917 was lost before its identity was established.

these two agents are sometimes described as the Eastern and Western subtypes of tick-borne encephalitis virus (TBEV). The next two decades established that the second virus was not confined to the western USSR, but was also responsible for many cases of encephalitis in Central Europe and in Scandinavia, where the vector was also *I. ricinus*; this virus, the western subtype of TBEV, is also known as Central European Encephalitis virus (CEEV).

In 1947 a third tick-transmitted virus was isolated in the Omsk region of central USSR. This virus is transmitted by *Dermacentor marginatus* and *Dermacentor pictus* ticks, and is responsible for a diphasic fever with gastrointestinal symptoms and hemorrhagic manifestations, but with little involvement of the central nervous system; it is known as Omsk Haemorrhagic Fever virus (OHFV).

In 1957 large numbers of monkeys were reported to have died in the forested region of Shimoga District, Mysore (now Karnataka) State, India, and severe, sometimes fatal disease also affected the human population of the area. A virus, now known as Kyasanur Forest disease virus (KFDV), was isolated from monkeys, humans and from a variety of ixodid ticks. KFDV is antigenically distinct from the tick-borne viruses mentioned above, although crossreactions can be demonstrated by sensitive methods.

Taxonomy and Classification

The seven viruses named above were first categorized as Group B arboviruses and were later placed in the genus *Flavivirus* (named after the type species yellow fever virus, from the Latin *flavus*, 'yellow') within the family *Togaviridae*. In 1985 the large (>70) genus *Flavivirus* was moved to the newly established family *Flaviviridae*.

Properties of the Virion

Virus particles are roughly spherical, 40–50 nm in diameter overall, and contain a 20–30 nm core. The genome contains single-stranded, positive sense RNA with a molecular weight of about 4×10^6. The three structural proteins: capsid (C), membrane (M), and envelope (E), are all encoded by the viral genome. For further details see entries under yellow fever virus and Japanese encephalitis virus.

Properties of the Genome

The complete genomic sequences are now known for WNV, SLEV, MVEV, CEEV and RSSEV, and partial sequence data are available for KFDV and OHFV. Genome lengths vary from 10 644 to 10 976 nucleotides. As with all other flaviviruses replication involves a single molecule of RNA which encodes a single open reading frame which directs the synthesis of a large polyprotein which is subsequently cleaved to form both structural and nonstructural proteins. Details of flavivirus replication are more thoroughly discussed in the entry for yellow fever virus.

Geographic and Seasonal Distribution

WNV has the widest geographic distribution, virus strains having been isolated in Africa (from Tunisia and Egypt in the north, through West, Central and East Africa to South Africa and Mozambique), in Europe (the Mediterranean area, Portugal, southern France, Cyprus, and western parts of the former USSR, and most recently (1996) Romania and Bulgaria) and in Asia (the Middle East, India, Borneo and Asiatic parts of CIS). WNV antibodies have also been found in Australia, Thailand and Malaysia, but these probably represent crossreactions from other closely related flaviviruses, notably Kunjin virus in Australia, and Japanese encephalitis virus in Malaysia and Thailand.

SLEV is responsible for epidemic disease in the USA and Canada, but is also endemic in Mexico, Panama, Trinidad and Jamaica, Surinam, French Guiana, Brazil and as far south as Argentina.

MVEV is confined to Australia and New Guinea.

RSSEV is endemic over a large area of the CIS corresponding to the distribution of *I. persulcatus*. OHFV is limited to the Omsk and Novosibirsk Oblasts of the CIS.

CEEV is something of a misnomer since it is now established that the virus is endemic in foci which provide a habitat suitable for the maintenance of the vector *I. ricinus* in virtually every country in Europe with the exception of the British Isles. There, *I. ricinus* ticks support the maintenance and transmission of a closely related flavivirus that is responsible for louping ill, a neurotropic disease affecting principally sheep, but also cattle, horses, deer, birds, notably grouse, and very occasionally, humans.

KFDV originally appeared to be confined to the Shimoga District of Mysore (now Karnataka) State, India, but the virus has more recently produced disease in a wider area of western coastal India.

The spring–summer seasonal incidence of disease recognized in the name 'Russian spring summer encephalitis', also applies to the other infections, whether these are tick-borne or mosquito-borne.

Host Range and Virus Propagation

All seven viruses are arboviruses, which, by definition, infect both vertebrate and invertebrate hosts.

The maintenance of an arbovirus in nature depends on the vertebrate host developing a sufficiently high level of viremia to infect a blood-sucking arthropod that can function as a permissive host for that particular arbovirus. All seven arboviruses under consideration are capable of infecting humans, and usually a wide range of other vertebrate species, but the principal vertebrate hosts responsible for virus maintenance are birds for WNV and MVEV, and small rodents for the others. The invertebrate hosts, which serve as vectors for the transmission of the viruses to vertebrates (including humans), are mosquitoes for SLEV, MVEV and WNV and ticks for RSSEV, CEEV, OHFV and KFDV. WNV is exceptional in that although normally mosquito-borne it is tick-transmitted between birds in arctic regions of the CIS. A single isolation of SLEV from ticks has been reported, but ticks play no role in the natural transmission cycle of this virus.

The properties of the seven viruses are similar to those of all other flaviviruses (*see* **Yellow fever virus**).

Genetics

Flavivirus susceptibility of laboratory mice has been shown to be genetically controlled, and although there is no firm evidence of a flavivirus-susceptibility gene in humans, the possibility cannot be dismissed. Genetic factors may also affect the susceptibility of mosquitoes or ticks, with consequent effects on the epidemiology of different viruses.

Regarding genetic variation in individual viruses, WNV has been shown to exist in at least two major antigenic types, one found in Africa, Europe and Central Pakistan and the other in India. However, studies with monoclonal antibodies have revealed antigenic drift within African strains, but also stability within strains isolated many years apart. Variant strains may be introduced into new areas by migratory birds.

Six different topotypes have been reported within strains of SLEV, four from within the USA and two from Central and South America. Topotype 1 occurs in western states and is of intermediate virulence for laboratory animals and probably also for humans; topotype 2 isolates are found in eastern and central states, and are of high virulence; topotypes 3 and 4 are limited to Florida, where one occurs in epidemics and has high virulence whereas the other occurs endemically and is of low virulence. The topotypes from Central and South America have variable virulence characteristics. Genetic drift has been detected within SLEV strains, and as with WNV, variants may be transported into new areas by migratory birds.

Analysis of MVEV strains has shown that isolates from the Australian mainland are generally similar and are distinguishable from strains from New Guinea. The earlier suggestion that Australian disease outbreaks were due to importations of virus from New Guinea now seems unlikely.

Tick-borne flaviviruses appear to be inherently more stable than mosquito-borne viruses. Analysis by peptide mapping of the envelope glycoproteins of a number of different strains of RSSEV and CEEV showed only insignificant differences; OHFV and KFDV have not been subjected to such detailed study.

Evolution

The high degree of conservation found within the nucleotide sequences of those flaviviruses that have been studied strongly suggests that they all evolved from a common, primitive precursor, possibly a virus infecting arthropods rather than vertebrates in the first instance, with involvement of vertebrates being a later step in the continuing process. Evolutionary links with certain insect viruses have been reported and it has been suggested that the cell fusing agent (CFA), a virus isolated from *Aedes aegypti* cells, may be the present day survivor of the primeval flavivirus. It is of note that about one-quarter of all flaviviruses now known appear not to depend on an arthropod for their maintenance in nature, but this subset has yet to be subjected to detailed molecular study. There is some evidence that the flaviviruses originated in the old world, perhaps as recently as within the past 10 000–20 000 years, and that the rate of evolution has been gradual for tick-borne viruses, and much more rapid for mosquito-borne members. WNV is unusual in its ability to replicate in both mosquitoes and in ticks; it may have originated in Africa and be the most ancient of the mosquito-borne flaviviruses, SLEV, JEV, MVEV and other Australian flaviviruses having evolved from it in different parts of the world. Future studies may throw more light on the phylogeny of this large and important family.

Serologic Relationships and Variability

Crossreactions between flaviviruses are most readily detected by hemagglutination–inhibition (HI) tests, followed by complement fixation (CF) tests; neutralization tests (NT) show the greatest specificity. Nevertheless, more closely related flaviviruses, such as WNV, SLEV and MVEV can be shown to crossreact by neutralization tests, particularly when antibodies prepared by multiple inoculations of viruses are used. Such crossreactions may result in difficulties in the interpretation of the results of

serological tests carried out on specimens collected in areas where two or more different flaviviruses co-exist.

As already mentioned under Genetics, considerable strain variation is apparent within the mosquito-borne viruses, but this is less evident with the tick-borne flaviviruses.

The envelope glycoprotein E is the dominant antigen in HI and NT, whereas the core protein C plays the major role in CF tests. Of the different viral antigens, the NS1 protein appears to be the most highly conserved between different viruses.

Epidemiology

These seven viruses are maintained in nature by complex cycles involving the principal vertebrate host and the arthropod vector that feeds upon that vertebrate. Infection of humans is a dead-end event as far as the virus is concerned and does not in any way contribute to the maintenance of the virus in nature.

Probably more is known about the epidemiology of SLEV than of any of the other viruses with which this section is concerned. With this virus, completely different mosquitoes have been shown to be involved in different parts of the USA. *Culex tarsalis* is the principal vector in the western USA, *Culex pipiens* dominates in northern and eastern parts of the USA, and in the southern parts of the country *Culex quinquefasciatus* is the most important species. In addition to these three species, *Culex nigripalpus, C. restuans, C. salinarius* and others also contribute to virus transmission. The virus remains endemic in many areas, and produces epidemics of human disease when environmental conditions permit the build-up of a substantial population of vector mosquitoes. Water accumulations in artificial containers, such as automobile tires, and natural containers, such as tree holes, are important in this respect. Vertical (transovarial) transmission of SLEV virus has been demonstrated under laboratory conditions, and virus has been recovered from overwintering female *C. pipiens* mosquitoes.

The epidemiology of the tick-borne flaviviruses is even more complex than that of the mosquito-borne viruses, since ticks serve as reservoirs of infection as well as acting as vectors. Virus can be transmitted both transtadially and transovarially within ticks, and infected male ticks are also capable of infecting vertebrates, although they are less efficient vectors than females, probably because the levels of virus found in salivary glands are lower in males. In addition to tick transmission, RSSEV and CEEV are both capable of causing infection in humans following the ingestion of milk from a virus-infected animal, usually a goat, but sometimes a cow.

The epidemiology of OHFV is not fully established, but it is clear that in addition to the role played by the ticks *Dermacentor pictus* and *Dermacentor marginatus*, which are responsible for most human infections, water-borne transmission occurs between muskrats, *Ondatra zibethica*, the principal vertebrate hosts in nature. However, the complete natural cycle probably involves rodents, birds and mosquitoes as well as musk-rats and ticks.

Transmission and Tissue Tropism

All seven viruses are vector transmitted, mosquitoes being responsible for the transmission of MVEV, SLEV and WNV and ticks for RSSEV, CEEV, OHFV and KFDV. As already noted, WNV is unusual in being transmitted in nature by both mosquitoes and ticks.

Five viruses, MVEV, SLEV, WNV, RSSEV, and CEEV have a marked tropism for the central nervous system, whereas OHFV and KFDV have tropism for vascular endothelial cells.

Pathogenicity

Of the neurotropic viruses, WNV is the least pathogenic, the majority of human infections being subclinical, and RSSEV is the most pathogenic, carrying a mortality of up to 5%, with CEEV, SLEV and MVEV being between these two extremes.

OHFV carries a mortality of up to 2.5% and that of KFDV is between 5 and 10%.

Clinical Features of Infection

Most infections with each of the seven viruses under consideration are entirely subclinical, and are diagnosed only on the basis of the detection of specific antiviral antibodies.

WNV is unusual in that it may be associated with a dengue-like rash and with arthralgia; more exceptionally, liver involvement with overt jaundice has been reported in Africa, where the condition may be confused with yellow fever. Meningitis or meningoencephalitis may occur, particularly in the elderly, as clearly seen in the 1996 outbreak in Romania.

Clinically overt St Louis encephalitis cases usually have encephalitis, but may also present as an aseptic meningitis or as a benign febrile headache. Manifest disease is rare in children, and fatalities in children are unknown. The onset is usually sudden, with fever, headache and a stiff neck. There is frequently mental confusion, and patients develop tremors, but remain

quiet and somnolent unless aroused. Convulsions are rare, but are indicative of a possible fatal outcome.

Unlike St Louis encephalitis, Murray Valley encephalitis affects children much more frequently than adults, and males more commonly than females in the proportion of about 2:1.

Russian spring summer encephalitis usually starts with a sudden onset of fever, severe headache, nausea and vomiting and severe back pains. The fever may be biphasic. Infection of the brain and spinal cord may result in signs of meningeal irritation, or there may be a focal, paralytic response, most frequently affecting muscles of the neck or the proximal regions of the arms.

Central European encephalitis may present as an influenza-like fever, which may be followed by a biphasic fever associated with meningitis, usually benign, but occasionally leading to a shoulder-girdle type of paralysis. A few patients may die with paralysis of all four limbs.

Hemorrhagic manifestations are prominent in both Omsk hemorrhagic fever and Kyasanur Forest disease, although they are more severe in the latter, occurring into the skin, the mouth, the gastrointestinal tract and the uterus. Central nervous system (CNS) involvement is uncommon in Kyasanur Forest disease, but there may be signs of meningeal irritation and neck rigidity.

Pathology and Histopathology

Experimental studies in mice and hamsters infected with WNV and SLEV have provided a substantial pool of information concerning the pathology of infection within the central nervous system. Prominent features seen by microscopy include neuronal necrosis, neuronophagia and perivascular cuffing, with infiltrations of mononuclear cells and the formation of microgleal nodules. In St Louis encephalitis, lesions may be found in the substantia nigra, the basal ganglia, brainstem, thalamus, spinal cord and cerebellum; the cerebral cortex is usually less heavily damaged. In hamsters infected with SLEV, other organs such as the liver and heart may show extensive damage, but such lesions are rare in human St Louis encephalitis infections. Interstitial myocarditis has been reported in humans and horses infected with WNV, and the same virus has also been responsible for a small number of cases of fatal hepatitis in humans, with the development of lesions very similar to those seen in the liver of fatal cases of yellow fever.

Laboratory studies show a pronounced effect of age, with neonatal mice and hamsters being highly susceptible to fatal encephalitis, whereas older animals become resistant to infection by peripheral routes, but retain a degree of susceptibility to intracerebral infection. The reverse pattern is seen in humans, severe encephalitis due to WNV, SLEV and tick-borne viruses being more common in the elderly than in children, except in the case of MVEV infections, where children have more serious disease. The reasons for the differences are unknown.

Immune Response

All seven viruses provoke a brisk immune response in humans, converting the great majority of infections into subclinical attacks. There is evidence that, whereas humoral antibodies are predominant in resistance to infection, both humoral and cell-mediated immune responses contribute to recovery from infection. Intracerebrally produced antibodies are found only when virus has invaded the CNS, and such antibodies are able, in some cases, to protect the patient from severe encephalitic disease. Passively transferred antibody can confer protection in experimental infections, even when given after as long as six days after peripheral inoculation of WNV, at which time the brain was already infected.

The prime antigen involved in the humoral immune response is the envelope glycoprotein, E, but antibodies are also produced against the matrix protein or its precursor, and against the nonstructural protein, NS1. Antibodies against the latter also contribute to protective immunity.

Prevention and Control

These may be divided into measures directed to limit exposure to the vector, and measures directed against the causative virus.

A highly efficacious inactivated vaccine is available against CEEV. In Austria, where around 500 cases used to occur each year, extensive use of this vaccine almost eliminated the disease. The same vaccine also confers protection against Russian spring summer encephalitis, but both eastern and western viruses still present a significant problem in the CIS.

A number of experimental vaccines have been prepared against KFDV, but no satisfactory vaccine is yet available. No vaccines are available against WNV, SLEV or MVEV.

Future Perspectives

Increasing knowledge of the molecular biology of the viruses under consideration and of the epitopes involved in protective immunity brings the prospect of improved vaccines, possibly based on noninfectious genetically engineered products.

See also: **Bovine diarrhea virus and Border disease virus (*Flaviviridae*); Chikungunya, O'nyong nyong and Mayaro viruses (*Togaviridae*); Dengue viruses (*Flaviviridae*); Encephalitis viruses (*Flaviviridae*): Tick-borne encephalitis and Wesselsbron viruses; Equine encephalitis viruses (*Togaviridae*); Hepatitis C virus (*Flaviviridae*); Hog cholera virus (*Flaviviridae*); Japanese encephalitis virus (*Flaviviridae*); Rubella virus (*Togaviridae*); Sindbis and Semliki Forest viruses (*Togaviridae*).**

Further Reading

Monath TP (1980) *St. Louis Encephalitis*. Washington, DC: American Public Health Association.

Monath TP (ed.) (1988) *The Arboviruses: Ecology and Epidemiology*, vol I. Boca Raton, Florida: CRC Press.

Porterfield JS (ed.) (1995) *Exotic Viral Infections*. London: Chapman & Hall Medical.

Saluzzo JF and Doder B (eds) (1997) *Factors in the Emergence of Arboviral Diseases*. Paris: Elsevier.

Schlesinger S and Schlesinger MJ (1986) *Togaviridae and Flaviviridae*. New York: Plenum Press.

Westaway EG, Brinton MA, Gaidamovich SY *et al.* (1985) Flaviviridae. *Intervirology* 24: 183–192.

Tick-Borne Encephalitis and Wesselsbron Viruses

EA Gould, Institute of Virology and Environmental Microbiology, Mansfield Road, Oxford, UK

History, Taxonomy and Classification

The classical clinical picture of yellow fever (YF), tick-borne encephalitis (TBE), dengue (DEN) fever and the close association of these diseases with mosquitoes or ticks have been recognized since the early part of the twentieth century. However, these diseases were accurately described hundreds of years previously. The first flaviviruses were isolated in the late 1920s and originally classified as group B *ar*thropod-*b*orne viruses (arboviruses) to distinguish them from other serologically unrelated arboviruses in groups A and C. There are approximately 70 recognized viruses in the genus *Flavivirus* (family *Flaviviridae*), of which about 20% are assigned to the TBE virus complex. Wesselsbron (WSL) virus is included amongst the 60% of flaviviruses transmitted by mosquitoes. Currently, there are no recognized invertebrate vectors for the remaining 20% of viruses in the genus. In contrast to encephalitis, which is the characteristic clinical picture induced in humans by most of the TBE complex viruses, Omsk hemorrhagic fever (OHF) and Kyasanur Forest disease (KFD) viruses usually cause haemorrhagic fever; nevertheless, they are firmly established both serologically and genetically as TBE complex viruses.

Subgrouping on the basis of serological cross-reactivity essentially confirms the close antigenic relationships between the TBE complex viruses and shows they are distinct from the mosquito-borne and nonvectored viruses. This is further supported by molecular phylogenetic analysis of flaviviruses in which the tick-borne, mosquito-borne and nonvectored flaviviruses form distinct phylogenetic groups which have evolved from a common ancestral virus. At the time of writing, there are insufficient data to demonstrate conclusively which group represents the ancestral line, although antigenic comparisons with monoclonal antibodies and limited sequence data imply that the nonvectored viruses have the most ancient lineage.

WSL virus is transmitted to vertebrate hosts by *Aedes* spp. mosquitoes which become infected when they take a bloodmeal from other infected animals. The virus is found in Southern and Central Africa in regions which provide sufficient vegetation to sustain farmed animals such as sheep and cattle. Humans, working with or associated with the animals, also frequently become infected but usually present with a mild febrile illness. WSL virus is antigenically closely related to YF, Zika and Uganda S (UGS) virus and immunity to WSL virus is believed to provide cross-protection against these other viruses.

Properties of the Virion

Virions are about 50 nm in diameter, spherical, with a single capsid (C) protein and a lipid envelope. Infectious virions contain two membrane-associated proteins: the envelope (E) and the membrane (M) protein. Intracellular virions are immature and contain a presursor membrane (prM) protein which is proteolytically cleaved at the end of the infectious cycle. The E protein forms a dimer which is orientated parallel to the membrane. The E and the C proteins probably form icosahedral structures. The virion M_r is about 6×10^7; mature virions sediment at about 200S and have a buoyant density of $1.19\,g\,ml^{-1}$ in sucrose. Viruses are stable at pH 8.0 but are readily inactivated at acidic pH, temperatures above 40°C, by ultraviolet light and gamma-irradiation. The genome of flaviviruses is a positive-sense single-stranded RNA of approximately 11 kb. The 5′ end of the genome possesses a type 1 cap followed by the conserved dinucleotide AG. The 3′ end lacks a terminal poly(A)

See also: **Bovine diarrhea virus and Border disease virus (*Flaviviridae*); Chikungunya, O'nyong nyong and Mayaro viruses (*Togaviridae*); Dengue viruses (*Flaviviridae*); Encephalitis viruses (*Flaviviridae*): Tick-borne encephalitis and Wesselsbron viruses; Equine encephalitis viruses (*Togaviridae*); Hepatitis C virus (*Flaviviridae*); Hog cholera virus (*Flaviviridae*); Japanese encephalitis virus (*Flaviviridae*); Rubella virus (*Togaviridae*); Sindbis and Semliki Forest viruses (*Togaviridae*).**

Further Reading

Monath TP (1980) *St. Louis Encephalitis*. Washington, DC: American Public Health Association.

Monath TP (ed.) (1988) *The Arboviruses: Ecology and Epidemiology*, vol I. Boca Raton, Florida: CRC Press.

Porterfield JS (ed.) (1995) *Exotic Viral Infections*. London: Chapman & Hall Medical.

Saluzzo JF and Doder B (eds) (1997) *Factors in the Emergence of Arboviral Diseases*. Paris: Elsevier.

Schlesinger S and Schlesinger MJ (1986) *Togaviridae and Flaviviridae*. New York: Plenum Press.

Westaway EG, Brinton MA, Gaidamovich SY *et al.* (1985) Flaviviridae. *Intervirology* 24: 183–192.

Tick-Borne Encephalitis and Wesselsbron Viruses

EA Gould, Institute of Virology and Environmental Microbiology, Mansfield Road, Oxford, UK

History, Taxonomy and Classification

The classical clinical picture of yellow fever (YF), tick-borne encephalitis (TBE), dengue (DEN) fever and the close association of these diseases with mosquitoes or ticks have been recognized since the early part of the twentieth century. However, these diseases were accurately described hundreds of years previously. The first flaviviruses were isolated in the late 1920s and originally classified as group B *ar*thropod-*bo*rne viruses (arboviruses) to distinguish them from other serologically unrelated arboviruses in groups A and C. There are approximately 70 recognized viruses in the genus *Flavivirus* (family *Flaviviridae*), of which about 20% are assigned to the TBE virus complex. Wesselsbron (WSL) virus is included amongst the 60% of flaviviruses transmitted by mosquitoes. Currently, there are no recognized invertebrate vectors for the remaining 20% of viruses in the genus. In contrast to encephalitis, which is the characteristic clinical picture induced in humans by most of the TBE complex viruses, Omsk hemorrhagic fever (OHF) and Kyasanur Forest disease (KFD) viruses usually cause haemorrhagic fever; nevertheless, they are firmly established both serologically and genetically as TBE complex viruses.

Subgrouping on the basis of serological cross-reactivity essentially confirms the close antigenic relationships between the TBE complex viruses and shows they are distinct from the mosquito-borne and nonvectored viruses. This is further supported by molecular phylogenetic analysis of flaviviruses in which the tick-borne, mosquito-borne and nonvectored flaviviruses form distinct phylogenetic groups which have evolved from a common ancestral virus. At the time of writing, there are insufficient data to demonstrate conclusively which group represents the ancestral line, although antigenic comparisons with monoclonal antibodies and limited sequence data imply that the nonvectored viruses have the most ancient lineage.

WSL virus is transmitted to vertebrate hosts by *Aedes* spp. mosquitoes which become infected when they take a bloodmeal from other infected animals. The virus is found in Southern and Central Africa in regions which provide sufficient vegetation to sustain farmed animals such as sheep and cattle. Humans, working with or associated with the animals, also frequently become infected but usually present with a mild febrile illness. WSL virus is antigenically closely related to YF, Zika and Uganda S (UGS) virus and immunity to WSL virus is believed to provide cross-protection against these other viruses.

Properties of the Virion

Virions are about 50 nm in diameter, spherical, with a single capsid (C) protein and a lipid envelope. Infectious virions contain two membrane-associated proteins: the envelope (E) and the membrane (M) protein. Intracellular virions are immature and contain a presursor membrane (prM) protein which is proteolytically cleaved at the end of the infectious cycle. The E protein forms a dimer which is orientated parallel to the membrane. The E and the C proteins probably form icosahedral structures. The virion M_r is about 6×10^7; mature virions sediment at about 200S and have a buoyant density of $1.19\,g\,ml^{-1}$ in sucrose. Viruses are stable at pH 8.0 but are readily inactivated at acidic pH, temperatures above 40°C, by ultraviolet light and gamma-irradiation. The genome of flaviviruses is a positive-sense single-stranded RNA of approximately 11 kb. The 5′ end of the genome possesses a type 1 cap followed by the conserved dinucleotide AG. The 3′ end lacks a terminal poly(A)

tract and terminates with the conserved dinucleotide CU. The E protein hemagglutinates red blood cells and mediates receptor binding and acid pH-dependent fusion activity. Seven nonstructural (NS) proteins are synthesized in infected cells: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. The N-terminal one-third of the NS3 protein forms the viral serine proteinase complex together with NS2B which is involved in proteolytic cleavage of the polyprotein. The C-terminal portion of NS3 contains an RNA helicase domain involved in RNA replication and RNA triphosphatase activity that is probably involved in formation of the 5′-terminal cap structure of the viral RNA. NS5 protein is the RNA-dependent RNA polymerase and probably encodes the methyltransferase activity necessary for methylation of the 5′ cap structure. Virions contain about 17% lipid by weight, which is derived from host cell membranes, and about 9% carbohydrate by weight; their composition and structure are dependent on the vertebrate or invertebrate host cell. *N*-glycosylation sites are present in the M, E and NS1 proteins.

Genome Organization and Replication

The genome RNA is the viral messenger RNA and is infectious when introduced into cells using either electroporesis or membrane-fusing agents such as lipofectin. The RNA consists of a long open reading frame of more than 10 000 bases encoding all structural and nonstructural proteins which is flanked by short untranslated regions (UTRs). About 300 nucleotides at the terminal part of the 3′ UTR show sequence divergence but conserved secondary structures among different flaviviruses. The 3′ UTR may contain internal poly(A) tracts. Virus particles attach to as yet unidentified receptors on the cell surface and are engulfed as virions in endocytoplasmic vesicles, where the slightly lower pH conditions induce conformational changes in the E protein and the viral envelope fuses with the membrane of the vesicle, thus releasing the RNA. This incoming messenger RNA is translated and RNA replication begins with synthesis of complementary negative strands which serve as templates for genome-length positive-stranded molecules. This process appears to occur on the membranes of the perinuclear endoplasmic reticulum by formation of replicative intermediates and replicative forms. A polyprotein is synthesized from the messenger RNA and is immediately processed by cellular proteases and the viral serine protease (NS2A–NS3), giving rise to the structural and nonstructural viral proteins. Immature virus particles appear in the rough endoplasmic reticulum, where they are probably assembled and transported as immature particles through the membrane systems of the secretory pathway to the cell surface where exocytosis occurs. Immediately before release from the cell, mature virions are generated by a furin-like cellular protease which cleaves the precursor premembrane (prM) protein.

Geographic Distribution and Evolution

Viruses in the TBE serocomplex are geographically widely dispersed. If we assume an African origin, their subsequent evolution and dispersal during the past 5000–10 000 years may have occurred as depicted in **Fig. 1**. It is possible that infected seabirds, or alternatively infected ticks, dispersed the viruses from Africa, across southeast Asia as far afield as the South Pacific Ocean and they then gradually dispersed and evolved in a northerly direction to far-east Asia and Canada in the northern hemisphere. During the past 2000–3000 years the more recently dispersed TBE complex viruses appear to have evolved in a westerly direction through the forests of Asia and Europe until, in the past 200–400 years, they reached the British Isles. This gradual evolutionary pattern takes the form of a cline showing a distinct correlation between geographic and genetic distance, as estimated from the most recently emerged virus, louping ill (LI), at the most westerly geographic location, i.e. the British Isles. The determining factor for the clinal distribution of the TBE complex viruses is probably the tick vector (primarily *Ixodes ricinus* and *I. persulcatus*), which has a very long (3–5 years) life cycle, feeding only once at each stage of the life cycle and moving only locally when carried by forest animals on which it feeds. The virus probably spends most of its time replicating or persisting in ticks rather than vertebrate hosts (i.e. rodents, deer, horses, etc.), which, in the wild, rarely develop significant viremia.

In contrast to the TBE complex viruses, WSL virus is found in south and central Africa. Serological evidence suggests that WSL virus may also be present in Thailand but it is not yet entirely clear if the Thai virus represents an antigenically closely related virus. WSL virus infections occur most frequently amongst sheep and the associated *Aedes* spp. mosquitoes that increase in density and feeding activity during rainy seasons. Recent molecular phylogenetic data support the results obtained using serology, showing that WSL virus is genetically most closely related to YF virus, which is also widespread throughout central Africa but, in contrast to WSL virus, has retained a sylvatic existence with periodic escapes from the jungle environment to cause human epidemics in rural and urban areas.

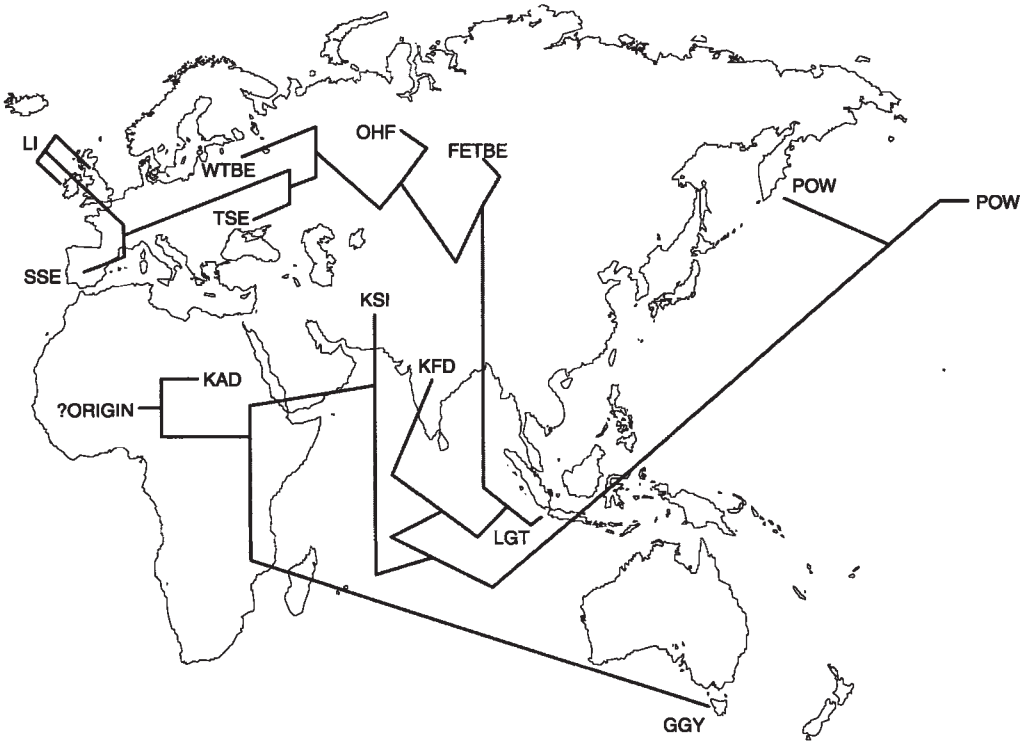


Figure 1 Predicted geographic dispersal pattern of the TBE virus complex during the past 5000–10 000 years. The illustration presumes an African origin for these viruses. The viruses can be identified from **Table 1**.

Host Range and Virus Propagation

Viruses in the TBE complex infect humans and a wide range of tick (*I. ornithodorus, Haemaphysalis, Dermacentor*) and wildlife vertebrate species, particularly forest rodents and birds. In many cases, indigenous vertebrates do not show clinical disease but produce antibodies in response to infection; however, severe disease often occurs in humans or animals encountering these viruses for the first time, such as livestock or domesticated animals. Louping ill disease first appeared as an epizootic sheep and grouse encephalomyelitis on the uplands of the British Isles, about 200–300 years ago when sheep and grouse were introduced on to the moors. Similar epizootics in sheep were observed on the Spanish, Turkish and Greek uplands, again following the introduction of sheep. The uplands, with their moist grassy undergrowth, provide an ideal environment for *I. ricinus* survival, and young nonimmune sheep provide a susceptible and readily available host for the tick. Tourists, hill-walkers, accompanying dogs and horses, etc. are all susceptible to infection if they encounter infected ticks.

WSL virus is found most commonly in sheep, to which it is transmitted by *Aedes* spp. mosquitoes. Cattle, goats and camels are also susceptible to infection when exposed. Human infections, obtained as an occupational disease, usually result in a febrile illness with rash. Seasonality of the disease is determined by the activity of the mosquitoes.

Propagation of TBE complex viruses or WSL virus can be achieved readily in the brains of newborn and young mice or hamsters and a wide range of vertebrate cell cultures, including Vero, LLC-MK2, BHK-21 and PS cells.

Genetics

Flavivirus genetic variability is constrained by the different structural and functional requirements of the two-host system, i.e. vertebrate and invertebrate. Amongst the seven nonstructural and three structural genes, NS5 is considered to be the most conserved, whereas the viral envelope (E) gene is believed to be one of the more variable genes, at least in part because of immune selection pressure. Nevertheless, phylogenetic trees constructed from sequence data based on any flavivirus gene are virtually identical, implying similar selective constraints on all flavivirus genes. The impact of the invertebrate host and its life cycle on evolutionary rates is revealed by the observation that mosquito-borne flaviviruses appear to be

Table 1 Subdivision of the flaviviruses based primarily on available sequence data

Tick-borne viruses	
Mammalian tick-borne virus group	
Louping ill	(LI)
Irish subtype	(ISE)
British subtype	(BSE)
Spanish subtype	(SSE)
Turkish subtype	(TSE)
Tick-borne encephalitis complex	(TBE)
European subtype	(WTBE)
Siberian subtype	(STBE)
Far Eastern subtype	(FETBE)
Omsk hemorrhagic fever	(OHF)
Langat	(LGT)
Kyasanur Forest disease	(KFD)
Karshi	(KSI)
Royal Farm	(RF)
Powassan	(POW)
Gadget's Gully	(GGY)
Kadam	(KAD)
Seabird tick-borne virus group	
Tyuleniy	(TYU)
Meaban	(MEA)
Saumaraez Reef	(SRE)
Mosquito-borne viruses (*Aedes* spp. associated)	
Yellow fever virus group	
Banzi	(BAN)
Bouboui	(BOU)
Edge Hill	(EH)
Jugra virus	(JUG)
Potiskum	(POT)
Saboya[a]	(SAB)
Sepik	(SEP)
Uganda S	(UGS)
Yellow fever	(YF)
Wesselsbron	(WSL)
Dengue virus group	
Dengue 1	(DEN-1)
Dengue 2	(DEN-2)
Dengue 3	(DEN-3)
Dengue 4	(DEN-4)
Spondweni virus group	
Spondweni	(SPO)
Kedougou[b]	(KED)
Zika	(ZIK)
Aroa virus group (*Culex* spp. associated)	
Aroa	(AROA)
Bussuquara	(BUS)
Iguape	(IGU)
Naranjal	(NJL)
Japanese encephalitis virus group	
Alfuy	(ALF)
Cacipacore	(CPC)
Koutango	(KOU)
Kunjin	(KUN)
Japanese encephalitis	(JE)
Murray Valley encephalitis	(MVE)
St Louis encephalitis	(SLE)
Usutu	(USU)
Langat	(LGT)
West Nile	(WN)
Kyasanur Forest disease	(KFD)
Yaounde	(YAO)
Ntaya virus group	
Bagaza	(BAG)
Ilheus	(ILH)
Israel turkey meningoencephalitis	(ITV)
Ntaya	(NTA)
Rocio	(ROC)
Tembusu	(TMU)
Kokobera virus group	
Kokobera	(KOK)
Stratford	(STR)
No-known vector viruses	
Rio Bravo group	
Batu Cave	(BC)
Bukalasa bat	(BB)
Carey Island	(CI)
Dakar bat	(DB)
Montana myotis leukoencephalitis	(MML)
Phnom-Penh bat	(PPB)
Rio Bravo	(RB)
Modoc virus group	
Apoi	(APOI)
Cowbone Ridge	(CR)
Jutiapa	(JUT)
Modoc	(MOD)
Sal Vieja	(SV)
San Perlita	(SP)
Yokose virus group	
Entebbe bat	(EB)
Sokoluk	(SOK)
Yokose	(YOK)

[a] No direct evidence of transmission in mosquitoes.

[b] Tentative assignment.

evolving at more than twice the rate of tick-borne flaviviruses.

The genetic determinants of flavivirus pathogenicity remain to be identified but their complexity is evident from the fact that both single and multiple nucleotide substitutions or deletions may result in attenuation of virus virulence. Whether or not genetic recombination contributes to virus virulence is not known but dual infections with different flaviviruses have been recorded, thus providing the potential for genetic interchange.

An interesting and practical application of comparative amino acid sequence analysis is the ability to diagnose viruses on the basis of genetic marker sequences. The E gene of flaviviruses possesses at least three characteristic domains which categorize the viruses. A hexapeptide sequence (EHLPTA for most TBE complex viruses) at amino acid position 207–212 is unique to the TBE viruses. A pentapeptide sequence (DSGHD) at amino acid position 320–332 (for the TBE virus complex) identifies TBE virus and other serotypes, whereas a unique tripeptide (position 232–234) identifies individual viruses (NPH for LI virus).

Serologic Relationships and Antigenic Variability

High titer hyperimmune antiserum against any individual flavivirus crossreacts, in sensitive serologic tests, with any other flavivirus. On the other hand, monoclonal antibodies show panflavivirus, subgroup or species-specific reactivity. These serologic properties formed the basis of flavivirus classification prior to the construction of phylogenies based on sequence data, and although there are inherent weaknesses in the serogroupings, the essential distinctions have proved to be quite robust. The TBE serocomplex comprises about 12 antigenically closely related viruses. An additional group of three tick-borne viruses (Tyuleniy, Meaban and Saumarez Reef) is associated with seabirds. These viruses crossreact in complement fixation tests. The mosquito-borne and nonvectored viruses were subdivided into a further six or seven antigenic groups, depending on the serological methods and other criteria employed. An updated scheme of classification for the flaviviruses, based on available serological and molecular sequence data, is presented in **Table 1**.

Antigenic variability of flaviviruses is most often detected in the E gene in the form of neutralization escape mutants that can be selected in the laboratory using neutralizing antibodies. Naturally occurring antibody escape mutants of LI virus have also been isolated from field samples collected in parts of the UK where vaccine was used to protect sheep from LI virus infection, implying that immune selection pressure could operate in the natural environment. Many of these and the experimentally selected escape mutants show attenuated virulence for mice.

Epidemiology

Flavivirus epidemics arise when large numbers of susceptible vertebrates encounter high densities of active infected invertebrates. Epidemics of TBE are seen characteristically in the European, Siberian and Asian forests during spring and early autumn, when ticks quest in order to obtain a bloodmeal. Forest workers, soldiers, local residents or tourists enter these tick-infested areas, are bitten and subsequently may become infected. In the spring, forest animals become active following hibernation and increase their likelihood of exposure to the questing infected ticks. In contrast to TBE in the forests, LI epidemics occur as encephalomyelitis in sheep grazing on the moist coarse grasses of the moorlands in Britain. The high moisture content of the grasses on the moorlands is essential for tick survival. Sheep are an abundant source of blood for *I. ricinus* which parasitize immunologically naive sheep and transmit the virus as the bloodmeal is imbibed. In Ireland, the situation is different because of the higher moisture levels in the grasses at lower elevations. Louping ill is therefore frequently seen in pigs, cattle and horses which graze on the tick-infested pastures. Epidemic OHF in Siberia is another example of the influence of environmental perturbation on disease incidence. Muskrats imported from Canada were introduced on to farmlands in Siberia, where OHF virus is known to circulate amongst the rodent population. In contrast to the local rodents, the introduced muskrats were highly susceptible to OHF virus. Epidemics amongst farm workers hunting the muskrats, and the families of the farm workers, were also observed. A different epidemiological picture was observed in the Shimoga district of Mysore in India. Hemorrhagic disease was first reported in 1957 in monkeys found dead in the Kyasanur Forest. Subsequent investigations revealed that *Hemaphysalis* spp. ticks had transmitted KFD virus to the monkeys when they came down from the trees to forage for food scraps left by local workers as they were clearing the edges of the forest for urbanization projects. Humans became infected when they entered the edges of the forest and were exposed to infected ticks.

WSL virus epidemics occur widely in regions of Africa where immunologically naive sheep are available at times when *Aedes* spp. are abundant. The factors that determine the dispersal of viruses such as

WSL are not fully understood but, in addition to the need for an environment that can sustain farm animals and the associated mosquitoes, immunological crossreactivity with other flaviviruses is probably also significant in restricting WSL virus to particular geographic regions. Moreover, as climate change impacts on the world, the dispersal of WSL and other viruses may be altered.

Transmission and Tissue Tropism

The main invertebrate vectors of TBE complex viruses are ixodid ticks. *I. ricinus* transmits LI virus and European strains of TBE virus, whereas *I. persulcatus* is usually associated with TBE complex viruses further east across the former Soviet Union. KFD virus, on the other hand, has been isolated from a variety of ixodid ticks, and Powassan (POW) virus also seems to have a catholic invertebrate host range. The virus has been isolated from *Dermacentor andersoni*, various *Ixodes* ticks and even mosquitoes. The classic picture of transmission to vertebrates involves larvae, nymphs or adults transmitting the virus during the feeding process or becoming infected as they take a bloodmeal from viremic animals. Replete ticks then drop off and molt to the next stage of their life cycle before taking another bloodmeal. Virus replicates and survives in the ticks through the trans-stadial phases of their life cycle. Thus, ticks that became infected at the larval (or nymphal) stage could potentially transmit the virus when feeding at the nymphal (or adult) stage. An alternative mechanism has been proposed to explain transmission on nonviremic animals. It has been shown in the laboratory that infected ticks can transmit the virus to noninfected ticks as they cofeed in clusters on animals which do not develop viremia. Saliva produced by the cofeeding ticks enhances the efficiency of nonviremic transmission. Nonvectored transmission of TBE complex viruses has also been reported. For example, biphasic milk fever has been seen in humans as the result of drinking milk from infected animals such as goats. Moreover, grouse chicks can develop fatal encephalitis from eating infected ticks. There is also evidence of vertical transmission of TBE complex viruses, albeit it at low frequency. It has been shown that the virus can survive in tick eggs and then be transmitted to vertebrates at the subsequent stages of development during the life cycle.

Virus enters the vertebrate host following the bite of an infected tick and probably replicates locally before being transported to lymph nodes via Langerhans and other cells. Further replication takes place in extraneural tissues and a viremia becomes detectable. When encephalitis develops, the major target of TBE complex viruses is the central nervous system. Viruses within the complex may target different but specific neuronal cells and, depending on the particular virus, the disease may be seen as an encephalitis or an encephalomyelitis. Viruses such as OHF and KFD may show a preference for vascular tissues in the final stages of the infection. WSL virus is transmitted by a variety of *Aedes* spp. which become infected when they feed on animals with a high viremia. Replication of the virus in the mosquito occurs rapidly to produce a high titre which can then be transmitted to susceptible animals such as sheep when the mosquito takes a second bloodmeal, usually within a few weeks. WSL infections involve the liver and skin, and in more severe cases the central nervous system.

Pathogenicity

There are significant differences in pathogenicity between viruses of the TBE complex, ranging from those which have not been shown to cause overt disease, such as Gadget's Gully virus, to those which cause encephalitis and/or hemorrhagic fever. In addition, differences in virulence for mice between strains of a virus are often seen; for example, highly virulent strains and almost totally avirulent strains of LI virus have been isolated. Human cases of encephalitis involve cerebral and cerebellar cortex, brainstem, basal ganglia and spinal cord. In general, the case fatality ratio of TBE is higher in the eastern and far eastern part of Russia than it is in western Europe but the reasons for these differences in neurovirulence have not been adequately explained. There is accumulating evidence, based on serology and virus isolation, of chronic progressive human encephalitis caused by TBE complex viruses that are sequestered in neuronal tissues, and this is supported by experimental evidence of chronic encephalitis in monkeys. WSL virus is not known to have caused fatal infections in humans; however, it causes abortion and death of newborn lambs and pregnant ewes.

Clinical Features

Tick-borne encephalitis in western and central Europe tends to take a milder form than that seen in eastern and far eastern Russia. The milder disease is often biphasic. The onset, 3–7 days after infection, is influenza-like with headache, mild fever and myalgia. There is then an asymptomatic (or recovery) period lasting 5–8 days before a proportion of those affected develop the second phase, which involves the central nervous system. Up to 2–3% of cases may be fatal, the fatality rate being higher in adults. Up to 20% of severe cases have long-lasting neuropsychiatric symp-

toms. The more severe eastern form of TBE presents a significantly different clinical picture. It has a higher case fatality rate (20%) and a gradual onset, with fever, headache, anorexia, vomiting and photophobia. This is followed by a severe deterioration to paralysis and convulsions. Deaths occur usually within a week of onset. The disease is more severe in children than in adults and neurologic sequelae are seen in 30–60% of survivors. WSL virus in humans has an incubation period of 2–4 days, followed by a sudden onset of fever, chills, myalgia, hyperesthesia of the skin and hepatosplenomegaly and maculopapular rash. Involvement of the central nervous system has been observed in severe cases of human infection. In sheep and goats the disease is severe, usually leading to death in newborn lambs and abortion and maternal death in pregnant ewes. A mild and usually nonfatal febrile disease occurs in cows.

Pathology and Histopathology

Severe infections by TBE complex viruses show swelling, congestion and petechial hemorrhages. Monkeys infected intracerebrally show degenerative spongiform lesions and astrocytic proliferation. Histopathologic alterations in humans include meningeal and perivascular inflammation, neuronal degeneration and necrosis, neuronophagia and glial nodule formation involving cerebral and cerebellar cortex, brainstem, basal ganglia and spinal cord. Kyasanur Forest disease in humans causes parenchymal degeneration of the liver and kidneys, hemorrhagic pneumonitis and an increase in reticuloendothelial tissue in liver and spleen, with marked erythrophagocytosis.

Immune Response

Infection with a TBE complex virus stimulates antibodies against each of the recognized structural and nonstructural proteins. The predominant protective antibodies are those stimulated by the E and NS1 proteins. Antibodies against the E protein neutralize virus infectivity, inhibit virus hemagglutination and are generally crossreactive with TBE complex and other flaviviruses in nonfunctional tests such as ELISA or immunofluorescence microscopy. NS1-specific antibodies are less broadly crossreactive with non-TBE complex flaviviruses. Nonfatal infections by TBE complex viruses induce long-lasting immunity to reinfection with TBE complex-related viruses but there is insufficient information to predict the extent to which crossprotection occurs amongst more distantly related mosquito-transmitted flaviviruses. Conventionally, immune animals are not thought to be hosts for virus transmission by infected vectors; however, there is experimental evidence of TBE virus transmission by ticks cofeeding on immune rodents.

Prevention and Control

Immunization with an inactivated vaccine prepared in Austria and derived from a strain of western European TBE virus affords good protection against many of the TBE complex viruses when tested under experimental conditions in laboratory mice. This vaccine has shown good efficacy in Austria and neighbouring European countries, where the incidence of encephalitis due to western European TBE virus has dropped significantly following intensive immunization programs. The immunization schedule recommends three doses within the first few months, followed by a booster immunization every 3–4 years. An inactivated virus vaccine is also available for protection of sheep against LI virus infection. While its efficacy is undoubtedly good, LI virus persistence in areas of Scotland where the vaccine has been correctly used demonstrates that sheep are not the only significant hosts for maintenance of the virus in the environment. Recent evidence has shown that virus transmission between ticks cofeeding on mountain hares may also contribute to virus persistence. Apart from vaccination, control strategies for TBE complex viruses are based on avoidance of exposure to infected ticks when they are actively questing during the spring and summer/autumn. Most of the TBE complex viruses are associated with forest environments, whereas LI virus occurs on the sheep-rearing uplands in the British Isles, Spain, Greece, Turkey and Bulgaria and can therefore be avoided accordingly.

There are no recognized vaccines for WSL virus and control of the disease is difficult in areas of Africa where mosquito populations increase during rainy seasons.

Future Perspectives

Current research associated with the TBE complex viruses is focused on the development of new, improved broad-spectrum vaccines based on the E and NS1 proteins using molecular biological techniques. Other major areas of research include the NS1, NS3, NS5 proteins and the untranslated regions of the viral genome. The major questions being addressed are the role of these proteins in virus replication and virus virulence. Understanding these complex characteristics of the viruses will ultimately lead to the development of antivirals. Nucleotide and amino acid sequencing is providing the basis for detailed molecular epidemiological and evolutionary

studies which will explain the origin and dispersal of these viruses.

See also: **Yellow fever virus (*Flaviviridae*); Dengue viruses (*Flaviviridae*); Japanese encephalitis virus (*Flaviviridae*).**

Further Reading

Gould EA, Zanotto PMA and Holmes EC (1997) The genetic evolution of flaviviruses. In: Saluzzo JF and Dodet B (eds) *Factors in the Emergence of Arbovirus Diseases*, p. 51. Amsterdam: Elsevier.

Monath TP and Heinz FX (1996) Flaviviruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology* 3rd edn, p. 961. Philadelphia: Lippincott-Raven.

Rice CM (1996) Flaviviridae: the viruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology* 3rd edn, p. 1931. Philadelphia: Lippincott-Raven.

ENDOGENOUS VIRUSES

Bernhard F. Benkel, Agriculture and Agri-Food Canada, Lethbridge Research Centre, Livestock Sciences Section, Lethbridge, Alberta, Canada

Introduction

Endogenous proviruses are commonly described as prehistoric sequences or retroviral fossils that are derived from ancient infections of germline cells. They may be considered intimate partners in long-term relationships. In contrast to their exogenous counterparts, endogenous viral loci are inherited as stable mendelian genetic elements, i.e. transmitted vertically from parents to offspring. Many endogenous proviral elements contain defective or incomplete viral genomes and are noninfective, and replication-incompetent. There is mounting evidence, however, that a small number of endogenous proviruses are complete and able to direct the production of infectious viral particles that spread by normal means of transmission. In this entry, the terms 'complete' or 'functional' are used to differentiate those endogenous proviruses that encode infectious virus from the bulk of endogenous viral loci, which are defective and/or transcriptionally silent.

The distribution limits of endogenous retroviruses remain to be established. However, to date endogenous viral sequences have been reported in all but the most primitive vertebrate taxa. In addition, the genomes of several invertebrate species have been shown to contain genetic elements that are remarkably retrovirus-like. Thus, it is conceivable that endogenous retroviruses are ubiquitous among eukaryotes.

In higher vertebrates, endogenous proviruses represent a truly prolific class of genetic elements. For example, it has been estimated that up to 2% of the human genome consists of retrovirus-derived sequences. This state of affairs has prompted some researchers to declare that humans are 'descended from viruses as well as from apes'. Since the available data suggest that infections of germline cells by retroviruses are rare events, the large number of some of these elements in higher vertebrate genomes is generally interpreted as evidence that endogenous proviruses are effective at intragenomic spread, i.e. RNA-mediated retrotransposition within the host genome. The intimate association between endogenous proviruses and their hosts over long periods of evolutionary time has resulted in complex patterns of interrelationships, many of which are still poorly understood.

The literature on endogenous retroviruses has been growing at an exponential rate during the past few years. In fact, there are currently close to a thousand reports pertaining to endogenous viruses in the human genome alone. The existence of such a wealth of information means that an all-inclusive review of the field is no longer practical. Instead, this review will focus on three topical themes in endogenous retrovirology: (1) patterns of evolution within endogenous proviral families; (2) the interactions of endogenous elements with their host genomes and their role in modulating physiological processes in the host; and (3) endogenous proviruses and their significance to viral transmission and disease.

Taxonomy and Classification

Retroviruses are currently classified as part of a large array of *retroid* elements, which also includes the elements known as long interspersed elements (LINEs), and short interspersed elements (SINEs).

studies which will explain the origin and dispersal of these viruses.

See also: **Yellow fever virus (*Flaviviridae*); Dengue viruses (*Flaviviridae*); Japanese encephalitis virus (*Flaviviridae*).**

Further Reading

Gould EA, Zanotto PMA and Holmes EC (1997) The genetic evolution of flaviviruses. In: Saluzzo JF and Dodet B (eds) *Factors in the Emergence of Arbovirus Diseases*, p. 51. Amsterdam: Elsevier.

Monath TP and Heinz FX (1996) Flaviviruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology* 3rd edn, p. 961. Philadelphia: Lippincott-Raven.

Rice CM (1996) Flaviviridae: the viruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology* 3rd edn, p. 1931. Philadelphia: Lippincott-Raven.

ENDOGENOUS VIRUSES

Bernhard F. Benkel, Agriculture and Agri-Food Canada, Lethbridge Research Centre, Livestock Sciences Section, Lethbridge, Alberta, Canada

Introduction

Endogenous proviruses are commonly described as prehistoric sequences or retroviral fossils that are derived from ancient infections of germline cells. They may be considered intimate partners in long-term relationships. In contrast to their exogenous counterparts, endogenous viral loci are inherited as stable mendelian genetic elements, i.e. transmitted vertically from parents to offspring. Many endogenous proviral elements contain defective or incomplete viral genomes and are noninfective, and replication-incompetent. There is mounting evidence, however, that a small number of endogenous proviruses are complete and able to direct the production of infectious viral particles that spread by normal means of transmission. In this entry, the terms 'complete' or 'functional' are used to differentiate those endogenous proviruses that encode infectious virus from the bulk of endogenous viral loci, which are defective and/or transcriptionally silent.

The distribution limits of endogenous retroviruses remain to be established. However, to date endogenous viral sequences have been reported in all but the most primitive vertebrate taxa. In addition, the genomes of several invertebrate species have been shown to contain genetic elements that are remarkably retrovirus-like. Thus, it is conceivable that endogenous retroviruses are ubiquitous among eukaryotes.

In higher vertebrates, endogenous proviruses represent a truly prolific class of genetic elements. For example, it has been estimated that up to 2% of the human genome consists of retrovirus-derived sequences. This state of affairs has prompted some researchers to declare that humans are 'descended from viruses as well as from apes'. Since the available data suggest that infections of germline cells by retroviruses are rare events, the large number of some of these elements in higher vertebrate genomes is generally interpreted as evidence that endogenous proviruses are effective at intragenomic spread, i.e. RNA-mediated retrotransposition within the host genome. The intimate association between endogenous proviruses and their hosts over long periods of evolutionary time has resulted in complex patterns of interrelationships, many of which are still poorly understood.

The literature on endogenous retroviruses has been growing at an exponential rate during the past few years. In fact, there are currently close to a thousand reports pertaining to endogenous viruses in the human genome alone. The existence of such a wealth of information means that an all-inclusive review of the field is no longer practical. Instead, this review will focus on three topical themes in endogenous retrovirology: (1) patterns of evolution within endogenous proviral families; (2) the interactions of endogenous elements with their host genomes and their role in modulating physiological processes in the host; and (3) endogenous proviruses and their significance to viral transmission and disease.

Taxonomy and Classification

Retroviruses are currently classified as part of a large array of *retroid* elements, which also includes the elements known as long interspersed elements (LINEs), and short interspersed elements (SINEs).

Table 1 Characteristics of retroid elements

Retroid element	*No. per genome*	*Size (bp)*	*Components*
SINE	Up to 1 million	100s	pro, polyA
LINE	Up to 100 000	1000s	pro, pol, polyA
Retrovirus	Up to 1000s	1000s	pro, gag, pol, env, LTR

pro, promoter; polyA, poly(A) tail; pol, polymerase; gag, group-specific antigen; env, envelope; LTR, long terminal repeat.

Table 1 compares the structures of prototypes of the major classes of eukaryote retroid elements, and gives estimates of the abundances of these elements within the genome of a typical higher eukaryote. The prototypes for both the long terminal repeat (LTR) elements and the LINE elements encode a reverse transcriptase enzyme. The prolific SINE elements lack such a coding region, and SINE element replication is dependent upon reverse transcriptase activity encoded elsewhere within the host genome.

Complete retroviruses are distinguished from LINEs and SINEs by their genetic structure and their ability to direct the production of infectious viral particles. Unlike complete retroviruses, which can spread horizontally from host to host, the non-LTR LINEs and SINEs are intracellular retroids that spread mainly by colonization of noncarrier chromosomes during sexual reproduction.

The established classification system divides retroviruses, both exogenous and endogenous members of the *Retroviridae*, into seven genera: avian leukosis viruses, human T cell leukemia viruses, lentiviruses, mammalian type B viruses, murine leukemia-type viruses, spumaviruses and type D viruses. A recent report on retroviral distribution provides general support for these groupings, but also strongly suggests that a number of new groupings constituting highly divergent groups of retroviruses, especially spumavirus-like and murine leukemia virus-related endogenous retroviruses, will emerge as more detailed information on these viruses is gathered.

The standardization of the nomenclature used to describe endogenous viral elements is an issue that has yet to be resolved. For exogenous viruses, the situation is somewhat simpler and the descriptors have frequently included information on the discoverer of the strain, the associated type of disease, and the common host species, e.g. mouse mammary tumor virus or Rous sarcoma virus. Endogenous viruses frequently lack a direct association with any particular pathological condition, and the global distribution patterns of families of endogenous elements are in many cases still unclear. In addition, for many endogenous viruses, only fragments of genomic sequence are known, resulting in phylogenetic assignments that are tentative at best. Also, although there are some notable exceptions, most endogenous proviral families lack exogenous, disease-inducing counterparts. For these reasons, the nomenclature applied to endogenous retroviruses can perhaps best be described as colourful. For example, *ev* is still commonly used to designate *a*vian *l*eukosis *v*irus-type (ALV) elements found in chickens, both *Mtv* and endogenous MMTV are used to describe *m*urine *m*ammary *t*umor *v*irus-related (MuMTV) proviruses in mice, whereas RD-114 has been used to describe a feline viral element family. For human endogenous viruses a variety of naming systems has been employed in the past, e.g. *h*uman *T* cell *l*eukemia *v*irus-like (HTLV-like).

Against this background, the recent trend towards adopting a common four-letter descriptor, e.g. HERV for *h*uman *e*ndogenous *r*etro*v*irus, followed by a letter representing the tRNA primer type, is a welcome improvement. However, as more information becomes available on individual loci, this descriptor will need to be extended in order to identify individual elements within proviral families (see below). For some of the more prolific proviral families, such as the HERV-H family which may contain as many as 10 000 members, the typing and classification of individual loci represents a huge undertaking. Furthermore, some elements are shared between humans and other primates, and this information is lost through the assignment of an HERV designation.

Another area where the taxonomy of endogenous viruses can be confusing is the designation of elements within a family. The first and most extensively characterized family of endogenous viral elements consists of the avian leukosis subgroup E proviruses in chickens, originally called *ev* genes (for *e*ndogenous *v*iral genes). At the time of their discovery, the all encompassing descriptor of *ev* genes may have seemed appropriate. However the characterization of at least three other families of endogenous viral elements (EAV), in addition to the ALV-type proviruses, has necessitated a revision of the nomenclature. Poultry researchers have recently adopted a four-letter designation modelled on the human system; in this case with an embedded descriptor identifying the element family, e.g. *ALVE-#*, where the # refers to a specific *ALVE* element.

The *ALVE* proviral family is a particularly informative example of the problematic nature of endogenous virus taxonomy. Although a total of roughly 50 different *ALVE* elements are known to

exist within the *Gallus* gene pool, the small number of elements per chicken (typically 1–12) and the ability to breed lines of chickens carrying a single element have allowed *ALVE* proviruses to be studied in great detail. Based on extensive characterization of *ALVE* elements, it has become clear that a complete description of an endogenous provirus must include information on both the proviral genome and the insertion site within the host genome (also known as the unoccupied site or the flanking sequences).

A case in point is the element *ALVE*21 of 'late feathering' chickens, a complete endogenous element that produces infective subgroup E viral particles. Chickens of the Nunukan line, which display a 'very-late feathering' phenotype, carry a proviral insert at a location identical to that of *ALVE*21; however, the 5′ half of the provirus plus about 650 bp of host DNA at the upstream flank of the insertion site are deleted in Nunukan chickens. A further complication is that the insertion site of the *ALVE*21 element is part of a large duplication on the Z chromosome of the chicken genome. Thus, there are two potential insertion sites for *ALVE*21 in female chickens and four in males.

Substantial polymorphisms within the genomes of endogenous proviruses at identical locations in the host genome have also been described in primates. For example, sequence variability has been described for the ERV-3 envelope gene, and a deletion in the HERV-K gag gene is evident in hominids. Such variations in proviral genomic sequences can be crucial elements in determining the effect of a particular endogenous element on host physiology. A definitive description of each element of a proviral family is a daunting task with current technologies, even for a family with relatively few members such as *ALVE*, and it becomes much more difficult for families with large numbers of members. However, new methodologies are becoming available that can dramatically speed up molecular typing and should help tremendously in providing the analytical power necessary to generate endogenous element profiles quickly and inexpensively (see Prevention and Control).

Evolution

At some point in time, the ancestors of endogenous viral elements of many of the classes that we recognize today were fully functioning, infectious exogenous retroviruses. Most extant proviruses, however, contain incomplete genomes and are no longer capable of coding for infectious viral particles. Thus, the evolutionary history and the biphasic lifestyle of functional endogenous retroviruses (infectious horizontal spread versus intragenomic retrotransposition) are reflected in current host distribution patterns as well as the structure and sequence composition of proviral families and their individual endogenous element members.

When a novel endogenous retrovirus arises (novel in the sense that it uses a cellular receptor that is different from those recognized by existing viruses in a particular host), the virus has an essentially unlimited supply of target host individuals. During the initial stages of the colonization of a species, therefore, the typical 'exogenous' form of spread involving horizontal transmission of infectious virus is likely to be the predominant factor in host conversion. However, as the colonization of the host population proceeds, the number of host individuals carrying at least one insert of the novel viral class increases. Viral interference, i.e. the inability of retrovirus to superinfect cells already carrying provirus of a related family, will begin to limit the efficiency of horizontal spread. Consequently, intragenomic spread through genome colonization during meiosis will become the dominant mode of viral replication.

Along with this switch in lifestyle to intragenomic replication and vertical transmission comes a major change in the selective pressures operating on the proviral genome. The 'hit and run' lifestyle of the exogenous virus, which depends on its ability to produce a large number of complete, infectious particles quickly, is replaced by an intimate, long-term association between the pathogen and its host. For its persistence, the endogenous virus becomes crucially dependent upon its host, and viral loci that have negative effects on host survival will tend to be evolutionarily unstable.

The factors limiting the intragenomic expansion of retroviral families are not understood at this time. Selection for attenuation in order to minimize the genetic load exerted on the host due to insertional mutagenesis is part of the explanation. However, the somewhat perplexing observation that some families of virus are able to amplify to thousands of copies, while others appear as single inserts within the same host genome, suggests that there are as yet unidentified factors at work. In parallel with intragenomic colonization, selection for completeness and the capacity to produce infectious particles diminishes, and proviral loci accumulate defects in the form of point mutations and small deletions, as well as sizable internal deletions. The result is the emergence of families of virus where most member proviruses are defective. Theoretically, given a sufficiently long period of evolutionary time, this process of sequence erosion would eventually obliterate the recognizable features of the endogenous provirus family. At this

point, novel viruses, even ones employing the same receptor as the obliterated elements, could begin the process of genome colonization afresh.

Expression and Effect on Host Immune Response

Each endogenous provirus has the capacity to affect the outcome of a viral infection of the host by related classes of virus in at least two ways: (1) by programming the immune response of the host organism; and (2) by providing a barrier to superinfection through receptor blocking. Definitive experimental evidence for programming of the immune response of the host comes from the avian leukosis system in chickens – the only system for which selected lines carrying single, defined endogenous elements are currently available. The results, however, have broad-ranging implications.

A number of studies using both semicongenic lines and commercial birds of defined genetic backgrounds have provided clear evidence that certain endogenous elements modulate the response of the carrier individuals to infection by exogenous retroviruses in a predictable manner. The endogenous *ALVE* elements that mediate this effect are structurally complete and express functional viral particles. The nature of the effect is a generally diminished host immune response to exogenous virus that includes a delayed-onset, low-level antibody response to exogenous *ALV* subgroup A, as well as increased shedding of exogenous ALV particles into the oviduct resulting in an increase in congenital infections. The basis for the modulation of the immune response is that peptides encoded by endogenous proviruses can take part in the programming of the host's immune system. Thus, individuals carrying expressed endogenous proviral elements typically recognize virus-encoded peptides as self. The result is the lack of an effective immune response to incoming virus with similar antigenic determinants.

At the other extreme of the spectrum are endogenous elements that, instead of predisposing their hosts to chronic infections, actually provide protection against related strains of incoming virus. A prime example of this effect is the transgenic line of chickens carrying the defective ALV subgroup A provirus, *alv6*. *Alv6* expresses the ALV subgroup A envelope proteins only, and provides resistance to infection from exogenous ALV subgroup A virus by blocking the relevant cell surface receptors. Receptor blocking also appears to be responsible for resistance to infection by endogenous ALV in chickens carrying the provirus *ALVE6*, and in Fv-4 mice carrying the Fv-4r resistance allele. Receptor blocking is not the only mechanism by which endogenous viral elements can afford a measure of protection against infection upon their hosts. The *Fv1* gene in mice encodes a *gag*-like protein that restricts murine leukemia virus replication after viral entry but before proviral integration.

The nature of the effect that any particular endogenous virus will have on a carrier individual depends on the structure and expression of the proviral genome. Many proviruses are defective and retain the capacity to code for only a portion of the peptides necessary to produce an infectious viral particle. Some apparently complete proviruses are not expressed, presumably due to the silencing effect of host DNA at the proviral integration site. Thus, each proviral element needs to be assessed individually for its effect on host physiology, and accurate diagnostics are essential for predicting host–virus interactions.

Pathogenicity

Endogenous proviruses typically show low levels of transcriptional activity and/or aberrant patterns of RNA and protein expression, which are often ascribed to the accumulation of mutations and deletions in proviral genomes as well as hypermethylation of viral genes. As a result, the effects on host physiology of endogenous viral elements typically stem from the action of viral LTRs on adjacent host DNA, e.g. proto-oncogenes, as well as from a host immune response directed toward viral-encoded peptides. Both of these mechanisms can lead to the progression of disease states in the host species and recent evidence has implicated endogenous viruses in a number of conditions in humans including breast cancer, multiple sclerosis and lupus erythematosus.

The pathogenicity of endogenous viruses has also become a prime concern in the field of xenotransplantation. Although it is true that the majority of extant endogenous proviruses are nonfunctional and noninfective, there is a short but expanding list of examples where, either through the expression of intact viral loci or by complementation between defective endogenous proviral elements, vertebrate cells have been shown to produce infectious viral particles. Examples of this phenomenon include several porcine PERVs, the chicken *ALVE* prototype RAV-0, endogenous murine leukemia virus and human RTLV-H. The plasticity of the retroviral genome and the proven ability of some endogenous retroviral strains to cross species boundaries, especially under the chronic high level exposure conditions afforded by xenotransplantation, should provide ample motivation for the development of endogenous virus-free donor cells.

Prevention and Control

Due to their intimate association with the germplasm of their hosts, the control of endogenous viral elements poses a unique set of problems. Obviously, although eradication has worked well for eliminating exogenous retroviruses from some experimental and commercial populations of animals, there is little chance of selectively breeding out the thousands of endogenous viral elements in the typical animal genome. Central to the issue of control, therefore, are the enabling tools of rapid diagnostics and a large database on the physiological roles of individual proviral elements. In terms of rapid diagnostics, the development of DNA chip technology has resulted in a massive increase in DNA-typing ability over previously available methods. Whereas in the past, establishing the profiles of endogenous elements was a painstakingly slow process, DNA chip technology has the potential to facilitate data acquisition at an unprecedented speed. Thus, it may soon be feasible to establish the complete genetic profile for endogenous viral elements in any individual on a routine basis. Rapid diagnostics will also aid the process of defining the interrelationships between individual endogenous proviruses and their hosts.

The potential benefit for endogenous viral profiling in humans lies in the identification of carriers of the particular endogenous elements that are associated with morbidity states. Once such genetic risk factors have been determined, lifestyle adjustments can be made to avoid environmental factors that might compound the risks, and available treatment programs can be fine tuned to address specific disease potentials. For applications in other areas, such as farm animal health, molecular profiling of endogenous viral elements can be integrated with existing selection programs to reduce viral particle prevalence in food products, and to increase natural resistance to viral infection.

See also: **Diagnostic techniques: Detection of viral antigens, nucleic acids and specific antibodies, Isolation and identification by culture and microscopy; Immune response: Cell mediated immune response, General features; Human immunodeficiency viruses (*Retroviridae*): Molecular biology, Anti-retroviral agents, General features; Retroviral Oncogenes.**

Further Reading

Herniou E, Martin J, Miller K *et al* (1998) Retroviral diversity and distribution in vertebrates. *J. Virol.* 72: 5955.

Loewer L, Loewere J and Kurth R (1996) The viruses in all of us: characteristics and biological significance of human endogenous retrovirus sequences. *Proc. Natl Acad. Sci. USA* 93: 5177.

Meisler MH and Ting CN (1993) The remarkable evolutionary history of the human amylase genes. *Crit. Rev. Oral Biol. Med.* 4: 503.

Patience C, Wilkinson DA and Weiss RA (1997) Our retroviral heritage. *Trends Genet.* 13: 116.

ENTERIC VIRUSES

Ruth F Bishop, Department of Gastroenterology and Clinical Nutrition, Royal Children's Hospital, Parkville, Victoria, Australia

Introduction

Viruses are amongst the first microorganisms to colonize the germ-free intestine after birth, where they encounter conditions ideal for growth, including warmth, an abundant supply of nutrients and continual replenishment of susceptible cells. Many viruses identified in feces, including reoviruses, adenoviruses and enteroviruses, have the capacity to replicate in the intestine without causing clinical symptoms. Other viruses detected in feces may be derived from swallowed secretions originating in the respiratory tract, from plant viruses ingested in food, or from phages released after bacterial lysis. Many viruses use the gastrointestinal tract as a portal of entry and may replicate there before initiating systemic disease (e.g. poliovirus, hepatitis A and E). A diverse group of viruses (rotaviruses, adenoviruses, caliciviruses, astroviruses, coronaviruses) replicate in the epithelial cells lining the small intestine, causing sufficient destruction to result in the symptoms of enteric infection, including diarrhea, vomiting, fever and dehydration. A further group of viruses of systemic origin (e.g. measles virus, cytomegalovirus

Prevention and Control

Due to their intimate association with the germplasm of their hosts, the control of endogenous viral elements poses a unique set of problems. Obviously, although eradication has worked well for eliminating exogenous retroviruses from some experimental and commercial populations of animals, there is little chance of selectively breeding out the thousands of endogenous viral elements in the typical animal genome. Central to the issue of control, therefore, are the enabling tools of rapid diagnostics and a large database on the physiological roles of individual proviral elements. In terms of rapid diagnostics, the development of DNA chip technology has resulted in a massive increase in DNA-typing ability over previously available methods. Whereas in the past, establishing the profiles of endogenous elements was a painstakingly slow process, DNA chip technology has the potential to facilitate data acquisition at an unprecedented speed. Thus, it may soon be feasible to establish the complete genetic profile for endogenous viral elements in any individual on a routine basis. Rapid diagnostics will also aid the process of defining the interrelationships between individual endogenous proviruses and their hosts.

The potential benefit for endogenous viral profiling in humans lies in the identification of carriers of the particular endogenous elements that are associated with morbidity states. Once such genetic risk factors have been determined, lifestyle adjustments can be made to avoid environmental factors that might compound the risks, and available treatment programs can be fine tuned to address specific disease potentials. For applications in other areas, such as farm animal health, molecular profiling of endogenous viral elements can be integrated with existing selection programs to reduce viral particle prevalence in food products, and to increase natural resistance to viral infection.

See also: **Diagnostic techniques: Detection of viral antigens, nucleic acids and specific antibodies, Isolation and identification by culture and microscopy; Immune response: Cell mediated immune response, General features; Human immunodeficiency viruses (*Retroviridae*): Molecular biology, Anti-retroviral agents, General features; Retroviral Oncogenes.**

Further Reading

Herniou E, Martin J, Miller K *et al* (1998) Retroviral diversity and distribution in vertebrates. *J. Virol.* 72: 5955.

Loewer L, Loewere J and Kurth R (1996) The viruses in all of us: characteristics and biological significance of human endogenous retrovirus sequences. *Proc. Natl Acad. Sci. USA* 93: 5177.

Meisler MH and Ting CN (1993) The remarkable evolutionary history of the human amylase genes. *Crit. Rev. Oral Biol. Med.* 4: 503.

Patience C, Wilkinson DA and Weiss RA (1997) Our retroviral heritage. *Trends Genet.* 13: 116.

ENTERIC VIRUSES

Ruth F Bishop, Department of Gastroenterology and Clinical Nutrition, Royal Children's Hospital, Parkville, Victoria, Australia

Introduction

Viruses are amongst the first microorganisms to colonize the germ-free intestine after birth, where they encounter conditions ideal for growth, including warmth, an abundant supply of nutrients and continual replenishment of susceptible cells. Many viruses identified in feces, including reoviruses, adenoviruses and enteroviruses, have the capacity to replicate in the intestine without causing clinical symptoms. Other viruses detected in feces may be derived from swallowed secretions originating in the respiratory tract, from plant viruses ingested in food, or from phages released after bacterial lysis. Many viruses use the gastrointestinal tract as a portal of entry and may replicate there before initiating systemic disease (e.g. poliovirus, hepatitis A and E). A diverse group of viruses (rotaviruses, adenoviruses, caliciviruses, astroviruses, coronaviruses) replicate in the epithelial cells lining the small intestine, causing sufficient destruction to result in the symptoms of enteric infection, including diarrhea, vomiting, fever and dehydration. A further group of viruses of systemic origin (e.g. measles virus, cytomegalovirus

Table 1 Viruses implicated in the etiology of acute diarrhea

Virus	*Family*	*Size (nm)*	*Genome*	*Host range*
Rotavirus	*Reoviridae*	70	dsRNA	
Group A				All mammalian and avian species
Group B				Pigs cattle, sheep, rats, humans
Group C				Pigs, ferrets, humans
Group D, F, G				Avian species
Group E				Pigs
Adenovirus Subgenus F Ad40, Ad41	*Adenoviridae*	70	dsDNA	Humans
Calicivirus	*Caliciviridae*	35–40	ssRNA	Humans, cattle, pigs, chickens, dogs
Astrovirus	*Astroviridae*	28–30	ssRNA	Humans, cattle, pigs, cats, dogs, avian species
Parvovirus	*Parvoviridae*	18–26	ssDNA	Cattle, cats, dogs, mink (humans)
Coronavirus	*Coronaviridae*	80–120 (pleomorphic fringed)	ssRNA	Pigs, cattle, foals, mice, rabbits, dogs, cats, turkeys (humans)
Torovirus	*Coronaviridae*	80–120 (pleomorphic fringed)	ssRNA	Cattle, horses (goats, sheep, pigs, rabbits, mice, humans)
Picobirnavirus	*Birnaviridae*	32–35	dsRNA	(Rats, cattle, pigs, hamsters, guinea pigs, humans)

ds, Double-stranded; ss, single-stranded. The role of the virus as an etiological agent of diarrhea is not proven in the species in parentheses.

(CMV), human immunodeficiency virus (HIV)) may multiply in gut-related cells, again causing symptoms of enteritis.

The diversity of viruses excreted in feces confused the interpretation of initial tissue culture-based surveys aimed at detecting etiological agents of acute nonbacterial gastroenteritis. It was only after electron microscopy revealed noncultivable viruses in diarrheal stools and not in control specimens that many viruses causing acute enteritis could be identified and studied in detail. Viruses belonging to at least seven different genera have now been implicated in the etiology of acute enteritis in animals, avian species and humans (**Table 1**).

History

The symptoms of acute diarrhea were described in the earliest medical writings, long before the concept of infectious agents was considered. Reports of viral enteritis are impossible to identify with accuracy because of the nonspecific nature of the symptoms resulting from injury to intestinal epithelial cells. However, nineteenth and early twentieth century accounts of 'pseudocholera infantum', as a severe watery diarrhea in young children during winter months, make it likely that rotavirus infection may have been implicated in this often fatal disease.

In 1943 a filtrate of stools from infants with diarrhea induced diarrhea in young calves. Many years later, rotaviruses were identified in calf feces obtained at the time. Similar experiments in the 1960s induced diarrhea in a proportion of human volunteers, giving evidence for involvement of an 'ultrafilterable agent' (presumed to be a virus) in the etiology of epidemic gastroenteritis. Eventually in 1972 the 27 nm Norwalk virus was identified, by immunoelectron microscopy, as a cause of gastroenteritis in older children and adults. In 1973 the 70 nm human rotavirus was identified by electron microscopy in duodenal tissue and stools from young children admitted to hospital for treatment of acute diarrhea. Similar particles had been described in 1963 as a cause of epidemic diarrhea of infant mice, and in 1967 as a cause of diarrhea in calves. Electron microscopy (**Fig. 1**) then revealed a great variety of other noncultivable viruses in stools, some of which have been shown to be etiological agents of acute enteritis in animals and humans, and others for which evidence of an etiological role is still not proven.

Rotaviruses (*Reoviridae*)

Rotaviruses are approximately 70 nm in diameter with a wheel-like appearance (*rota* is Latin for wheel) (**Fig. 2**). The genome is composed of double-stranded

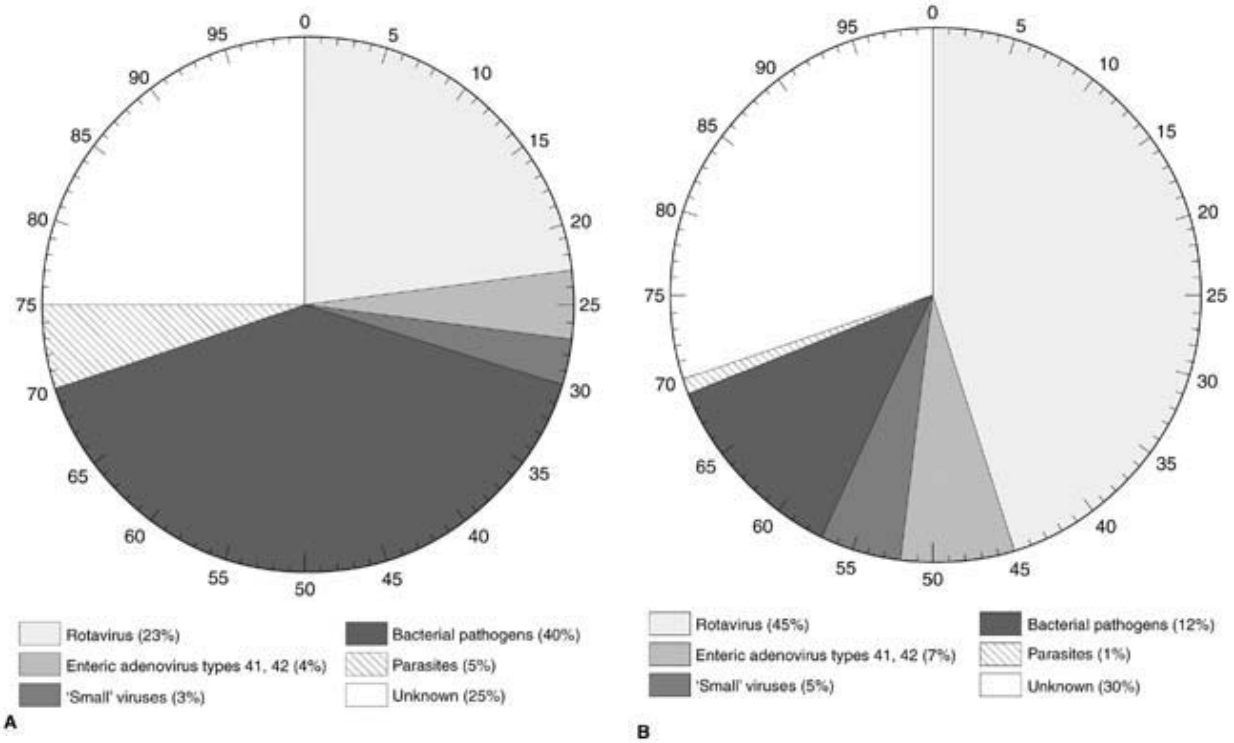


Figure 1 Etiology of severe acute diarrhea in young children in (**A**) developing and (**B**) developed countries (data compiled from published surveys).

RNA which can be separated into 11 segments (genes) by polyacrylamide gel electrophoresis. The inner capsid protein bears non-neutralizing antigenic epitopes that allow serological subdivision of the strains into groups A–F. The majority of human infections studied to date are caused by group A rotaviruses.

Group A rotaviruses

These are ubiquitous in nature and infect the young of all mammalian and avian species. They cause life-threatening diarrhea, predominantly in young children aged 6–24 months, and epidemics of mild to severe diarrhea in newborn animals and birds. Newborn babies are susceptible to infection, but this is often asymptomatic, either because rotaviruses adapted to growth in the neonatal intestine are naturally attenuated and/or because maternally derived antibody reduces the severity of symptoms. Reinfections occur regularly thoughout life. Group A rotaviruses have a worldwide distribution and cause 20–45% of disease in children admitted to hospital. Seasonal epidemics occur in children during the colder months of the year. Infection is endemic throughout the year in tropical countries. Group A rotaviruses present an almost limitless spectrum of genetic variation, but antigenic variation appears more restricted. Rotaviruses are species-specific, although cross-species infections can be induced under experimental conditions. When this occurs, rotavirus replication is minimal, thus reducing the likelihood of horizontal transmission of infection. Occasional zoonotic infections have occurred in humans. There is

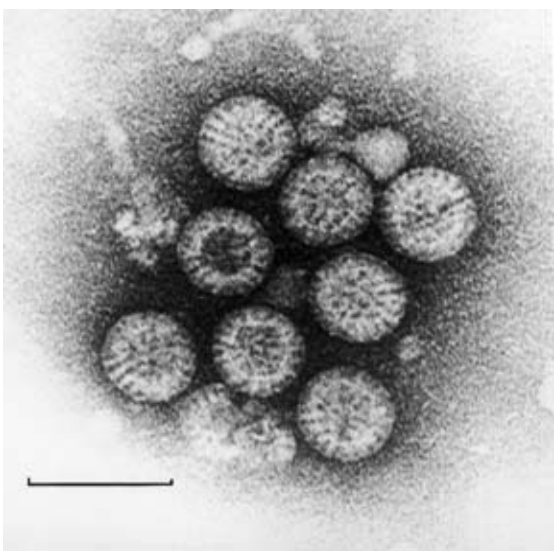


Figure 2 Electron microscopic appearance of negatively stained rotavirus particles. Bar = 100 nm.

a potential for human and animal strains to reassort in nature. During natural group A rotavirus infections, as many as 10^{10} particles ml^{-1} are shed in feces. The virus is highly infectious, spreads rapidly to contacts, and can retain infectivity for several months in the environment, and in sewage.

Group A rotaviruses are divided into serotypes by identification of neutralizing antigens on the two outer capsid proteins VP7 (G-type) and V4 (P-type). To date 14 G-types and 20 P-types have been identified. G1P1A[8] infections have been overwhelmingly predominant in humans worldwide during the past two decades.

The non-group A rotaviruses

Also referred to as 'novel' rotaviruses, pararotaviruses or atypical rotaviruses, these are morphologically identical with group A rotaviruses, although some disintegrate more readily and appear in negatively stained preparations as thin-walled featureless core particles. They show differing patterns of mobility of the 11 genome segments, and to date all have lacked the 7-8-9 gene triplet characteristic of group A rotaviruses. They do not react serologically in assays to identify group A rotaviruses. Attempts to produce reassortants between group A and non-group A rotavirus *in vitro* have been unsuccessful. All appear to be species-specific. All have been difficult to adapt to cell culture, with limited success using swine kidney cells (group B), swine testicular cells and CaCO-2 cells (group C).

Non-group A rotaviruses can be subdivided into serogroups B–G. Group B rotaviruses have been identified in pigs, cattle, sheep, rats and humans. Infections in farm animals may often be asymptomatic or cause only mild symptoms. The numbers of particles shed during infection appear to be less than in group A infections. Their distribution worldwide is uncertain. Serum surveys indicate a moderate prevalence (10–20%) of antibodies to group B rotaviruses in humans in China, the USA and Australia, and group B rotaviruses caused a large epidemic of severe enteritis in older children and adults in China in 1982–1983.

Group C rotavirus infections have been recorded in pigs, ferrets and humans. Infection is thought to be relatively common in pigs in the UK and USA (as judged by serum surveys) but severe disease may be comparatively rare. A similar situation may occur with humans. Antibodies have been detected in 43% of sera from humans (0–75 years), with the highest incidence (60%) in the 71–75 year age group, but severe infections are rare (<2% of children admitted to hospital worldwide). Most symptomatic infections have been associated with family or community outbreaks. Infection with group C rotaviruses has been suggested as a cause of biliary atresia in infant humans.

Groups D, F and G have been identified frequently in chickens, pheasants and turkeys in the UK and USA, where they appear to be more common than group A infections. Group E rotaviruses have been identified in pigs.

The importance of non-group A rotavirus infections in animals and humans will be debatable until suitable diagnostic tests are developed and used widely.

'Enteric' Adenoviruses (*Adenoviridae*)

Adenoviruses are nonenveloped icosahedral particles of 75–80 nm in diameter (**Fig. 3**). The virion contains a genome composed of double-stranded DNA coding for 10 different structural polypeptides. Human adenoviruses are classified into six subgenera, A–F (with different trophisms), each containing one or more serotypes. 'Enteric' adenoviruses causing disease in humans belong to subgenus F, serotypes 40 (Ad40) or 41 (Ad41). Restriction enzyme analysis indicates heterogeneity within both serotypes. Neither replicate in conventional cell culture but can

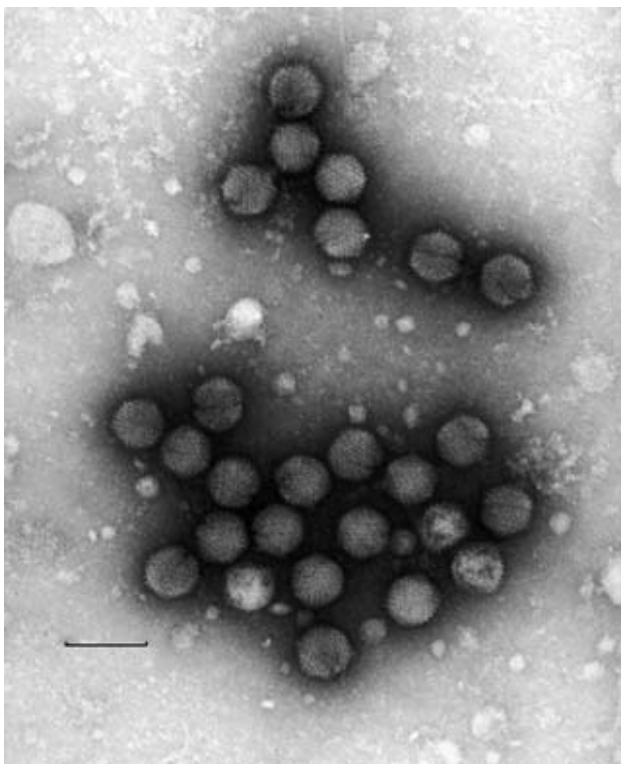


Figure 3 Enteric adenoviruses. Bar = 100 nm.

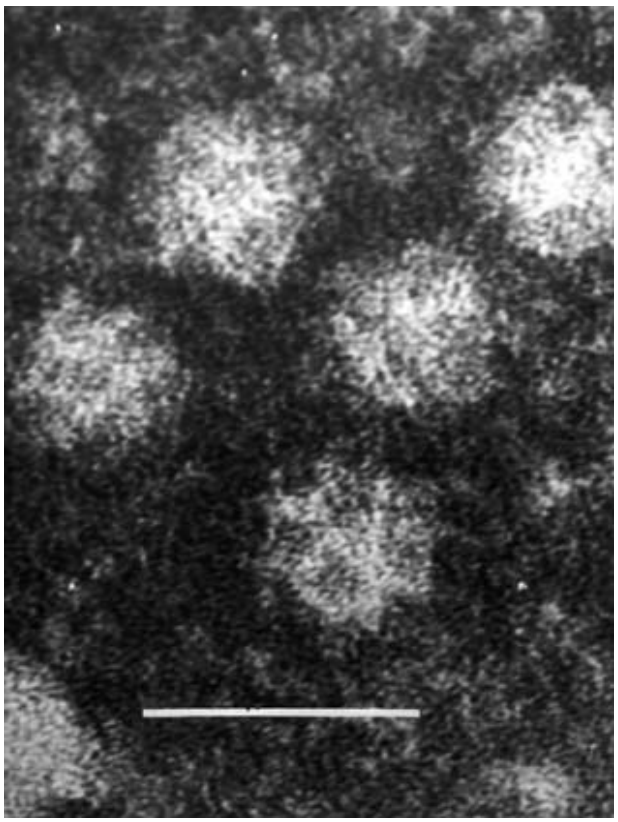


Figure 4 Caliciviruses (HuCV). Bar = 50 nm.

be coaxed to grow in Graham 293 cells (transfected with regions of Ad5).

Enteric adenoviruses (Ad40, Ad41) have been identified worldwide as a cause of severe diarrhea in 7–17% of children admitted to hospital. The relative incidence of Ad40 has declined since 1981, and Ad41 infections have shown a rapid rise in prevalence in North America, Europe and Australia. Enteric adenoviruses are a common cause of nosocomial infection, with an incubation period of 7–8 days. Diarrhea is often protracted, lasting up to 12 days, and viral excretion can be prolonged. Immunodeficiency may enhance enteric adenovirus infections because these viruses have been found more frequently than other enteric viruses in bone marrow transplant patients, and in patients with HIV-associated diarrhea. They cause fatal enteritis in immunodeficient foals.

Adenoviruses (of other serotypes) have been reported to cause hemorrhagic enteritis in turkeys, enteritis in mice and mild diarrhea in weaned piglets. Infections in calves or lambs are rare. 'Nonenteric' adenoviruses are common in human feces, perhaps derived from swallowed respiratory secretions. The ability of adenovirus 7 to colonize the human intestine asymptomatically has led to its suggested use as an oral vaccine vector.

Caliciviruses (*Caliciviridae*)

These are small (35–40 nm) single stranded RNA viruses (**Fig. 4**) 'Classical' caliciviruses show characteristic surface cup-shape indentations (calices), but many other members of this family are described as small round structureless viruses (SRSV) with an amorphous surface structure and a feathery ragged outline. Some of the size variations noted by electron microscopy and/or the relative scarcity of particles detected in feces may be due to proteolytic degradation of particles shed in feces. Caliciviruses possess only a single major structural polypeptide and comprise at least five genogroups. Caliciviruses associated with enteritis appear to be highly species-specific, and have been described in cattle (Newbury agent), pigs, chickens, dogs and humans.

The disease due to human caliciviruses (HuCV) was initially studied in adult volunteers using oral administration of fecal filtrates, leading to the discovery of Norwalk virus in 1972 by immune electron microscopy. This was followed by the description of many other small viruses implicated in gastroenteritis outbreaks. Most of these viruses were named after their geographical origin, e.g. Hawaii agent, Snow Mountain virus, Sapporo virus, Mexico virus, but now are known to have worldwide distribution. Identification and classification within HuCV took an enormous leap forward with the development of reverse transcriptase polymerase chain reaction primer-based hybridization assays, and enzyme immunoassays using recombinant antigens. Preliminary genetically based classification now recognizes three HuCV genogroups represented by Norwalk virus (NV), Snow Mountain agent (SMA) and Sapporo virus. The Sapporo virus genogroup has been detected in 0.5–6.6% of sporadic gastroenteritis in hospitalized children. The NV and SMA genogroups have predominantly caused outbreaks of diarrhea (in all age groups) as a result of ingestion of contaminated food/fluids. Etiological surveys undertaken in the USA have shown that approximately 65% of outbreaks of gastroenteritis in camps, cruise ships, nursing homes, schools and families have been associated with excretion of HuCV. HuCV are still recalcitrant to growth *in vitro*, and these viruses have not been visualized in intestinal tissue, despite repeated attempts.

Astroviruses (*Astroviridae*)

These viruses have a diameter of 28–30 nm and possess a positive-strand RNA genome coding for four structural polypeptides. A surface structure with a five- or six-pointed star-like appearance (hence

astrovirus) can be seen by electron microscopy on 5–10% of particles (**Fig. 5**). These viruses were first described in association with an outbreak of mild diarrhea in newborn infants. Strains have been identified in many animals including calf, lamb, pig, cat, dog, duck and turkey. Experimental infections are usually asymptomatic or produce only mild symptoms, although infected ducklings can develop fatal hepatitis. Infections appear to be host-specific. Seven human serotypes that are serologically distinct from animal strains have been described. Serotype 1 has been responsible for >50% of all human astrovirus infections described to date.

Human astrovirus infections occur worldwide, with peak incidence in winter/spring in temperate climates. Illness is usually mild, with an incubation period of 3–4 days, and is accompanied by seroconversion. Symptomatic astrovirus infection is mainly restricted to young children and can account for severe diarrhea in ≤5% of hospitalized children. Outbreaks of astrovirus gastroenteritis have been observed in nursing homes for the elderly (Marin county agent), among military recruits, and in immunocompromised patients. An enzyme immunoassay incorporating monoclonal antibodies and an RNA probe hybridization assay have been developed recently to facilitate diagnosis. Antibody prevalence surveys show that 65% of 3–4-year-old children and 87% of 5–10-year-old children possess serum antibody. The virus can be propagated in human embryo kidney cells in the presence of trypsin, and in human colonic carcinoma cell lines (CaC02, HT-29).

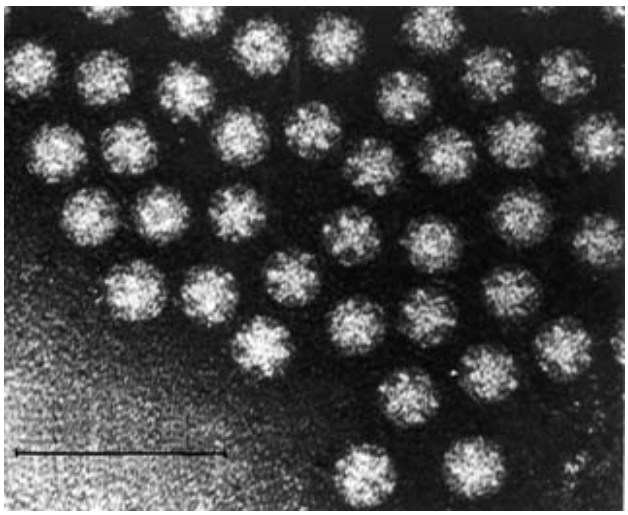


Figure 5 Astroviruses. Bar = 100 nm.

Parvoviruses (*Parvoviridae*)

These are small structureless single-stranded DNA viruses of 18–26 nm diameter. Some have caused severe acute gastroenteritis in cats, dogs, mink and calves. Pathogenesis clearly differs from most other enteric infections, as primary virus replication after ingestion occurs in lymphoid tissues, followed by viremia. Secondary replication (within a few days) occurs within the intestinal epithelium, where infection of crypt cells is widespread. No equivalent infectious agents have been described in humans. A heterogeneous collection of small round featureless viral particles of similar size has been described in diarrheal feces from humans. The particles have a size range of 22–26 nm diameter, with no discernible surface features and no sharply delineated outer edge. They resemble parvoviruses in morphology, size and buoyant density. These particles (Ditchling, W, Paramatta 'cockle agents') have been detected in stools of patients involved in approximately one-third of outbreaks of gastroenteritis attributed to ingestion of shellfish or cold foods. There has been frequent dual infection with other viruses confirmed as etiological agents of enteritis. No serological evidence of infection with these small viruses has been demonstrated. It is possible that these parvovirus-like particles are endemic 'intestinal' viruses that replicate to low titer in crypt cells, and that infection by other enteric viruses stimulates replication of crypt cells, favoring increased replication and fecal shedding of these particles.

Coronavirus/Coronavirus-like Particles (*Coronaviridae*)

Coronaviruses are enveloped single-stranded RNA viruses, visible in feces by electron microscopy as large pleomorphic particles (80–120 nm diameter) with a characteristic corona (crown) projecting as a single or double fringe of 10–20 nm length (**Fig. 6**). Coronaviruses are undisputed causes of epidemic diarrhea in newborn piglets *t*ransmissible gastro*e*nteritis *v*irus (TGEV), calves, foals, mice, rabbits, turkeys, dogs and cats. Large pleomorphic fringed particles resembling coronaviruses have been identified in humans in outbreaks of gastroenteritis among army personnel and hospital nurses, and in newborn babies with bloody diarrhea and necrotizing enterocolitis. Pleomorphic particles resembling coronaviruses have frequently been observed in symptomatic and asymptomatic adults and children living in crowded unhygienic conditions. The significance of many of the fringed particles seen in human feces in relation to enteric symptoms is controversial. In some circumstances, the particles may arise from degenerate fragments of intestinal tissue or they may be

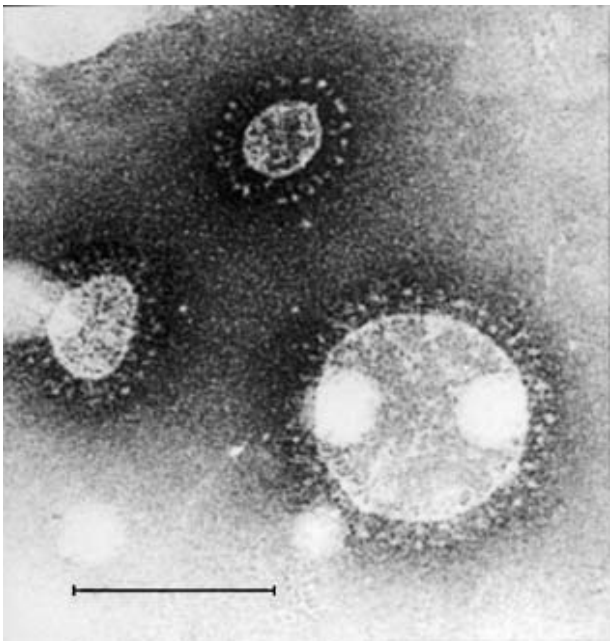


Figure 6 Coronavirus-like particle. Bar = 100 nm.

immunologically related to human respiratory coronaviruses. However, coronavirus-like particles shed in feces by babies with necrotizing enterocolitis replicated in human fetal intestinal organ culture, and homologous seroconversions were demonstrated in paired sera from these infants. Recognition and identification of human enteric coronaviruses requires development of specific diagnostic techniques. Only then will their importance in human infections be resolved.

Toroviruses (*Coronaviridae*)

These are positive-strand RNA viruses classified as a genus within the family *Coronaviridae*. They are visible by electron microscopy of feces as 100–140 nm pleomorphic fringed particles (spherical, kidney-shaped or elongated) with 10–20 nm long peplomers. These viruses infect a number of animal species but appear to be most common in horses (Berne virus) and calves (Breda viruses). Equine infections appear to be asymptomatic. Diarrhea due to Breda viruses has been reproduced experimentally in gnotobiotic calves. With the exception of Berne virus (successfully cultivated in equine dermal cells), none have been adapted to growth *in vitro*. Serum antibody surveys indicate that torovirus infection, but not disease, is widespread among cattle in the UK, Europe and the USA. Antibody has been detected in humans, goats, sheep, pigs, laboratory rabbits and wild mice. Toroviruses have been identified as a cause of diarrhea in humans (including children) in the UK, France, the USA and Canada. Etiological and epidemiological studies require improved methods for detection, and strict criteria to differentiate toroviruses from other 'fringed particles' seen in feces. These viruses may prove to be widespread and of low pathogenicity in the intestinal tract.

'Picobirnaviruses'

These are double-stranded RNA viruses (assumed to be members of the family *Birnaviridae*) initially detected by polyacrylamide gel electrophoresis (PAGE) in 14 of 3134 fecal samples from Brazilian patients with acute gastroenteritis. The 32–35 nm particles resemble viruses found in intestinal contents of rats, calves, pigs, hamsters and guinea pigs. Similar particles have since been reported in approximately 10% of humans (aged 3–70 years) in the UK, and in 10% of HIV-infected patients in the USA. 'Picobirnaviruses' appear to be widespread in humans but their role in human disease is not known.

Enteroviruses/Reoviruses

Routine cell culture of human feces will frequently show reoviruses and enteroviruses (including poliovirus, echoviruses, coxsackieviruses) excreted by patients with and without diarrhea. Most are coincidental infections, unrelated to an etiology of diarrhea, but there is evidence implicating group A coxsackieviruses and echoviruses types 11 and 18 as causes of diarrhea in normal and in immunodeficient patients. Reoviruses can produce diarrhea in animals, including mice, where the intestine is a portal of entry for systemic infection.

Other Viruses

Diarrhea is associated with a variety of other viruses that more characteristically produce systemic infections. Viremia can result in infection of gut epithelium or of cells in the lamina propria. For example, diarrhea is a common and severe complication of *measles* infection in children in tropical countries. CMV infection (in patients with HIV infection or immunoincompetence due to other causes) produces changes ranging from mild inflammation to ulceration and necrosis at all levels of the intestinal tract, associated with gastrointestinal symptoms relative to the level of gut infected. HIV may directly cause an enteropathy, with symptoms of weight loss, diarrhea, vomiting and abdominal pain.

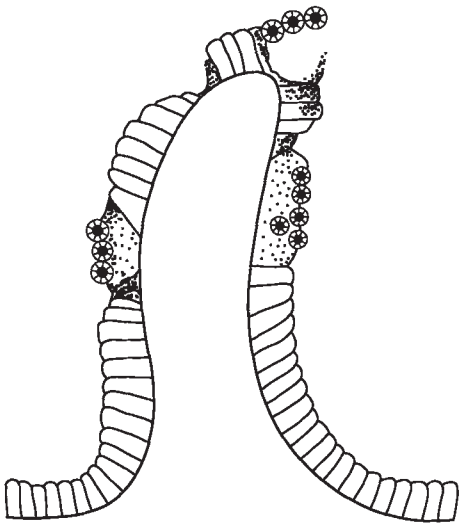


Figure 7 Small intestinal villus infected with rotavirus.

Clinical Features of Enteric Infection

Clinical features of viral enteric infection (regardless of agent) include an abrupt onset of vomiting, fever and diarrhea. Infections in adult humans (particularly with caliciviruses) predominantly involve nausea and vomiting. Profuse watery diarrhea in young children (and other young animals) can lead to dehydration, acidosis and electrolyte imbalance. Feces do not contain blood, white cells or mucus. Abdominal cramps occur but are much less frequent than with bacterial infections. Respiratory symptoms are present in 20–40% of children but may be coincidental. Vomiting usually persists for 24–48 h, and diarrhea for 2–7 days. Depression of mucosal disaccharidase activity leads to lactose malabsorption in a proportion of young children, and may persist and require dietary modification.

Pathology and Histopathology

Most enteric viruses implicated in the etiology of acute diarrhea replicate only in the mature absorptive epithelial cells of the villi of the small intestine (**Fig. 7**). The inability of these cells to undergo further replication *in vitro* has been the main barrier to adaptation of many enteric viruses to cell culture. *In vivo* replication in these cells produces similar histopathological changes in the intestine of animals and humans. Rotaviruses, Norwalk viruses, astroviruses and caliciviruses preferentially infect the upper small intestine, whereas adenoviruses, toroviruses and coronaviruses commonly infect the lower small intestine. Toroviruses also infect the colon. Most enteric viruses have been detected in M cells overlying lymphoid tissue, but do not appear to replicate in lamina propria or lymph nodes. Parvoviruses (and Breda viruses) also infect crypt cells. Only parvoviruses have been conclusively shown to have a viremic phase.

Histological changes observed in gut mucosa at all levels are similar. Initially, epithelial cells (infected and uninfected) are stripped from the sides and tips of villi, which may be denuded. Villi become stunted, and are covered with nonabsorptive cells derived from mitosis of crypt cells. The intestine is rapidly repopulated by mitosis of crypt cells, resulting in restitution of normal villus architecture covered by new mature absorptive villus cells. Crypt cell hyperplasia may be mediated via the hormone enteroglucagon, levels of which are raised in calves after enteric virus infections. The availability of newly regenerated absorptive cells may lead to a brief second wave of viral replication.

Treatment

Treatment consists of replacement of fluid losses, usually by administration of oral fluids containing glucose and electrolytes. Early resumption of normal feeding is encouraged, particularly in malnourished children. Breast-feeding can usually be continued throughout the illness. Lactose malabsorption may be a problem in a proportion of young children: reduction of lactose content in feeds may be required. There is no place for use of antibiotics or antiperistaltic agents in treatment of viral enteritis.

Immune Responses

The humoral immune response has been analyzed in great detail for human rotavirus infection. Specific antibodies detectable in intestinal contents and feces (of immunoglobulin IgA, IgM class) and serum antibodies (IgG, IgA, IgM class) appear during the first week after onset of infection. Serum antibody levels cannot be used as predictors of immunity to reinfection. The level of ingested or persisting antibody in the intestinal lumen can be predictive of immunity. Longitudinal studies of rotavirus infection in children (in developed and developing countries) show that primary infection (symptomatic or asymptomatic) does not confer immunity to reinfections, which are frequent, but does result in clinical immunity to development of severe symptoms during reinfection (**Fig. 8**). Immunity to caliciviruses following infection appears to be influenced by the infecting agent. Sporadic pediatric infections (due to Sapporo viruses) are associated with long-lived immunity. However, immunity to HuCV after epidemic out-

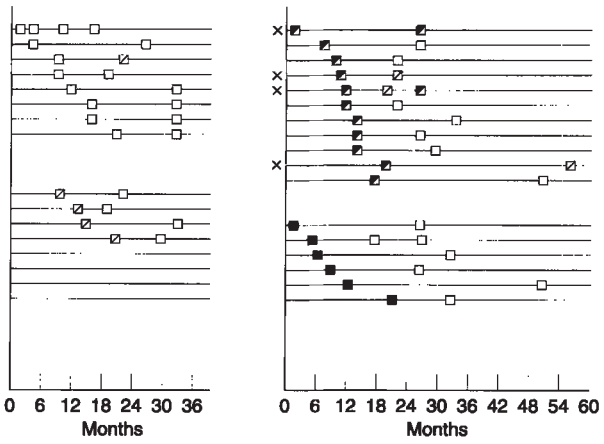


Figure 8 Longitudinal serological surveillance of rotavirus infection in 29 children recruited at birth and studied for 36–60 months (each line represents one child). □, Asymptomatic seroconversion; ▨, mild diarrhea on seroconversion; ◩, moderate diarrhea on seroconversion; ■, severe diarrhea on seroconversion; ×, children with more than one symptomatic infection.

breaks (in older age groups) is short-lived with no apparent protection against reinfection with the same viruses. Adult volunteers given Norwalk virus orally on more than one occasion were either repeatedly ill or persistently well. Volunteer studies with Norwalk virus show that approximately 50% of adults have an inherent resistance to infection (and disease) that has no detectable immunological basis. Cell-mediated immune responses have been demonstrated after rotavirus infection, and are important for cessation of virus excretion. Their role in long-term immunity is not known.

Prevention and Control

Some viral enteric infections, e.g. epidemics of waterborne or food-borne disease, could be controlled by improved sanitation and hygiene. Public health departments need to remain on the alert to monitor outbreaks and identify sources of infection. Infections where fecal/oral person-to-person spread is common are less likely to be controlled by improvements in sanitation. Implementation of strict crossinfection control measures could limit nosocomial spread of some infectious agents in hospitals, daycare centers, etc. However, the development of vaccines, particularly against rotavirus infection, is likely to have the greatest impact in both animals and humans on reduction of morbidity and mortality resulting from enteric viral infections.

See also: **Adenoviruses (*Adenoviridae*): General features; Astroviruses (*Astroviridae*); Birnaviruses – animal (*Birnaviridae*); Coronaviruses (*Coronaviridae*); Diagnostic techniques: Isolation and identification by culture and microscopy; Epidemiology of viral diseases; Immune response: General features; Norwalk and related viruses (*Caliciviridae*); Parvoviruses (*Parvoviridae*): Cats, dogs and mink; Pathogenesis: Animal viruses; Rotaviruses (*Reoviridae*): General features, Molecular biology; Toroviruses (*Coronaviridae*).**

Further Reading

Bishop RF (1993) Development of candidate rotavirus vaccines. *Vaccine* 11: 247.

Chiba S, Estes MK, Nakata S and Calisher CH (1996) Viral gastroenteritis. *Arch. Virol. Suppl.* 12.

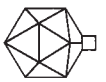
Ciba Foundation Symposium 128 (1987) *Novel Diarrhea Viruses*. New York: Wiley.

Estes MK (1996) Rotaviruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Field's Virology* 3rd edn, pp 1625–1655. Philadelphia: Lippincott-Raven.

Kapikian AZ (1994) *Viral Infections of the Gastrointestinal Tract*, 2nd edn. New York: Marcel Dekker.

Tiemessen CT and Kidd AH (1995) The subgroup F adenoviruses. *J. Gen. Virol.* 76: 481.

ENTEROBACTERIA PHAGE N4 (*PODOVIRIDAE*)



Lucia B Rothman-Denes, Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, Illinois, USA

Introduction

Coliphage N4 is a lytic phage specific for *Escherichia coli* K12 with a linear double-stranded genome 72 kb in length. The phage, originally isolated from the sewers of Genoa by G. C. Schito, displays several unique features, which include the sequential use of three different DNA-dependent RNA polymerases to transcribe its genome, a virion-encapsulated RNA polymerase which is injected into the host cell along with the phage DNA upon infection, the use of single-stranded DNA-binding proteins as transcriptional activators, and the presence of 3′ extensions at the ends of its linear genome.

Taxonomy and Classification

The phages of Enterobacteriaceae are classified with the *Podoviridae* in the family containing the T7-like phages. Phage N4 is assigned to the same genus, 'T7-like viruses' as the better-studied T7, based upon the size of its icosahedral head and short noncontractile tail. P22 has recently been assigned to a separate genus, 'P22-like viruses'.

Virion Structure

Electron microscopy of negatively stained N4 virions reveals particles with an icosahedral head 70 nm in diameter, a base plate, a small noncontractile tail, and short tail fibers originating at the neck. The particle weight is about 83×10^6, half of which is contributed by DNA. Virions contain at least 11 proteins, ranging in denatured molecular mass from 350 kDa to 16.5 kDa. The major capsid protein, which makes up 65% of the total protein of the virion, is 48 kDa.

The genome is a linear double-stranded DNA molecule approximately 72 kb in length with terminal direct repeats varying in length from 390 to 440 bp. The left and is relatively unique with the 5- or 6-base 3′ protruding sequences 3′-CATAA or 3′-CATAAA. In contrast, at least six discrete families are present at the right-hand end, differing in length by 10 bp and giving rise to the variability in the length of the terminal repeats. Each of these ends exhibits 1- to 3-base 3′ extensions.

Growth Cycle/Morphogenesis

Upon adsorption, the phage injects its DNA and a virion-encapsulated, DNA-dependent RNA polymerase which transcribes the early region of the genome. Synthesis of N4 middle transcripts, which appear 3 min after infection, requires the activities of three early proteins (p4, p7 and p17). Middle transcripts encode, in part, proteins required for N4 DNA replication. N4 DNA synthesis begins approximately 7 min after infection. Late RNA synthesis requires the activity of the *E. coli* RNA polymerase and begins 13–15 min after infection. A middle gene product, the N4 single-stranded DNA-binding protein (N4SSB), activates the host polymerase to initiate transcription at late promoters. Late RNAs code for morphogenetic proteins, including the virion RNA polymerase. Approximately 30 min after infection, the first progeny appear. N4-infected cells are lysis-inhibited, with up to 3000 N4 particles per infected cell accumulating intracellularly over a 3 h period. Enlarged cells filled with paracrystalline arrays of virions are observed under the electron microscope.

Gene Expression

N4 transcription is regulated in a hierarchical manner, with phage transcripts falling into three classes (early, middle and late) based on their time of appearance following infection. Early and middle transcription occur at the left half of the genome and proceed with a rightward polarity, whereas late transcription occurs at the right half of the genome with a leftward polarity.

Early transcription

Phage with temperature-sensitive mutations in the virion RNA polymerase are defective in early transcription, indicating a requirement for the virion RNA polymerase in this process. The virion RNA polymerase, which consists of a single, 3244 amino acid polypeptide, has been purified to homogeneity from N4 virions, where it is present in 1–2 copies. *In vitro,* virion RNA polymerase uses denatured N4 DNA as a template and exhibits little activity on native N4 DNA or heterologous denatured or native DNAs. Initiation of transcription on denatured N4

DNA occurs at unique sites which coincide with the sites of *in vivo* early transcription initiation. N4 virion RNA polymerase utilizes these sites efficiently and specifically when present on single-stranded vectors, indicating unequivocally that all the determinants of RNA polymerase–promoter interaction are present on the template DNA strand.

The three N4 early promoters share two features: interrupted sequence homology between −18 and +1 with the longest region being a GC-rich heptamer centered at −12, and a set of 5–7 base inverted repeats composed of both conserved and nonconserved bases, also centered at −12. Templates containing mutations at conserved positions have reduced *in vitro* activity, demonstrating that these bases are required for RNA polymerase recognition. Templates containing mutations that disrupt the inverted repeats at the nonconserved positions also have reduced activity, indicating that template single-strandedness is required for the formation of a stem–loop structure (hairpin) at the inverted repeats and that this hairpin and specific bases are required for productive RNA polymerase–promoter recognition.

However, single-stranded DNA is not the *in vivo* template for virion RNA polymerase. *In vivo,* early transcription requires the activity of two host gene products, *E. coli* DNA gyrase and *E. coli* single-stranded DNA binding protein (*Eco* SSB). *In vitro, Eco* SSB activates transcription on double-stranded, supercoiled but not on relaxed DNA templates. Transcriptional activation by *Eco* SSB is specific, as other single-stranded DNA binding proteins (i.e. T7 gene 2.5 protein, T4 gene 32 protein and N4SSB) cannot activate. These observations led to a model for virion RNA polymerase–promoter recognition whereby supercoiling elicits hairpin formation at the promoter region. *Eco* SSB subsequently activates by stabilizing a single-stranded region and allowing virion RNA polymerase to recognize the hairpin and specific bases at the promoters. This model predicts that the promoter hairpin must extrude in a supercoil-dependent manner and that *Eco* SSB must interact without destabilization of the extruded hairpin. Results from recent experiments indicate that both predictions are fulfilled. Contrary to certain theoretical predictions, hairpin extrusion at the promoters occurs at physiological superhelical densities in a Mg^{2+}-dependent manner. Specific sequences on the template strand, which define unusually stable DNA hairpins, are required for hairpin extrusion. In addition, DNA footprinting indicates that *Eco* SSB stabilizes the template-strand hairpin while the non-template strand hairpin is destabilized. Other single-stranded DNA binding proteins destabilize the template hairpin, explaining the specificity of *Eco* SSB activation. Therefore, *Eco* SSB acts as an activator of transcription by providing the appropriate DNA structure at the promoter required for N4 virion RNA polymerase binding.

The availability of an *in vitro* transcription system which recognizes the promoters on a double-stranded template allowed the identification of transcription termination sites for the N4 virion RNA polymerase. Sequencing of the template at the site of termination revealed a nine-base palindrome separated by five bases, followed by a row of five thymidines. These results suggest that the sequence determinants for N4 virion RNA polymerase termination may be similar to those for eubacterial factor-independent termination.

Middle transcription

N4 middle RNA synthesis requires the activity of three N4 early gene products. Two of these gene products, p4 (40 kDa) and p7 (30 kDa), are soluble, have been purified to homogeneity using an *in vitro* complementation assay, and constitute a heterodimeric, rifampicin-resistant RNA polymerase (N4 RNA polymerase II). This enzyme, although composed of two polypeptides, shares sequence homology with the single-subunit phage T7–T3 and the yeast mitochondrial RNA polymerases. However, purified N4 RNA polymerase II does not transcribe N4 DNA with *in vivo* specificity.

Conserved regions at seven sites of middle transcription initiation share the sequence 3′-t/aAAAT-5′ at the +1 region while the sequence 3′-Tt/aCTGGA-Ca/t-5′ is present 14–21 nucleotides upstream. *In vitro* utilization of these sites is dependent on purified N4 RNA polymerase II and a DNA-membrane salt-wash complex from N4-infected cells containing p17 (15 kDa), the third protein required for N4 middle RNA synthesis. However, purified p17 does not allow N4 RNA polymerase II to utilize middle promoters specifically, indicating that an additional factor is required for promoter recognition.

Late transcription

N4 late transcription is catalyzed by the host σ70-RNA polymerase. Indeed, temperature-sensitive *E. coli* mutants in the σ, β, or β' subunits of RNA polymerase are able to support N4 growth at the permissive but not the restrictive temperature. Late N4 transcripts appear about 8 min after N4 DNA replication has started, but their synthesis is not dependent on concurrent or previous N4 DNA replication. N4 mutants defective in the N4-coded DNA polymerase (*dnp*) or in *dns,* another N4 gene required for replication, are able to produce late transcripts.

Interestingly, N4 mutants defective in N4SSB are defective not only in N4 DNA replication but also in late transcription. Sites of late N4 transcription initiation have been identified by S1-nuclease mapping. The sequences of five such regions show weak homology to the *E. coli* promoter consensus sequence at the −10 and −35 regions. No strong sequence homology is shared by the late promoters at the −10 or −35 regions. A conserved sequence (5′AGTCGGTT3′) is present around the site of transcription initiation but its function is unknown. *In vitro* utilization of these sites by *E. coli* RNA polymerase requires the presence of N4SSB, demonstrating that N4SSB is a transcriptional activator.

N4SSB does not detectably bind double-stranded DNA. Therefore, the mechanism of N4SSB transcriptional activation must involve its single-stranded DNA-binding activity (for example, to facilitate isomerization from closed complex to open complex), or protein–protein interactions with *E. coli* RNA polymerase, or both. The isolation of N4SSB mutants defective in single-stranded DNA binding (and therefore in recombination and DNA replication), but proficient in transcriptional activation indicates that the single-stranded DNA binding activity of N4SSB is not required for transcription activation. In contrast, certain carboxy-terminal N4SSB mutants proficient in single-stranded DNA binding, recombination and replication are deficient in transcription activation, indicating that the carboxy-terminus of N4SSB comprises part or all of an 'activating region' that makes direct protein–protein contacts with RNA polymerase. Results of affinity chromatography experiments utilizing immobilized *E. coli* RNA polymerase indicate that N4SSB interacts specifically (activation-defective mutants do not interact) with both RNA polymerase core and holoenzyme.

N4 late transcription is blocked in an *E. coli* mutant that lacks the carboxy-terminal 52 amino acids of β' and has in their place 23 nonnative amino acids, indicating that the extreme carboxy-terminal region of β' plays a specific role in transcriptional activation by N4SSB. Results of site-specific protein–protein photocrosslinking showed that the interaction between N4SSB and *E. coli* RNA polymerase occurs exclusively within the carboxy-terminal 111 amino acids of the β' subunit. This region contains region H, which is conserved in sequence, followed by a negatively charged segment, which is conserved in charge, in the largest subunits of procaryotic RNA polymerase and eucaryotic RNA polymerase II. Since both these regions of the largest subunit of eucaryotic RNA polymerase II interact with transcription activators, it will be interesting to determine whether activation involving this region of the largest subunits of both procaryotic and eucaryotic RNA polymerases has similar mechanistic consequences.

The finding that N4SSB activates *E. coli* RNA polymerase through direct protein–protein interactions in the absence of DNA binding raises two questions. How does N4SSB achieve sufficient occupancy at target promoters? DNA binding to achieve occupancy might not be required since N4SSB is produced at very high levels ($\sim 10^{-5}$ M) during N4 infection. How does N4SSB achieve specificity for target promoters? It is possible that specificity is achieved through 'indirect readout' of promoter sequence, with N4SSB preferentially recognizing a specific conformation that RNA polymerase adopts only at N4SSB-dependent promoters. Alternatively, the specificity might be achieved kinetically, with N4SSB affecting a step in transcription initiation that is limiting only at N4SSB-dependent promoters. Finally, N4SSB might have only a limited specificity for target promoters; although limited specificity would be unsuitable for most activators, it might be suitable for a late-phase, lytic-viral activator.

Recombination

Very high levels of recombination have prevented the generation of an N4 linkage map. Recombination is independent of host recombination genes, suggesting that N4 encodes its own recombination functions. N4SSB is required for recombination.

Mutants/Genetic and Physical Map

The availability of cloned N4 fragments allowed the mapping of a large collection of suppressor-sensitive and temperature-sensitive mutants by marker rescue. The genes for the components of the N4 middle transcription apparatus, the N4 DNA polymerase, N4SSB and the N4 virion RNA polymerase have been mapped. The gene for the N4 virion RNA polymerase, which is part of the phage particle, is located with other morphogenetic proteins in the late region of the genome.

The isolation of heat- and citrate-induced deletions defined a 6 kb nonessential region in the middle transcriptional region. The ability to isolate heat- and citrate-resistant N4 phage implies that N4 does not encapsidate its DNA by full-head packaging but through the recognition of specific sequences at both ends of the genome. This is corroborated by the finding that deleted phages have wild-type terminal sequences.

The recent development of a method for constructing gene replacements, disruptions and deletions on the N4 chromosome by means of site-specific recom-

bination, analogous to the two-step gene replacement method in yeast, will be useful in investigating the role of N4 open reading frames of unknown function.

Effect on Host Cell

Immediately after infection, an as of yet unidentified early gene product inhibits host DNA replication. The host chromosome is not degraded and its transcription remains unaffected except for the shut-off of cAMP-dependent operons.

Replication

The functions of three host genes, *dnaF* (ribonucleotide reductase), *lig* (DNA ligase) and *gyrB* (DNA gyrase), are required for N4 replication. Cells defective in the polymerization activity of DNA polymerase I (*polA1*) support N4 growth; however, the 5′–3′ exonuclease activity of DNA polymerase I is required for processing of N4 Okazaki fragments. The functions of the *dnaA, dnaB, dnaC, dnaE* and *dnaG* genes of *E. coli* are not required for N4 DNA synthesis, suggesting that the phage must code for functions required for initiation, priming, polymerization and strand-displacement during replication.

Five phage-encoded functions involved in N4 replication, the N4 DNA polymerase (*dnp*), a single-stranded DNA binding protein (*dbp*), a 5′–3′ exonuclease (*exo*), the virion-encapsulated RNA polymerase and an unknown function (*dns*), have been identified through the isolation of phage containing amber- and temperature-sensitive mutations in each of these genes. Besides the virion-encapsulated RNA polymerase (see Early transcription), the N4 DNA polymerase, the single-stranded DNA binding protein and the 5′–3′ exonuclease have been purified to apparent homogeneity. The DNA polymerase (87 kDa) catalyzes accurate DNA synthesis due to an active 3′–5′ exonuclease activity. As with other replicative polymerases, the N4 enzyme lacks 5′–3′ exonuclease activity, cannot strand-displace and is nonprocessive. These results suggest that a processivity factor(s) and helicase activities must be N4-encoded. N4 DNA polymerase activity is greatly stimulated by N4SSB (32 kDa), which binds with moderate cooperativity to single-stranded DNA, covering 10 bp per protein monomer. The 5′–3′ N4-coded exonuclease (a 45 kDa polypeptide) exists as a dimer. Its preferred substrate is duplex DNA containing 3′ extensions (i.e. N4 DNA), which it degrades by a distributive mechanism. It is inactive on nicks or gaps. These properties suggest that the 5′–3′ exonuclease might play a role in recombination or replication of the ends of the N4 genome.

An *in vitro* replication system has been developed, which shows a marked preference for double-stranded N4 DNA as a template with little activity on heterologous or denatured templates. The system uses extracts from N4-infected *polA1* cells. Extracts from mutant N4-infected cells demonstrated that the *dnp, dbp* and *exo* gene products, but not the *dns* gene product, are required in the *in vitro* replication system. The *in vitro* system, therefore, does not possess all the properties of *in vivo* N4 replication. Replication in the *in vitro* system initiates at the ends of the N4 genome and proceeds towards the center. This is in agreement with electron microscopic analysis of *in vivo* replicating N4 DNA molecules, which shows Y-shaped molecules and molecules with single-stranded tails rather than replication bubbles, suggesting that origins of replication are located near the ends of the genome. Two-dimensional gel electrophoresis of the terminal restriction fragments from *in vitro* replicating N4 DNA molecules suggests that initiation occurs through hairpin priming of the single-stranded ends. Since the *in vivo* origin of N4 replication is not yet known, the relevance of the *in vitro* mode of initiation remains to be determined.

The utilization of an RNA primer has not been ruled out, and indeed the role that the N4 virion RNA polymerase plays in replication has not been elucidated. A possible role of this enzyme in replication initiation is suggested by the structure of 'activated' N4 virion RNA polymerase promoters, which is reminiscent of activated origins of replication. The accumulation of Okazaki-like fragments after infection of *polAex1* or *lig* mutants suggests discontinuous DNA synthesis and it is possible that the virion RNA polymerase synthesizes primers for this process as well.

The 3′ extensions of the N4 genome are unusual and, as all known DNA polymerases synthesize in a 5′ to 3′ direction, N4 must have a novel mechanism for replicating these sequences. Analysis of restriction products of intracellular N4 DNA detects a restriction fragment containing one copy of the terminal redundancy flanked by the right and left ends of the genome (joint fragment). Furthermore, *in vivo* pulse-chase experiments show that label accumulates in the joint fragment and chases into both the right and left genome ends. We have not yet identified the *in vivo* structure that contains the joint fragment but it is likely that this fragment originates through homologous recombination at the direct terminal repeats. Moreover, analysis of *in vivo* replicating N4 DNA by electron microscopy reveals longer than unit length molecules, suggesting concatemer formation. The enzyme or enzymes and the mechanism responsible for the generation of N4 DNA ends from processing of concatemers are unknown.

Host Range

The N4 genome is resistant to cleavage by a wide range of restriction endonucleases including *Ava*I, *BamH*I, *Ban*I, *Ban*II, *Cla*I, *Eco*RI, *Eco*RV, *Hind*III, *Kpn*I, *Nae*I, *Nar*I, *Nco*I, *Pst*I, *Pvu*I, *Pvu*II, *Sal*I, *Sma*I, *Sph*I, *Sst*I and *Sst*II, and *Xho*II, *Hae*II, *Mlu*I and *Nru*I cleave the 72 kb genome only once. Fragments of N4 cloned into *E. coli* vectors remain resistant to these restriction enzymes, indicating that resistance is due to the absence of enzyme recognition sequences and not to phage-induced base modifications or an N4-encoded antirestriction activity. The resistance of the N4 genome to restriction enzymes present in enterobacteria might reflect the host range of N4 in nature.

N4 infection is initiated by the attachment of the phage to, at most, five sites per bacterium. To characterize the N4 receptor, we have isolated and mapped mutations in *E. coli* that prevent adsorption of bacteriophage N4. The mutations map to four loci designated *nfrA, nfrB, nfrC* and *nfrD*. *NfrA* and *nfrB* map at 12′ on the *E. coli* map. Both genes have been cloned and sequenced. *NfrA* encodes a 96 kDa outer membrane protein which is presumed to be the bacteriophage receptor. The neighboring *nfrB* gene encodes a 70 kDa inner membrane protein whose exact role in N4 adsorption remains to be defined. We speculate that it may form a multicomponent receptor complex with the *nfrA* gene product or may serve as a channel through which the N4 genome and virion-encapsulated RNA polymerase pass during infection. The discovery of these genes has explained the restricted N4 host range. *E. coli* B is naturally N4-resistant because it lacks *nfrA* and *nfrB*. When a plasmid carrying these genes is introduced into *E. coli* B, it becomes sensitive to N4 infection.

The *nfrC* gene, which maps between *cya* and *ilv*, has been cloned and sequenced; maxicell experiments show that it encodes a 40 kDa polypeptide with UDP-*N*-acetylglucosamine-2-epimerase activity. *NfrD* maps to 50′ on the *E. coli* linkage map. The roles of these proteins in N4 adsorption and DNA and virion RNA polymerase injection remain to be elucidated.

Future Perspectives

N4 is unique among the lytic phages. The study of its transcription has provided novel mechanisms for RNA polymerase–promoter recognition, activation and the supercoil-dependent generation of noncanonical DNA structures. N4 RNA polymerase II is the smallest DNA-dependent RNA polymerase described to date and an excellent candidate for structure–function studies. The discovery of single-stranded DNA binding proteins behaving as transcriptional activators has endowed these proteins with a novel activity beyond their role in replication and recombination.

Little is known about the injection of proteins into bacterial cells. The study of the localization of the RNA polymerase in the virion and its injection into the host will provide clues as to the mechanism of this process. The generation of the 3′ single-stranded ends present on mature N4 DNA cannot be explained easily using known mechanisms of replication of linear genomes. Identification of the *in vivo* origin of N4 DNA replication and further characterization of *in vivo* replication intermediates will allow delineation of what could be a novel pathway for replicating linear genomes.

Finally, the imminent availability of the complete sequence of the N4 genome will provide clues as to its evolutionary origins.

See also: **Salmonella phage P22 (*Podoviridae*); T7-like phages (*Podoviridae*).**

Further Reading

Dai X, Greizerstein M, Nadas-Chinni K and Rothman-Denes LB (1997) Supercoil-induced extrusion of a regulatory DNA hairpin. *Proc. Natl Acad. Sci. USA* 94: 2174.

Glucksmann-Kuis AM, Dai X, Markiewicz PM and Rothman-Denes LB (1996) *E. coli* SSB activation of N4 virion RNA polymerase: specific stabilization of an essential DNA hairpin required for promoter recognition. *Cell* 84: 147.

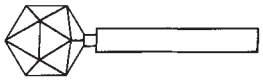
Kiino D and Rothman-Denes LB (1989) Genetic analysis of bacteriophage N4 adsorption. *J. Bacteriol.* 171: 4595.

Lindberg G, Pearle MS and Rothman-Denes LB (1988) N4 DNA replication in vivo and in vitro. In: Moses RE and Summers WC (eds) *DNA Replication and Mutagenesis*, p. 130. ASM: Washington, DC.

Miller A, Wood D, Ebright RH and Rothman-Denes LB (1997) RNA polymerase β' subunit: target of DNA-binding-independent transcriptional activation. *Science* 275: 1655.

Rothman-Denes LB (1995) DNA supercoiling, DNA hairpins and single-stranded DNA binding proteins in bacteriophage N4 transcription regulation. *Semin. Virol: Recent Adv. Bacterial Virol.* 6: 15.

ENTEROBACTERIA PHAGE P1 (*MYOVIRIDAE*)



Hansjörg Lehnherr, University of Odense, Institute for Molecular Biology, Odense, Denmark

Jürg Meyer, University of Basel, Dental Center, Basel, Switzerland

History

Bacteriophage P1 was one of three phages isolated in 1951 by Bertani from the lysogenic *Escherichia coli* strain of Lisbonne and Carrère. The phages, called P1, P2 and P3, had different plaque sizes on a particular strain of *Shigella dysenteriae* and were serologically distinguishable. P1 did not attract much attention until Lennox discovered the P1-mediated generalized transduction of chromosomal markers between strains of *E. coli* and *Shigella*. Furthermore, Lennox isolated a mutant, P1*kc*, which produced slightly larger (but still rather small) plaques, from which the strains studied later were derived.

P1 gained tremendous practical importance as a transducing phage and is still extensively exploited in bacterial research. It also has served as a model system for the study of several fundamental aspects of bacterial and phage biology:

- P1 specifies its own DNA-restriction-modification system, which was extensively studied by Arber and provided first insights into the biology of such systems. Thus, P1 was at the root of genetic engineering.
- The P1 genome encodes two site-specific recombination systems, which play important roles in its life cycle. In addition, P1 DNA carries a copy of the insertion element IS1. These systems contribute to the formation of specialized transducing phages and may be involved in generalized transduction.
- The P1 prophage exists extrachromosomally as a stably maintained low copy number plasmid. Studies of replication, partition, incompatibility and plasmid addiction (postsegregational host killing) have been fruitful for the understanding of the plasmid way of (prophage) life.
- P1 specifies a complex immunity system, intricately balancing the decision between lytic and lysogenic growth. Important elements of the immunity systems have been revealed by comparison to P7 (originally called ϕAmp), a heteroimmune phage which is otherwise closely related to P1.

Classification

P1 is in the genus 'P1-like viruses' of the *Myoviridae* family.

Virion Structure and Proteins

Three phage particles with similar tails, but with icosahedral heads of different diameters, are present in P1 lysates: B (big) 85 nm, S (small) 65 nm and M (minute) 47 nm. The inflexible tail is composed of a head–neck connector, a tail tube and a contractile sheath, and ends in a baseplate with six kinked tail fibers (**Fig. 1**). The proportion of the three head variants in a population of P1 virions depends on the P1 genotype and on the host (for P1 wild-type in *E. coli* K12 P1B 70–94%, P1S 6–27%, P1M 0.2–4%). All three phage particles can adsorb to host cells and infect their DNA, but only P1B particles are infectious because they contain the entire P1 genome. They hold slightly more than 100 kb of double-stranded linear DNA, which is circularly permuted with a terminal redundancy of about 10%. P1S particles contain about 36–40 kb of circularly permuted P1 DNA. At high multiplicities of infection, P1S virions can be infectious, due to recombination to a complete genome.

The phage head is composed of one major and 14 auxiliary proteins, while two major and seven auxiliary proteins contribute to the tail structure. The location of four additional virion proteins has not been assigned so far.

Genome Structure and Organization

Phage P1 has a genome of about 100 kb, thus having a coding capacity of about 100 genes. Fewer than 50 have been identified by genetic analyses. Interestingly, the genetic map based on recombination frequencies is linear, even though the phage DNA circularizes upon infection and the prophage is a plasmid. This discontinuity comes about by the action of the P1 *lox-cre* site-specific recombination system. The re-

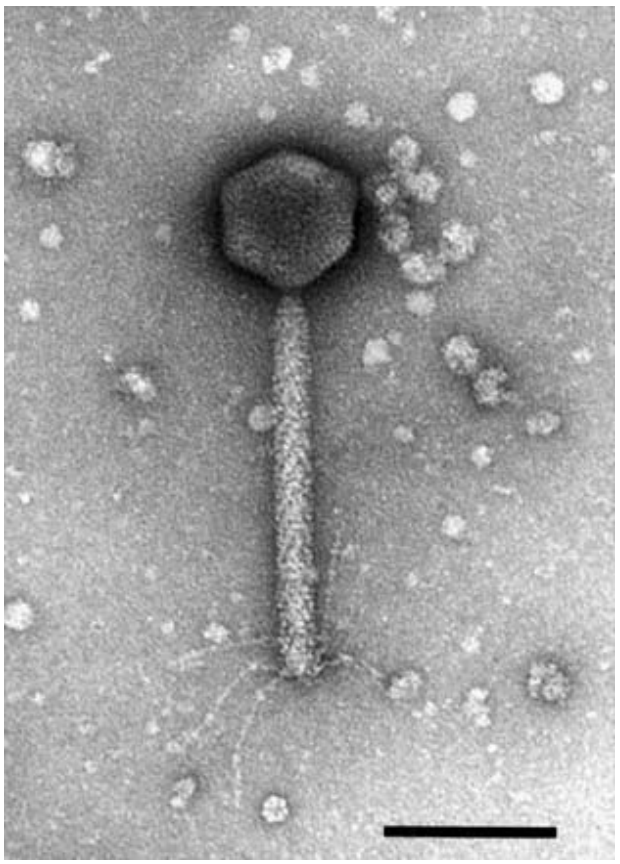


Figure 1 Electron micrograph of a phage P1 particle. Bar=100 nm. (Kindly supplied by Dr Michel Wurtz.)

combinational hot spot (*loxP*) marks the end of the genetic map (**Fig. 2**).

Packaging of P1 is initiated at the *pac* site, which is located about 5 kb from *loxP*, and proceeds in this direction. If a P1B particle results from the first round of headful packaging, it will contain two *loxP* sites in the terminally redundant DNA.

P1 DNA contains a 4.2 kb invertible segment, which is composed of 3 kb of unique sequence, flanked by 0.62 kb of inverted repeats. This segment, called the C-segment, encodes two sets of tail fiber functions and provides P1 with two alternative host ranges by changing its orientation in the prophage.

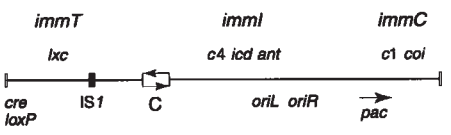


Figure 2 Simplified genome map of phage P1. The regions and functions shown are discussed in the text. The boxes at the termini represent *loxP*. The arrow above *pac* indicates the direction of DNA packaging.

Related functions are scattered on the P1 genome. Immunity determinants are found in three well-separated regions. Similarly, head and tail genes are each located in at least three distinct regions. This dispersion is reminiscent of the genome organization of the T-even phages.

Replication Cycle

The lytic cycle of phage P1 in *E. coli* and *S. dysenteriae* hosts takes about 60 min and yields 100–200 progeny phages per bacterium.

Phage P1 attaches by its tail fibers to its receptors, which have been postulated to be a terminal or subterminal glucose or galactose moiety of the lipopolysaccharide core of the bacterial outer membrane. How the linear DNA is injected into the host cell has not been studied; however, within a few minutes it is converted into a closed circular form.

Presumably, this rapid circularization is important to protect the DNA against degradation by cellular nucleases, e.g. the RecBCD enzyme, against which P1 perhaps expresses some activity, as has been established for other phages. The cyclization of P1 DNA is mediated efficiently by the *lox-cre* system. The Cre recombinase mediates a site-specific recombination reaction between two 34 bp *loxP* sites. It functions *in vivo* and *in vitro* without requiring any accessory factors. The *lox-cre* system can be replaced by other recombination systems dependent on the host *recA* function, which might be stimulated by the P1 function *ref* and/or by DNA ends. It is interesting to note that phage P7 DNA is circularized efficiently in a *recA* host, even though its terminal redundancy is less than 1 kb, and is unlikely to contain two *loxP* sites.

Replication of P1 DNA can be detected as early as 5 min after infection, and by 30 min reaches a level of about 95% of bacterial DNA synthesis. While in the early phase, σ forms and θ forms are found with equal frequencies, σ forms are found almost exclusively in the late phase, as was revealed by electron microscopy of replication intermediates. The σ forms appear to result from rolling circle replication, rather than from recombination, and they produce concatemeric DNA. Thus, like λ, P1 seems to be able to replicate by two different modes.

The σ and θ replication are predominantly initiated at *oriL*. However, vegetative DNA replication is clearly independent of the plasmid replication system, *oriR*, used in the prophage state. There are P1 mutants defective for vegetative replication, which are unaffected in their plasmid replication. Conversely, there are others with intact vegetative growth, but no plasmid replication. Furthermore, vegetative

and plasmid replication are affected differently by mutations of host functions (e.g. *recA* or *dnaA*). *oriL* encodes one essential protein, RepL, and is activated by transcription from the upstream promoter Pr53. This promoter is negatively regulated by the major repressor protein C1 and by a 180 bp antisense RNA, which is expressed from Pr53as, initiating transcription in opposite and convergent direction to Pr53.

The morphogenetic pathways have not been studied extensively. Only a few head genes have been identified, and the function of most of them remains to be determined. Head morphogenesis proceeds via a prohead and requires a maturation protease, involved in the processing of phage proteins. A nonessential gene, *vad,* modulates the ratio of P1B to P1S particles. Most of the tail genes have probably been identified by mutation, but their interactions during the assembly process have yet to be clarified.

Detailed information has been obtained on the phage-specific DNA-recognition and cleavage functions, *pac,* mediating the packaging of viral DNA into proheads. Mature P1B and P1S particles appear to result from 'headful' packaging, starting from the same concatemeric DNA. Filling of the first head starts at the *pac* site and proceeds unidirectionally. Three to four 'headfuls' were estimated to be processed sequentially, but the number may be two to three times higher, depending on the length of the P1 concatemer substrate.

The *pac* cleavage site is located within the 5′ part of *pacA,* which together with the adjacent gene, *pacB,* specifies the *pac* endonuclease. Almost symmetrically located around the major cleavage site are eight copies of the hexanucleotide sequence 5′-TGATCA(G), seven of which are contained in the 162 bp minimal *pac* segment. They are the binding sites for the *pac* endonuclease. The actual cleavage termini are distributed nonrandomly around one helical turn of DNA. Preferred cleavage sites on the two strands are staggered by 2 bp. The hexanucleotides also contain DNA adenine methyltransferase (*dam*) methylation sites (5′-GATC). As *pac* endonuclease activity is found as early as 10 min after initiation of the lytic growth cycle, expressed first from the *c*1-regulated promoter Pr94 and later from the late stage-specific promoter LPr95, further regulation of *pac* cleavage occurs via adenine methylation. Methylated *pac* sites are cleavable, whereas unmethylated sites are not. These *dam* methylation sites are not only a substrate for the host chromosomal enzyme, but also for a P1-encoded adenine methylase, the expression of which is regulated by the *c*1 repressor.

Host cell lysis is dependent on three genes, *lydA, lydB* and gene *17.* The last specifies a T4-like lysozyme and is essential for cell lysis, while the first two are dispensable. The product of *lydA* is a holin, thought to provide the lysozyme with improved access to the bacterial cell wall. The product of *lydB* antagonizes the holin, thus fine tuning the timing of cell lysis.

Mutations

By 1980 two collections of about 100 amber mutations which affect P1 plaque formation had been assigned to 18 and 31 complementation groups, respectively, and many have been mapped genetically. The two nonessential genes *res* and *mod* specifying the P1 restriction-modification system have been mapped by transposon mutagenesis. Thus, of the estimated coding capacity of the P1 genome of about 100 genes, less than half are defined by mutation. A project to determine the complete sequence of the P1 genome is near completion. It is expected to reveal a wealth of new genes, the functions of which have yet to be determined.

Lysogeny and Immunity

Phage P1 encodes a tripartite immunity system composed of the *immC, immI* and *immT* regions (**Fig. 2**). The *immC* region encodes the *c*1 repressor and the C1 inactivator function *coi.* The major repressor protein C1 is responsible for maintaining the lysogenic state. It binds to at least 17 operator sequences, which show a 17 bp asymmetrical consensus sequence and are scattered widely over the P1 genome. Upon infection both C1 and Coi are expressed simultaneously. To establish lysogeny C1 has to repress the expression of Coi by binding to an operator sequence located in the *coi* promoter. Coi, in turn, promotes lytic growth by inactivating C1 upon the formation of a 1:1 complex with C1.

To maintain the lysogenic state the major repressor C1 is assisted by the auxiliary repressor protein Lxc (*l*owers e*x*pression of *c*1, formerly called Bof), encoded in the *immT* region. Lxc interacts directly with C1 and increases the binding activity of C1 for all operator sequences. However, as C1 autoregulates its own expression and the interaction with Lxc results in increased autorepression, the total concentration of C1 in the cell is reduced. This lower C1 concentration in turn results in decreased repression of several *c*1-controlled genes. Having these two antagonistic effects Lxc modulates C1 activity, stabilizing the lysogenic state, but keeping the repression level low and thus facilitating a potential shift to the lytic mode of growth.

The three functions *c*4, *icd* and *ant,* located in the *immI* region, are coexpressed from two promoters, the strong *c*1-regulated promoter Pr51a and the

weaker, constitutive promoter Pr51b. The product of the *c*4 gene is a 77 bp antisense RNA, acting as a translational repressor of the adjacent *icd* gene and indirectly also of the *ant* gene, as *icd* and *ant* are translationally coupled. This blockage of *icd-ant* translation also leads to premature termination of *icd-ant* transcription, via a *rho*-dependent transcriptional terminator. The *c*4 RNA is synthesized as a precursor and needs to be processed by RNaseP in order to be functional. C4 is essential for establishing lysogeny and is solely responsible for the heteroimmunity of P1 and P7. Only in the absence of functional C4 are *icd* and *ant* synthesized. The Icd product interferes with host cell division and eventually leads to cell death, but *per se* is not required for *ant* expression. The functional antirepressor molecule, Ant, is a heterodimer composed of Ant1 and Ant2, the latter being initiated at an in-frame start codon in the *ant* gene. Ant in 2–4-fold molar excess forms a complex with and precipitates C1, thereby inactivating it. Differences between P1 and P7 indicate that a phage-specific loading site, called *sas* (for *s*ite of *a*nt *s*pecificity) might be essential for Ant to interact with DNA-bound C1 repressor molecules.

P1 is inducible by UV irradiation and other agents causing DNA damage, but the induction is not mediated by *recA*-assisted cleavage of an immunity repressor. It remains an enigma why bacteriophage P1 maintains such a complex immunity system in order to regulate the choice between lytic and lysogenic growth.

Plasmid Replication and Partition

P1 prophages replicate autonomously as plasmids and regulate their copy number at 1–2 copies per host chromosome. Like other plasmids with low copy number, P1 carries functions which ensure reliable partition of prophages to the daughter cells. Two P1 prophages cannot stably coexist in the same host cell, that is, they display – like plasmids in general – incompatibility behavior.

Two incompatibility determinants, *incA* and *incB*, were identified in separate regions of the P1 genome (**Fig. 3**). Either one is sufficient to destabilize P1, but only *incA* also destabilizes P7, and thus represents the incompatibility determinant of the IncY plasmids. Based on the fact that incompatibility determinants are involved in the replication and/or partition of plasmids, these P1 genome regions were searched for stable plasmid replicons. Between *incB* and the *imml* region, the L-replicon (*oriL*) was found, which is under *c*1 control and is used for lytic replication. As repression is incomplete, the lytic replicon may occasionally act as an auxiliary in plasmid maintenance.

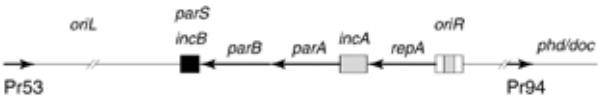


Figure 3 Organization of the replication and partition region of P1. See text for details.

On a 1.5 kb DNA segment including *incA*, the R-replicon was identified. This replicon is sufficient for P1 plasmid replication, because it replicates with the same low copy number as intact P1. The R-replicon consists of an origin (*oriR*) of not more than 245 bp, a gene (*repA*) encoding an essential replication initiator protein, and the control element *incA* of 285 bp, which is dispensable for DNA replication. The R-replicon also contains recognition sequences for the *E. coli* DnaA initiator protein, which is required for *oriR* replication together with several other host proteins (DnaB helicase, DnaC, DNA gyrase, RNA polymerase). Plasmid replication is also modulated by DNA methylation. Several *dam* methylation sites (5′-GATC) are located in *oriR* and the host-encoded protein SeqA binds the hemimethylated form of the origin, exerting negative control (preventing premature reinitiation) by sequestration.

The *repA* promoter located within *oriR* straddles one of the five directly repeated copies of a 19 bp sequence called 'iterons', to which RepA protein binds, resulting in autoregulation of *repA* expression. Nine additional iterons constitute *incA*, located at the 3′ end of the *repA* gene. According to a current model of replication control, involving protein-mediated DNA looping (handcuffing), RepA mediates the binding of *incA* to *oriR* so as to sterically block initiation of DNA replication at *oriR*.

Analysis of *in vitro* P1 DNA replication showed that RepA monomers efficiently bind *oriR* and *incA*, while RepA dimers are inactive. The *E. coli* heat shock proteins DnaJ, DnaK and GrpE stimulate RepA binding by monomerization of RepA dimers. The three heat shock proteins act coordinately as molecular chaperones in an ATP- and temperature-dependent reaction in the absence of *oriR* DNA.

The P1 prophage is rarely lost from its host cell (about 10^{-5} per generation). Several mechanisms contribute to this high stability. The site-specific *lox-cre* recombination system efficiently resolves plasmid dimers, ensuring that monomeric plasmid copies are present at the moment of cell division. Mutations in the partition functions *par* may lead to about a 100-fold increase in loss rate. The *par* region is located adjacent to *incA* and contains a promoter

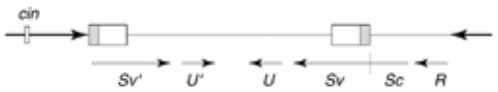


Figure 4 Organization of the DNA inversion system of P1. The *cin* recombinase gene contains the recombinational enhancer sequence (blank rectangle). The large boxes represent the 0.62 kb inverted repeats carrying the recombination sites *cix* (hatched part) at the termini. Arrows indicate the direction of transcription of the genes, the synthesis of which is controlled by DNA inversion. In the opposite orientation Sc is fused to *Sv'* resulting in *S'*.

expressing two genes, *parA* and *parB*, and the *cis*-acting (centromere-like) site, *parS*, which coincides with *incB*. The formation of a functional partition complex requires binding of the ParB protein to *parS*, a reaction which is assisted by the host protein IHF (*i*ntegration *h*ost *f*actor). The *parS* site contains a 13 bp inverted repeat sequence, which overlaps with the heptamer and hexamer sequences defining ParB-binding sites.

While the plasmid replication functions of P1 are interchangeable with P7, the partition functions are not, although they are clearly related. The organization of the *rep-par* region of P1 is very similar to that of the functional homologue of the F plasmid, but the DNA sequences are considerably divergent. In addition to the *par* functions, P1 also encodes a postsegregational host killing system, stabilizing the prophage by killing plasmid-free cells. Two small proteins, called Phd and Doc, are expressed from an autoregulated promoter. Phd is an antidote, forming a neutralizing complex with the cell toxin, Doc. Phd is slowly degraded by the host encoded protease ClpXP, while Doc is stable. Upon loss of P1 the antidote protein is degraded and the toxin is able to kill the plasmid-free cell.

Gene Expression

The few studies of gene expression indicate that some genome segments of the prophage are transcribed and are thus not repressed by the *c*1 repressor. According to a model proposed by Sternberg, early phage functions may be activated by replicative induction: at low concentration of *c*1 repressor, the lytic origin *oriL* is expressed from the strong *c*1-controlled promoter Pr53, and lytic DNA replication is initiated. Expression of the genes required for the lytic cycle is stimulated on one hand by an increased gene dosage; on the other hand transient undermethylation may increase the strength of some promoters, as has been shown for the *cre* promoter.

The host core RNA polymerase is probably used throughout the replication cycle. A single phage encoded gene, *lpa* (*l*ate *p*romoter *a*ctivator), mediates the transition from early to late gene expression. The *lpa* gene is *c*1-regulated and is expressed as soon as the C1 concentration in the cell drops below a certain threshold level. Lpa then activates transcription from P1-specific late promoter sequences, which, in common with the operator sequences, are scattered over the entire genome. The late promoters show a conserved *E. coli*-like −10 region and a 9 bp palindromic sequence centered around position −22, but they lack homology to the *E. coli* −35 region. Whether Lpa is a DNA-binding activator or modifies the host RNA polymerase remains to be determined.

DNA-Restriction-Modification and Antirestriction Systems

Phages P1 and P7 encode the type III restriction-modification enzyme *Eco*P1, which is composed of two subunits, the products of the *res* and *mod* genes. The Mod protein is responsible for both recognition of the specific DNA sequence 5′-AGACC and its modification methylation (A becomes N^6-methyladenine). It forms a complex with the Res protein which cuts DNA about 25 bp to the 3′ side of the recognition sequence. The *Eco*P1 enzyme displays both restriction and modification activities simultaneously so that unmodified DNA is not completely restricted as a result of modification of some sites. Because of the asymmetry of the recognition sequence, a fully modified site contains a methyl group in one strand only. The totally unmodified recognition sequence in newly synthesized DNA escapes restriction, because restriction requires two unmodified sites in inverse orientation (all of the nonmodified sites in newly replicated DNA are in the same orientation).

The two genes, *mod* and *res*, are organized in one operon. One would expect that, after P1 infection, the *mod* function would be expressed before the *res* function, in order to protect the host chromosome. Indeed, modification activity can be detected within a few minutes after infection, while the restriction function is fully developed only after 3–4 hours. How this regulation is achieved is at present still unclear, but the regulation is not at the transcriptional level, because by 30 min after infection both Mod and Res proteins can be detected.

Phage P1 also encodes an antirestriction function, which protects P1 DNA against type I restriction enzymes, i.e. the chromosomally encoded restriction endonucleases of the *Enterobacteriaceae*. It is based on the action *in cis* of two phage proteins, DarA and

DarB, which are incorporated into the phage head and then injected into host cells along with the phage genome.

Control of Host Range by DNA Inversion

Inversion of the 4.2 kb C-segment of the P1 genome (**Fig. 4**), leads to the expression of alternative tail fiber genes (*R, S, U* or *R, S′, U′*) and thus to (mixed) populations of two different phage particles with different host ranges. The C-segment is largely homologous to the smaller G-segment of phage Mu, and by analogy its orientation is indicated as C(+) or C(−). The correlation of type of tail fiber with host bacterium has not been studied as extensively as for phage Mu, but P1-C(+) particles infect *E. coli* K-12, while P1-C(−) virions do not; both phages form plaques on an *E. coli* C mutant. The receptors for both particles are the lipopolysaccharides of the outer membrane. Phages derived from infection carry the C-segment predominantly (>99%) in the original orientation, while, after induction of lysogens, both orientations are represented with equal frequency.

Recombination occurs between 26 bp imperfect inverted repeat sequences, *cix*, which flank the C-segment, and is mediated by the phage Cin protein, a site-specific recombinase acting as DNA invertase. The *cin* gene is located immediately adjacent to the inverted repeats flanking the C-segment and contains an approximately 75 bp DNA sequence, which stimulates the DNA inversion reaction *in cis* and controls the topology of the reaction. This recombinational enhancer sequence contains two binding sites for the host factor FIS (*f*actor for *i*nversion *s*timulation). Recombination sites and DNA invertase are nearly identical between P1 and P7 and also closely related to those of phage Mu and the multiple inversion system of the P1-related plasmid p15B. The different invertases complement each other.

Occasional Integration of P1 into the Host Chromosome: Significance for Transduction

The P1 and P7 prophages can integrate into the host chromosome, although with low probability. This integration is documented by the fact that P1 and P7 can suppress the replication defect of some *E. coli dnaA*ts mutants. This phenomenon is known as integrative suppression. Under nonpermissive conditions, the bacterial chromosome can be replicated from an integrated prophage origin. This implies that *oriR* replication is less sensitive to the impaired DnaA function than the chromosomal origin and/or that replication initiates from a *dnaA*-independent origin, (e.g. *oriL*). Another consequence of prophage integration may be the production of specialized transducing phage particles.

The integration is mediated in most cases by the *lox-cre* system and occurs at a distinct site, *loxB*, of the host chromosome, which has a high degree of homology to *loxP*. Also the other site-specific recombination system, *cin-cix*, can lead to integrative recombination. Furthermore, co-integrate formation with the host chromosome – or with other plasmids – can result from the activity of the IS*1* element of P1. Homologous recombination between different copies of IS*1* is more frequently observed than integration mediated by the transposition functions of IS*1*. Subsequent IS*1*-mediated recombination events may result in specialized transducing P1 genomes (e.g. P1*argF*, P1*raf*, P1Cm), which carry the host DNA flanked by IS*1* elements. The Tn*902* element of P7 may be implicated in similar processes.

Generalized transducing P1 particles contain very little, if any, phage DNA and the cellular DNA encapsidated appears to represent all genome regions equivalently. This suggests that packaging is not exclusively initiated at *pac* sites of integrated prophages or at pseudo-*pac* sites in the bacterial chromosome, but might start at additional sites. For a long time P1 transduction has been applied to fine-structure genetic mapping in *E. coli* and other enterobacteria.

Applications in Genetic Engineering

A DNA cloning system based on phage P1 has been developed, which is capable of accepting inserts of up to 100 kb. The vectors have a size of about 13 kb and are composed of a P1 *pac* site for *in vitro* packaging of recombinant DNA into phage particles, two P1 *loxP* sites for circularization of the recombinant DNA upon injection into an *E. coli* expressing the P1 *cre* recombinase gene, a P1 plasmid replicon for stable maintenance, a kanamycin-resistance gene for selection and a P1 lytic replicon under the control of the Lac repressor for inducible amplification of recombinant DNA before isolation and *in vitro* packaging. The system yields up to 10^5 clones carrying large inserts per microgram of vector DNA.

The *lox-cre* recombination system has also been combined with λ cloning vectors, which *in vivo* automatically lead to the conversion of recombinant phages to plasmid subclones. Furthermore, it has been used for manipulating DNA, i.e. producing deletions, inversions and co-integrates, in plant and animal cells, for example in the genetic engineering of 'knockout' mice.

See also: **Phage ecology, evolution and speciation; Phage Homologous Recombination; Phage transduction; Phages as cloning vehicles; Host-controlled modification and restriction; Coliphage lambda (*Siphoviridae*).**

Further Reading

Brendler T, Abeles A and Austin SJ (1995) A protein that binds to the P1 origin core and the *oriC* 13mer region in a methylation-specific fashion is the product of the host *seqA* gene. *EMBO J.* 14: 4083.

Citron M and Schuster H (1992) The C4 repressor of bacteriophage P1 is a processed 77-base antisense RNA. *Nucleic Acids Res.* 20: 3085.

Guo F, Gopaul DN and Van Duyne GD (1997) Structure of Cre recombinase complexed with DNA in a site-specific recombination synapse. *Nature* 389: 40.

Hayes F and Austin SJ (1994) Topological scanning of the P1 plasmid partition site. *J. Mol. Biol.* 243: 190.

Lehnherr H, Maguin E, Jafri S and Yarmolinsky M (1993) Plasmid addiction genes of bacteriophage P1: *doc,* which causes cell death on curing of prophage, and *phd,* which prevents host death when prophage is retained. *J. Mol. Biol.* 233: 414.

Sandmeier H, Iida S, Arber W (1992) DNA inversion regions Min of plasmid P15B and Cin of bacteriophage P1 – evolution of bacteriophage tail fiber genes. *J. Bacteriol.* 174: 3936.

Sauer B (1993) Manipulation of transgenes by site-specific recombination: use of Cre recombinase. *Methods Enzymol.* 225: 890.

Yarmolinsky MB and Sternberg N (1988) Bacteriophage P1. In: Calendar R (ed.) *The Bacteriophages,* vol. 1, p. 291. New York: Plenum Press.

ENTEROVIRUSES (*PICORNAVIRIDAE*)

Contents

Animal and Related Viruses

Elizabeth M Hoey and **Samuel J Martin**, School of Biology and Biochemistry, The Queen's University of Belfast, Belfast, UK

Introduction

This entry deals with animal viruses that up to recently have been referred to as enteroviruses. However, as discussed below, the taxonomic status of some of them remains to be determined. This is particularly true of the avian viruses that will be considered, and such viruses will be referred to as enterovirus-related viruses.

History

Over the past 70 years a number of diseases caused by animal enteroviruses and enterovirus-like agents have been described in the literature. For example, the virulent form of pig polioencephalomyelitis (Teschen disease) was first reported in 1929 in Czechoslovakia and avian encephalomyelitis was first recorded in the USA in 1932. It is probable that the viruses causing these and other diseases had been circulating in animal populations for considerable lengths of time. However, with the advent of more intense farming practices their disease-causing potential has become more apparent and their spread facilitated. With the introduction of tissue culture and egg culture techniques it has been possible to isolate and biologically characterize some of these viruses but few, as yet, have been biochemically characterized.

Classification

Bovine enteroviruses (BEVs), porcine enteroviruses (PEVs), simian enteroviruses (SEVs) and swine vesicular disease virus (SVDV) are currently classified as belonging to the genus *Enterovirus* of the family *Picornaviridae*. Such viruses are small in size (25–30 nm), resistant to ether and are relatively stable over a wide pH range (3.0–10.0). They have a buoyant density of 1.30–1.34 g ml^{-1} in caesium chloride gradients. Mature virus particles are nonenveloped and possess icosahedral symmetry. Empty protein shells are sometimes observed. These enteroviruses have been categorized further into serotypes.

Avian encephalomyelitis virus (AEV), avian nephritis virus (ANV), duck hepatitis virus (DHV) and

See also: **Phage ecology, evolution and speciation; Phage Homologous Recombination; Phage transduction; Phages as cloning vehicles; Host-controlled modification and restriction; Coliphage lambda (*Siphoviridae*).**

Further Reading

Brendler T, Abeles A and Austin SJ (1995) A protein that binds to the P1 origin core and the *oriC* 13mer region in a methylation-specific fashion is the product of the host *seqA* gene. *EMBO J.* 14: 4083.

Citron M and Schuster H (1992) The C4 repressor of bacteriophage P1 is a processed 77-base antisense RNA. *Nucleic Acids Res.* 20: 3085.

Guo F, Gopaul DN and Van Duyne GD (1997) Structure of Cre recombinase complexed with DNA in a site-specific recombination synapse. *Nature* 389: 40.

Hayes F and Austin SJ (1994) Topological scanning of the P1 plasmid partition site. *J. Mol. Biol.* 243: 190.

Lehnherr H, Maguin E, Jafri S and Yarmolinsky M (1993) Plasmid addiction genes of bacteriophage P1: *doc,* which causes cell death on curing of prophage, and *phd,* which prevents host death when prophage is retained. *J. Mol. Biol.* 233: 414.

Sandmeier H, Iida S, Arber W (1992) DNA inversion regions Min of plasmid P15B and Cin of bacteriophage P1 – evolution of bacteriophage tail fiber genes. *J. Bacteriol.* 174: 3936.

Sauer B (1993) Manipulation of transgenes by site-specific recombination: use of Cre recombinase. *Methods Enzymol.* 225: 890.

Yarmolinsky MB and Sternberg N (1988) Bacteriophage P1. In: Calendar R (ed.) *The Bacteriophages,* vol. 1, p. 291. New York: Plenum Press.

ENTEROVIRUSES (*PICORNAVIRIDAE*)

Contents

Animal and Related Viruses

Elizabeth M Hoey and **Samuel J Martin**, School of Biology and Biochemistry, The Queen's University of Belfast, Belfast, UK

Introduction

This entry deals with animal viruses that up to recently have been referred to as enteroviruses. However, as discussed below, the taxonomic status of some of them remains to be determined. This is particularly true of the avian viruses that will be considered, and such viruses will be referred to as enterovirus-related viruses.

History

Over the past 70 years a number of diseases caused by animal enteroviruses and enterovirus-like agents have been described in the literature. For example, the virulent form of pig polioencephalomyelitis (Teschen disease) was first reported in 1929 in Czechoslovakia and avian encephalomyelitis was first recorded in the USA in 1932. It is probable that the viruses causing these and other diseases had been circulating in animal populations for considerable lengths of time. However, with the advent of more intense farming practices their disease-causing potential has become more apparent and their spread facilitated. With the introduction of tissue culture and egg culture techniques it has been possible to isolate and biologically characterize some of these viruses but few, as yet, have been biochemically characterized.

Classification

Bovine enteroviruses (BEVs), porcine enteroviruses (PEVs), simian enteroviruses (SEVs) and swine vesicular disease virus (SVDV) are currently classified as belonging to the genus *Enterovirus* of the family *Picornaviridae*. Such viruses are small in size (25–30 nm), resistant to ether and are relatively stable over a wide pH range (3.0–10.0). They have a buoyant density of 1.30–1.34 g ml^{-1} in caesium chloride gradients. Mature virus particles are nonenveloped and possess icosahedral symmetry. Empty protein shells are sometimes observed. These enteroviruses have been categorized further into serotypes.

Avian encephalomyelitis virus (AEV), avian nephritis virus (ANV), duck hepatitis virus (DHV) and

turkey hepatitis virus (THV) have previously been referred to as enteroviruses because they share some of the above features. However, the genome sequence is not available for any of them as yet and little is known about their biochemical properties. Thus the ICTV currently have not assigned them to any genus of the family *Picornaviridae*. Indeed, although at present all 13 serotypes of porcine enteroviruses are classified as belonging to the genus *Enterovirus*, evidence is emerging in our laboratory that at least one of these serotypes may be misclassified.

Properties of the Virion

The animal enteroviruses belong to the family *Picornaviridae*. Virions are nonenveloped and exhibit icosahedral symmetry (pseudo $T = 3$). Reported sizes vary but most estimates are 25–30 nm in diameter. They show little surface morphology. All enteroviruses so far examined are comprised of a single-stranded positive-sense RNA genome surrounded by a protein shell. This is made up of 60 structural units (protomers), the majority of which contain one copy of the four virus-encoded structural polypeptides (VP1–4). The consistent presence of a small proportion of VP0 in purified BEV preparations suggests that in a few structural units this polypeptide, which normally gives rise to VP2 and VP4, remains uncleaved.

The avian viruses are of similar size and purified AEV particles have been reported to contain three polypeptides.

Properties of the Genome

Few molecular studies have been performed with the majority of these viruses; however, the characterization of the genomes of those studied (BEVs, SVDV and a few PEV serotypes) indicates that they are closely related to the human enteroviruses. The genome consists of a single species of single-stranded positive-sense RNA of approximately 7–8 kb. The 3′ end is polyadenylated. The 'A' tract is heterogeneous in length and is genetically encoded. The 5′ end is covalently linked to a small virus-encoded protein, VPg. The naked RNA of BEV is infectious when transfected into tissue culture cells. The genome contains a single open reading frame (ORF) from which the various virus-encoded proteins are derived. This is preceded by a long 5′ untranslated region (UTR). In the SVDV genome this is of approximately the same length as in the human enteroviruses (750 bases). However, in four sequenced BEV strains it is longer (818–821 bases). This region shows high sequence conservation when comparisons are made between the human and animal enteroviruses, and analyses of sequences indicate that it assumes an extensive secondary conformation. The ORF is followed by another shorter noncoding region (70–100 bases). This shows little sequence conservation between viruses. The ORF is approximately 6500 bases in length, potentially giving rise to a polyprotein of 230 kDa. The generation of the various virus proteins from this and their functions are described below.

The detailed structures of the avian virus genomes have not as yet been assessed.

Properties of the Viral Proteins

The genome layout of the animal enteroviruses so far examined is essentially the same as that of the human enteroviruses. The four structural capsid proteins VP1–4 are derived from the P1 coding region at the 5′ end of the ORF. These have been alternatively called proteins 1A (VP4), 1B (VP2), 1C (VP3) and 1D (VP1) and are encoded in this order in the genome. VP4 and VP2 arise from the cleavage of a precursor protein VP0. Proteins VP1, 2 and 3 are the major structural proteins and have molecular masses of 25–33 kDa. The minor structural protein VP4 is about 7 kDa. This protein has been shown to have a myristic acid molecule covalently attached to it in BEV. The VP1 amino acid sequence is the most variable when comparisons are made between the proteins of animal and human enteroviruses. Purified AEV particles have been reported to contain three structural polypeptides with molecular masses of 35, 30 and 26 kDa; however, the sequence of these has not been determined. Three nonstructural proteins (2A, 2B and 2C) are derived from the P2 coding region in the central part of the genome and four (3A, 3B, 3C and 3D) from the P3 coding regions at the 3′ end of the ORF. Although very little investigative work has been performed on these proteins, because of the high levels of sequence identity at the amino acid level it is assumed that they perform the same function as their human enterovirus counterparts. Thus there would appear to be two virus proteases, 2A and 3C, and a polymerase protein, 3D. Prior to cleavage the protein 3CD may be involved in a number of proteolytic processing events. In poliovirus, this protein has also been shown to bind to the cloverleaf structure formed by the first 95 bases of the genome. The protein 3B is the small genome-linked protein VPg and is linked to the replicative complex by 3A prior to being donated to the genome. The precise function of the 2B protein remains to be fully elucidated; 2C is involved in replication processes.

A high-resolution structure is available for a serotype 1 strain of BEV. This indicates that the three virus proteins assume the same basic conformation as those of other picornaviruses, with each protein having an eight-stranded antiparallel β-barrel core. However, there are few protrusions emanating from the capsid shell, giving the virion a smooth appearance. The minor structural protein, VP4, is internally situated in the virion. The canyon, which surrounds the pentameric axes, is partially filled in when compared to the other enteroviruses and rhinoviruses for which crystal structures have been determined. This feature lies at the junction between the three major structural proteins and it has been suggested that this is the receptor-binding site. The hydrophobic pocket below it is occupied by a pocket factor which has been modeled to be myristic acid.

Physical Properties

The molecular weights of animal enteroviruses are $8–9 \times 10^6$. They have a sedimentation coefficient of 145–165S and a buoyant density in caesium chloride of approximately 1.34 g ml^{-1}. Viruses are stable over a wide pH range (3–10) and are insensitive to ether, chloroform and nonionic detergents. They can be stabilized against heat inactivation by divalent cations.

The buoyant density of AEV has been reported to be 1.31 g ml^{-1}. It is stable at pH 2.8 but the titer drops when the virus is incubated at pH 9–10. The sedimentation coefficient is 160S. It can be stabilized against heat inactivation by magnesium ions.

Replication

To replicate, viruses need to attach to their cellular receptor, penetrate into the cell and uncoat their genome. Translation of this is necessary to provide the proteins involved in replication processes. Little is known about the receptors to which animal enteroviruses bind. However, as with other picornaviruses, the binding site is thought to be the canyon area in BEV. It is assumed that the animal enteroviruses enter the cell and uncoat their genome in a similar manner to poliovirus, which has been studied in much more depth. Three species of viral RNA can be extracted from BEV infected cells. The first is single-stranded RNA, which is identical in sequence to the RNA found in mature virions. The second is replicative intermediate, which has a double-stranded core with nascent single strands growing from it. The third is the completely double-stranded replicative form. The enzyme responsible for replication of the genome is the virus encoded protein 3D, which functions as an RNA-dependent RNA polymerase. Synthesis of a negative-sense complementary strand is initiated at the 3′ end of the genome. The completed negative strand acts as template for progeny viral RNA. The rates of initiation may allow more of this to be formed relative to the minus strands. The progeny positive-sense molecules are either translated by polysomes or are encapsidated. It is likely that the other viral agents replicate their genomes in a similar way; however, further molecular studies will be necessary to confirm this.

Characterization of Translation

Like other picornaviruses, analyses of the available enterovirus sequences indicate that they contain a single large ORF. This is preceded by the long 5′ UTR described above. With poliovirus, part of this region has been shown to contain an internal ribosome entry site (IRES), to which ribosomes attach directly. This is in contrast to the situation with other eucaryotic mRNAs where ribosomes attach at the 5′ end and scan the sequence until the first AUG in the proper context is met, signifying the translation start site. The sequence similarities between the 5′ UTR of SVDV, BEV and the polioviruses indicate that these two animal viruses also contain IRES sequences. Preliminary translation studies with one strain of BEV suggest that this is the case. Translation starts at the AUG following the 5′ noncoding region, although with both viruses this is not the first AUG in the genome sequence. The long ORF frame covers about 85% of the genome. During BEV infection, translation of host mRNAs is shut off and viral RNA is translated preferentially. This facilitates analysis of virus-induced protein products following labeling of infected cells with radioactive amino acid(s). These are considered below.

Post-Translational Processing

Post-translational processing of the long polypeptide occurs *in situ* and has been demonstrated in BEV-infected cells using pulse chase experiments. The enzymes responsible are probably the two virus-encoded proteases 2A and 3C, although 3CD may play a role in the cleavage of the structural region. 2A is responsible for cleaving between VP1 and P2A at a T/G site in SVDV and at a Y/G or A/G site in BEV serotype 1 or 2 strains, respectively. 3C or 3CD are responsible for all but one of the remaining cleavages, which are mainly at Q/G sites in both viruses. Exceptions are the cleavage sites between 2B and 2C, which is Q/N in SVDV and Q/S in BEV, and the VP1/VP3 cleavage site in BEV which has not

accurately been determined. Sequence alignments indicate that it is either between Q/N or Q/T. The final cleavage event occurs on maturation of the virion when RNA enters the viral particle. VP0 is cleaved to generate VP2 and VP4 at an N/S bond in SVDV and a K/S bond in BEV. None of the virus proteins are glycosylated post-translationally; however, it has been shown that the N-terminal residue of VP4 of BEV is modified with a myristic acid moiety.

Assembly Site, Uptake, Release, Cytopathology

Few studies have been performed on the assembly and release of any of the animal viruses discussed here but it is likely that the mechanisms used are similar to those occurring in poliovirus-infected cells. The reader is referred to the entry dealing with this virus.

Most of the animal enteroviruses, which grow in cell cultures, produce marked cytopathic effect. Following BEV infection, BHK cells round up and eventually shrivel. The 13 serotypes of PEV have been divided into three groups, each of which produce a different type of cytopathic effects on pig kidney cell lines. Group I consists of serotypes 1–7, 11–13 and produces rounded refractile cells. Serotype 8 strains are the only members of group 2 and produce granular cells with cytoplasmic protrusions. Group III contains serotypes 9 and 10 and produces a cytopathic effect typical of SVDV and the coxsackieviruses. Group 1 viruses grow only in pig kidney cell lines, while group II strains are also cytopathogenic in the BHK21 cell line and type III are cytopathogenic in BHK21, Vero and HeLa cell lines.

Geographic and Seasonal Distribution

Many animal enteroviruses occur worldwide and may be endemic where host populations are sufficient to sustain them. However, the virulent serotype 1 PEV strain, Teschen disease virus, appears to be geographically restricted to parts of Africa and Eastern Europe, whereas the less virulent serotype 1 strains have a global distribution. Swine vesicular disease was first observed in Italy in 1966, since when outbreaks have been reported in most European countries and in Malta, Hong Kong and Japan. The continents of Africa, America and Australia have remained free of the disease. Most outbreaks have occurred in Great Britain, where they have been reported to peak in December and January. Avian encephalomyelitis was first identified in New England in 1932 and the disease has since been confirmed in many countries of the world. Duck virus hepatitis was initially reported in the USA in 1945 and then again in 1949. Since then the disease has spread to many countries in Europe, Asia, North Africa and America. The first reports of turkey virus hepatitis came from Canada and Massachusetts. It has since been reported in Italy. The true incidence of the disease is not known because it tends to be subclinical in nature. In many other instances it has not proved possible to link enteroviruses and related agents to defined disease symptoms; evidence of such infections have therefore come from serological conversion studies.

Host Range and Virus Propagation

Enteroviruses and enterovirus-like viruses have been isolated from a large variety of animal and avian species. Some animal enteroviruses only infect the species, or closely related species, to that from which they were isolated. A notable exception to this is SVDV which is antigenically similar to the human coxsackievirus B5 and has been shown to have infected laboratory personnel, causing a coxsackievirus-like illness. However, coxsackievirus B5 infection of pigs does not cause clinical signs of disease. SVDV also causes a progressive neurological disease in infected experimental newborn mice. Antibodies, which neutralize some BEV and PEV strains, have been demonstrated in human sera, although this may be a nonspecific reaction. In addition, some BEVs belonging to serotype 1 have been isolated from a variety of other animals, including goats and sheep; serotype 2 viruses have only been isolated from domestic cattle. Chickens, turkeys, pheasants and Japanese quail are the natural hosts of AEV but ducklings, pigeons and guinea fowl can be experimentally infected. ANV infects mainly chickens. Ducks are the natural hosts of the two DHV picornaviruses but experimental infection of other avian species has been achieved. THV infection has only been observed in turkeys.

Most animal enteroviruses can be propagated in primary kidney cell lines from the host species of origin. Many PEVs can be grown in established pig kidney cell lines although they may need to be isolated and adapted on primary cultures. They have been divided into three groups on the basis of the different types of cytopathic effect they produce in established pig kidney cultures. Viruses from group I and II are isolated more frequently from feces than from epithelium cells, whereas with group III viruses the reverse is the case. The majority of BEV strains grow in a range of primary kidney cell lines and also in a variety of established cell lines such as BHK-21, HeLa, Vero and L cells. Few avian enteroviruses grow well on primary avian cell cultures. Growth of ANV has been demonstrated in chicken kidney and embryo

liver cultures and two antigenically related viruses have also been shown to grow in chicken kidney cells, forming irregular plaques and giving rise to a cytopathic effect untypical of enteroviruses. In most cases propagation of avian viruses can be achieved by inoculation of chick embryos via the yolk sacs of eggs. AEV isolates have also been propagated by intracerebral inoculation of day-old chicks.

Genetics

No studies have been made of mutation rates in animal enteroviruses; however, as these are RNA viruses, the polymerases of which are error prone, it is likely that the mutation rate is high. In addition, picornavirus genomes undergo recombination events leading to variability.

Evolution

The genomes of only a few animal enteroviruses have been sequenced. Those that have show considerable homology to the human enteroviruses, suggesting that these viruses may have arisen from a common ancestor. SVDV shares over 90% sequence identity at the amino acid level with some coxsackie B viruses (CBVs), and in a recent phylogenetic analysis they have been clustered with the CBVs, several CAVs and several echoviruses. The human enteroviruses included in the cluster vary with the region of the genome that was analysed. However BEV (strain VG-5-27) is more distinct, with 30–50% amino acid sequence identity with human enteroviruses in the structural proteins and 30–70% in the nonstructural proteins. Thus these viruses appear to have diverged much earlier in evolutionary history. They have been placed in a separate cluster from the human enteroviruses when both the 5′ NCR and 3′ NCR sequences were considered. However, CAV16 was included in the cluster containing them following analysis of the coding region. The capsid regions of two serotype 1 and two serotype 2 BEVs have been sequenced. As expected, sequence identity is greatest within serotypes and suggests that two branches of these viruses have evolved. The serotypes are more closely related to each other than to any other sequenced picornavirus, implying that the two BEV serotypes have emerged from each other relatively lately in evolution or, alternatively, that there is some constraint on the evolution of these viruses.

Serologic Relationships and Variability

Enteroviruses from various different animal species have been comparatively characterized using a variety of immunological techniques and placed into serotypes. There are 18 different prototypes of simian enteroviruses (1–18) which do not appear to be related to any of the human enteroviruses. However, many simian species exist and it is likely that further isolation of enteroviruses from these will give rise to other distinct viruses. Numerous attempts have been made at serologically classifying the PEVs. Currently 13 serotypes are recognized (not including SVDV) with designated prototype viruses. Other work indicates that there may be as many as 15 serotypes. The BEVs were initially classified into seven serotypes but later reclassified into only two, based on data obtained from virus neutralization and complement fixation studies. Those in serotype 1 are more closely related serologically than those in serotype 2. There is only one serotype of SVDV. However, strains are divided into distinct phylogenetic groups. All strains of AEV are antigenically similar but they can vary in neurovirulence. ANV is antigenically unrelated to AEV. DHV types I and III are also antigenically distinct. DHV type II, which was once thought to be a picornavirus, is now classified as an astrovirus.

Epidemiology

Many animal enterovirus infections are subclinical and transient and therefore their true incidence are not known. Serological methods have indicated that many enteroviruses are widespread; for example, in one study, antibodies against porcine enteroviruses were found in serum samples from all herds tested from throughout the world. Feral pigs living in Georgian swamps have also been demonstrated to have antibodies to several porcine enteroviruses. Pigs are most susceptible to infection at 4–5 weeks when maternal antibody has dropped to below protective levels, although infection may occur *in utero* and at any age after birth. The spread of swine vesicular disease in pigs to various European countries and Japan may have been linked to the importation of pork products from infected countries. Some salami sausages, for example, use nonheat-treated pork and may harbor virus for considerable lengths of time. In England, pigs may have become infected because they were transported in vehicles previously used for infected animals, even though the trucks were cleaned between cargoes. The stable nature of this virus makes decontamination of infected premises very difficult and virus has been isolated from earthworms collected from soil above burial places for carcasses of slaughtered diseased animals.

Serological studies have indicated that BEVs are also widespread, with as much as 80% of tested cattle sera in certain herds having been reported to have antibodies against such viruses. Cattle may be

infected *in utero* and at any time thereafter. The simian enteroviruses, also on the basis of serological evidence, appear to be widespread.

Avian encephalomyelitis has been reported from most countries of the world where there is a commercial poultry industry. Embryos may be infected and young chicks are also susceptible. When the virus is first introduced into flocks, the morbidity and mortality rates are high, but once established both drop considerably due to protection of young chicks by maternal antibody. ANV occurs worldwide in domestic fowl. It is probably spread via ingestion of fecally contaminated food or water. DHV occurs in most countries where ducks are reared commercially. The virus is very stable and may remain viable in feces for considerable lengths of time, allowing ducklings to become infected following ingestion of contaminated food. The disease spreads rapidly through the flock.

Transmission and Tissue Tropism

The major mode of transmission of the animal enteroviruses is by the faecal–oral route, and in most instances infection is confined to the gastrointestinal tract. SVDV infection is thought to occur mainly though damaged skin, particularly around the feet, or in some instances through pigs eating contaminated garbage. During the viremic stage all tissues contain virus. Other porcine enteroviruses infect through the mouth and nose. In addition to showing a tropism for intestinal tissues, one such virus has been isolated from the spiral colon, ileum and tonsils following experimental infection of pigs. Various serotypes have been isolated from fetuses and serological evidence has implicated most of the others in such infections. BEVs have been isolated from blood, semen, vaginal washings, placenta, liver, kidney, pancreas, spleen, hard palate, epithelium and lymph node of infected animals and from fetal fluids and fetuses. AEV is spread by the faecal–oral route. It can also be transmitted through the egg. In young infected chicks, virus can be found in a variety of organs, including the central nervous system. Following infection, ANV can be recovered from a variety of organs including the kidney. DHV and THV are both spread by the faecal–oral route. The principal organ affected with both is the liver.

Pathogenicity

Most enterovirus infections are subclinical and inapparent and only the porcine and avian enteroviruses cause diseases of economic importance. Certain porcine enterovirus strains are neurotropic, giving rise to pig polioencephalomyelitis. The most severe form of the disease was first reported from a town called Teschen in Czechoslovakia and proved highly fatal. This was caused by a virulent strain of serotype 1. Although such cases are still reported, less severe forms of the disease, such as Talfan disease, which was originally reported in the UK, are now more common. These are caused by less virulent strains of serotype 1. In addition, serotypes 2, 3 and 5 strains have been implicated in some less severe cases of polioencephalomyelitis. Variations in pathogenicity of strains within serotypes have been observed. The frequent isolation of these viruses from stillborn and aborted fetuses has implicated them in the SMEDI *s*tillbirth, *m*ummification, *e*mbryonic *d*eath, *i*nfertility) syndrome which results in reproductive failure. SVDV causes a vesicular disease of pigs; however, subclinical infections have also been reported. In general, the disease in itself is not severe and most animals recover. Induced serum antibody prevents reinfection of these animals.

Strains of various avian enterovirus-related viruses have been reported to vary in pathogenicity. Field isolates, which normally give signs of infection only after hatching, have been adapted for growth in embryos. The adapted strains induce gross symptoms following infection.

Clinical Features of Infection

The clinical features of disease caused by animal enteroviruses are wide and varied. The initial symptoms of classical Teschen disease, a neurological disease of pigs caused by virulent strains of PEV-1, are high temperature, some lack of coordination in the back limbs, listlessness and loss of appetite. This is usually associated with mild diarrhea. Extreme stiffness generally follows, resulting in pigs walking with a limping gait and falling frequently. Mortality may be high, with death occurring at about 3–4 days after the onset of neurological symptoms. Surviving pigs show residual paralysis and muscular wasting. Teschen disease is also referred to as pig polioencephalomyelitis. The Teschen viruses cause disease in animals over a wider age range than the less virulent viruses such as Talfan virus. The latter mainly affect young pigs, resulting in similar but less severe symptoms than in Teschen disease. Morbidity and mortality rates are much lower in these cases. Porcine enteroviruses associated with the SMEDI syndrome produce different pathogenic effects depending on the stage of gestation. At early stages, infection may cause embryonic death and return to estrus. Fetuses infected between 40 and 70 days die, whereas those infected at a later stage may survive, although some are stillborn.

Viruses from other families such as the parvovirus family can also give rise to the SMEDI syndrome.

The clinical features of swine vesicular disease are virtually indistinguishable from foot-and-mouth disease. The disease is often first detected when several animals in the same herd go lame and is characterized by the appearance of vesicles of various sizes on one or more feet, usually about the coronary band, and later spreading to the metacarpals, metatarsals and soles of the feet. In the most severe cases there may be so many lesions that claws may slough off. In up to 10% of the cases vesicles also occur on the snout, tongue and lips. The vesicles develop into raw ulcers, which heal quickly, with most animals returning to normal within 3 weeks; however, if secondary infection of lesions occurs, the pig may become chronically lame. Animals on soft bedding tend to show less serious disease symptoms than animals kept on concrete floors. In general, the morbidity rates are moderate and the mortality rates are low.

AEV produces disease only in young birds. Clinical signs include weight loss, ataxia, head and neck tremors, blindness, paralysis and, in severe cases, coma and death. ANV causes growth depression but little or no signs of illness. DHV causes a severe disease in ducks of less than 21 days. Affected ducks stop moving, partially close their eyes and eventually fall on their sides and kick their legs. Death may follow rapidly. Morbidity is 100% and, depending on the age of the flock, mortality rates vary. It has approached 95% in birds of less than a week old, whereas in birds older than 5 weeks it is negligible.

Pathology and Histopathology

The intestinal phase of porcine enterovirus infections is generally not accompanied by histological changes, although atrophy of villi has been reported for recently weaned scouring pigs. Changes have been reported in the lung, liver, kidney, spleen, heart and in adrenal and thyroid glands. Teschen/Talfan viruses have the ability to invade the central nervous system. The histological changes in Teschen disease are more severe than those of Talfan disease. These include varying degrees of neuronal degradation, neuronophagia, lymphocytic perivascular cuffing and the development of focal areas of gliosis. The endoplasmic reticulum appears to be the target organelle in the infected degenerating neuron. Central nervous system changes are most prevalent in the gray matter of the spinal cord, with the cerebellum being the next most affected region. At the height of the disease the meninges above the cerebellum are severely infiltrated with lymphocytes. The pons and medulla oblongata are also often affected, followed by other areas of the brain to lesser extents. The gross lesions of swine vesicular disease are very similar to those caused by the foot-and-mouth disease viruses and it is the differentiation between these two diseases that is important. SVDV infection often causes microscopic neurological lesions and produces a mild or moderate, diffuse encephalitis. Neuroglia cell foci and lymphocytic perivascular cuffing occur to varying extents.

Macroscopic lesions are seen only rarely as a result of infection by AEV. Histological changes in the central nervous system include lymphocytic perivascular cuffing, evidence of gliosis and axonal-type neuronal degradation. In addition, hyperplasia of the lymphoid follicles in visceral tissue occurs. The livers of ducks suffering from duck virus hepatitis are enlarged and contain hemorrhages. The spleen is often also enlarged and the kidneys may be swollen. Histological signs include hepatic necrosis, varying degrees of inflammatory cell infiltration and proliferation of bile duct epithelium. Gross lesions are observed in the liver and pancreas of turkeys suffering from turkey virus hepatitis. The histological changes include necrotic foci, hemorrhage and mononuclear foci. Focal parenchymal foci of necrosis may also be observed in the pancreas.

Immune Response

In general, enterovirus infection results in a strong immune response. PEV infection results in the appearance of serum virus neutralizing antibody at about 6 days postinfection and intestinal antibody at about 14–15 days postinfection. The former is predominantly IgG and persists for a long period, whereas the latter is IgA and transient. Neutralizing antibody has also been demonstrated in sow milk and may provide protection to piglets until they are weaned. The cell-mediated response appears to be weak and to provide little antiviral protection. Fetuses infected with SMEDI enteroviruses at 65–70 days gestation produce IgM antibody initially and start producing IgG antibodies at 1 month from term. Pigs infected with SVDV produce neutralizing antibody at 4 days. This is initially IgM but later IgG predominates. Birds recovering from avian encephalomyelitis develop neutralizing antibody and this is transferred vertically into yolk sacs.

Prevention and Control of Disease

Economic loss is not considerable with most of the milder forms of pig encephalomyelitis, and therefore vaccination is not considered justifiable. In addition, subclinical infections prevent the success of a slaughter policy. Both oral and parenteral vaccines have been used successfully against Teschen disease.

Eradication has been achieved in one area by a combination of improved sanitary methods, slaughtering of diseased animals and ring vaccination. Virulent serotype 1 viruses have been prevented from spreading to disease-free countries by restrictions on the importation of pork products and swine. Vaccines have been prepared against SVDV, which in most countries is a notifiable disease. However, the preferred method of control is by containment and stamping out using a slaughter policy. The stability of the virus makes the eradication of the disease difficult. Following slaughter of an infected herd, premises have to be cleansed and disinfected thoroughly and possible infected pigs that have left the premises have to be tracked, with particular attention paid to vehicles used in their transport. In Great Britain, a small number of pigs are placed in previously infected farms and are observed for 3 weeks for evidence of disease development before full restocking is allowed. In the past, garbage containing pork products harboring virus has been a major source of infection. Careful monitoring of food products has helped to limit spread of infection in some instances. The banning of pork and swine imports has prevented the spread of the virus to disease-free countries.

Live vaccines are available against AEV and control has been achieved by administering these in drinking water to chicks that are more than 10 weeks old. Younger chicks are not vaccinated directly because the vaccine is not sufficiently attenuated. Immunization of laying hens is usually sufficient to pass on specific immunity to their progeny chicks in the critical 3 weeks after hatching. A killed vaccine is also available. Chicken egg-adapted DHV vaccines are also available and are used in a similar manner to that described above.

Future

Apart from the BEVs, SVDV and a few PEV serotypes, few biochemical studies or modern molecular biology techniques have been applied to the viruses discussed. Indeed it has not been confirmed that the enterovirus-related viruses are typical members of any of the recognized genera of the picornavirus family. Only the application of cloning and sequencing techniques and the analyses of generated data will help clarify the taxonomic relationships of this large group of agents that have previously been considered to be the animal enteroviruses.

See also: **Enteroviruses (*Picornaviridae*): Human enteroviruses (serotypes 68–71); Polioviruses (*Picornaviridae*): General features; Molecular biology; Coxsackieviruses (*Picornaviridae*); Echoviruses (*Picornaviridae*); History of virology: Polio, coxsackie, echo and other enteroviruses; Rhinoviruses (*Picornaviridae*).**

Further Reading

Derbyshire JB (1989) Porcine enteroviruses (polioencephalomyelitis). In: Pensaert MB (ed.) *Virus Infections of Porcines*, p. 225. Amsterdam: Elsevier.

Hypia T, Hovi T, Knowles NJ and Stanway G (1997) Classification of enteroviruses based on molecular and biological properties. *J. Gen. Virol.* 78: 1.

Knowles NJ and McCauley JW (1997) Coxsackievirus B5 and the relationship to swine vesicular disease virus. *Curr. Top. Microbiol. Immunol.* 223: 153.

Knowles NJ and Mann JA (1990) Bovine enteroviruses. In: Dinter Z and Morein B (eds) *Virus Infections of Ruminants*, p. 513. Amsterdam: Elsevier.

McNulty MS and McFerran JB (1996) Diseases associated with the *Picornaviridae*. In: Jordan FTW and Pattison M (eds) *Poultry Diseases*, p. 187. London: WB Saunders.

Rueckert RR (1996) Picornaviridae: the viruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 609. New York: Lippincott-Raven.

Tannock GA and Shafren DR (1994) Avian encephalomyelitis: a review. *Avian Pathol.* 23: 603.

Human Enteroviruses (Serotypes 68–71)

Jeffrey W Almond, School of Animal and Microbial Sciences, University of Reading, Reading, UK

Margeruite Yin-Murphy, Raffles Park, Singapore

Introduction

Because the subdivision of human enteroviruses into the polioviruses, coxsackieviruses group A, coxsackieviruses group B and echoviruses was not always unambiguous, it was decided in 1969 that enterovirus serotypes not previously assigned to the above groups be designated enterovirus (EV) followed by a number starting from 68. Since that time four enteroviruses, EV68–71, have been described and are in the *Enterovirus* genus of the *Picornaviridae* family. In addition, hepatitis A virus was provisionally identified as EV72, but this virus is now classified in a distinct genus (*Hepatovirus*). EV68 and EV69 seem to be of relatively minor importance in terms of human disease. Reports of their isolation and associated illnesses are infrequent and the viruses have not been studied extensively. By contrast, EV70, a cause of

Eradication has been achieved in one area by a combination of improved sanitary methods, slaughtering of diseased animals and ring vaccination. Virulent serotype 1 viruses have been prevented from spreading to disease-free countries by restrictions on the importation of pork products and swine. Vaccines have been prepared against SVDV, which in most countries is a notifiable disease. However, the preferred method of control is by containment and stamping out using a slaughter policy. The stability of the virus makes the eradication of the disease difficult. Following slaughter of an infected herd, premises have to be cleansed and disinfected thoroughly and possible infected pigs that have left the premises have to be tracked, with particular attention paid to vehicles used in their transport. In Great Britain, a small number of pigs are placed in previously infected farms and are observed for 3 weeks for evidence of disease development before full restocking is allowed. In the past, garbage containing pork products harboring virus has been a major source of infection. Careful monitoring of food products has helped to limit spread of infection in some instances. The banning of pork and swine imports has prevented the spread of the virus to disease-free countries.

Live vaccines are available against AEV and control has been achieved by administering these in drinking water to chicks that are more than 10 weeks old. Younger chicks are not vaccinated directly because the vaccine is not sufficiently attenuated. Immunization of laying hens is usually sufficient to pass on specific immunity to their progeny chicks in the critical 3 weeks after hatching. A killed vaccine is also available. Chicken egg-adapted DHV vaccines are also available and are used in a similar manner to that described above.

Future

Apart from the BEVs, SVDV and a few PEV serotypes, few biochemical studies or modern molecular biology techniques have been applied to the viruses discussed. Indeed it has not been confirmed that the enterovirus-related viruses are typical members of any of the recognized genera of the picornavirus family. Only the application of cloning and sequencing techniques and the analyses of generated data will help clarify the taxonomic relationships of this large group of agents that have previously been considered to be the animal enteroviruses.

See also: **Enteroviruses (*Picornaviridae*): Human enteroviruses (serotypes 68–71); Polioviruses (*Picornaviridae*): General features; Molecular biology; Coxsackieviruses (*Picornaviridae*); Echoviruses (*Picornaviridae*); History of virology: Polio, coxsackie, echo and other enteroviruses; Rhinoviruses (*Picornaviridae*).**

Further Reading

Derbyshire JB (1989) Porcine enteroviruses (polioencephalomyelitis). In: Pensaert MB (ed.) *Virus Infections of Porcines*, p. 225. Amsterdam: Elsevier.

Hypia T, Hovi T, Knowles NJ and Stanway G (1997) Classification of enteroviruses based on molecular and biological properties. *J. Gen. Virol.* 78: 1.

Knowles NJ and McCauley JW (1997) Coxsackievirus B5 and the relationship to swine vesicular disease virus. *Curr. Top. Microbiol. Immunol.* 223: 153.

Knowles NJ and Mann JA (1990) Bovine enteroviruses. In: Dinter Z and Morein B (eds) *Virus Infections of Ruminants*, p. 513. Amsterdam: Elsevier.

McNulty MS and McFerran JB (1996) Diseases associated with the *Picornaviridae*. In: Jordan FTW and Pattison M (eds) *Poultry Diseases*, p. 187. London: WB Saunders.

Rueckert RR (1996) Picornaviridae: the viruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 609. New York: Lippincott-Raven.

Tannock GA and Shafren DR (1994) Avian encephalomyelitis: a review. *Avian Pathol.* 23: 603.

Human Enteroviruses (Serotypes 68–71)

Jeffrey W Almond, School of Animal and Microbial Sciences, University of Reading, Reading, UK

Margeruite Yin-Murphy, Raffles Park, Singapore

Introduction

Because the subdivision of human enteroviruses into the polioviruses, coxsackieviruses group A, coxsackieviruses group B and echoviruses was not always unambiguous, it was decided in 1969 that enterovirus serotypes not previously assigned to the above groups be designated enterovirus (EV) followed by a number starting from 68. Since that time four enteroviruses, EV68–71, have been described and are in the *Enterovirus* genus of the *Picornaviridae* family. In addition, hepatitis A virus was provisionally identified as EV72, but this virus is now classified in a distinct genus (*Hepatovirus*). EV68 and EV69 seem to be of relatively minor importance in terms of human disease. Reports of their isolation and associated illnesses are infrequent and the viruses have not been studied extensively. By contrast, EV70, a cause of

epidemic and occasionally pandemic acute conjunctivitis, and EV71, a major cause of aseptic meningitis, have attracted considerable attention. In terms of their acid stability, size, morphology, protein structure, RNA genome organization and structure, EV68–71 have characteristics typical of the enteroviruses, as described elsewhere in this volume.

Enterovirus Serotype 68 (EV68)

The first and prototype isolate of EV68 is known as 'Fermon virus'. This virus was one of four similar agents isolated in California in 1962 from oropharyngeal swabs of infants with lower respiratory tract illnesses. The isolation of these viruses is relatively rare: apart from the California isolates, countries that reported to the World Health Organization (WHO) in Geneva on a surveillance of virus diseases for the years 1975–1983 showed that, of 864 infections caused by the new human enteroviruses, only three were EV68. The California strains were isolated from infants aged between 10 months and 3 years with pneumonia and bronchiolitis. In addition, one isolate was obtained from a young adult with upper respiratory tract infection in Australia. It appears therefore that virus transmission is via the respiratory–oral route with virus affinity for epithelial cells of the oropharynx and respiratory tract producing pneumonia and bronchiolitis. EV68 in clinical specimens produces enteroviral cytopathic effects (CPEs) in primary monkey kidney but not human fetal kidney cell cultures incubated at 33°C. Only the Rhyne strain of E68 is pathogenic for mice; the Fermon, Franklin and Robinson strains are not. The histopathological picture of tissue from infected mice is similar to that of group A coxsackievirus. Zenker's necrosis occurs as swellings in short segments of muscle fibers accompanied by increased basophilia, loss of striations and pyknosis of nuclei. Some muscle lesions are characterized by infiltration of neutrophils into the endomysium and perimysium. Lesions are generalized throughout the skeletal muscles but the intrascapular fat pads and cardiac muscles are not affected. Recent partial sequencing of EV68 in the capsid encoding region of its genome suggests that it is most closely related genetically to EV70. These two serotypes appear to form a distinct genetic cluster among the enteroviruses.

Enterovirus Serotype 69 (EV69)

The prototype, Toluca-1 virus (obtained from a rectal swab of a healthy 4-year-old child), together with two other isolates were recovered from Toluca, Mexico in 1959. EV69 is also a relatively uncommon virus: only seven isolations of this virus were made from countries reporting to WHO on a surveillance of virus diseases in the years 1975–1983. The Fairfield Hospital, Victoria, Australia recorded one isolation in 1979 and the WHO Collaborating Centre for Virus Reference and Research, Singapore, has identified only two strains, both in 1984. EV69 can be isolated from feces and from nasopharyngeal aspirates, and virus from both sources produces enteroviral CPEs in primary rhesus kidney, Hep-2 and Hela cell cultures. Genetically, on the basis of partial nucleotide sequence data, EV69 clusters with the coxsackie B viruses, coxsackie A9 and the major group of echoviruses. Some crossreaction antigenically with one of this group, echovirus 6, had previously been reported, viz: Toluca-1 antiserum, with homologous hemagglutination inhibition (HI) and neutralization (N) titers of 1:320 and 1:500 respectively, had an HI titer of 1:40 and an N titer of 1:50 against echo 6 D1 (Cox) strain. Conversely, D1 (Cox) antiserum, with homologous N and HI titers of 1:5000 and 1:50 respectively, had HI titer of $<$1:10 and N titer of $<$1:50 against Toluca-1. Whereas E6 agglutinates human erythrocytes to much higher titers at 37°C than 4°C, the reverse was observed for Toluca-1. Although the Toluca EV69 strains were obtained from feces of apparently healthy children, the Australian (1979) and Singapore (1984) strains were isolated from nasopharyngeal aspirates of infants with respiratory illnesses. EV69 was implicated in cases of two infants hospitalized with symptoms of pharyngitis and bronchiolitis in Singapore and a child treated for pertussis in Australia. The respiratory illnesses had no distinctive feature. Transmission of virus via oral–fecal and respiratory routes would be expected, with virus tropism for cells of alimentary and respiratory tracts.

Enterovirus Type 70 (EV70)

History

EV70 is the second human enterovirus isolated from extensive epidemics where the dominant clinical manifestations are in the eye, the first being coxsackievirus A24 variant (CA24v). Together these viruses are universally recognized as causative agents of acute hemorrhagic conjunctivitis (AHC). EV70 was first isolated in 1971 during the 1969–1971 wave of AHC in Morocco, Singapore, Hong Kong (concurrently with CA24v) and Japan. Explosive outbreaks of acute conjunctivitis, thought intitially to be due to a virulent strain of adenovirus, were reported from Ghana in June 1969 and the disease extended rapidly along the coastal areas of west, east and north Africa. Clinically

similar epidemics of conjunctivitis involved major cities in the Indian subcontinent, southeast Asian countries and Japan, and small localized outbreaks occurred in London, Holland and Moscow in 1970–1971. The extent of the involvement of EV70 as compared with CA24v during the 1969–1972 AHC pandemic cannot be ascertained because most reports were based on clinical findings. Furthermore, the agent responsible for the African 1969–1970 epidemics has not been available for comparative investigations. It is clear that EV70 played a prominent role in the 1980–1982 AHC pandemic, during which not only Africa, southeast Asian countries, India and the Middle East were affected but the western hemisphere also became involved for the first time. A small localized outbreak in Oakland, California occurred in July 1980 and extensive epidemics which originated in Brazil spread rapidly through South and Central America, Oceania and Islands of the Western Pacific Ocean from mid-1981 to the beginning of 1982.

J670/71, the most extensively studied strain and the one that grows best in cell lines, has been classified as the prototype EV70.

Geographic and seasonal distribution

Australia is the only continent that has not reported an outbreak of AHC caused by EV70 or by CA24v. Extensive epidemics have occurred mainly in countries which are hot and humid. Unhygienic conditions, poor personal hygiene and overcrowding are conducive to the spread of conjunctivitis. There is no apparent seasonal influence on outbreaks but increased incidences are observed during the warmer humid months. In temperate countries, localized outbreaks occur in all seasons.

Properties of the virion

EV70 has the general physicochemical properties of enteroviruses. Electron micrographs reveal a typical nonenveloped virion of 22–27 nm in diameter with icosahedral symmetry. The virions have a buoyant density of $1.34\,g\,ml^{-1}$ in cesium chloride and a sedimentation coefficient of 160 S in sucrose. EV70 is inactivated by phenol, cresol, formaldehyde, chlorine at a concentration of 1 p.p.m. in distilled water, 50% methanol, 70% phenol, 0.0125% free iodine in disinfectant PA10DO and 0.004% free iodine in DIASAN. Replication of EV70 is inhibited in *in vitro* experiments by interferon β, arildone {4-[6-2-chloro-4-methoxyphenoxy)hexyl]-3,5-heptanedione}, Ro 09-0179 (4′,5-dihydroxy-3,3′,7-trimethoxyflavone), the benzimidazoles – enviroxime (LY-122772) and enviradone (LY-127123) – and guanidine hydrochloride but not actinomycin D. Benzalkonium chloride, benzethonium chloride, chlorhexidine gluconate and hexachlorophene at concentrations applied as germicides have no effect. EV70 retains infectivity at 40°C for at least 30 min but is largely inactivated at 50°C within 30 min. EV70 hemagglutinates human erythrocytes by virtue of its receptor (CD55) being expressed on these cells.

Properties of the viral genome

The complete nucleotide sequence of EV70 (J670/71) has been determined. The positive-sense single-stranded RNA has a typical enterovirus organization. It contains a 5′ noncoding region (NCR) of 726 nucleotides, a long open frame of 6582 nucleotides and a 3′ noncoding region of 82 nucleotides prior to the poly(A) tract. The genome encodes a VPg protein which by implication, in common with other picornaviruses, is covalently attached to virion RNA. The 5′ NCR is predicted to contain a high degree of secondary structure, consistent with it containing an internal ribosome entry site (IRES) to allow initiation of protein synthesis. The 3′ NCR also contains sequences likely to form secondary structures, although the function(s) of this region remains obscure. EV70 has been included in a genetic and phylogenetic analysis of a substantial number of partial and complete enterovirus sequences. This indicates that EV70 is most closely related to EV68 and that these two viruses form a distinct genetic cluster fairly well separated from other enteroviruses. Paradoxically, over most of its genome, EV70 does not appear to be closely related to coxsackie A24 variant, even though these viruses cause indistinguishable disease. It is interesting however, that, in its 5′NCR, EV70 does appear to be CA24v-like, clustering also with poliovirus type 1 and coxsackie A21. This raises the possibility that a recombination event may have occurred at some point in its evolution.

Viral proteins

In common with other enteroviruses, the icosahedral virion of EV70 is made up of 60 copies each of four virus-encoded proteins 1A (Vp4, 9kD), 1B (Vp2, 28kD), 1C (Vp3, 27kD) and 1D (Vp1). Comparison of the predicted amino acid sequence of the EV70 polyprotein with published findings for other enteroviruses permits prediction of some antigenic regions and most of the polyprotein cleavage sites. The organization and cleavage pathway of virus-encoded proteins appear to be in accordance with the L434 system, with glutamine–glycine as the most common cleavage site. Occasionally, for example at the 2B/2C junction and possibly also at the 1C/1D junction,

glutamine–serine appears to be used. Alignment of the structural proteins with those from related viruses whose crystal structures are known suggests that the VP1 protein contains an antigenic loop corresponding to the 'site 1' observed in poliovirus type 3. Other surface loops or 'puffs' can be predicted, although their significance remains to be determined.

Replication

The receptor on Hela cells for EV70 has been recently identified as CD55, otherwise known as decay-accelerating factor (DAF). This GPI-anchored cell surface protein is a natural regulator of complement action which functions through accelerating the decay of C3 and C5 convertases in both the classical and alternative pathways of complement fixation. In using this protein as its cellular receptor, EV70 is similar to a range of echoviruses, including types E6, 7, 13, 21, 29 and 33. This interaction with DAF explains the ability of some isolates of the virus to agglutinate human red blood cells. After attaching to DAF on permissive cells, EV70 enters the cell and replicates in the cytoplasm of infected cells. The optimum growth temperature is 33°C. Above 39°C (the nonpermissive temperature), viral RNA synthesis is blocked and replication stops. *In vitro* experiments suggest that the temperature-sensitive step is the uridylation of VPg. Little specific information is available about the steps in virus replication such as translation, RNA synthesis and assembly, but EV70 is believed to conform to the general strategy best understood for poliovirus.

Host range and virus propagation

EV70 from conjunctival swabs and scrapings, throat swabs and feces produces CPEs in tissue cell cultures of human and simian origin, with Hela cells being the preferred cell line. Other cell lines and organ cultures vary in sensitivity. Generally, 3–5 cell culture passes are required to attain characteristic CPEs. Optimum incubation temperature is 33°C but EV70 grows well between 33 and 37°C.

Genetics and evolution

EV70 strains isolated from Asia and the Americas during the 1980–1981 AHC pandemic are closely related to each other but differ from the 1981 Japanese endemic and the 1971 strains by many nucleotides. This finding suggests that only one basic EV70 genotype circulates worldwide at any one time but that clusters of EV70 strains emerge and evolve independently from each other. Partial nucleotide sequence information is available for a large number of isolates taken from various geographical regions in the period 1971–1981. These sequences infer a phylogenetic tree with an origin for all branches occurring in approximately 1967, suggesting that the virus first arose in the human population, giving a recognizable AHC, at that time. The rate of nucleotide substitution was estimated to be 3.8×10^{-3} per year, higher than that seen in influenza virus and human immunodeficiency virus.

Serologic properties

EV70 is antigenically distinct from existing members of the human enteroviruses by crossneutralization tests. Neutralization kinetics revealed only slight antigenic variations between strains isolated in 1971 and strains isolated from subsequent epidemics. The major antigenic sites remained unchanged. Epitopes reacting in microneutralization test with monoclonal antibodies are also relatively stable and conserved.

Epidemiology

Alternating and together with CA24V, EV70 has been responsible for sporadic and epidemic outbreaks of AHC globally. AHC affects all age, social-income and ethnic groups. However, incidences in children under 10 years of age, in particular infants, and the over-60 age group have been markedly low. AHC is most prevalent in young adults and teenagers, and more males than females are affected. The overall attack rates vary from 20% to 100% within members of families.

Transmission and pathogenicity

EV70 is transmitted by the fecal–oral route, ocular and respiratory secretions and contact with contaminated objects such as facecloths, bath towels, handkerchiefs and bedding. In ophthalmic clinics, crossinfections occur when the eye is infected with contaminated fingers of attending physicians and nurses, ophthalmic instruments and solutions. Although water stored in containers and basins and spring water have been identified as sources of virus transmission, contaminated utensils used to bale water and contaminated water taps and door knobs are also possible vehicles of virus spread.

EV70 has affinity for epithelial cells of the conjunctiva, which is the primary site of replication. Only a few strains have been isolated from the throat and feces. Virus neurotropism is suggested in patients with central nervous system (CNS) involvement and polio-like radiculomyelitis accompanying or following AHC, and in patients who have been in contact with cases of AHC. However, to date, EV70 has not been isolated from the CNS.

EV70 produces an enteroviral CPE in cell cultures of primate origin and some strains can be adapted to grow in nonprimate cells. The virus is not pathogenic for mice. Monkeys inoculated with EV70 via the intraspinal and intrathalamic routes develop paraplegia or monoplegia of lower limbs. However, feeding and instillation of virus into the eyes does not cause pathological changes. Rabbits with experimentally induced conjunctivitis develop clinical disease and virologic and immunologic responses similar to AHC in humans. Conflicting reports on animal studies may be attributed to differences in the preparation of virus inoculum, choice and origin of EV70 strains, and the method of inoculation.

Clinical features of infection

Acute conjunctivitis caused by EV70 is clinically indistinguishable from conjunctivitis caused by CA24v, poliovirus and adenovirus type 2. The eye affliction is characterized by a short incubation of 1–2 days and high secondary attack rate. Common features are sore red eyes with a 'foreign body' sensation, swelling of the eyelid, lacrimation, chemosis, edema and hyperemia of the conjunctiva, photophobia, blurring of vision and preauricular gland enlargement. Seromucous and purulent discharge due to secondary bacterial infection are common. Follicular hypertrophy of the conjunctiva is most prominent in the upper and lower fornix. Small petechiae to large blotches of subconjunctival hemorrhage have been reported in 10–100% of patients in various countries. Subconjunctival hemorrhage usually initiates in the bulbar conjunctiva near the upper fornix and spreads to the upper half of the bulbar conjunctiva and to the lower fornix. It is this striking appearance of subconjunctival hemorrhage which prompted the coining of the name acute hemorrhagic conjunctivitis. Corneal lesions cause blurring of vision and pain. Iritis is rare. Uni- or bilateral, the conjunctivitis varies in severity and usually resolves within 1–2 weeks without sequelae. With few exceptions, cases of radiculomyelitis involve adults. Three clinical forms of CNS manifestations have been identified. The most common is the spinal form, which is characterized by acute asymmetrical flaccid motor paralysis or paresis in one or more limbs, followed by atrophy in severely affected muscles. Pain in the limbs, back and neck are present during the early phase of disease. The next most common form is the cranial form, which is characterized by acute motor cranial nerve palsy and involves a single nerve or groups of nerves. The third form features a combination of spinal and cranial involvements. The preparalytic manifestations, prognosis of flaccid motor paralysis, changes in cell count and total protein level in cerebrospinal fluid (CSF), resemble poliomyelitis. The distribution of flaccid paralysis and development of muscular wasting indicate involvement of anterior horn cells of the spinal cord. Rare incidences of facial palsy, palatal paresis, Bell's palsy, bladder paralysis, vertigo, parotitis resembling mumps and sensory loss have also been reported. Respiratory and gastrointestinal symptoms accompany AHC in some patients.

Pathology and histology

Histological examinations of the conjunctiva reveal a predominance of mononuclear cells with diffuse lymphocytic infiltration into the adenoid layer and accumulation of lymphocytes in areas corresponding to lymph follicles. The tarsal conjunctiva is hyperemic, with small petechiae. Small follicles in the temporal portion of the lower tarsal conjunctiva are seen in 10–100% of patients. Subconjunctival hemorrhages vary in severity. Large areas of blotchy bleeding may extend over the whole bulbar conjunctiva. Hemorrhages usually disappear within 1 week but in severe cases a crescent-shaped hemorrhage remains longer. Fine pinpoint epithelial opacities in the cornea are common but punctate subepithelial keratitis is rarely seen. Sections of the spinal cord of three patients with polio-like paralysis revealed degeneration of cells of the anterior horns, hemorrhages and neuroglial cell proliferation. EV70-specific antigen was demonstrated in the microglial and/or neuronal cells by immunofluorescent testing.

Immune response

EV70-specific IgM and IgG antibodies and interferon are found in response to infection. When sera are taken at the acute stage of infection and again 3–4 weeks later, a $\geq$ fourfold increase in antibody is frequently discernible. However, the absence of antibody from patients from whom virus has been isolated has also been encountered. Neutralizing antibody has been detected in the CSF of patients with radiculomyelitis. In a few patients, blood serum to CSF neutralizing antibody ratios of <20 have been observed and are indicative of direct invasion of the CNS by EV70. Despite two major EV70 AHC epidemics in 1971 and 1980 and sporadic interepidemic incidences of EV70 conjunctivitis in Singapore, sera sampled at the acute stage of conjunctivitis do not appear to have neutralizing antibody ($<1/10$) to EV70. A similar seronegativeness was observed in Hong Kong. This transient immunity may be attributed to the predominantly localized nature of the infection.

Prevention and control

Intrafamilial and epidemic spread of AHC is controlled by public education through mass media on the mode of virus transmission, practice of good personal hygiene, exclusion of persons with conjunctivitis from schools, institutions, offices, factories, recreation centers and crowded locations. Nosocomial transmission of the disease can be prevented by segregation of patients with conjunctivitis, thorough hand washing before and after contact with infected eyes, use of separate eye-droppers for treatment and proper sterilization of instruments used for the eye. The future prospects for treatment of AHC include application of interferon β, arildone and chemically related compounds that have been shown to inhibit replication of EV70 and CA24v *in vitro*.

Enterovirus Serotype 71 (EV71)

History and classification

CNS disease caused by EV71 was first recognized in California (1969–1972) when patients presented with aseptic meningitis (AM) and encephalitis. BrCr/1970, the prototype EV71 strain, was isolated from the brain of a fatal case of encephalitis. Since then, EV71 has been isolated from patients with neurological involvement and various clinical conditions in many parts of the world. Indeed, EV71 seems to be increasing in importance as a cause of, sometimes severe, neurological disease.

Geographic and seasonal distribution

EV71 seems to be widespread in the world. It was implicated in cases of AM and encephalitis in New York State in 1972 and in Australia in 1972–1973, 1978, 1979, 1982–1983 and 1986 in similar cases of CNS involvement and additionally hand–foot–mouth disease (HFMD), respiratory illness and polyneuritis. Similar EV71 outbreaks occurred in Sweden (1972), Japan (1973, 1978) and Bulgaria (1975) where poliomyelitis-like paralysis occurred with a high rate of fatality in children. EV71 has been associated with a concurrent epidemic of tick-borne encephalitis in Hungary (1978), an outbreak of acute respiratory illness with CNS involvement in Lyon, France (1979), a case of HFMD in Singapore (1984), an outbreak of monoplegia in Hong Kong (1987), a case of HFMD in China (1989) and two cases of acute viral encephalitis in Japan (1996). More recently (April–August 1997), EV71 was implicated, possibly with concurrent coxsackie B virus, in an extensive epidemic of HFMD in Malaysia in which 30 children died of cardiac failure, with or without CNS involvement.

There is no apparent seasonal influence on outbreaks.

The virus

EV71 shares the physicochemical characteristics of the enterovirus group. Like the other viruses in this group, EV71 is a typical enterovirus. The complete nucleotide sequence of strain MS7423/87 has been determined. This reveals that it is very closely related to coxsackieviruses A16 and A2 and clusters genetically in a group that contains many other coxsackie A viruses, including 3, 5, 7, 8, 10, 12 and 14. Little specific information on replication, receptor recognition and three-dimensional structure is available, but the virus probably does not differ significantly in these properties from the better-studied enteroviruses such as polio.

Host range and viral propagation

EV71 in feces, throat swabs, tissue from brain, spinal cord, mesenteric lymph nodes, CSF and skin vesicles produce CPEs in monkey kidney cell cultures, human fetal kidney, Hela and HEL cell lines. Some strains require several tissue culture passes to produce CPEs. Strains from patients with encephalitis produce CPEs at incubation temperatures of 35°C and 39°C, while strains from patients with HFMD grow at 35°C but not 39°C. EV71 strains vary in pathogenicity for suckling mice.

Serologic relationships and variability

BrCr/71 and related strains are antigenically distinct from previously classified enteroviruses as determined by neutralization and immunodiffusion tests.

Clinical features

EV71 is a prime example of an enterovirus capable of causing a variety of illnesses in sporadic or epidemic proportion. It is a major cause of aseptic meningitis often accompanied by encephalitis and bulbar–spinal dysfunction and polyneuritis. It can cause outbreaks of HFMD with or without neurological complications and has also been associated with cases of encephalomyocarditis and respiratory illnesses.

Epidemiology, transmission and tissue tropism

Generally, infants and young children are most frequently and severely affected. In the Bulgarian 1975 encephalitis epidemic, the highest incidence and mortality rates were in the 5 years and below age group.

EV71 can be transmitted by oral–fecal and respiratory routes and also in vesicular fluids from skin lesions. It has tropism for epithelial cells of the

alimentary and respiratory tracts, the skin and cells of the CNS.

Pathogenicity

Primate cell cultures inoculated with EV71 produce characteristic enteroviral CPEs, and EV71 strains vary in pathogenicity for suckling mice, cotton rats and hamsters, where they induce group A coxsackievirus-type myositis and paralysis. Strains isolated from patients with encephalitis are neurovirulent for monkeys, while strains from HMFD cases are less so.

Pathology and histology

In monkeys, neurotropic strains produce marked chromatolysis, degeneration and necrosis of the neurons and neuronophagia by polymorphonuclear or mononuclear leukocytes. Perivascular cuffings and petechiae are found in the lumbar and cervical cord, medulla oblongata, midbrain, pons and cerebral stem.

Immune response

Neutralizing antibody is produced in response to infection. Because of the relatively long incubation period in CNS disease, high antibody titers may be observed at the time of neurologic manifestations, and the diagnostic fourfold antibody rise between paired sera may be absent. However, persistence of EV71-specific immunoglobulin (Ig)M is transient and is indicative of current infection.

See also: **Coxsackieviruses (*Picornaviridae*); Eye infections; History of virology: Polio, coxsackie, echo and other enteroviruses; Pathogenesis: Animal viruses, Plant viruses; Polioviruses (*Picornaviridae*): General features, Molecular biology.**

Further Reading

Ishii K, Uchida Y, Miyamura K and Yamazaki S (eds) (1989) *Acute Hemorrhagic Conjunctivitis, Etiology, Epidemiology and Clinical Manifestations*. Tokyo: University of Tokyo Press.

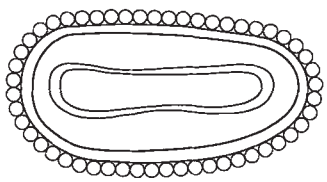
Melnick JL (1984) Enterovirus 71 infections: a varied clinical pattern sometimes mimicking paralytic poliomyelitis. *Rev. Infect. Dis.* 6: S387.

Melnick JL (1996) Enteroviruses: polioviruses, coxsackieviruses, echoviruses and the newer enteroviruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn. p. 655. Philadelphia: Lippincott-Raven.

Pöyry T, Kinnunen L, Hyypiä T *et al* (1966) Genetic and phylogenetic clustering of enteroviruses. *J. Gen. Virol.* 77: 1699.

Yin-Murphy M (1984) Acute hemorrhagic conjunctivitis. *Prog. Med. Virol.* 29: 23.

ENTOMOPOXVIRUSES (*POXVIRIDAE*)



Richard W Moyer, Department of Immunology and Medical Microbiology, University of Florida, Gainesville, Florida, USA

Introduction

The entomopoxviruses (EPVs) and vertebrate poxviruses comprise the two subfamilies of the poxvirus family. The EPV subfamily includes a varied collection of insect viruses which share features associated with the more commonly known and more intensively studied vertebrate poxviruses such as vaccinia, fowlpox, variola (smallpox) and myxoma viruses. While EPVs and the vertebrate poxviruses have some properties in common, there are also some striking distinctions. Similarities include: general virion morphology, a large linear double-stranded DNA genome comprising by weight about 5% of the particle; common sequence motifs that are associated (at least in the vertebrate viruses) with regulation of transcription; nonspliced transcripts; a cytoplasmic site of replication and the encoding of a wide variety of cytoplasmic enzymes necessary to support transcription and replication of the virus within the cytoplasm. Differences include: the G + C content of the viral DNA (EPV G + C content is quite low; 17–27% as compared to the 32–39% observed for vertebrate poxviruses); length of the replication cycle and optimal growth temperature (26–28°C as compared to ~37°C for vertebrate poxviruses) and host range.

alimentary and respiratory tracts, the skin and cells of the CNS.

Pathogenicity

Primate cell cultures inoculated with EV71 produce characteristic enteroviral CPEs, and EV71 strains vary in pathogenicity for suckling mice, cotton rats and hamsters, where they induce group A coxsackievirus-type myositis and paralysis. Strains isolated from patients with encephalitis are neurovirulent for monkeys, while strains from HMFD cases are less so.

Pathology and histology

In monkeys, neurotropic strains produce marked chromatolysis, degeneration and necrosis of the neurons and neuronophagia by polymorphonuclear or mononuclear leukocytes. Perivascular cuffings and petechiae are found in the lumbar and cervical cord, medulla oblongata, midbrain, pons and cerebral stem.

Immune response

Neutralizing antibody is produced in response to infection. Because of the relatively long incubation period in CNS disease, high antibody titers may be observed at the time of neurologic manifestations, and the diagnostic fourfold antibody rise between paired sera may be absent. However, persistence of EV71-specific immunoglobulin (Ig)M is transient and is indicative of current infection.

See also: **Coxsackieviruses (*Picornaviridae*); Eye infections; History of virology: Polio, coxsackie, echo and other enteroviruses; Pathogenesis: Animal viruses, Plant viruses; Polioviruses (*Picornaviridae*): General features, Molecular biology.**

Further Reading

Ishii K, Uchida Y, Miyamura K and Yamazaki S (eds) (1989) *Acute Hemorrhagic Conjunctivitis, Etiology, Epidemiology and Clinical Manifestations*. Tokyo: University of Tokyo Press.

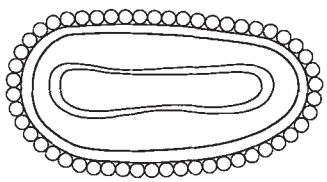
Melnick JL (1984) Enterovirus 71 infections: a varied clinical pattern sometimes mimicking paralytic poliomyelitis. *Rev. Infect. Dis.* 6: S387.

Melnick JL (1996) Enteroviruses: polioviruses, coxsackieviruses, echoviruses and the newer enteroviruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn. p. 655. Philadelphia: Lippincott-Raven.

Pöyry T, Kinnunen L, Hyypiä T *et al* (1966) Genetic and phylogenetic clustering of enteroviruses. *J. Gen. Virol.* 77: 1699.

Yin-Murphy M (1984) Acute hemorrhagic conjunctivitis. *Prog. Med. Virol.* 29: 23.

ENTOMOPOXVIRUSES (*POXVIRIDAE*)



Richard W Moyer, Department of Immunology and Medical Microbiology, University of Florida, Gainesville, Florida, USA

Introduction

The entomopoxviruses (EPVs) and vertebrate poxviruses comprise the two subfamilies of the poxvirus family. The EPV subfamily includes a varied collection of insect viruses which share features associated with the more commonly known and more intensively studied vertebrate poxviruses such as vaccinia, fowlpox, variola (smallpox) and myxoma viruses. While EPVs and the vertebrate poxviruses have some properties in common, there are also some striking distinctions. Similarities include: general virion morphology, a large linear double-stranded DNA genome comprising by weight about 5% of the particle; common sequence motifs that are associated (at least in the vertebrate viruses) with regulation of transcription; nonspliced transcripts; a cytoplasmic site of replication and the encoding of a wide variety of cytoplasmic enzymes necessary to support transcription and replication of the virus within the cytoplasm. Differences include: the G + C content of the viral DNA (EPV G + C content is quite low; 17–27% as compared to the 32–39% observed for vertebrate poxviruses); length of the replication cycle and optimal growth temperature (26–28°C as compared to ~37°C for vertebrate poxviruses) and host range.

The limited serology available suggests that there is little immunological relationship either among the insect poxviruses or with the vertebrate poxviruses. Using an ELISA, antibodies made against purified *Amsacta moorei* entomopoxvirus (AmEPV) detected little antigenic relatedness between the structural proteins of the *Amsacta, Euoxa* or *Melanoplus* EPVs or vaccinia virus. However, antibodies against the *A. moorei* (red hairy caterpillar) EPV occlusion body protein crossreacted extensively with the occlusion body protein of the *E. auxiliaris* (army cutworm) EPV. The EPVs are relatively recent discoveries, the first being observed in 1963 as a disease of *Melolontha* (the common European cockchaffer). Since that time, these viruses have been discovered in well over 60 species of insects, which to date are the only known hosts to give a totally permissive infection. Distribution of these viruses is throughout the world – the Americas, Europe, Asia, Australia and Africa – and they are found in both agricultural and aquatic insect populations. These viruses have been found to infect primarily, if not exclusively, four orders of insects, Orthoptera, Diptera, Coleoptera and Lepidoptera, with most members being associated with coleopteran and lepidopteran hosts. More recently, poxvirus-like particles have been discovered in Hymenoptera (bumblebees and parasitic wasps) but with seemingly little pathological effects. A distinctive property of these viruses is the presence of occluded virions within a protein matrix consisting primarily of a single protein, spheroidin. Presumably spheroidin provides environmental protection and virion stability until exposure, after ingestion, to the alkaline midgut of the insect. While a wealth of biological studies on the behavior of these viruses in their natural hosts is available, molecular studies have been extremely limited due to the paucity of *in vitro* cell culture systems, and the failure to perceive that these viruses have the potential to be generally useful as biopesticides and for expression systems. There are currently only two EPVs which can be readily grown in cell culture. The first, AmEPV, a virus originally isolated from the red hairy caterpillar of India and adapted to the saltmarsh caterpillar (in the US); and more recently HaEPV isolated from *Heliothis armigera.*

Taxonomy

The family *Poxviridae* is divided into two subfamilies: (1) the *Chordopoxvirinae,* comprising the vertebrate poxviruses; and (2) the *Entomopoxvirinae,* which comprises the viruses of insects. The most recent proposals of the International Committee on Taxonomy of Viruses define the genera *Orthopoxvirus* (vaccinia subgroup), *Parapoxvirus* (orf subgroup), *Avipoxvirus* (fowlpox subgroup), *Capripoxvirus* (sheeppox subgroup), *Leporipoxvirus* (myxoma subgroup), *Suipoxvirus* (swinepox subgroup), *Molluscipoxvirus* (molluscum subgroup) and *Yatapoxvirus* (Yaba/tanapox subgroup) within the *Chordopoxvirinae* subfamily. The *Entomopoxvirinae* contains three proposed genera, designated as *Entomopoxvirus A, B* and *C,* devised primarily on observed structural differences between virions. *Entomopoxvirus A* viruses infect the order Coleoptera (beetles). *Entomopoxvirus B* viruses infect insects of the orders Lepidoptera (moths) and Orthoptera (grasshoppers and locusts), whereas *Entomopoxvirus C* infects insects of the order Diptera (flies). The prototype viruses from each genera are those from *Melolontha* (genus A), *A. moorei* (genus B) and *Chironomus luridus* (midge) (genus C). Whether these genera, which were devised primarily on morphological considerations, are substantiated by similarities at the molecular level must await further detailed characterization of individual viruses. However, Southern hybridizations between viruses (see below, Viral DNA) suggest revision at some later date may be necessary.

Virion Structure

The virions are quite large, brick- or oval-shaped, exhibiting a rich, complex and somewhat varied structural morphology reminiscent of the vertebrate poxviruses (**Fig. 1**). Lengths observed vary from 150 to 470 nm and widths from 165 to 300 nm. General structural characteristics shared with the vertebrate poxviruses include a surface envelope, lateral bodies and an internal core located at the center of the virion. The surface characteristics of the envelope of the virions are beaded and 'raspberry-like', resulting from the folding pattern of an external membrane. Similar convolutions at the external surface of vaccinia virus are not evident. Within the virion is located the well-defined electron-dense core which contains the DNA. As in the orthopoxviruses, the lateral bodies are located within the virion, external to the core but inside the envelope. The lateral bodies are distinct from the core and no function has yet been assigned to them. One property distinguishing different genera is the presence of either one or two lateral bodies.

The largest (250 × 450 nm) are the coleopteran EPVs, which contain a single lateral body and a core of concave appearance. The lepidopteran and orthopteran EPVs are intermediate in size (250 × 350 nm) and are characterized by a symmetrical cylindrical core and a cylindrical lateral body. The dipteran viruses are the smallest (230 × 320 nm) and are characterized by a vaccinia-like biconcave core and

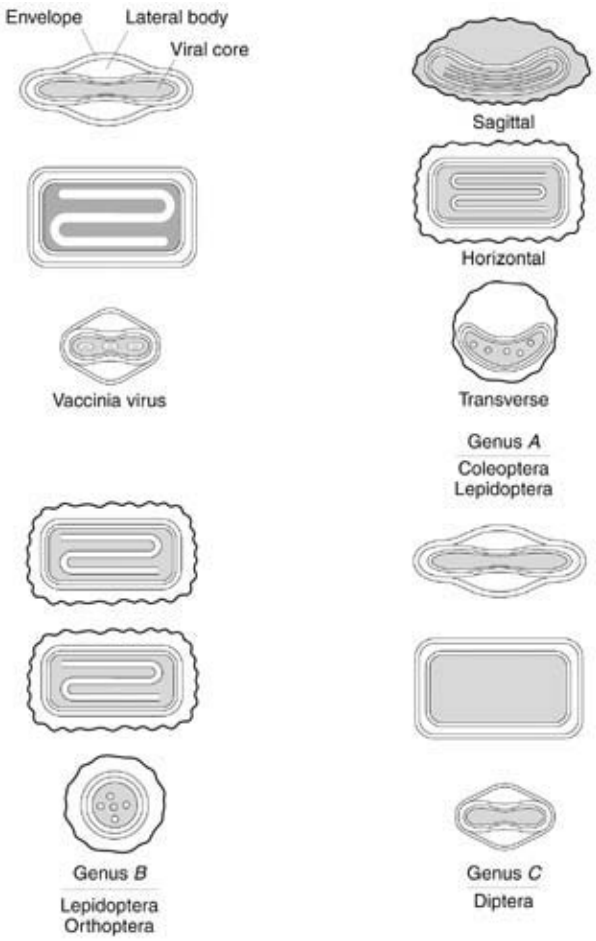


Figure 1 EPV structures from the three genera as compared to vaccinia virus showing the relevant morphological features of each (taken from Arif B (1995) *J. Gen. Virol.* 76: 1.

two lateral bodies. Indeed, the dipteran viruses most closely resemble vaccinia virus in appearance.

Virion and Genome Composition

Virion

Analysis of polypeptide composition by PAGE reveals an extraordinarily complex pattern of polypeptides with molecular weights from approximately 12 kDa to 250 kDa. Many viral particles are occluded within a proteinaceous mass, not an integral component of the virion *per se*, which consists predominately of a single protein, spheroidin. These so-called occlusion bodies are reminiscent of A-type inclusions (ATIs), formed by the vertebrate cowpox virus and the occlusion bodies formed by the nucleopolyhedrosis viruses, structures which again in each case are predominately derived from a single protein (the ATI and polyhedrin proteins respectively). Spheroidins are approximately 115 kDa in size and typically very high in both cysteine and methionine residues, which could explain why carbonate and a reducing agent are required to effect dissolution of occlusion bodies and release of the free virions. The spheroidin genes of the EPVs of *A. moorei* (AmEPV), *Choristoneura fumiferana* (CfEPV), *Melolontha melolontha* (MmEPV) and *Heliothis armigera* (HaEPV) are highly conserved. Identity to the AmEPV spheroidin gene ranges from 81% and 78% for the CfEPV and HaEPV genes, respectively (genus *B* viruses), to 43% for the MeEPV gene (genus *A* virus). There is little significant homology between the spheroidin genes and the ATI gene of cowpox virus. The amounts of spheroidin synthesized within infected cells are large and spheroidin constitutes a significant percentage of total cell mass.

Frequently, but not in every case, EPVs that infect Lepidoptera and Coleoptera (genus *B* and *A* viruses) produce a second very abundant 350–380 amino acid protein (fusolin or spindle body protein) which forms paracrystalline, spindle-shaped structures. Although

the fusolin-containing spindle structures themselves do not contain virions, fusolin can in many cases become occluded together with virions into occlusion bodies (spheroids). AmEPV lacks this gene (hence it is nonessential), but it is present within the genomes of MmEPV, HaEPV, CfEPV, as well as in the EPVs from *C. biennis* (CbEPV) and the armyworm *Pseudaletia separata* (PsEPV). The degree of identity of this protein ranges from about 70–90% among the genus *B* viruses (HaEPV, CfEPV, CbEPV and PsEPV) to 60% between HaEPV and MmEPV (a genus *A* virus). There are two other noteworthy features of this protein. First, it exhibits significant homology (37–42% identity) with a 37 kDa glycoprotein from *Orgyia pseudotsugata* and *Autographa californica* nucleopolyhedrosis viruses. This relationship extends to five conserved regions within the baculovirus glycoprotein which are conserved within the EPV fusolin or spindle protein. The second feature is that the PsEPV fusolin (spindle) protein is reported to serve as a 'virulence enhancing factor' which increases the susceptibility of the armyworm larvae host to a nucleopolyhedrosis virus several thousand-fold.

A third abundantly expressed ~25 kDa protein (filament-associated late protein of EPV, FALPE) has been described in AmEPV infected cells. Fibrils containing this protein are closely associated with occlusion bodies and may play a role in the morphogenesis and/or occlusion process.

As a consequence of a cytoplasmic site of development within the infected cell, EPVs, like their vertebrate counterparts, both encode and contain a number of enzymes within the virion. Enzymes identified to date include a DNA-dependent RNA polymerase, poly(A) polymerase, alkaline protease, topoisomerase, nucleotide phosphohydrolase and acid and neutral DNases. The alkaline protease has been associated with occlusion bodies and may be responsible, in part, for degradation and dissolution of occlusion bodies within the insect gut. The properties of the AmEPV encoded topoisomerase have been compared with those of the vaccinia-encoded enzyme and found to be quite similar, despite significant sequence divergence (only 36% identity).

AmEPV contains a homologue of an unusual vertebrate poxvirus gene first reported in vaccinia virus. The vaccinia virus gene, when mutated, can confer resistance of the virus infection to the inhibitory action of rifampicin.

Viral DNA

The DNA of the vertebrate poxviruses is a linear, large duplex (130–300 kb) and is characterized by a high degree of colinearity amongst all genes located within the conserved central core comprising 60% of the viral genome, as well as terminal inverted repetitions, and crosslinked telomeric termini. The DNAs of EPVs range in size from 200 to 300 kb and are uniformly characterized by an unusually low G+C content ranging from 17 to 27% (compared to 32–39% for the vertebrate poxviruses). The DNA of AmEPV is 225 kb in size and has a G+C content of 18.5%. There is some evidence both for the presence of terminal inverted repetitions in AmEPV DNA, and for the existence of crosslinked telomeric termini, typical of the vertebrate poxviruses. As might be expected, DNA restriction enzyme digests of EPV DNAs from various sources differ significantly and studies of the relative homologies of entomopoxvirus genomes to each other, and to vaccinia have yielded only limited information. Hybridization studies with various entomopoxviruses show no obvious homology to vaccinia virus based on Southern hybridizations. Likewise, no hybridization was observed between EPVs which infect different insect orders. Within the same insect order, extensive hybridization was observed between the lepidopteran *A. moorei* and *E. auxiliaris* viruses. However, the lepidopteran viruses CbEPV and the virus from *Oncopera albogutatta* failed to show hybridization with either the *A. moorei* or *E. auxiliaris* viruses. Hybridization was also observed among some orthopteran viruses: *Melanoplus sanguinipes, Arphia conspersa, Phoetaliotes nebrascensis* and *Oedaleus senegalensis*. However, the two grasshopper viruses from *Aeropedellus clavatus* and *Cataloipus cymbiferus* failed to hybridize to any of the other four orthopteran viruses. These results must be interpreted with caution because significant homologies can still exist in the final protein products and at the DNA level, yet not be detected through Southern hybridizations. Indeed there are now many examples amongst EPVs and vertebrate poxviruses where direct sequencing has shown this to be the case.

Based on available sequence information, it has also been shown that the order of core genes of the AmEPV genome is not colinear with the conserved arrangement of core genes characteristic of the vertebrate viruses (**Fig. 2**). The significance of this observation is not clear but argues that the conserved arrangement of genes observed in the vertebrate poxviruses is not essential for viability nor a feature to be expected of all poxviruses.

Characteristics and Pathology of Viral Infections

These viruses collectively show a wide variety of cell, tissue and host specificity. No productive crossinfec-

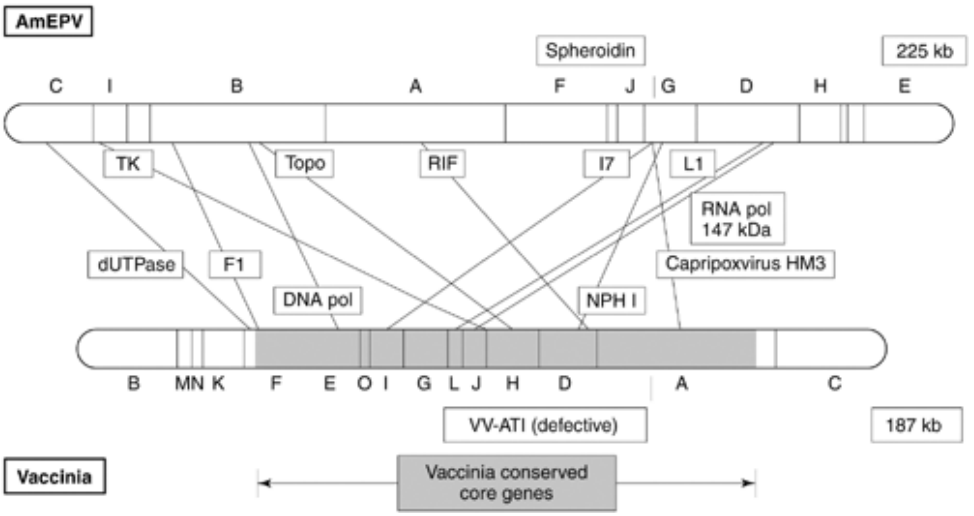


Figure 2 *Hin*dIII restriction maps drawn to scale showing the relative location of similar genes in the vaccinia and *A. moorei* genomes. TK, thymidine kinase vaccinia, open reading frame (ORF) J2R; Topo, DNA topoisomerase, vaccinia ORF H6R; RIF, rifampicin resistance gene, vaccinia ORF D13L; I7, vaccinia I7L ORF; L1, vaccinia L1R ORF; RNA pol 147 kDa, vaccinia ORF J6R; Capripoxvirus HM3, ORF HM3 of capripoxvirus; NPH 1, nucleoside triphosphate phosphohydrolase 1, vaccinia ORF D11L; DNA pol, vaccinia DNA polymerase ORF D9R; F1, vaccinia ORF F1L; dUTPase, deoxyuridine triphosphatase, vaccinia ORF F2L.

tions have yet been produced between the two insect subfamilies. Most viruses replicate within the cytoplasm of cells of the fat body, with the infections being characterized by the appearance of large elliptical occlusions and occasionally microspindles. Some viruses are restricted to the fat body, while others infect, in addition, hemocytes and the epidermis. Some viruses can produce systemic infections. The pathology therefore varies somewhat depending on the order of insect. It has been well described for specific viruses by Granados and Arif (see Further Reading) and is reproduced and summarized here.

Lepidopteran infections are relatively short, generally less than 3 weeks in duration. Infection of larvae is common, of pupae is rare and of adults unknown. Some species become 'white' after infection. Infected larvae may become discoordinated, lethargic and show significant loss of mobility during later phases of the infection. The larvae of *E. acrea* show paralysis in the posterior two-thirds of the body. Death of some species is preceded by regurgitation or defecation of material containing occlusion bodies. Infection of *C. fumiferana* larvae with *C. biennis* virus was characterized by hormonal imbalance. Wing buds were present in insects that died in the sixth instar. Infected pupae retained prolegs. Diseased larvae were approximately twice the size of controls and the last larval stadium was around 15 days instead of the usual 7. Infection by the prototypic *A. moorei* virus is promiscuous, in the sense that many cells and tissues are involved. The fat body, hemocytes, nerve cells, midgut cells, hypodermis, tracheoblasts and muscle cells are all involved. Other lepidopteran virus infections are much more limited and involve primarily the fat body. Midgut infection has been reported infrequently for both the *C. biennis* and *A. moorei* viruses, but may probably be relatively common because the natural port of entry into the insect is through the midgut. The presence of occlusion bodies is typical, whereas the presence of spindles is sporadic, depending on the virus. Indeed, spindles are much more typical of EPV infections of lepidopteran and coleopteran than orthopteran or dipteran species. However, the *C. biennis* virus, a second intensively studied lepidopteran virus, forms extremely large spindles (100 × 500 nm). Antigenically, spindles and the spheroidin derived from occlusion bodies are unrelated, consistent with sequence information which shows them to be distinct.

In the case of the orthopteran EPV from *Melanoplus sanguinipes* (MsEPV), an interesting relationship between the pattern of mortality and infectious dose has been noted. Infections with high or low dosages of virus resulted in early or late death of the insect respectively. Insects that died early (<12 days postinoculation) were devoid of spheroids (occlusions), and homogenates of cadavers were not infectious. However, cadavers of insects that died late (>16 days postinfection) contained many spheroids and were infectious to other grasshoppers. Hence, either nonoccluded virus or a viral toxin or toxic factor are critical elements of early insect mortality.

Coleopteran infections are unusually prolonged and may persist for as long as 30–40 weeks. Early signs of disease in *M. melolontha* larvae include

distension or proliferation of diseased fat body tissue with the lobes of fat body tissue exhibiting a foamy white appearance, a chalky white appearance of the larvae and softening of the integument. Infected larvae lose turgidity and become lethargic. The only cells infected are hemocytes and fat body cells. Late in disease, the hemolymph becomes chalky. All stages of larval development are susceptible to infection, with the first two instars being most susceptible. While fat body and the hemocytes are the most common cell types involved, the intestinal tract and hypodermis can all be infected. Viral infections are characterized by the presence of fusiform spindles and oval occlusion bodies in the cytoplasm of the infected cells. Spindles are smaller, in cross-section, and commonly 15–23 μm in length. Occlusion bodies are 5–20 μm in diameter but larger overall. Virions are only found within occlusion bodies and the function and/or significance of the spindles is unclear.

Dipteran infections are again characterized by a whitening of the larvae as masses of opaque white hemocytes and fat body accumulate. In the case of chironomid larvae, the presence of irregular white areas is observed beneath the integument. The course of the infection is generally from 3 to 8 weeks. Infected hemocytes accumulate early in infection in the dorsal region of the ninth abdominal segment just posterior to the heart. Infected first instar larvae may die as soon as 24–78 h postinfection. The pathology again varies depending on the virus. The EPV of *C. luridus* appears to infect many cells and tissues, including fat bodies, hemocytes, epidermis, oenocytes, imaginal disks of the leg and genital organs, muscles, nervous system and the intestinal tract. Others infect only the hemocytes. Signs of infection include the presence of elliptical cytoplasmic occlusion bodies ranging in diameter from 3 to 6 μm.

Orthopteran infections are characterized (in the grasshopper) by lethargy, slow development and a high mortality. Abdomens of infected nymphs become distended, and show protruding cervical membranes as occlusion bodies accumulate in the fat bodies. Diseased fat bodies become hypertrophied, grayish and take on an irregular shape. Severely infected nymphs rarely develop past the first instar, and become essentially moribund. In grasshoppers, the fat body is certainly the main target of the infection and appears to be the only tissue infected. Again, the presence of occlusion bodies is characteristic of infected insects.

Virus Replication

Infection of the organisms ensues following dissolution of occlusion bodies in the midgut after ingestion. The infectious process initiates with attachment of the released virions to epithelial cells of the midgut. The observed pattern and strategy of infection at the cellular level is quite similar to that noted for the vertebrate poxviruses. Electron micrographs indicate that fusion of the virion envelope to the membrane of the microvilli allows entry of the partially uncoated virion (core and lateral bodies) into the cell cytoplasm. In intrahemocoelic infections, penetration of the free virus occurs via viropexis. In this case, partial uncoating of the internalized virion takes place within the phagosome derived from the process of viropexis, followed by release of the core into the cytoplasm where additional steps of the infectious cycle ensue. Viropexis does not appear to play a significant role during *per os* infections of larvae. Similar to the vertebrate poxviruses, the further, more complete uncoating likely requires early viral gene expression. One of the first signs of replication is the appearance of viroplasm (factories) consisting of irregularly shaped masses of DNA, some of which is coated by or within semicircular bilayer membranes. Later in infection, one observes a series of completely enveloped assembly intermediates (immature particles or virions) consisting of amorphous spheres and spheres with electron-dense cores, as well as other less common forms that accumulate in the cytoplasm. For some viruses, a second type of viroplasm (type II) has been noted that is characterized by aggregated granular material interspersed with spherical vesicles. Once immature particles form, the virus undergoes further maturation, assumed to occur from within, leading to formation of the mature cores and lateral bodies. Only fully mature virions are occluded. Nonoccluded virions bud through the cytoplasm into the hemocele, a process accompanied by the acquisition of a second membrane. It is likely that it is the nonoccluded form of the virus that accounts for generalized dissemination throughout the insect. Occluded virus tends to concentrate in the fat bodies.

While replication of these viruses has been studied intensively in the host organisms, fewer data are available on replication in cell culture, particularly at the molecular level, as there is a paucity of insect cell lines permissive for infection with these viruses. Until recently, only the *A. moorei* virus could be readily propagated in culture using a cell line derived from *E. acrea* (salt marsh caterpillar) hemocytes (BTI-EAA cells) or from the gypsy moth, *Lymantria dispar* (IPLB-LD-652 cells). In cell culture, AmEPV DNA replication can be detected as soon as 6 h postinfection, with a rapid increase in synthesis being observed between 12 and 24 h. Extracellular virus and virus-containing occlusion bodies appear 18 h after infection. Thirty-seven virus proteins ranging from 13 to

200 kDa were found in occluded and nonoccluded forms of the virus. Structural proteins are synthesized from 18 to 34 h postinfection and the occlusion body protein can be detected as early as 24 h after inoculation (see Marlow *et al*, Further Reading). Similar results have been found more recently with HaEPV (see Fernon *et al*, Further Reading). Another lepidopteran virus isolated from *Adoxophyes* (smaller tea tortrix) reportedly can be propagated in about 50% of cells in a cell line derived from *Homona magnanima*. Recently, a virus isolated from *Pseudaletia separata* larvae has been reported to propagate on a cell line derived from *P. separata* or *Bombyx mori* larvae. The replicative potential of the *A. moorei* virus has been examined in mammalian (L) cells. No newly synthesized AmEPV proteins were observed at 37°C. However, AmEPV virions could apparently induce cell fusion and the formation of polykaryocytes in either L or HeLa cells and reduce the numbers of superinfecting vaccinia plaques. More recent studies have shown that AmEPV enters vertebrate cells, expresses early genes, but that viral DNA synthesis fails to occur and late viral protein expression is absent. Most surprising is that the vertebrate cells survive the infection and continue to divide.

Information relevant to the regulation of gene expression in EPVs is now becoming available. This information suggests that while many regulatory signals are likely to be universal amongst all poxviruses, detailed study of the AmEPV spheroidin gene promoter has revealed features unique to the insect virus. Many of the sequences associated with regulatory signals, typical of the vertebrate (vaccinia) poxviruses, appear to be conserved and present in the EPVs. DNA sequence derived from both the *C. biennis* and *A. moorei* viruses suggests that the TAAATG motif, typical of late vertebrate poxvirus promotors is also present at the beginning of at least some EPV late genes, including both the spheroidin and nucleoside triphosphate phosphohydrolase I genes. Promotor elements isolated from the *C. biennis* virus have been shown to generate appropriately regulated gene expression (early or late) when cloned into appropriate vertebrate (vaccinia) constructs. Most late vertebrate poxvirus transcripts are polydisperse with heterogenous 3′ ends, an exception being the transcript for the cowpox ATI inclusion protein gene. The spheroidin transcript derived from the *A. moorei* virus appears, like that for the ATI gene of cowpox virus, also to be of discrete size and not polydisperse. The spheroidin transcript also contains a 5′ noncoded poly(A) tract typical of late transcripts from vertebrate poxviruses which is a feature of transcripts unique to poxviruses. Transcription of the AmEPV spheroidin gene has been shown to initiate within the TAAATG motif. However, despite these apparently conserved motifs, the AmEPV spheroidin gene promoter functions very poorly, if at all, in vertebrate cells. Analysis of recombinant vaccinia virus containing AmEPV-spheroidin promoter *lacZ* fusions also revealed that the expected transcription initiation site observed from similar AmEPV recombinants in insect cells was ignored (Hall *et al*, Further Reading). Thus, the AmEPV spheroidin promoter is the first known example of a promoter active uniquely within the insect environment. On the other hand, it has been reported that the 5′ upstream (promoter) region of the CbEPV spheroidin gene can function as a late promoter in vaccinia when cloned upstream of an appropriate reporter gene. However, expression was not as high as reported for other strong promoters of the vertebrate poxviruses such as the ATI promoter, nor as high as would have been expected based on comparative activity observed in infected insect cells.

At least one early gene product (thymidine kinase) is derived from a transcript of discrete size and the enzyme is functional when cloned into vaccinia virus. The heptanucleotide TTTTTNT, a sequence associated with the termination of early vertebrate poxvirus transcripts, has also been noted downstream of EPV early open reading frames, consistent with the hypothesis that this motif performs a similar function in the insect poxviruses.

Economic Importance

The ready genetic manipulation of EPVs is now possible. This advance required the detection, mapping and sequencing of the thymidine kinase and other non-essential genes from AmEPV for use as sites for insertion of foreign genes. The many similarities between the entomo- and vertebrate poxviruses have facilitated the adaptation of molecular techniques, originally designed for vaccinia virus, to the EPVs. Molecular biopesticides based on AmEPV can now be generated, as well as other potentially useful recombinants. Although traditionally insect control has been through the use of chemical pesticides, increasing environmental concerns are calling many of these chemicals and procedures into question. While the cost of biopesticides today may be higher, the environmental benefits are theoretically large. The potential market for such biopesticides is substantial, as EPVs have been described as causing disease in many insects of economic importance, including the spruce budworm (*C. biennis*), the lesser cornstalk borer (*Elasmopalpus lignosellus*), grasshoppers and locusts. The desert locust, *Schistocerca gregaria*, is

believed to be the most devastating crop pest in the world. Killing of insects following EPV infection in nature has been observed, but usually a second infectious agent was also present, which complicated evaluation of the specific effects of the EPV. At present, evidence suggesting that either laboratory or wild insect populations can be reduced by infection with wild-type EPVs is inconclusive. However, recombinant EPVs containing insect-specific toxins should make these viruses much more effective.

Another potential possibility is that 'shuttle' vectors for insertion of genes into either vertebrate or EPVs can now be developed. Although similarities between EPVs and vertebrate poxviruses permit common technology to facilitate genetic manipulation of both classes of viruses, one concern should be kept in mind – namely, the unintended exchanges of genetic material between the vertebrate and invertebrate families of viruses. Whether this issue is real or imagined, the concern may limit ultimate deployment of such recombinant viruses. Unintended recombination is less of an issue in the case of baculoviruses, as they do not resemble viruses of any other plant or vertebrate group.

An unanticipated application of the EPVs to the area of gene therapy was first suggested by the lack of any observable cytopathology during EPV infections of vertebrate cells. It was subsequently found that such cells survive and continue to grow despite entry of the virus and the expression of early viral genes. These results clearly suggest that rapid, intracellular transient expression of foreign genes, e.g. those encoding anti-inflammatory proteins or proteins which inhibit or induce apoptosis, may be possible in vertebrate cells and tissues through the use of recombinant EPVs as gene delivery systems. If so, such recombinant EPVs could be quite efficacious in treating certain acute clinical disorders.

If EPVs are to be seriously considered as tools for the control of insect populations or as gene therapy vectors, many more in-depth studies related to safety towards unintentional hosts, increasing growth and ease of manipulation *in vitro,* host specificity and basic biology are needed to separate real from perceived problems. Indeed, much useful information can be gained from these studies. In the past, the current limits in technology and lack of interest were the primary reasons the EPVs were not accorded the scientific attention they deserve. Based on more recent studies, the potential uses of EPVs now appear much greater, based on their biological, molecular and morphological similarities to the vertebrate poxviruses, together with the disappearance of many of the technical barriers to their genetic manipulation.

See also: **Poxviruses (*Poxviridae*): Capripoxviruses, Leporipoviruses and suipoxviruses; Smallpox and monkeypox viruses (*Poxviridae*); Vectors: Animal viruses, Plant viruses.**

Further Reading

Arif B (1995) Recent advances in the molecular biology of entomopoxviruses. *J. Gen. Virol.* 76: 1.

Arif BM and Kurstak E (1991) The entomopoxviruses. In: Kurstak E (ed.) *Viruses of Invertebrates,* p. 179. New York: Marcel Dekker.

Fernon CA, Vera AP, Crnov R *et al* (1995) Replication of *Heliothis armigera* entomopoxvirus *in vitro. J. Invertebr. Pathol.* 66: 216.

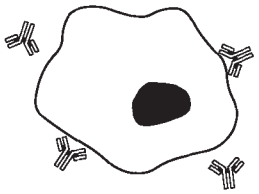
Granados R (1981) Entomopoxvirus infections in insects. In: Davidson EW (ed.) *Pathogenesis of Invertebrate Microbial Diseases,* p. 101. Mont Clair, NJ: Allanheld, Osmun.

Hall RL, Li Y, Feller JA and Moyer RW (1996) The *Amsacta moorei* entomopoxvirus spheroidin gene is improperly transcribed in vertebrate poxviruses. *Virology* 224: 427.

Li Y, Hall RL and Moyer RW (1997) Transient, non-lethal expression of genes in vertebrate cells using recombinant entomopoxviruses. *J. Virol.* 71: 9557.

Marlow SA, Billam LJ, Palmer CP and King LA (1993) Replication and morphogenesis of *Amsacta moorei* entomopoxvirus in cultured cells of *Estigmene acrea* (salt marsh caterpillar). *J. Gen. Virol.* 74: 1457.

EPIDEMIOLOGY OF VIRAL DISEASES



Frederick A Murphy, School of Veterinary Medicine, University of California, Davis, California, USA

Introduction

Viral disease epidemiology is the study of the determinants, dynamics and distribution of viral diseases in populations. The risk of infection or disease in a population is determined by characteristics of the virus, the host and the host population, as well as behavioral, environmental and ecological factors that affect virus transmission from one host to another. Epidemiology attempts to meld these factors into a unified whole.

The foundations of epidemiology predate the microbiological and virological sciences, starting with Hippocrates, the Greek physician and father of medicine, who in the fourth century BC made important epidemiologic observations on infectious diseases. John Snow is called the father of modern epidemiology because he developed excellent quantitative methods while studying the source of a cholera outbreak at the Broad Street pump in London in 1849. Snow was followed by William Farr, who in the 1870s advanced the use of vital statistics and clarified many of the principles of risk assessment and retrospective and prospective studies. Their vision is reflected in the fast-changing science of epidemiology which is now supported by advanced computer technology, sophisticated statistical methods and very sensitive and specific diagnostic systems.

Assessment of Disease Occurrence and Outcome

By introducing quantitative measurements of disease trends, epidemiology has come to have a major role in improving our understanding of the overall nature of disease and in alerting and directing disease control activities. Epidemiology is also effective in: (1) clarifying the role of particular viruses and viral variants as the cause of disease; (2) clarifying the interaction of viruses with environmental determinants of disease; (3) determining factors affecting host susceptibility; (4) unraveling modes of transmission; and (5) field testing of vaccines and antiviral drugs.

The comparison of disease experience between populations is expressed in the form of *rates*. The terms *incidence rate* and *prevalence rate* are used to describe quantitatively the frequency of occurrence of infection or disease in populations. Incidence rate is defined as the ratio of new cases occurring in a population to the size of the population during a specified period of time. Prevalence rate is the ratio of the total number of cases occurring in a population to the size of the population during a specified period of time. *Seroprevalence rate* relates to the occurrence of antibody to a particular virus in a population. Because viral antibodies, especially neutralizing antibodies, often last a lifetime, seroprevalence rates usually represent cumulative experience with the virus. The term *case-fatality rate* is used to indicate the percentage of subjects with a particular disease that die from the disease. All these rates may be affected by various attributes that distinguish one individual from another: age, sex, genetic constitution, immune status, nutritional status and various behavioral and medical care and patient management parameters. The most widely applicable attribute is age, which may encompass immune status as well as various physiological variables.

An infectious disease is characterized as *endemic* when there are multiple or continuous chains of transmission resulting in continuous occurrence of disease in a population over a period of time. *Epidemics* are peaks in disease incidence that exceed the endemic baseline or expected rate of disease. The size of the peak required to constitute an epidemic is arbitrary and is related to the background endemic rate and the anxiety that the disease arouses (e.g. a few cases of rabies is regarded as an epidemic, whereas a few cases of influenza is not). A *pandemic* is a worldwide epidemic.

Epidemiologic Studies

Case control studies and cohort studies

There are two basic analytic techniques used to investigate relationships between cause and effect and to evaluate risk factors of disease. These are the *case-control study* and the *cohort study*. In the case-control study, investigation starts after the disease has occurred – it is a retrospective study, going back in time to determine causative events. Although this kind of study does not require the creation of new data or records, it does require careful selection of the control group, matched to the test group so as to avoid bias. The retrospective case-control study lends

itself to quick analysis and is relatively inexpensive to carry out. In the cohort study, the prospective study, investigation entails the gathering of new data to identify cause–effect relationships. This kind of study is expensive and does not lend itself to quick analysis as groups must be followed until disease is observed. However, when cohort studies are successful, proof of cause–effect relationship is often incontrovertible.

Molecular epidemiologic studies

The term *molecular epidemiology* is used to denote the use of any of a large number of molecular biological methods in support of epidemiologic investigations. For example, with herpesviruses, restriction endonuclease mapping provides a means of identification of unique viral genotypes – in an epidemiologic study recognized as the first based upon viral molecular characterization, the source of herpes simplex virus 1 causing disease in a hospital newborn nursery was traced to one persistently infected nurse rather than any of several other possible shedders. With rotaviruses and bluetongue viruses, polyacrylamide gel electrophoresis of the segmented viral RNA is used epidemiologically, for example to unravel outbreaks involving multiple viral variants.Partial sequencing is rapidly becoming the most commonly used molecular epidemiologic method: partial sequencing of poliovirus isolates recovered from patients indicates whether they are wild-type (even local or introduced wild-type), attenuated vaccine type, or a vaccine type that has reacquired neurovirulence during human passage. Panels of monoclonal antibodies are used similarly to distinguish virus variants for epidemiologic purposes; they have been particularly useful in elucidating host-range and geographic variants of rabies virus.

Seroepidemiologic studies

Seroepidemiology is useful in public health investigations and in research to determine the prevalence or incidence of particular infections, to evaluate control and immunization programs, and to assess past history when a 'new' virus is discovered. When paired serum specimens are obtained from individuals several weeks apart, the initial appearance of antibody in the second specimen or a rise in antibody titer indicates recent infection. Likewise, the presence of specific immunoglobulin M (IgM) antibody in single serum samples, indicating recent infection, may be used in seroepidemiologic studies. Correlation of serologic tests with clinical observations makes it possible to determine the ratio of clinical to subclinical infections.

Sentinel studies

Because of advanced diagnostic/serologic methods, sentinel studies can yield many valuable data in timely fashion about impending disease risks. For example, sentinel chicken flocks are set out for the early detection of the presence of arboviruses such as St Louis encephalitis virus in the southern and western United States. These flocks are bled and tested weekly for the presence of virus or antiviral antibody; they provide an early warning of the levels of virus amplification that occur before epidemics.

Vaccine trials

The immunogenicity, potency, safety and efficacy of vaccines are first studied in laboratory animals, followed by small-scale closed trials, and finally in large-scale open trials. Such studies employ epidemiologic methods, rather like those of the cohort (prospective) study. In most cases, there is no alternative way to evaluate new vaccines, and the design of trials has now been developed so that they yield maximum information with minimum risk and cost.

Virus Transmission among Individuals

Viruses survive in nature only if they are able to be transmitted from one host to another, whether of the same or another species. Transmission cycles require virus entry into the body, replication and shedding with subsequent spread to another host.

Virus entry

Portals of virus entry into the body include the skin, respiratory tract, intestinal tract, oropharynx, urogenital tract and conjunctiva. In some cases, viruses use a particular portal of entry because of particular environmental or host behavior factors and in other cases because of specific viral ligands and host cell receptors. In many cases, disruption of normal host defense mechanisms leads to entry that might otherwise be thwarted; for example, papillomaviruses may enter the deep layers of the skin via abrasions, acid-labile coronaviruses may enter the intestine protected by the buffering capacity of milk, and influenza viruses may enter the lower respiratory tract because a drug has dampened cilial action of the respiratory epithelium.

Virus shedding

The exit of virus from an infected host is just as important as entry in maintaining its transmission cycle. All portals used by viruses to gain entry are used for exit. The important elements in virus

shedding are virus yield (from the standpoint of the virus, the more shedding the better) and timeliness of yield (again, the earlier the shedding the better). Viruses that cause persistent infections often employ remarkable means to avoid host inflammatory and immune responses so as to continue shedding. For example, the epidemiologically important shedding of herpes simplex viruses 1 and 2 that perpetuates the viruses in populations requires recrudescence of persistent ganglionic infection, centrifugal viral genomic transit to peripheral nerve endings and productive infection of mucosal epithelium, all in the face of established host immunity.

Modes of Virus Transmission

Virus transmission may be *horizontal* or *vertical*. The vast majority of transmission is horizontal, that is between individuals within the population at risk. Modes of horizontal transmission of viruses can be characterized as *direct contact, indirect contact, common vehicle, airborne, vector-borne, iatrogenic* and *nosocomial*. *Vertical* or *transplacental* transmission occurs between the mother and her fetus or newborn. Some viruses are transmitted in nature via several modes, others exclusively via one mode (**Table 1**).

Direct contact transmission involves actual physical contact between an infected subject and a susceptible subject [e.g. kissing (Epstein–Barr virus, the cause of mononucleosis), biting (rabies), coitus (sexually transmitted viral diseases)]. Indirect contact transmission occurs via *fomites*, such as shared eating utensils, improperly sterilized surgical equipment or improperly sterilized nondisposable syringes and needles. Common vehicle transmission pertains to fecal contamination of food and water supplies (e.g. Norwalk virus diarrhea). Common vehicle transmission commonly results in epidemic disease. Airborne transmission typically results in respiratory infections (and less typically in intestinal infections), but these infections may also be transmitted by direct and indirect contact. Airborne transmission occurs via droplet nuclei (aerosols) emitted from infected persons during coughing or sneezing (e.g. influenza) or from environmental sources. Large droplets settle quickly, but microdroplets evaporate forming dry droplet nuclei (less than 5 nm in diameter) which remain suspended in the air for extended periods. Droplets may travel only a meter or so whereas droplet nuclei may travel much longer distances. Vector-borne transmission involves the bites of arthropod vectors (e.g. mosquitoes, ticks, sandflies). Iatrogenic transmission involves health care procedures, materials and workers (e.g. physicians, nurses, dentists). Nosocomial transmission pertains to infections acquired while a patient is in hospital.

Vertical or transplacental transmission occurs from mother to fetus prior to or during parturition. Certain retroviruses are vertically transmitted in animals via the integration of viral DNA directly into the DNA of the germ-line of the fertilized egg. Other viruses are transmitted to the fetus across the placenta, yet others are transmitted when the fetus passes through the birth canal. Another vertical transmission route is via colostrum and milk. Vertical transmission of a virus may or may not be associated with *congenital disease* (i.e. disease that is present at birth) which may be lethal (and the cause of abortion or stillbirth) or the cause of congenital abnormalities. The herpesviruses, especially cytomegaloviruses, and rubella virus cause important congenital diseases.

Common patterns of virus transmission

Enteric infections are most often transmitted by direct contact and by fomites in a *fecal–oral cycle* that may include fecal contamination of food and water supplies; diarrheic feces may also splash to give rise to aerosols. Respiratory infections are most often transmitted by the airborne route or by indirect contact via fomites in a *respiratory cycle*, i.e. virus is shed in respiratory secretions and enters its next host through the nares during inhalation. The respiratory cycle is responsible for the most explosive patterns of epidemic disease.

Perpetuation of Viruses in Nature

Perpetuation of a virus in nature depends on the maintenance of serial infections, i.e. a chain of transmission; the occurrence of disease is neither required nor necessarily advantageous.

Influence of the clinical status of the host

Infection without recognizable disease is called *subclinical* or *clinically inapparent*. Overall, subclinical infections are much more common than those that result in disease. Their relative frequency accounts for the difficulty of tracing chains of transmission, even with the help of laboratory diagnostics. Although clinical cases may be somewhat more productive sources of virus than subclinical infections, because the latter do not restrict the movement of the infected host, they can be most important as sources of viral dissemination. In most acute infections, whether clinically apparent or not, virus is shed in highest titers during the late stages of the incubation period, before the influence of the host immune response

Table 1 Examples of virus transmission patterns

Infectious agent/disease	*Mode of transmission*	*Portal of entry*
Influenza virus/influenza	Contact/direct/indirect via droplets and aerosol	Respiratory tract
Rhinoviruses/common cold	Contact/direct/indirect via droplets, aerosols and fomites	Respiratory tract
Rubella virus/congenital rubella	Contact/direct/indirect via droplets, aerosol	Respiratory tract
	Vertical/congenital	Transplacental
Rotaviruses/diarrhea	Contact/direct/indirect via fomites	Intestinal tract (oral)
Poliovirus/poliomyelitis	Contact/direct	Intestinal tract (oral)
Norwalk virus/diarrhea	Common vehicle/fecal contamination of water	Intestinal tract (oral)
Hepatitis A virus/hepatitis	Common vehicle/fecal contamination of food	Intestinal tract (oral)
New-variant Creutzfeldt–Jakob disease prion/prion disease (spongiform encephalopathy)	Common vehicle/bovine spongiform encephalopathy prion contamination of beef or beef products	Intestinal tract (oral)
Herpes simplex virus/genital herpes	Contact/direct (sexual)	Genital tract
Human immunodeficiency virus 1/ acquired immunodeficiency syndrome (AIDS)	Contact/direct (sexual), contact/direct (blood), vertical/congenital	Genital tract, bloodstream, transplacental, at birth and via breast feeding
Rabies virus/rabies	Zoonotic/contact/direct (saliva)	Skin (bite wound)
Russian spring summer encephalitis virus/encephalitis	Zoonotic/arthropod-borne	Skin (tick bite)
Dengue viruses/dengue	Zoonotic/arthropod-borne	Skin (mosquito bite)
Sin Nombre and related viruses	Zoonotic/contact/direct (rodent urine, saliva and feces)	Respiratory tract
Lassa virus	Zoonotic/contact/direct (rodent urine, saliva and feces)	Respiratory tract and intestinal tract (oral)
Ebola and Marburg viruses	Zoonotic/index cases unknown; secondary cases contact/direct/ nosocomial and iatrogenic	Index cases unknown, likely respiratory tract or skin and mucous membranes; secondary cases, contact and iatrogenic (injection)
Leukemia viruses/leukemias (proven only in animals)	Vertical/germ-line	Transmitted as genetic trait

takes effect. Persistent infections, whether or not they are associated with episodes of clinical disease, also play an important role in the perpetuation of many viruses in nature. For example, prolonged virus shedding can reintroduce virus into a population of susceptibles all of which have been born since the last clinically apparent episode of infection. This is important in the survival of rubella virus in some isolated populations. Sometimes the persistence of infection, the production of disease and the transmission of virus are dissociated; for example, togavirus and arenavirus infections have little adverse effect on their reservoir hosts (arthropods, birds and rodents) but transmission is very efficient. On the other hand, the persistence of infection in the central nervous system, as with measles virus in subacute sclerosing panencephalitis (SSPE), is of no epidemiological significance, since no infectious virus is shed from this site.

Influence of virulence of the virus

The virulence of the infecting virus may directly affect the probability of its transmission. The classic example of this is rabbit myxomatosis. In Australia, mosquito-borne transmission of myxoma virus was found to be most effective when infected rabbits maintained highly infectious skin lesions for several days before death. Highly virulent strains of the virus were found to kill rabbits so quickly that transmission did not occur, and naturally attenuated strains were found to produce minimal lesions that healed quickly and did not permit transmission. Virus strains at either extreme of this virulence spectrum were found

not to survive in nature, but virus strains of intermediate virulence have circulated for many years.

Influence of host population immunity

With most viruses, endemic or epidemic transmission leads to a level of immunity in the host population that affects or even interrupts further transmission. The 'herd immunity' effect is countered in some cases by viral antigenic variation. For example, influenza viruses undergo genetic variations ('shift' and 'drift') such that persons immune to previously circulating virus strains are susceptible to new strains. Assessing these genetic changes is the main objective of laboratory-based surveillance programs, which in turn are used to decide the formulation of new influenza vaccines.

Influence of population size

It is self-evident that the long-term survival of a virus requires that it be continuously transmitted from one host to another. In general, for rapidly and efficiently transmitted viruses such as many respiratory viruses, local survival of the virus requires that the susceptible host population be very large. A virus may disappear from a population because it exhausts its potential supply of susceptible hosts as they acquire immunity to reinfection with the same virus. Depending on duration of immunity and the pattern of virus shedding, the *critical population size* varies considerably with different viruses and with different host species. The most precise data on the importance of population size in acute nonpersistent infections come from studies of measles. Persistence of measles virus in a population depends on a continuous supply of susceptible children. Analyses of the incidence of measles in large cities and in island communities have shown that a population of about half a million persons is needed to ensure a large enough annual input of new susceptible hosts, by birth or immigration, to maintain measles virus in the population. Because infection depends on respiratory transmission, the duration of epidemics of measles is correlated inversely with population density. If a population is dispersed over a large area, the rate of spread is reduced and the epidemic will last longer, so that the number of susceptible persons needed to maintain transmission chains is reduced. On the other hand, in such a situation a break in the transmission chain is much more likely. When a large proportion of the population is initially susceptible, the intensity of the epidemic builds up very quickly and attack rates are almost 100% (*virgin-soil epidemic*).

Influence of zoonotic transmission cycles

Because most viruses are host-restricted, most viral infections are maintained in nature within populations of the same or related species. However, there are a number of viruses that may have multiple hosts and spread naturally between several different species of vertebrate host, e.g. rabies and eastern equine encephalitis viruses. The term *zoonosis* is used to describe multiple-host infections that are transmissible from animals to man. The zoonoses, whether involving domestic or wild animals or arthropods, usually represent important problems only under conditions where humans are engaged in activities involving close contact with animals or exposure to arthropod habitats.

Influence of arthropod transmission cycles

Many viral zoonoses are caused by arboviruses. Arboviruses have two classes of host, vertebrate and invertebrate. Over 500 arboviruses are known, of which about 100 cause disease in humans and 40 in domestic animals; some of these are transmitted by ticks, some by mosquitoes and yet others by phlebotomine flies (sandflies) or *Culicoides* spp. (midges). Arthropod transmission may be mechanical, where the arthropod acts as a 'flying pin', or more commonly, biological, involving replication of the virus in the arthropod vector. The arthropod vector acquires virus by feeding on the blood of a viremic person or animal. Replication of the ingested virus, initially in the arthropod's gut, and its spread to the salivary glands takes several days; the interval varies with different viruses and is influenced by ambient temperature. Virions in the salivary secretions of the vector are injected into animal hosts during subsequent blood meals. Most arboviruses have localized natural habitats in which specific receptive arthropod and vertebrate hosts are involved in the viral life cycle. Vertebrate reservoir hosts are usually wild mammals or birds; humans are rarely involved in primary transmission cycles, although the exceptions to this generalization are important (e.g. Venezuelan equine encephalitis, yellow fever and dengue viruses). Humans are in most cases infected incidentally, for example by the geographic extension of a reservoir vertebrate host and/or a vector arthropod. Ecological changes produced by human activities disturb natural arbovirus life cycles and have been incriminated in the geographic spread or increased prevalence of arbovirus diseases.

Mathematical Modeling

From the time of William Farr, who studied epidemic disease problems in the 1870s, mathematicians have

been interested in 'epidemic curves' and secular trends in the incidence of infectious diseases. With the development of computer-based mathematical modeling techniques, there has been a resurgence of interest in the population dynamics of infectious diseases. There has also been a resurgence in controversies surrounding the use of models: critics say 'for every model there is an equal and opposite model'. Models are used to determine: (1) patterns of disease transmission; (2) critical population sizes to support the continuous transmission of viruses with short and long incubation periods; (3) the dynamics of endemicity of viruses that become persistent in their hosts; and (4) the variables in age-dependent viral pathogenicity. Computer modeling also provides useful insights into the effectiveness of disease control programs. Much attention has been given to modeling the future of the acquired immunodeficiency disease (AIDS) epidemic in the United States and the rest of the world. Such models usually start with historical data on the introduction of the etiologic virus, HIV1, proceed to the present stage of the epidemic where the disease has become well established in many countries and in fewer subject to prevention and treatment strategies, and then proceed to project its course into the future. During the first 10 years of the AIDS epidemic in the United States, African countries and then in Asian countries most models underestimated developing trends; more recently models have become more accurately predictive.

Implications for Disease Prevention

Knowledge of the epidemiology and modes of transmission of infectious diseases is critical to the development and implementation of prevention and control strategies. Data on incidence, prevalence and mortality contribute directly to the establishment of priorities for prevention and control programs whereas knowledge of viral characteristics and modes of transmission are used in deciding prevention strategies focusing on vaccine development and delivery, environmental improvements, enhancement of nutritional status, improvement in personal hygiene and behavioral changes.

See also: **Emerging and re-emerging virus diseases; Human immunodeficiency viruses (*Retroviridae*): Molecular biology, Anti-retroviral agents, General features; Influenza viruses (*Orthomyxoviridae*): General features, Molecular biology, Structure of antigens; Pathogenesis: Animal viruses, Plant viruses; Vaccines and immune response; Zoonoses.**

Further Reading

Anderson RM and May RM (1991) *Infectious Diseases of Humans. Dynamics and Control.* Oxford: Oxford University Press.

Benenson AS (ed.) (1995) *Control of Communicable Diseases in Man,* 16th edn. Washington, DC: American Public Health Association.

Evans AS and Kaslow RA (eds) (1997) *Viral Infections of Humans. Epidemiology and Control,* 4th edn. New York: Plenum Medical Book Company.

Mandell GL, Bennett JE and Dolin R (eds) (1995) *Mandell, Douglas and Bennett's Principles and Practice of Infectious Disease,* 4th edn. New York: Churchill Livingstone.

Nathanson N (1996) Epidemiology. In: Fields BN, Knipe DM, Howley RM *et al* (eds) *Fields Virology,* 3rd edn. Philadelphia: Lippincott-Raven.

Nathanson N and Murphy FA (1997) Evolution of viral diseases. In: Nathanson N, Ahmed R, Griffin D *et al* *Viral pathogenesis.* Philadelphia: Lippincott-Raven Press.

EPSTEIN–BARR VIRUS (*HERPESVIRIDAE*)

Contents

General Features

Lawrence S Young, CRC Institute for Cancer Studies, University of Birmingham Medical School, Birmingham, UK

History

In 1958 Dennis Burkitt described a lymphoma that represented the most common tumor affecting children in certain parts of East Africa. The geographical distribution of this malignancy suggested that the development of Burkitt's lymphoma (BL) might be

been interested in 'epidemic curves' and secular trends in the incidence of infectious diseases. With the development of computer-based mathematical modeling techniques, there has been a resurgence of interest in the population dynamics of infectious diseases. There has also been a resurgence in controversies surrounding the use of models: critics say 'for every model there is an equal and opposite model'. Models are used to determine: (1) patterns of disease transmission; (2) critical population sizes to support the continuous transmission of viruses with short and long incubation periods; (3) the dynamics of endemicity of viruses that become persistent in their hosts; and (4) the variables in age-dependent viral pathogenicity. Computer modeling also provides useful insights into the effectiveness of disease control programs. Much attention has been given to modeling the future of the acquired immunodeficiency disease (AIDS) epidemic in the United States and the rest of the world. Such models usually start with historical data on the introduction of the etiologic virus, HIV1, proceed to the present stage of the epidemic where the disease has become well established in many countries and in fewer subject to prevention and treatment strategies, and then proceed to project its course into the future. During the first 10 years of the AIDS epidemic in the United States, African countries and then in Asian countries most models underestimated developing trends; more recently models have become more accurately predictive.

Implications for Disease Prevention

Knowledge of the epidemiology and modes of transmission of infectious diseases is critical to the development and implementation of prevention and control strategies. Data on incidence, prevalence and mortality contribute directly to the establishment of priorities for prevention and control programs whereas knowledge of viral characteristics and modes of transmission are used in deciding prevention strategies focusing on vaccine development and delivery, environmental improvements, enhancement of nutritional status, improvement in personal hygiene and behavioral changes.

See also: **Emerging and re-emerging virus diseases; Human immunodeficiency viruses (*Retroviridae*): Molecular biology, Anti-retroviral agents, General features; Influenza viruses (*Orthomyxoviridae*): General features, Molecular biology, Structure of antigens; Pathogenesis: Animal viruses, Plant viruses; Vaccines and immune response; Zoonoses.**

Further Reading

Anderson RM and May RM (1991) *Infectious Diseases of Humans. Dynamics and Control.* Oxford: Oxford University Press.

Benenson AS (ed.) (1995) *Control of Communicable Diseases in Man,* 16th edn. Washington, DC: American Public Health Association.

Evans AS and Kaslow RA (eds) (1997) *Viral Infections of Humans. Epidemiology and Control,* 4th edn. New York: Plenum Medical Book Company.

Mandell GL, Bennett JE and Dolin R (eds) (1995) *Mandell, Douglas and Bennett's Principles and Practice of Infectious Disease,* 4th edn. New York: Churchill Livingstone.

Nathanson N (1996) Epidemiology. In: Fields BN, Knipe DM, Howley RM *et al* (eds) *Fields Virology,* 3rd edn. Philadelphia: Lippincott-Raven.

Nathanson N and Murphy FA (1997) Evolution of viral diseases. In: Nathanson N, Ahmed R, Griffin D *et al* *Viral pathogenesis.* Philadelphia: Lippincott-Raven Press.

EPSTEIN–BARR VIRUS (*HERPESVIRIDAE*)

Contents

General Features

Lawrence S Young, CRC Institute for Cancer Studies, University of Birmingham Medical School, Birmingham, UK

History

In 1958 Dennis Burkitt described a lymphoma that represented the most common tumor affecting children in certain parts of East Africa. The geographical distribution of this malignancy suggested that the development of Burkitt's lymphoma (BL) might be

Table 1 EBV-associated tumors

Tumor	*Subtype*	*EBV genome +VE (%)*
Burkitt's lymphoma	Endemic	100
	Sporadic	10–20
	AIDS	30–40
Nasopharyngeal carcinoma	Undifferentiated	100
	SCC	40
Hodgkin's disease	Mixed cellularity	>80
	Nodular sclerosing	30–40
	Lymphocyte predominant	<10
T cell lymphoma	Nasal	100
	Others	10–40
Immunoblastic lymphoma	Transplant	100
	AIDS	>90

SCC, squamous cell carcinoma.

due to an infectious agent. In 1964 the successful establishment of cell lines from explants of BL enabled Epstein and Barr to identify herpesvirus-type particles by electron microscopy within a subpopulation of tumor cells *in vitro*. W. and G. Henle subsequently demonstrated that BL-derived cell lines expressed antigens that were recognized not only by sera from patients with BL but also by sera from patients with infectious mononucleosis (IM). Similar seroepidemiological studies also suggested a link between the so-called Epstein–Barr virus (EBV) and undifferentiated nasopharyngeal carcinoma (NPC) leading to the subsequent direct demonstration of EBV DNA in the tumor cells of NPC. The ability of this virus to efficiently immortalize B lymphocytes *in vitro* and to induce lymphomas in nonhuman primates established EBV as a putative oncogenic agent in humans. More recent studies have implicated EBV in a variety of other lymphoid and epithelial malignancies (**Table 1**).

Taxonomy and Classification

EBV is a member of the genus *Lymphocryptovirus* which belongs to the lymphotropic *Gammaherpesvirinae* subfamily of the family *Herpesviridae*. The lymphocryptoviruses exhibit a similar genomic structure and can infect the B lymphocytes of Old World primate species, resulting in latent infection often associated with lymphoproliferation. The 172 kbp EBV genome resembles other lymphocryptovirus genomes in that it contains (1) tandemly reiterated 0.5 kbp terminal direct repeats, and (2) tandemly reiterated 3 kbp internal direct repeats which divide the genome into unique short and long regions.

Geographic and Seasonal Distribution

EBV is ubiquitous being found as a widespread and largely asymptomatic infection in all human communities. Primary infection often occurs early in life particularly in tropical areas and in persons of lower socioeconomic class. Thus, in tropical Africa and New Guinea primary EBV infection is common in the first year of life whereas in Western communities infection is continually acquired throughout childhood and early adulthood. Although the EBV-associated malignancies BL and NPC exhibit an unusual geographic distribution this appears not to be due to differences in EBV infection but to additional cofactors.

Host Range and Virus Propagation

Humans are the natural host for EBV infection. Certain nonhuman primates, particularly the cotton-top tamarin, have been used as experimental hosts for EBV but in these animals virus infection is associated with the induction of lymphomas. Lymphoblastoid cell lines (LCLs) generated by EBV immortalization of cotton-top tamarin B lymphocytes *in vitro* produce large amounts of virus compared with their human counterparts and as such have been used as a source of EBV, e.g. the prototype strain of EBV is B95.8 which is produced from a tamarin LCL originally immortalized with EBV from a patient with IM. However, the lack of a fully permissive system for propagating EBV *in vitro* has hampered our understanding of virus replication and prevented the generation of EBV mutants. New culture systems for efficient replication of EBV include anti-immunoglobulin-treated BL cell

lines and epithelial cell lines transfected with the EBV receptor.

Genetics

EBV isolates from different regions of the world or from patients with different virus-associated diseases are remarkably similar when their genomes are compared by restriction fragment length polymorphism (RFLP) analysis. However, variations in repeat regions of the EBV genome are observed among different EBV isolates. Analysis of the EBV genome in a number of BL cell lines has revealed gross deletions in the viral genome some of which account for biological differences, i.e. P3HR-1 virus which is non-transforming contains a deletion of the EBNA 2-encoding gene.

Strain variation over the EBNA 2-encoding (*Bam*HI WYH) region of the viral genome has permitted all EBV isolates to be classified as either 'type 1' (EBV-1, B95.8-like) or 'type 2'(EBV-2 Jijoye-like). This genomic variation results in the production of two antigenically distinct forms of the EBNA 2 protein which share only 50% amino acid homology. Similar strain-specific variation occurs in the EBNA 3-encoding region of the viral genome. These differences have functional consequences as EBV-2 isolates are less efficient in *in vitro* B lymphocyte transformation assays compared with EBV-1 isolates. Recent work demonstrates that sequence variation over the *LMP*1 gene, particularly a 30 bp deletion in the C-terminus of the protein, is a common feature of EBV isolates in certain regions (i.e. China) and may predispose to the development of virus-associated tumors.

Evolution

EBV, like other herpesviruses, has probably evolved with humans. The relatedness of the lymphocryptoviruses at both the genomic and protein levels does not correlate with the evolutionary relatedness of their Old World primate hosts. This implies that the selective pressures governing the evolution of these viruses are different from those responsible for the evolution of primate species. It has been suggested that the specific tissue tropism of lymphocryptoviruses may have constrained their evolutionary divergence.

The evolutionary relationship between the type 1 and type 2 strains of EBV remains obscure. They may have evolved from a common progenitor virus or through recombination of either EBV strain with one of the lymphocryptoviruses infecting Old World primates. The pronounced (but not exclusive) segregation of EBV-2 isolates within equatorial regions suggests that environmental factors may have influenced EBV evolution and may still be responsible for the effective competition between EBV-2 isolates and the ubiquitous EBV-1 family.

Serologic Relationships and Variability

EBV isolates can be classified as type 1 or type 2 on the basis of allelic polymorphism of virus-encoded nuclear antigens EBNAs 2, 3A, 3B and 3C. Both seroprevalence studies and typing of EBV in BL cell lines and LCLs have demonstrated that the majority of wild-type isolates in Western communities are of the EBV-1 strain whereas EBV-2 strains appear to be largely restricted to areas of equatorial Africa and New Guinea. However, in immunosuppressed HIV-positive individuals infection with both EBV-1 and EBV-2 can be found and EBV-2 is frequently detected in the lymphomas arising in these patients.

Further microheterogeneity exists among EBV isolates, particularly over the repeat regions of the viral genome. These give rise to variations in the size of the individual EBNA proteins which are apparent after their electrophoretic separation on polyacrylamide gels. Thus, the EBNA 1, 2, 3a, 3b and 3c proteins encoded by any one virus isolate display a unique size combination in gels which has been termed the 'EBNA type' of that particular isolate. This characteristic of EBV isolates can be used to trace the origin and transmission of EBV within families.

Epidemiology of EBV-associated Diseases

Infectious mononucleosis

Infection with EBV is widespread and, once infected, individuals become life-long virus carriers. Primary infection with EBV in childhood is usually asymptomatic but when delayed until adolescence or early adulthood can manifest clinically as IM, a self-limiting lymphoproliferative disease. The incidence of IM is low in Third World countries where primary infection predominantly occurs in childhood. In certain poorly defined situations IM-like symptoms can persist, resulting in chronic active EBV infection associated with elevated antibody titers to virus lytic antigens but low titers to the EBNAs.

Burkitt's lymphoma (BL)

The endemic form of BL which is found in areas of equatorial Africa and New Guinea represents the most common childhood cancer (peak age 7–9 years) in these regions with an incidence of up to 10 cases per 100 000 people per year. This high incidence of BL is

associated with holoendemic malaria accounting for the climatic variation in tumor incidence first recognized by Dennis Burkitt. More than 95% of these endemic BL tumors are EBV-positive compared with 20% of the low-incidence, sporadic form of BL which occurs worldwide (**Table 1**). In areas of intermediate BL incidence, such as Algeria and Malaysia, the increased number of cases correlates with an increased proportion of EBV-positive tumors. A consistent feature of all BL tumors, irrespective of geographical location, is chromosomal translocations involving the long arm of chromosome 8 (8q24) in the region of c-*myc* proto-oncogene and either chromosome 14 in the region of the immunoglobulin heavy-chain gene or, less frequently, chromosomes 2 or 22 in the region of the immunoglobulin light-chain genes. Seroepidemiological studies have demonstrated elevated antibody titers to EBV capsid antigen (VCA) and early antigens (EA) in BL patients compared to children without the tumor. These elevated antibody titers have been found to precede the development of BL and can therefore be used to screen 'at risk' individuals.

Nasopharyngeal carcinoma (NPC)

The association of EBV with undifferentiated NPC was first shown by serological evidence and later confirmed by the demonstration of EBV DNA in NPC biopsy material. NPC is particularly common in areas of China and Southeast Asia reaching a peak incidence of around 20–30 per 100 000. Incidence rates are high in individuals of Chinese descent, irrespective of where they live, and particularly in Cantonese males. In addition to this genetic predisposition, environmental cofactors such as dietary components (e.g. salted fish) are thought to be important in the etiology of NPC. Extensive serological screening the EBV-specific antibody titers in high incidence areas, in particular IgA antibodies to EBV capsid antigen (VCA) and early antigens (EA), have proved useful in diagnosis and in monitoring the effectiveness of therapy. The association between EBV and undifferentiated NPC has been confirmed for many different racial groups, whether these exhibit a high, intermediate or low incidence of the tumor. Undifferentiated NPC is associated with a prominent lymphoid stroma and tumors arising at other anatomical sites (e.g. lung, stomach, salivary gland) with this morphology (lymphoepitheliomas) tend to be EBV-positive (**Table 1**). EBV infection is also associated with the more differentiated squamous cell NPCs particularly those occurring in the Far East. EBV infection has been detected in a proportion of common gastric adenocarcinomas.

Lymphomas in patients with immunodeficiency

Patients with primary immunodeficiency diseases such as X-linked lymphoproliferative syndrome (XLP) and Wiscott–Aldrich syndrome are at increased risk of developing EBV-associated B cell lymphomas. Because these tumors are extremely rare little is known of their association with EBV infection. Mortality from XLP is high with around 50% of patients developing fatal IM after primary infection with EBV and an additional 30% of patients developing malignant lymphoma.

Allograft recipients receiving immunosuppressive therapy and patients receiving immunosuppressive therapy patients with AIDS are also at increased risk for development of EBV-associated lymphoproliferative disease and lymphomas. The incidence of B cell lymphomas in allograft recipients varies with the type of organ transplanted and with the type of immunosuppressive regimen used. Allogeneic bone marrow transplantation into EBV seronegative children is a particular risk factor for the development of virus-associated B cell lymphomas.

The incidence of non-Hodgkin lymphoma in AIDS patients is increased approximately 60-fold compared to the normal population. Around 60% of these tumors are large-cell lymphomas like those found in allograft recipients, 20% are primary brain lymphomas and 20% are of the BL type. Recent studies have demonstrated that 50% of AIDS lymphomas are EBV-positive and that this association varies with the histological tumor type. Thus, only 38% of the BL tumors are EBV-positive compared with 65% of the large-cell lymphomas.

T cell lymphomas

Recent studies have demonstrated EBV infection in a considerable proportion of T cell non-Hodgkin's lymphomas (**Table 1**). Nasal T cell lymphomas, a tumor which is more common in the Far East, is invariably EBV-positive whereas around 20% of T cell lymphomas arising at other sites (gastrointestinal, lung, lymph nodes) are associated with EBV.

Hodgkin's disease

Epidemiological studies originally suggested a possible role for EBV in the etiology of Hodgkin's disease (HD). Thus, elevated antibody titers to EBV antigens have been detected in patients with HD and these increased antibody levels are present before the diagnosis of disease. Furthermore, there is an increased risk of HD following IM. EBV has been demonstrated in around 50% of HD cases with both viral nucleic acid (DNA/RNA) and virus latent antigens localized to the malignant component of

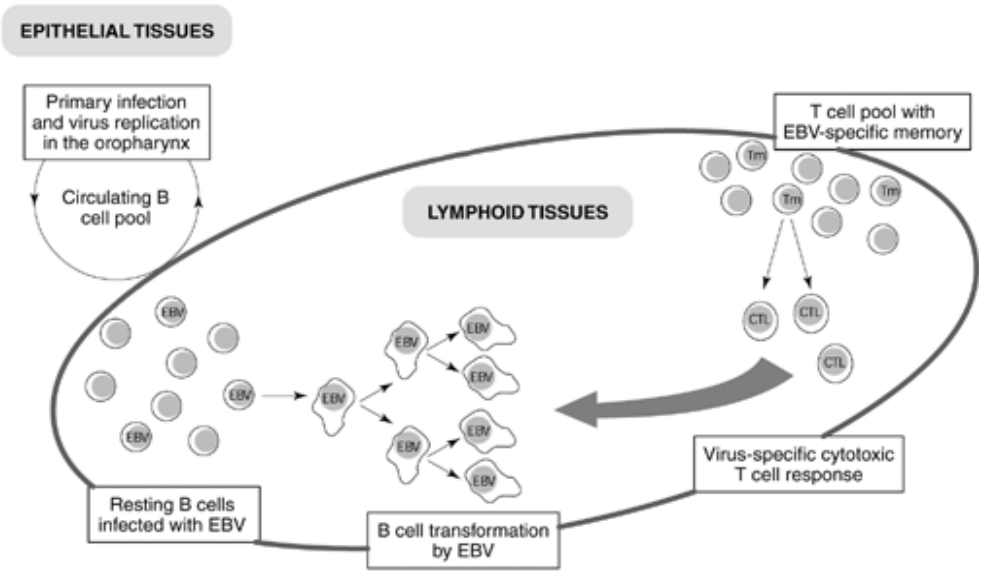


Figure 1 A model for EBV infection in normal healthy virus carriers. Primary EBV infection occurs in the oropharynx and is probably mediated via B cells. Following primary infection, a chronic virus carrier state is established in which EBV infection persists in resting B cells which may only express the EBNA1 protein. Occasional reactivation of EBV in this compartment results in the outgrowth of EBV-infected B cells which resemble EBV-transformed lymphoblastoid cell lines *in vitro*. The growth of these transformed cells is controlled by an EBV-specific cytotoxic T lymphocyte (CTL) response. At certain sites such as the oropharynx, latently infected B cells may become permissive for lytic EBV infection shedding infectious virus (i.e. into the saliva) thereby resulting in infection of other cells in the vicinity such as B cells and epithelial cells.

HD, the so-called Reed–Sternberg cells and their variants. The association of HD with EBV is age-related; pediatric and older adult cases are usually EBV-associated whereas HD in young adults is less frequently virus-positive. The proportion of EBV-positive HD in developing countries is high consistent with a greater incidence of HD in children and more frequent prevalence of the mixed cellularity histiotype (**Table 1**). Although the incidence of HD is relatively low (1–3/100 000 per year) this tumor is not geographically restricted, making its association with EBV significant in world health terms.

Transmission and Tissue Tropism

The usual route of EBV transmission is via saliva, although rare cases of infections transmitted by blood transfusion have been reported. EBV, measured by its ability to transform B lymphocytes *in vitro*, can be detected in oropharyngeal secretions from IM patients, from patients receiving immunosuppressive drugs and, at a lower level, from normal asymptomatic EBV-positive individuals. These observations, together with the fact that EBV-transformed LCLs *in vitro* tend to be poor producers of the virus and B lymphocytes permissive of viral replication have not been demonstrated *in vivo*, suggest that EBV replicates and is shed at epithelial sites in the oropharynx and/or from salivary glands. This is supported by the demonstration of replicating EBV in the more differentiated epithelial cell layers of oral 'hairy' leukoplakia, a benign lesion of the tongue found in patients with AIDS. However, the inability to detect EBV in normal epithelial cells and the demonstration that EBV can be completely eradicated by irradiation in bone marrow transplant recipients indicates that B cells are likely to be the main site of EBV persistence and may also represent the target of the virus in primary infection (**Fig. 1**).

The B lymphotropism of EBV is due to the expression of a specific cell surface receptor on B cells, the CD21 antigen or CR2, which can bind EBV and the C3d component of complement. The EBV-encoded membrane antigen (MA) is a 340 kDa glycoprotein which is expressed on the outer membrane of the virion and has been identified as the viral protein that binds to CR2. The expression of CR2 has also been demonstrated on immature thymocytes and follicular dendritic cells suggesting that these cell types may also be susceptible to EBV infection. The lack of CR2 expression on epithelial cells implies that an alternative receptor or mechanism is responsible for their infection. Under certain circumstances EBV can infect other tissues (i.e. smooth muscle, endothelial cells) but the relevance of this effect to primary and persistent virus infection is unknown.

Pathogenicity

As EBV strain variation does not appear to have a significant effect on the pathogenicity of the virus, attention has concentrated on the patterns of viral gene expression in the different virus-associated diseases. The *in vitro* ability of EBV to transform resting B cells into permanent LCLs is associated with the constitutive expression of a limited number of EBV-encoded latent proteins; the nuclear antigens EBNA 1, EBNA 2, EBNAs 3A/3B/3C and EBNA-LP and the latent membrane proteins, LMP1 and LMP2. Analysis of the expression and function of these viral gene products suggests that the EBNA 2 protein and LMP1 play central roles in EBV-induced cell transformation. EBNA 2 is a transcriptional regulator whereas LMP1 functions as a constitutively activated receptor.

The pattern of EBV latent gene expression varies among the different virus-related diseases. Thus, the proliferation of B cells characteristic of IM and XLP is associated with the expression of all the EBV latent proteins as seen in LCLs. However, in BL and NPC there is a downregulation of the EBV latent protein expression: only EBNA 1 is found in BL whereas in NPC both EBNA 1 and the LMPs are expressed. Recent studies suggest that the pattern of EBV latent gene expression in HD is similar to that in NPC. The large-cell lymphomas of post-transplant patients resemble LCLs in expressing the full range of EBV latent genes whereas the phenotypic heterogeneity of EBV-positive AIDS-related lymphomas is reflected at the EBV latent protein level providing a spectrum of tumors from LCL-like to BL-like. These different patterns of EBV latent gene expression are the result of different virus transcriptional programs influenced by factors such as methylation and the host cell environment. The downregulation of certain EBV latent proteins in BL, NPC and HD does not preclude a role for these gene products at an earlier stage in the oncogenic process. Thus, differences in viral protein expression appear to influence the pathogenicity of EBV as it relates to the development of the various virus-associated malignancies.

Clinical Features of Infection

Although the majority of primary EBV infections are asymptomatic, those resulting in IM can initiate a range of clinical symptoms which may last for weeks or even months. IM occurs predominantly in the adolescent and young adult with rare cases in infants and individuals of greater than 30 years of age. The acute illness is associated with a sore throat characterized by hyperplasia of lymphoid tissue in the oropharynx, fever and generalized lymphoadenopathy. There is lymphocytosis accompanied by the presence of atypical lymphocytes. Enlargement of the spleen is found in around 50% of IM patients at 2–3 weeks after the onset of symptoms. Hepatosplenomegaly is occasionally found and is associated with elevated levels of serum liver enzymes. Resolution of these symptoms results in the establishment of the life-long EBV carrier state as seen after normal, asymptomatic primary infection. Failure of this to occur can precipitate a chronic active EBV infection where the symptoms of IM persist. The fatal IM associated with XLP takes a rapid course usually associated with progressive failure of the lungs, kidneys, liver and bone marrow due to an overwhelming infiltration of these organs with EBV-infected B cells.

In the endemic areas of Africa 60% of children with BL present with jaw tumors. These jaw tumors are common in younger BL patients whereas older children are more likely to present with abdominal tumors similar to those found in the sporadic form of the disease. Whereas bone marrow involvement is frequently found in patients with sporadic BL, it is rarely a feature of endemic BL. Over half of NPC cases present with a cervical mass resulting from lymphoid spread of the primary tumor from its common site of origin in the lateral nasopharynx (fossa of Rosenmuller). Common symptoms resulting from the location of the primary NPC tumor include nasal obstruction and bleeding as well as those due to malfunction of the eustachian tube such as ear blockage, otitis media and conductive hearing loss. Lymphomas in patients with immunodeficiency can present at a number of different anatomic sites including the mediastinum, lungs, central nervous system and abdomen. The site of presentation and rate of development of these tumors appears to be influenced by the type of immunodeficiency. The lymphomas associated with patients on immunosuppressive therapy often develop within the grafted organ. Lymphadenopathy is the most common presentation of HD and in around 30% of patients is accompanied by fever, night sweats and weight loss.

Pathology and Histopathology

The polyclonal activation of B cells and resultant T cell response account for the pathology associated with IM which is evident in the lymph nodes, peripheral blood, liver and spleen. In IM and the lymphoproliferative disorders associated with immunosuppression EBV-infected B cells (immunoblasts) are found as infiltrates in solid organs as well as bone marrow. This is more pronounced in immunodefi-

cient patients where the polymorphic B cell hyperplasia can evolve into a high-grade B cell lymphoma of immunoblastic or undifferentiated large cell type. The jaw tumors characteristic of endemic BL are usually associated with multifocal disease involving liver, kidney and gut. BL is classified as a high-grade malignant lymphoma of small noncleaved follicle center B cell type with a 'starry sky' morphology resulting from infiltrating histiocytes and macrophages. The EBV-associated anaplastic or undifferentiated type of NPC is an aggressive tumor which tends to metastasize to cervical lymph nodes. NPC has been classed as a 'lymphoepithelioma' on account of the associated heavy T lymphoid infiltrate. The histopathology of HD is complicated by the paucity of the malignant Reed–Sternberg cells in tumor biopsies. Histological subtypes of HD reflect the cellular composition of the affected lymph nodes and may correlate with clinical features as well as with EBV association.

Immune Response

EBV illicits both humoral and cell-mediated immune responses in infected hosts. Primary infection with EBV is associated with the rapid appearance of antibodies to replicative viral antigens such as VCA, EA and MA with a later serological response to the EBNA proteins. In IM these responses are accentuated and are accompanied by autoantibodies such as rheumatoid factor as well as a heterophile antibody response directed against antigens on the surface of sheep erythrocytes. These autoantibodies are the result of EBV-induced polyclonal B cell activation. In the chronic, asymptomatic virus carrier, antibodies to VCA, MA and the EBNAs are found, the titers of which remain remarkably stable. Of these antibodies those against MA are particularly important as they have virus neutralizing ability and can also mediate antibody-dependent cellular cytotoxicity. As discussed previously the levels of these EBV-specific antibodies are elevated in the different virally associated diseases.

As with other persistent viruses, cell-mediated immunity plays an important part in controlling EBV infection (**Fig. 1**). This is evidenced by the effect of immunosuppression on EBV-infected individuals which results in increased excretion of the virus and is associated with an increased risk of developing EBV-positive lymphomas. The development and maintenance of class I MHC restricted EBV-specific cytotoxic T lymphocytes (CTLs) is of particular importance in the control of virus infection (**Fig. 1**). Virus-specific CTLs are able to efficiently recognize the EBV latent antigens and thus prevent the unlimited proliferation of EBV-infected B cells. More recent work demonstrates that CTL responses to virus lytic antigens are a common feature of both primary and persistent EBV infection. Although impairment of CTL function is responsible for lymphomagenesis in immunosuppressed patients, the development of BL, NPC and HD is more complex. The growth and survival of these tumors in immunocompetent individuals implies that the tumor cells can evade EBV-specific CTL surveillance. This may be achieved by restricting EBV latent gene expression to those viral proteins not recognized by EBV-specific CTLs and/or by the downregulation of target cell antigens required for immune recognition such as class I MHC and lymphocyte adhesion molecules. Interestingly, EBNA1 which is consistently expressed in all EBV-associated tumors is not a target for CD8-positive CTLs.

Prevention and Control of EBV Infection

The importance of the EBV-associated malignancies in world health terms has prompted the development of a vaccine against viral infection. As the MA glycoprotein on the EBV virion is a target for neutralizing antibodies during normal infection, this molecule has been proposed as a suitable immogen for eliciting protective immunity. In the experimental cotton-top tamarin model, immunization with various preparations of MA is able to protect the animals against lymphomagenic doses of EBV. Recombinant soluble MA has now been produced and clinical trials should take place in the next few years. An important patient group to target will be those children awaiting transplantation where virus-neutralizing antibodies raised in response to MA vaccination may provide protection from the development of EBV-associated lymphomas.

As the nature of primary EBV infection may make prophylactic vaccination extremely difficult, the use of therapeutic vaccines, perhaps directed to other EBV-encoded proteins, should be considered. The possibility of peptide vaccination with well-defined CTL epitopes is attractive particularly in NPC and HD where EBV latent gene expression is restricted. Adoptive CTL therapy using *ex vivo* expanded EBV-specific CTL preparations has proven successful in treating virus-associated lymphoproliferative disease and lymphomas in bone marrow transplant patients. A similar approach to the treatment of NPC and HD is currently being explored.

The antiviral agent acyclovir is a potent inhibitor of EBV DNA polymerase thus preventing virus replication. Acyclovir does not significantly alter the clinical course of IM or XLP. A few reports suggest that this

drug used in conjunction with interferon α may resolve the polyclonal B cell lymphomas found in transplant recipients but in this group withdrawal or reduction in immunosuppression is more effective. Although chemotherapy is extremely effective against BL, recent epidemiological evidence shows that the incidence of disease can be significantly reduced by malaria eradication. NPC can be successfully treated by surgery and local radiotherapy only at early stages of the disease. Early diagnosis using serologic screening for EBV-specific IgA antibodies and CAT scans has helped in identifying those patients with treatable disease. Combined chemotherapy is successful in 70% of HD patients. The EBV-positive form of HD may be more difficult to treat and thus the early identification of these cases using EBV-specific monoclonal antibodies could be useful.

Future Perspectives

The use of a variety of molecular virological techniques has unequivocally identified EBV as oncogenic in humans. However, the precise nature of primary infection and the subsequent establishment of lifelong viral persistence is poorly understood and will require the further refinement of techniques for identifying individual EBV-infected cells *in vivo*. Any prophylactic vaccine must be effective at preventing primary infection and requires a better knowledge of the immune control of EBV infection and replication at the site of primary infection. More efficient *in vitro* systems for studying EBV infection and replication will help in this regard. The use of EBV recombinants has already yielded valuable information on the function of certain viral genes and will continue to help identify the cellular and viral factors responsible for tumor development. Understanding the host cell:virus interaction will be dependent on the generation of appropriate *in vitro* models which are currently lacking for NPC and HD. The application of adoptive CTL therapy for EBV-associated tumors will depend on a better understanding of the tumor microenvironment and may require approaches aimed at manipulating the local cytokine milieu. A more detailed knowledge of the functions of individual EBV latent proteins will allow the development of sophisticated pharmacological approaches to the treatment of virus-associated malignancies.

See also: **Herpesvirus 8 – Human (*Herpesviridae*); Immune response: Cell mediated Immune response, General features; Transformation: Animal viruses.**

Further Reading

Niedobitek G and Young LS (1997) Epstein–Barr virus and non-Hodgkin lymphomas. In: Magrath I (ed.) *The Non-Hodgkin's Lymphomas*, 2nd edn, p. 309. London: Edward Arnold.

Rickinson AB and Kieff E (1996) Epstein–Barr virus. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 2497. Philadelphia: Lippincott-Raven.

Young LS (ed.) (1996) Epstein–Barr virus and non-B-cell tumours. *Semin. Cancer Biology*, vol. 7. Cambridge: Academic Press.

Molecular Biology

Jeffery T Sample and **Clare E Sample**, Department of Virology and Molecular Biology, St Jude Children's Research Hospital, Memphis, Tennessee, USA

Properties of the Virion

The Epstein–Barr virus (EBV) virion has a morphology typical of all herpesviruses and comprises four major substructures. The core of the virion contains the DNA genome and associated proteins in a tightly packed torus, which is encased within an icosadeltahedral nucleocapsid that contains 162 capsomeres and is approximately 100 nm in diameter. Between the nucleocapsid and membrane envelope of the virus is a poorly defined amorphous region known as the tegument. The envelope, which is largely derived from cellular membrane, contains several virus-encoded glycoproteins. The most abundant of these glycoproteins are gp350 and gp220, related proteins encoded by alternatively spliced mRNAs expressed from the same gene, that bind to the major cellular receptor for EBV attachment, CD21, and help mediate entry of the virus into the host cell.

Properties of the Genome

The genome of EBV is a double-stranded DNA molecule of approximately 185 kb. The complete genomic nucleotide sequence of the prototype isolate B95-8 has been determined, as have substantial segments of the genomes of other commonly studied isolates. The EBV genome contains approximately 90 genes, of which 12 are variably expressed during latent (nonproductive) infection. The remaining genes encode proteins that are expressed exclusively during the viral replication cycle. As indicated in the previous entry, two distinct strains of EBV (EBV-1 and EBV-2)

drug used in conjunction with interferon α may resolve the polyclonal B cell lymphomas found in transplant recipients but in this group withdrawal or reduction in immunosuppression is more effective. Although chemotherapy is extremely effective against BL, recent epidemiological evidence shows that the incidence of disease can be significantly reduced by malaria eradication. NPC can be successfully treated by surgery and local radiotherapy only at early stages of the disease. Early diagnosis using serologic screening for EBV-specific IgA antibodies and CAT scans has helped in identifying those patients with treatable disease. Combined chemotherapy is successful in 70% of HD patients. The EBV-positive form of HD may be more difficult to treat and thus the early identification of these cases using EBV-specific monoclonal antibodies could be useful.

Future Perspectives

The use of a variety of molecular virological techniques has unequivocally identified EBV as oncogenic in humans. However, the precise nature of primary infection and the subsequent establishment of lifelong viral persistence is poorly understood and will require the further refinement of techniques for identifying individual EBV-infected cells *in vivo*. Any prophylactic vaccine must be effective at preventing primary infection and requires a better knowledge of the immune control of EBV infection and replication at the site of primary infection. More efficient *in vitro* systems for studying EBV infection and replication will help in this regard. The use of EBV recombinants has already yielded valuable information on the function of certain viral genes and will continue to help identify the cellular and viral factors responsible for tumor development. Understanding the host cell:virus interaction will be dependent on the generation of appropriate *in vitro* models which are currently lacking for NPC and HD. The application of adoptive CTL therapy for EBV-associated tumors will depend on a better understanding of the tumor microenvironment and may require approaches aimed at manipulating the local cytokine milieu. A more detailed knowledge of the functions of individual EBV latent proteins will allow the development of sophisticated pharmacological approaches to the treatment of virus-associated malignancies.

See also: **Herpesvirus 8 – Human (*Herpesviridae*); Immune response: Cell mediated Immune response, General features; Transformation: Animal viruses.**

Further Reading

Niedobitek G and Young LS (1997) Epstein–Barr virus and non-Hodgkin lymphomas. In: Magrath I (ed.) *The Non-Hodgkin's Lymphomas*, 2nd edn, p. 309. London: Edward Arnold.

Rickinson AB and Kieff E (1996) Epstein–Barr virus. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 2497. Philadelphia: Lippincott-Raven.

Young LS (ed.) (1996) Epstein–Barr virus and non-B-cell tumours. *Semin. Cancer Biology*, vol. 7. Cambridge: Academic Press.

Molecular Biology

Jeffery T Sample and **Clare E Sample**, Department of Virology and Molecular Biology, St Jude Children's Research Hospital, Memphis, Tennessee, USA

Properties of the Virion

The Epstein–Barr virus (EBV) virion has a morphology typical of all herpesviruses and comprises four major substructures. The core of the virion contains the DNA genome and associated proteins in a tightly packed torus, which is encased within an icosadeltahedral nucleocapsid that contains 162 capsomeres and is approximately 100 nm in diameter. Between the nucleocapsid and membrane envelope of the virus is a poorly defined amorphous region known as the tegument. The envelope, which is largely derived from cellular membrane, contains several virus-encoded glycoproteins. The most abundant of these glycoproteins are gp350 and gp220, related proteins encoded by alternatively spliced mRNAs expressed from the same gene, that bind to the major cellular receptor for EBV attachment, CD21, and help mediate entry of the virus into the host cell.

Properties of the Genome

The genome of EBV is a double-stranded DNA molecule of approximately 185 kb. The complete genomic nucleotide sequence of the prototype isolate B95-8 has been determined, as have substantial segments of the genomes of other commonly studied isolates. The EBV genome contains approximately 90 genes, of which 12 are variably expressed during latent (nonproductive) infection. The remaining genes encode proteins that are expressed exclusively during the viral replication cycle. As indicated in the previous entry, two distinct strains of EBV (EBV-1 and EBV-2)

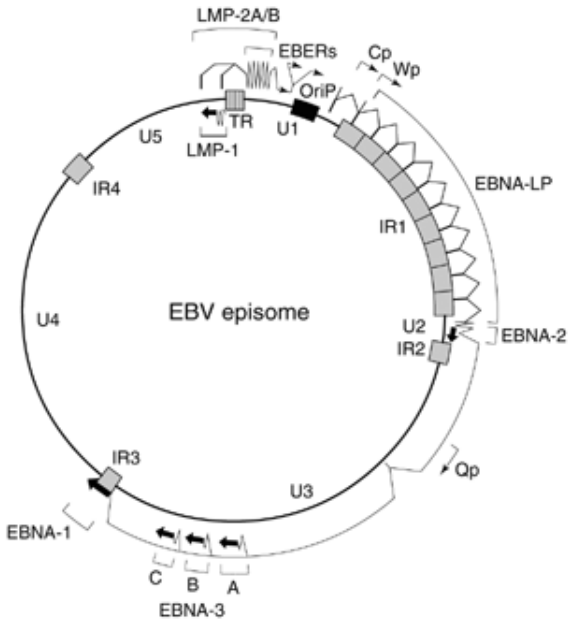


Figure 1 Structure and latency-associated transcription of the EBV genome. The repeat elements (TR, IR1–IR4) and unique sequence domains (U1–U5) of the genome are indicated as are the latency-associated mRNAs and EBERs. Exons that contain open reading frames are bracketed. Note that the EBNA mRNAs are expressed from a common transcription unit under the control of the promoter Cp or Wp during the growth program of latency, and that individual EBNA mRNAs are generated through alternative RNA processing. Exclusive expression of EBNA-1 during restricted latency is mediated by the promoter Qp. LMP-2A and LMP-2B differ in that their expression is controlled by separate promoters; LMP-2A is 119 amino acids longer than LMP-2B due to additional coding sequence in its unique 5′ exon. *OriP* is the latent infection origin of DNA replication. Not shown are the *Bam*HI-A rightward transcripts (BARTs), a complex family of alternatively spliced polyadenylated RNAs that initiate in U4 and terminate in U5.

exist that are defined by specific sequence differences within several of the viral genes expressed during latent infection, most notably those encoding the EBV nuclear antigen (EBNA) -2 and -3 proteins. In its linear configuration within the virion, the genome is bounded by terminal repeats of 538 bp that contain the DNA packaging signals. Internally, the genome is separated into short (12 kb) and long (134 kb) predominantly unique sequence domains by 8–10 copies of a 3 kb direct repeat, IR1. Historically, the long unique domain has been divided into smaller domains by short repeat elements, as illustrated in **Fig. 1**.

Shortly after infection of a B lymphocyte, the terminal repeats of the EBV genome fuse to generate an episome. A variable but relatively stable number of episomes (10–100) are maintained within the nuclei of infected B cells, which do not actively support virus replication. In response to the establishment of a latent EBV infection *in vitro*, B cells undergo immortalization and thus acquire the potential to proliferate indefinitely in cell culture. Replication of the EBV episome in latently infected B cells is mediated by the cellular replication machinery, occurs once per cell division cycle, and is initiated within an origin of DNA replication, *oriP*, located in the short unique domain of the viral genome. Although maintenance of EBV DNA during latency requires only one viral protein, the origin-binding protein EBNA-1, the processes of genome replication is poorly understood. Two separate but highly homologous origins of DNA replication, *oriLyt*, are active during the virus replicative cycle. These lie 100 kb apart within the left and right one-third of the long unique sequence domain, and enable production of the linear genomes that ultimately are packaged into the virion. In contrast to latent infection, replication of EBV DNA during the lytic cycle is predominantly, if not solely, regulated by viral proteins.

Virus Replication

Although the latent state of EBV infection is most often associated with human disease, the replicative component of the virus life cycle is essential for the maintenance of EBV in the general population and therefore ultimately for the pathogenic potential of EBV. Unfortunately, delineation of the specific mechanisms through which mature EBV virions are produced has been hampered by the lack of cell lines that support EBV replication. Although some latently infected B cell lines can be induced to replicate EBV in response to chemical agents such as phorbol esters or by the crosslinking of cell-surface immunoglobulin molecules, the efficiency of induction is generally poor, in that only 20–30% of the treated cells, at best, will actually produce virus. Although epithelial cells are believed to be primary sites of EBV replication *in vivo*, attempts to establish epithelial cell lines readily infectable by EBV and which actively support virus replication have not been successful. Nonetheless, the general process of EBV replication is reasonably well understood, in large part due to parallels that can be drawn to the replication of herpes simplex virus, for which an extensive body of knowledge exists. Furthermore, comparative sequence analyses of the genomes of several mammalian herpesviruses have been identified in each a set of conserved replicative genes which encode viral structural as well as regulatory proteins. Thus, the replication of EBV is

believed to occur through a basic process common to all herpesviruses.

Herpesvirus replication progresses through a highly ordered cascade of viral gene expression that is initiated by the immediate early class of genes (those that do not require *de novo* protein synthesis to activate their expression), followed by expression of the early and then late genes. In general, immediate early and early proteins regulate viral gene expression and DNA replication, whereas late gene products are either structural components of the virion or are required for functions such as virus assembly and egress. Although a thorough description of EBV replication is beyond the scope of this text, there are several unique aspects of the EBV lytic cycle that warrant additional discussion.

Activation of the lytic cycle

Much of the focus on EBV replication has been directed towards the mechanisms that disrupt latency, ultimately enabling the transmission of virus to naive hosts. Although the physiologic signals that trigger activation of EBV replication *in vivo* are poorly understood, the critical event downstream of such signals is the activation of expression of the EBV protein Zta, which alone is capable of initiating the lytic cycle cascade of gene expression. Zta is a transcriptional activator related to members of the cellular AP-1 family of transcription factors, and is able to activate gene expression through binding to AP-1 and closely related Zta response elements. Zta activates the lytic cycle in cooperation with a second EBV transactivator, Rta, itself activated by Zta, through transactivation of EBV early genes which contain Zta and Rta response elements within their promoters. Zta and Rta also function in the replication of EBV DNA, presumably through mediating the assembly of EBV replication proteins into the replication complex at *oriLyt*, which contains Zta and Rta response elements. Additionally, Zta and Rta interact with or induce the expression of a number of cellular proteins that regulate cellular proliferation and differentiation, such as p53, NFκB, retinoic acid receptor, pRB, p21, TGF-β and c-*MYC*. These properties of Zta and Rta likely provide an optimal cellular environment for virus replication. Interestingly, although expression of Rta alone is not sufficient to activate the lytic cycle within latently infected B cells, it may do so within epithelial cells, although this most likely requires Rta-mediated activation of Zta expression. This suggests that different signals may exist for the activation of EBV replication in B lymphocytes and epithelial cells.

Auxiliary functions of lytic cycle genes

The EBV genome encodes several lytic cycle proteins that are not directly involved in the production of infectious virus, but instead influence the intra- or extracellular milieu to enhance the efficiency of replication or promote the infection process by subverting host immune mechanisms that have evolved to control virus infections. The principal example of an EBV gene that may promote efficiency of replication is BHRF1, which encodes a homologue of the cellular protein BCL-2 capable of inhibiting programed cell death or apoptosis. By inhibiting induction of apoptosis in response to stresses associated with lytic EBV infection, the BHRF1 protein presumably prolongs the life of the cell, enhancing virus production.

EBV expresses at least three genes during the lytic cycle that are capable of modulating the host immune response. Two of these encode homologues of human interleukin 10 (hIL-10) and the receptor for colony-stimulating factor 1 (CSF-1). EBV IL-10 (vIL-10), encoded by the BCRF1 gene, displays a colinear homology of 78% with hIL-10, and is functionally equivalent in many (but not all) respects to its cellular counterpart. Because hIL-10 has numerous immunosuppressive effects, expression of vIL-10 is viewed as a mechanism to potentiate EBV infection and establishment of the carrier state. The EBV CSF-1 receptor homologue, encoded by the BARF1 gene, is a secreted protein able to bind and neutralize soluble CSF-1, though a role for CSF-1 in the host defense against EBV infection has not been defined. The third EBV gene, BZLF2, encodes gp42, a glycoprotein that plays an important role in the infection of B lymphocytes through interaction with the cell-surface class II antigen HLA-DR. Additionally, gp42 bound to HLA-DR on the surface of cells supporting virus replication is able to inhibit class II mediated antigen presentation, and is thus another potential mechanism adopted by EBV to interfere with the host immune response.

Latency

During initial exposure to EBV a latent infection is established within B lymphocytes. Whether latency is initiated through direct infection of resident or circulating B cells within tissue of the oropharyngeal cavity, or secondary infection following initial replication of virus in epithelial cells, is currently a matter of contention. Regardless, once latency has been established the virus is maintained within the B cell pool for the life of the host, presumably without further requirement for virus replication. The *in vitro*

Table 1 Functions of the EBV latency-associated genes

Gene/protein	*Properties*	*Required for B cell immortalization*
EBNA-1	Required for genome replication/maintenance; activates EBNA transcription via *oriP* (Cp/Wp); autorepression of Qp; oncogenic potential (?)	+[a]
EBNA-2	Transcriptional activation of EBNA (Cp), LMP-1, LMP-2B and cellular genes	+
EBNA-3A	Transcriptional repression of LMP-1 and LMP-2B; possible regulation of cellular genes	+
EBNA-3B	Transcriptional repression of LMP-1 and LMP-2B; possible regulation of cellular genes	−
BNA-3C	Transcriptional repression of LMP-1 and LMP-2B; activator of LMP-1 and cellular genes	+
EBNA-LP	Potentiator of transactivation by EBNA-2	+/−[b]
LMP-1	Oncogenic; constitutive activation of TNF signaling pathway	+
LMP-2A LMP-2B	LMP-2A affects BCR signaling to inhibit reactivation of virus; provides survival signal; isoform LMP-2B has no known function	−
BARTs	Complex spliced transcripts, may encode at least two polypeptides (RK-BARF0 and RPMS1); function unknown	−
EBER-1 EBER-2	Small nuclear RNAs; bind autoantigen La, dsRNA-dependent protein kinase (EBER-1), ribosomal protein L22 (EBER-1); regulation of translation (?)	−

[a] Assumed to be essential due to requirement for viral DNA maintenance.

[b] Enhances immortalization but not essential (see text).

models of EBV latency have been long-term cultures of Burkitt's lymphoma (BL) cells and EBV-immortalized B lymphoblastoid cell lines (LCLs) which express a subset of 12 EBV genes, collectively known as the latency-associated genes. These genes encode six EBV nuclear antigens (EBNA-1, -2, -3A, -3B, -3C and -LP), three membrane proteins (LMP-1, -2A and -2B), a complex family of alternatively spliced transcripts (BARTs) from the *Bam*HI-A region of the genome, and two small noncoding nuclear RNAs (EBER-1 and EBER-2). The known functions and properties of these genes are summarized in **Table 1**.

For two decades following the discovery of EBV in BL biopsies, EBV latency *in vitro* within LCLs and BL cell lines was widely accepted as the model of EBV persistence *in vivo*. Increasing evidence indicated, however, that EBV-infected B cell lines, and subsequently the latency-associated proteins themselves, were targets of immune cytotoxic T lymphocytes (CTLs). It seemed unlikely, therefore, that EBV could persist solely within an LCL-like cell in a healthy EBV-infected individual, but instead might adopt an alternative, less immunogenic state of latency. Evidence of this was first provided by observations that early-passage BL cells, but not HLA-matched LCLs or late-passage BL cells, were resistant to killing by HLA class I antigen-restricted EBV-specific CTLs. This was determined to be predominantly the result of a downregulation of EBV latent-gene expression in BL tumors, particularly those viral proteins, such as the EBNA-3 molecules, that contain dominant CTL epitopes. In contrast to LCLs, BL tumor cells limit expression of EBV genes to EBNA-1, the BARTs and EBERs (type I latency). Shortly after the discovery of a restricted program of EBV latent-gene expression in BL tumors, similar patterns of EBV gene expression were detected in other EBV-associated tumors, most notably nasopharyngeal carcinoma and EBV-positive Hodgkin's lymphoma, which also express LMP-1 and LMP-2A (type II latency). Recent analyses of EBV gene expression in B cells of otherwise healthy donors have revealed that EBV gene expression progresses from an LCL-like or growth program of latency-associated gene expression during acute (primary) infection, to a restricted program of latency similar to BL upon establishment of the carrier state.

Growth program of latency

During the growth program of EBV latency (alternatively referred to as type III latency), the full complement of latency-associated genes is expressed (**Fig. 1** and **Table 1**). However, the highly proliferative nature of B cells that maintain the growth program is due to the influence of only a subset of these proteins, namely EBNA-2, EBNA-LP, the EBNA-3 proteins and LMP-1, which target, either directly or indirectly, cellular signaling pathways

involved in the regulation of cell growth and differentiation. Discussion of the growth program of latency, therefore, will focus on the functions of these proteins. The remaining EBV latency-associated genes will be discussed in the context of restricted latency.

EBNA-2 EBNA-2 is one of six proteins essential for EBV-mediated B cell immortalization and sustained cell proliferation (**Table 1**). Studies evaluating the effect of EBNA-2 expression in EBV-negative B cell lines provided the first indication that EBNA-2 functions as a transcriptional regulator. EBNA-2 is known to activate expression of several cellular genes: the B cell growth factor CD23, the c-fgr proto-oncogene, and CD21, the EBV receptor and a component of the CD19 signaling pathway. EBNA-2 also activates expression from the EBV promoters for LMP-1 and -2B, as well as the EBNA gene promoter Cp (**Fig. 1**). EBNA-2 does not bind directly to DNA, but is targeted to the promoters of EBNA-2-responsive genes through interactions with cellular DNA-binding proteins. Because EBNA-2 contains a strong acidic transcriptional activation domain within its C-terminus that interacts with a number of basal transcription factors, targeting of EBNA-2 to a promoter leads to increased transcription.

One protein through which EBNA-2 is targeted to a promoter is the ubiquitous cellular transcription factor Jκ. Jκ has been highly conserved throughout evolution, and its importance is underscored by the finding that deletion of the Jκ gene results in embryonic lethality in such diverse species as the mouse and fruit fly. Jκ itself is a component of the Notch signaling pathway, and binds to the intracellular domain of the receptor Notch. Ligand-mediated activation of Notch results in cleavage and release of its intracellular domain, which then translocates to the nucleus and indirectly binds to DNA through its interaction with Jκ. Thus, EBNA-2 mimics activated Notch. Interestingly, Notch is activated or overexpressed in several malignancies, including leukemias, though the role of Notch signaling in EBV-induced cell proliferation is unclear. Since Jκ binding sites are present in numerous cellular promoters, in addition to the EBV Cp and LMP-1 and -2B promoters, a large number of cellular genes are likely to be heretofore unidentified targets for transactivation by EBNA-2.

A second mechanism for targeting EBNA-2 to DNA, particularly the LMP-1 promoter, is interaction with the cellular factor Spi-1 or the highly related Spi-B. Both proteins are members of the Ets family of transcription factors, which recognize the same DNA sequence element within responsive promoters. Whereas expression of Spi-1 is restricted to and plays an important role in the development of B lymphocytes and myeloid cells, less is known of Spi-B, the expression of which is limited to B and T lymphocytes. Of particular interest with respect to the role of EBNA-2 in the regulation of B cell proliferation is the fact that the murine homologue of Spi-1, PU.1, was first identified as an oncoprotein. The importance of these proteins is further highlighted by the embryonic lethality of mice nullizygous for Spi-1, and defective B cell receptor-mediated responses in Spi-B knockout mice. The Ets family of transcription factors, which are clearly important for B cell development and differentiation, regulate a wide variety of cellular genes. The expression of at least some of these genes, therefore, is likely to be regulated by EBNA-2 and important in the EBV-mediated control of B cell growth and differentiation.

EBNA-3 family The EBNA-3 family comprises three genes, EBNA-3A, -3B and -3C, whose tandem arrangement within the EBV genome and similar gene structure (**Fig. 1**) suggests that they may have arisen by gene duplication. There is, however, little notable sequence homology between these proteins. EBNA-3A and -3C are essential for B cell immortalization by EBV, whereas EBNA-3B is dispensable. One common function of the EBNA-3 proteins is their ability to bind to Jκ, which also interacts with EBNA-2. The interaction of the EBNA-3 proteins with Jκ is mediated through a homologous motif in the N-terminus of each EBNA-3 protein, lending further support to the hypothesis that these proteins are distantly related. By contrast to the interaction of Jκ with EBNA-2, interaction with the EBNA-3 proteins inhibits transcriptional activation mediated through Jκ response elements by preventing the binding of Jκ to DNA. Interestingly, the function of the *Drosophila* homologue of Jκ, Suppressor of Hairless, is regulated in an analogous fashion by Hairless, in an analogous fashion to the EBNA-3s, suggesting that the activity of Jκ must be tightly regulated. Conservation of the mechanisms that regulate Jκ-mediated transcription, in a manner requiring four viral proteins, further suggests that modulation of this pathway is critical to the control of cell proliferation by EBV.

The fact that both EBNA-3A and EBNA-3C are required for B cell immortalization by EBV suggests that they possess unique functions in addition to their ability to regulate Jκ. Like EBNA-2, EBNA-3C increases the expression of CD21 in EBV-negative B cell lines. EBNA-3C, in the presence of EBNA-2, can also activate expression of LMP-1. This activation occurs at the level of transcription through a Jκ-independent mechanism. While EBNA-3C does

possess a transcriptional activation domain in its C-terminus, it has not yet been demonstrated to bind to a specific DNA sequence. Furthermore, cotransformation assays using rat embryo fibroblasts have demonstrated that EBNA-3C can immortalize these cells in collaboration with an activated *ras* gene, suggesting that EBNA-3C contributes to the growth program in a manner independent of its ability to regulate LMP-1 expression.

Expression of EBNA-3B in EBV-negative B cell lines results in increased expression of CD40 and vimentin, an intermediate filament of the cytoskeletal network. The combined expression of all three family members induces the expression of pleckstrin, a hematopoietic-cell protein containing two pleckstrin homology domains proposed to mediate protein–protein or protein–lipid interactions. The fact that these domains are found in many proteins that participate in cell signaling or cytoskeletal regulation suggests that the pleckstrin gene may be a significant target of the EBNA-3 proteins. Like EBNA-3C, EBNA-3A and -3B possess a C-terminal transcriptional activation domain, and EBNA-3A also contains a transcriptional repression domain. Thus, all members of the EBNA-3 family clearly function as transcriptional regulators, and have the potential to influence gene expression through Jκ-dependent as well as Jκ-independent mechanisms.

EBNA-LP Relatively little is known of the function(s) of EBNA-LP (leader protein), named for the fact that it is encoded by the common leader sequence of the EBNA mRNAs expressed during the growth program (**Fig. 1**). Although EBNA-LP is critical for the efficient immortalization of B lymphocytes by EBV, it is not absolutely essential. EBNA-LP has been demonstrated to interact with a member of the heat shock family of proteins, p73, and can activate gene expression through the transcriptional activation domain of EBNA-2. This latter property of EBNA-LP suggests that it may act as an adaptor molecule to promote interaction between the activation domain of EBNA-2 and the basal transcriptional machinery. Coexpression of EBNA-2 and EBNA-LP in primary B lymphocytes results in increased expression of the G_1 cyclin D2, and progression from the G_0 to G_1 stage of the cell cycle, clearly a property consistent with a role in promoting cell growth.

LMP-1 LMP-1 is unique among the EBV proteins expressed during the growth program in that it alone induces the classic phenotypic changes associated with transformation when expressed in rodent fibroblasts. In B cells, LMP-1 expression results in the increased levels of cellular adhesion molecules associated with either EBV infection or antigen activation of primary B lymphocytes. Expression of the anti-apoptotic cellular proteins bcl-2 and A20 is also activated by LMP-1, promoting the survival of infected cells. Interestingly, although LMP-1 transgenic mice develop B cell lymphomas, LMP-1 alone is not sufficient to sustain B cell proliferation, underscoring the importance of the contributions of the other EBV proteins expressed during the growth program.

LMP-1 is composed of a small cytoplasmic domain at its N-terminus, and a larger cytoplasmic domain at the C-terminus separated by six transmembrane domains. Genetic studies have demonstrated that two regions of the LMP-1 C-terminal domain, termed transformation effector sites (TES)-1 and -2, are essential for efficient B cell immortalization. Through these domains, LMP-1 directly associates with the intracellular signaling proteins of the tumor necrosis factor receptor (TNFR). Since LMP-1 is a highly hydrophobic membrane protein localized to patches in the plasma membrane, this association results in aggregation of these signaling proteins and constitutive activation of the TNF signal transduction pathway, a critical outcome of which is activation of the transcription factor NFκB. TES-1, located proximal to the membrane, interacts with the TNFR-associated factors (TRAFs) to mediate low-level activation of NFκB that drives the initial proliferation of B lymphocytes following EBV infection. TES-2, located at the very C-terminus of LMP-1, mediates the majority of NFκB activation through binding to the TNFR-associated death domain protein TRADD, and is required for the continued growth of EBV-infected B lymphocytes in culture. TNFR is one of a family of receptors whose members are associated with the promotion of cell growth (CD40) or cell death (FAS receptor), and thus LMP-1 appears to play a direct role in the immortalization or growth-transformation process associated with EBV infection of B lymphocytes.

Within epithelial cells, LMP-1 is able to activate expression of the epidermal growth factor receptor through TRAF signaling, resulting in the inhibition of terminal differentiation. This pathway is likely to mediate the development of epithelial hyperplasia observed in LMP-1 transgenic mice, and suggests a significant role for LMP-1 in the more restricted (type II) latency found in undifferentiated nasopharyngeal carcinoma.

Restricted program of latency

Whereas the growth program of EBV latency drives a rapid expansion of EBV-infected B cells, restricted

latency, commonly referred to as the latency program, is believed to promote the long-term maintenance of infection once a critical pool of infected cells has been established. Within an uncertain time frame following primary infection, but presumably before the maturation of the CTL response to EBV proteins exclusively associated with the growth program, there is a downregulation of the expression of LMP-1, LMP-2B and each of the EBNA proteins except EBNA-1. In the absence of EBV proteins that act in concert to stimulate cell proliferation, infected B cells are thought to re-enter the pool of resting B lymphocytes to become the primary reservoir of EBV-infected cells. Even though several EBV proteins continue to be expressed, namely EBNA-1, LMP-2A and possibly at least two proteins encoded by the BARTs, it is apparent that the host does not effectively perceive these cells as infected, thus permitting them to persist in the face of an otherwise potent anti-EBV immune surveillance. Although LMP-2A and proteins encoded by the BARTs may simply be poor stimulators of a CTL response, EBNA-1 contributes directly to immune escape by blocking its own degradation and subsequent presentation on the cell surface in association with HLA class I antigens. Once restricted latency has been established, it is unclear whether the EBV growth program is subsequently reactivated. Periodic reactivation of the growth program, presumably in response to physiologic signals, may serve to expand and thus sustain the pool of infected B cells. Alternatively, physiologically-induced B cell proliferation might occur in the context of a restricted latency, without induction of the growth program, which presumably would enable limited expansion without risking significant loss of infected cells due to reactivation of the anti-EBV CTL response.

Regulation and function of the latency program

Approximately one B lymphocyte in 10^5 is latently infected with EBV within the peripheral blood of EBV-positive individuals. Although this is a sufficient number of cells to both detect the presence of viral DNA and assess the pattern of EBV gene expression *in vivo* using polymerase chain reaction (PCR)-based technology, these cells cannot be propagated *in vitro* for more detailed analysis unless the growth program of latency is reactivated. Thus, much of what is known about the restricted latency program is based on the analysis of a subset of BL cell lines that, for unknown reasons, continue to maintain restricted latency *in vitro*. In contrast to EBV-infected LCLs, which have not undergone malignant transformation and require EBV for continued growth, deregulation of the c-*MYC* proto-oncogene in BL is able to sustain cell growth in culture independent of EBV. Although these so-called group I BL cell lines do not consistently express LMP-2A, they have nonetheless proven to be a reasonably accurate model of the latency program in normal B lymphocytes.

The apparent mechanism through which a subset of the EBV latency-associated genes are repressed during restricted latency is the selective methylation of EBV promoters by cellular DNA methyltransferases. Methylation of the cytosine moiety of CpG dinucleotide pairs within the targeted EBV promoters results in transcriptional inactivation of the gene. How the selective methylation of EBV promoters is regulated, and whether this is in fact the primary mechanism responsible for restricting EBV gene expression, is unknown. Despite inactivation of the EBNA promoters Cp and Wp during restricted latency, BL and other EBV-infected tumor cells continue to express EBNA-1, which is required for maintenance of the EBV episome in proliferating cells. Exclusive expression of EBNA-1 in these cells, as well as in latently infected B cells *in vivo*, is mediated through the activation of an EBNA-1-specific promoter, Qp, that is not active during the growth program of EBV latency (**Fig. 1**).

With the exception of EBNA-1, which performs an essential but as yet undefined role in the replication and maintenance of the EBV genome, the EBV gene products expressed during the latency program are not required for the immortalization and sustained growth of B lymphocytes *in vitro*. This suggests that the functions of LMP-2A, the BARTs and EBERs may be critical in the context of the latency program *in vivo*. In fact, a number of observations indicate that these EBV genes do indeed target cellular pathways in a manner that would contribute positively to the maintenance of long-term latency. Recent examination of LMP-2A function in transgenic mice indicates that LMP-2A provides a survival signal in B lymphocytes that mimics such a signal provided by the B cell receptor (BCR) that is essential for B cell maintenance. Earlier studies, furthermore, demonstrated that LMP-2A is capable of inhibiting activation of the EBV lytic cycle through inhibition of BCR-mediated signal transduction. Thus, one would predict that LMP-2A contributes to long-term latency by promoting B cell survival through at least two mechanisms: first, by providing a constitutive survival signal to help ensure an extended lifespan of the infected B cell; and second, by preventing the activation of EBV replication and the cellular destruction that would occur as a result.

Further evidence that the genes expressed during restricted latency are important *in vivo* comes from recent studies with the group I BL cell line Akata. A small percentage of Akata cells in culture undergo a

spontaneous loss of the EBV genome that is concomitant with a loss of tumorigenic potential. Reinfection and establishment of restricted latency in these cells restores tumorigenicity, confirming that tumorigenic potential in this BL cell line is dependent on EBV. Whether EBV is universally essential for the tumorigenicity of BL, however, remains to be demonstrated. EBV promotes tumorigenicity in Akata BL cells by inhibiting apoptosis driven by the dominant oncoprotein in this tumor, c-MYC, which is overexpressed in BL due to a chromosomal translocation leading to transcriptional deregulation of the c-MYC gene. Thus, although none of the EBV genes normally expressed in Akata cells (EBNA-1, the BARTs and EBERs) are known to directly promote cell growth, it is clear that one or more of these genes does impart a strong survival signal that is most likely also operational within the context of the latency program in normal B cells. Interestingly, EBNA-1, which has been linked to B cell lymphoma in some lines of mice carrying an active EBNA-1 transgene, does not restore tumorigenicity or inhibit apoptosis in Akata cells when expressed in an EBV-negative background. This suggests that the EBERs or a protein(s) encoded by the BARTs, or a previously unidentified viral protein, is responsible for promoting B cell survival.

In summary, analyses of EBV gene expression and function have defined two predominant programs of EBV latency in B lymphocytes. These are the growth program characterized by rapid virus-induced proliferation of cells that is associated with expression of the full complement of EBV latency-associated genes, including the oncogenic LMP-1 protein, and the more restricted latency program in which the primary function of the limited set of EBV genes expressed is not to direct cell growth, but to maintain the viral episome, promote B cell survival and prevent activation of the virus replication cycle. Retrospective studies of EBV infection *in vivo* have indicated that the growth program is associated with and possibly limited to the acute phase of EBV infection, and thus likely serves to establish a critical mass of infected B cells during primary infection. By contrast, the latency program, though less-well understood, contributes primarily to the long-term maintenance of latency following the resolution of primary infection and in the face of a potent anti-EBV immune surveillance capable of eliminating infected cells should they sustain or reactivate the growth program.

See also: **Epstein-Barr virus (*Herpesviridae*): General features; Herpesviruses – baboon and chimpanzee (*Herpesviridae*); Latency; Tumor viruses – human; Transformation: Animal viruses; Transplantation and virus infections.**

Further Reading

Kieff E (1996) Epstein–Barr virus and its replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Virology*, 3rd edn, p. 2343. Philadelphia: Lippincott-Raven.

Rickinson AB and Kieff E (1996) Epstein–Barr virus. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Virology*, 3rd edn, p. 2397. Philadelphia: Lipincott-Raven.

Equine arteritis virus *see* **Arteriviruses (*Arteriviridae*)**

EQUINE ENCEPHALITIS VIRUSES (*TOGAVIRIDAE*)

Diane E Griffin, Molecular Microbiology and Immunology, Johns Hopkins School of Public Health, Baltimore, Maryland, USA

History

Nomenclature for the encephalitic alphaviruses recognizes the characteristic geographic distribution of each and the initial identification of the viruses as causes of encephalitis in horses. The first clear record of epidemic equine encephalitis comes from 1831 when an outbreak in Massachussetts, USA, resulted in the deaths of 75 horses. Over the next 100 years several local epidemics of encephalitis in horses

spontaneous loss of the EBV genome that is concomitant with a loss of tumorigenic potential. Reinfection and establishment of restricted latency in these cells restores tumorigenicity, confirming that tumorigenic potential in this BL cell line is dependent on EBV. Whether EBV is universally essential for the tumorigenicity of BL, however, remains to be demonstrated. EBV promotes tumorigenicity in Akata BL cells by inhibiting apoptosis driven by the dominant oncoprotein in this tumor, c-MYC, which is overexpressed in BL due to a chromosomal translocation leading to transcriptional deregulation of the c-MYC gene. Thus, although none of the EBV genes normally expressed in Akata cells (EBNA-1, the BARTs and EBERs) are known to directly promote cell growth, it is clear that one or more of these genes does impart a strong survival signal that is most likely also operational within the context of the latency program in normal B cells. Interestingly, EBNA-1, which has been linked to B cell lymphoma in some lines of mice carrying an active EBNA-1 transgene, does not restore tumorigenicity or inhibit apoptosis in Akata cells when expressed in an EBV-negative background. This suggests that the EBERs or a protein(s) encoded by the BARTs, or a previously unidentified viral protein, is responsible for promoting B cell survival.

In summary, analyses of EBV gene expression and function have defined two predominant programs of EBV latency in B lymphocytes. These are the growth program characterized by rapid virus-induced proliferation of cells that is associated with expression of the full complement of EBV latency-associated genes, including the oncogenic LMP-1 protein, and the more restricted latency program in which the primary function of the limited set of EBV genes expressed is not to direct cell growth, but to maintain the viral episome, promote B cell survival and prevent activation of the virus replication cycle. Retrospective studies of EBV infection *in vivo* have indicated that the growth program is associated with and possibly limited to the acute phase of EBV infection, and thus likely serves to establish a critical mass of infected B cells during primary infection. By contrast, the latency program, though less-well understood, contributes primarily to the long-term maintenance of latency following the resolution of primary infection and in the face of a potent anti-EBV immune surveillance capable of eliminating infected cells should they sustain or reactivate the growth program.

See also: **Epstein-Barr virus (*Herpesviridae*): General features; Herpesviruses – baboon and chimpanzee (*Herpesviridae*); Latency; Tumor viruses – human; Transformation: Animal viruses; Transplantation and virus infections.**

Further Reading

Kieff E (1996) Epstein–Barr virus and its replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Virology*, 3rd edn, p. 2343. Philadelphia: Lippincott-Raven.

Rickinson AB and Kieff E (1996) Epstein–Barr virus. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Virology*, 3rd edn, p. 2397. Philadelphia: Lipincott-Raven.

Equine arteritis virus *see* **Arteriviruses (*Arteriviridae*)**

EQUINE ENCEPHALITIS VIRUSES (*TOGAVIRIDAE*)

Diane E Griffin, Molecular Microbiology and Immunology, Johns Hopkins School of Public Health, Baltimore, Maryland, USA

History

Nomenclature for the encephalitic alphaviruses recognizes the characteristic geographic distribution of each and the initial identification of the viruses as causes of encephalitis in horses. The first clear record of epidemic equine encephalitis comes from 1831 when an outbreak in Massachussetts, USA, resulted in the deaths of 75 horses. Over the next 100 years several local epidemics of encephalitis in horses

occurred along the Atlantic seaboard. The virus of this eastern form of encephalitis (EEE) was first isolated from the brain of an affected horse in 1933 by Tenbroek and Merrill. However, 2 years earlier Meyer, Haring and Howitt had isolated a virus from the central nervous system (CNS) tissues of two horses involved in an epidemic of equine encephalitis in the San Joaquin Valley of California. The eastern isolate and the western equine encephalitis (WEE) virus were serologically distinct. Both diseases occurred in summertime epidemics; in 1933 Kelsor showed that WEE could be transmitted by mosquitoes and shortly thereafter mosquito transmission of EEE was shown. In 1938 both the EEE and WEE viruses were isolated from human cases of encephalitis occurring in the same regions as the equine cases. In 1936 an epidemic of equine encephalitis occurred in the Goajira region of Venezuela and the virus, isolated independently by Beck and Wyckoff and Kubes and Rios, was not neutralized by sera from animals immunized with EEE or WEE viruses and was designated Venezuelan equine encephalitis (VEE) virus.

Taxonomy and Classification

In 1954 arthropod-borne viruses were divided by Casals and Brown into three serologic groups, A, B and C, based on crossreactivity in hemagglutination inhibition (HI) tests. EEE, WEE and VEE viruses constituted the original group A arboviruses. A second crossreacting set, including dengue, St Louis encephalitis and yellow fever viruses, constituted the group B arboviruses; the nonreactive viruses were designated group C. As viruses became classified on the fundamental properties of the virion, the group A viruses became the genus *Alphavirus* within the *Togaviridae* family of enveloped RNA viruses. HI has been used to classify alphaviruses into six broad antigenic complexes. EEE, WEE, and VEE form three of these complexes. The EEE and VEE complexes contain only EEE and VEE viruses, while the WEE complex includes several viruses in addition to WEE (Y62-33, Highlands J, Fort Morgan, Aura and Sindbis) that are distinguished from each other by complement fixation and neutralization.

Viruses within each complex can be subtyped using reactivity with monoclonal antibodies, kinetic HI (EEE, VEE) or neutralization (WEE, VEE) assays. EEE and WEE viruses both have North American and South American subtypes. WEE and VEE viruses can also be subdivided into epizootic and enzootic strains. The VEE viruses are differentiated by HI and neutralization tests into subtypes I–VI. Epizootic strains are equine-virulent and fall within subtype I, which has been further subdivided serologically into IAB, IC (epizootic strains) and ID, IE and IF (enzootic strains). Sequence information, where available, has confirmed the validity of these distinctions.

Properties of the Virion

The virions are 60–65 nm in diameter. The RNA is enclosed in a capsid formed by a single protein arranged as an icosahedron with a $T = 4$ symmetry. The nucleocapsid is enclosed in a lipid envelope derived from the host cell plasma membrane that contains the viral-encoded glycoproteins, E1 and E2. These proteins form heterodimers that are grouped as trimers to form knobs on the virion surface that are arranged with a $T = 4$ symmetry. Glycoproteins are arranged with 240 copies of each protein interacting with 240 copies of capsid protein.

Properties of the Genome

The 49S genome is a single-stranded, nonsegmented, capped and polyadenylated message-sense RNA that is infectious. Complete sequence information is available for representative members of all three virus complexes. The genomes contain approximately 11 700 nucleotides and are organized with the nonstructural proteins at the 5′ end and the structural proteins at the 3′ end. Highly conserved regions of the genome are the 19 nucleotides at the 3′ terminus, important for negative-strand synthesis, and 21 nucleotides at the junction between the nonstructural and structural genes, important for synthesis of the subgenomic RNA. Analysis of codon usage shows an underutilization of the dinucleotide CpG.

Properties of the Viral Proteins

Five potential structural proteins (C, E3, E2, 6K and E1) are encoded in the 3′ end of the genome. The capsid protein is 259 (EEE, WEE) to 275 (VEE) amino acids long. The N-terminal portion is conserved, basic and is presumed to bind the RNA, while the C-terminal portion is presumed to interact with the cytoplasmic tail of E2 and with capsid proteins. The E3 protein is 59 (VEE), 60 (WEE) or 63 (EEE) amino acids long, probably serves as a signal sequence for E2 and is shed into the supernatant fluid after cleavage. The E2 glycoprotein is a transmembrane protein that is 420 (EEE) or 423 (WEE, VEE) amino acids long and has two (EEE) or three (WEE, VEE) N-linked glycosylation sites. The intracytoplasmic portion has a second stretch of hydrophobic amino acids. The 6K protein is 55 (WEE, VEE) or 56 (EEE) amino acids long and probably serves as a signal peptide for E1. By analogy to Sindbis virus, small amounts are

probably incorporated into the virion. The E1 protein is 439 (WEE), 441 (EEE) or 442 (VEE) amino acids long and has one or two *N*-linked glycosylation sites. E1 has a short (one or two residue) intracytoplasmic tail and a positionally conserved hydrophobic stretch of amino acids in the N-terminal portion that is postulated to serve as the fusion peptide for virion entry into the cell. By analogy with other alphaviruses it is presumed that the 5′ portion of the genome encodes four nonstructural proteins that function in replication of the viral RNA and production of the subgenomic RNA.

Physical Properties

The virion is sensitive to ether and detergent. Infectivity can be inactivated by heat or acid. The viruses are stable at −70°C for long periods.

Replication

For the most part, knowledge of the replication of EEE, WEE and VEE is based on the extensive studies of Sindbis and Semliki Forest viruses. Initial attachment to the cell may involve interaction of the E1 glycoprotein, which has hemagglutination activity, with phospholipids on the cell surface. Efficient subsequent entry involves binding of E2 to cellular proteins followed by receptor-mediated endocytosis. Within the acidified endosomal compartment the glycoprotein spikes undergo a conformational change that results in fusion with the endosomal membrane and release of the nucleocapsid into the cytoplasm. Acidified endosomes may not be essential for infection of mosquito cells. Once released from the capsid matrix, the virion RNA serves as mRNA and translation of the nonstructural proteins is initiated. Cytopathic vacuoles form and negative-strand 49S, positive-strand 49S and positive-strand 26S subgenomic RNAs are transcribed. The 26S RNA represents the 3′ portion of the genome from which the structural proteins are translated and synthesized as a polyprotein. After translation of the capsid protein is complete, it is autocatalytically cleaved from the nascent chain. E2 and E1 are transported into the endoplasmic reticulum, cleaved by cellular proteases and processed through the Golgi. At the plasma membrane nucleocapsids align with regions containing the E1–E2 heterodimers and bud from the cell surface. The process may be different in mosquito cells, where intracytoplasmic compartments containing mature virus particles are seen *in vitro*; however, these structures have not been seen during ultrastructural studies of alphavirus infection in mosquitoes.

Geographic and Seasonal Distribution

The encephalitic alphaviruses are geographically restricted. All of the viruses are transmitted primarily by mosquitoes and, therefore, transmission is restricted to warm months of the year. EEE is endemic from New Hampshire along the Atlantic seaboard and Gulf Coast to Texas in North America, in the Caribbean, Central America, and along the north and east coasts of South America. Inland foci exist in the Great Lakes region and South Dakota. In the northern part of the range cases occur between July and October, while in the southern region cases can occur throughout the year. Viruses of the WEE complex are widely distributed throughout the Americas as three closely related, but serologically distinct, viruses (WEE, Highlands J and Ft Morgan). Sindbis, Y62-33 and Aura viruses are Old World viruses. Highlands J virus is endemic on the East Coast of the United States in the same areas as EEE virus. WEE virus is widely distributed in the western plains and valleys of the USA and Canada. A subtype of WEE, AG87-646, is found in South America. Enzootic VEE viruses are perennially active in subtropical and tropical areas of the Americas (subtype II in Florida; ID and IE in Central America; IF and III–VI in South America). Epizootics have appeared in Venezuela, Colombia, Peru and Ecuador at approximately 10 year intervals.

Host Range and Virus Propagation

The endemic life cycle of the encephalitic alphaviruses involves replication in vectors, primarily mosquitoes, and in reservoir hosts, primarily birds (EEE and WEE) and small mammals (VEE). In epidemic periods additional hosts such as horses and humans are infected. EEE virus causes encephalitis in humans, horses, pigeons and pheasants. Many birds are susceptible to infection, but remain asymptomatic despite prolonged viremia. The amplifying species for EEE virus in North America are wading birds and migratory song birds. In South America, forest-dwelling rodents and marsupials are frequently infected and may provide an additional reservoir. Infection has also been reported in turtles and snakes. In invertebrates, virus is most consistently recovered from *Culiseta melanura* mosquitoes but the first arthropod isolations were from chicken mites and lice.

WEE virus causes encephalitis in horses and humans. The enzootic cycle in North America involves domestic and wild birds and *Culex tarsalis* for WEE virus in the western USA and songbirds and *Culiseta melanura* for Highlands J virus in the eastern USA. Serosurveys and virus isolations demonstrate

evidence of natural infection in chickens and other domestic birds, passerine birds, pheasants, rodents, rabbits, ungulates and snakes.

Epizootic strains of VEE virus cause disease in horses and in humans. The maintenance cycle in nature is unclear. The enzootic strains of VEE can infect horses but these infections are asymptomatic and may protectively immunize horses against epizootic strains. Enzootic strains produce mild disease in humans. Many wild birds are susceptible to infection. Rodents serve as an important reservoir with efficient transmission of infection by *Culex (Melanconion)* mosquitoes.

In addition to the native hosts, a number of laboratory animals are susceptible to infection. All three viruses cause encephalitis in monkeys, mice, rats, guinea pigs and hamsters. Disease is generally age-dependent so that young animals more often develop fatal infections than adult animals. Primary isolates of these viruses are often made in newborn mice.

In vitro, the viruses are routinely propagated in cultures of chick embryo fibroblast, BHK-21 or Vero cells. Most strains will form plaques on these cell lines and plaque assays provide the usual basis for virus quantitation. Mosquito cell lines support replication, often without cytopathic effect. Many other cell lines (e.g. L cells, HeLa cells) support replication as well.

Genetics

Alphaviruses show genetic changes by accumulation of point mutations in the genomic RNA, but this occurs at a rate that is much slower than that predicted for other RNA viruses. Recombination is infrequent, but can be demonstrated *in vitro* and occurs at least occasionally in nature, as sequence analysis has shown that WEE virus is the result of recombination between EEE and Sindbis-like viruses.

Evolution

The alphaviruses, which replicate in mosquitoes, birds and mammals, derive from a single unknown protoalphavirus and are part of the alphavirus superfamily of viruses. Viruses in this superfamily have a similar genetic organization and include many RNA plant viruses. In general, amino acids important in secondary structure (e.g. cysteine) have been conserved for the structural glycoproteins E1 and E2, suggesting that the three-dimensional structure, is similar, for all alphaviruses. Oligonucleotide fingerprinting and sequence analysis suggest that the encephalitic alphaviruses evolve slowly in nature. Comparative sequence information indicates that the capsid protein and E1 glycoprotein are most conserved of the structural proteins, while the E2 glycoprotein is more divergent. Sequence comparisons show that EEE virus has evolved independently in North and South America over the last 1000 years. Currently there is one group in North America and the Caribbean and two groups in South America, one in the Amazon basin and the other on the coast of South and Central America. North American EEE virus isolates spanning 52 years show considerable overall conservation, with an estimated 0.7% divergence per year. Rates of divergence of WEE and Highlands J viruses of 0.1–0.2% per year have been estimated. It is hypothesized that the short transmission season and limited host mobility constrain genetic diversity in a geographic region.

Epidemiology

EEE virus is endemic in the Great Lakes region of North America and along the Altantic and Gulf coasts of North, Central and South America and causes localized outbreaks of equine, pheasant and human encephalitis in the summer. Cases of equine encephalitis are usually the first indicators of an outbreak. In North America the primary enzootic cycle is maintained in shaded freshwater swamps where the vector is the ornithophilic swamp mosquito *Culiseta melanura* and the reservoir hosts are migratory passerine songbirds. Young birds are probably most important for virus amplification, as they are more susceptible to infection, have a prolonged viremia and are less defensive towards mosquitoes. Human and equine cases usually occur within 5 miles of the swamp, with virus being transmitted by epizootic vectors such as *Coquillittidia perturbani, Aedes sollicitans*, and potentially *Aedes albopictus*. The enzootic vector in the Caribbean is probably *Culex taeniopus* and in South America *Culex (Melanoconion)*. Epizootics appear approximately every 5–10 years and are usually associated with heavy rainfall that increases the populations of enzootic and epizootic mosquito vectors. The mechanism of overwintering in northern areas is not known. The virus may be reintroduced annually by migratory viremic birds or wind-borne infected mosquitoes coming from subtropical areas of year-round transmission. There is no evidence for overwintering in mosquitoes. Human infections are unusual, with a median of five cases per year in the USA. Serological surveys in the northeastern USA suggest that there are approximately 23 inapparent infections for every case of encephalitis, but children are more susceptible and this declines to only 8:1 for children under 4 years of age.

In the western USA, WEE virus is maintained in an endemic cycle involving domestic and passerine birds and *Culex tarsalis*, a mosquito particularly adapted to irrigated agricultural areas. Interseasonal persistence occurs in salt water marshes where vertical transmission of WEEV in *Cx. tarsalis* has been demonstrated. Occasional isolations have been made from *Culex stigmatosoma, Aedes melanimon and Ae. dorsalis*, also competent vectors. Transmission from this endemic cycle has resulted in small numbers of cases of encephalitis in humans. However, periodically there are large, often widespread, epidemics of equine encephalitis occurring from mid-June to late-September in North America with significant spillover into the human population. The estimated case to infection ratio is 1:58 in children under five years and 1:1150 in adults. In the eastern USA, Highlands J virus has been isolated from freshwater swamp habitats along the Atlantic coast from birds and the ornithophilic *Culiseta melanura*, the endemic vector for EEE. Ecologic restriction and limited pathogenicity may explain the paucity of human and equine disease due to this virus. The lack of significant human disease during equine outbreaks of WEE in South America may be related to the feeding habits of the vector or to a difference in virulence for humans of the South American strains.

VEE is maintained in enzootic and epizootic cycles by distinct virus strains. Enzootic strains are maintained by *Culex* (*Melanoconion*) mosquitoes that live in tropical and subtropical swamps throughout the Americas and breed near aquatic plants. They feed at dawn and dusk on a wide variety of rodents, birds and other vertebrates. Humans living in these areas have a high prevalence of antibody, but little recognized disease. The epidemiology of the epizootic strains of VEE is less clear because they have been isolated only during outbreaks. Epizootics have occurred primarily in Latin America in cattle ranching areas during the rainy season. Formalinized vaccines containing residual live virus were responsible for initiating the 1969–1972 outbreak in Central America and Texas. During epizootics horses are an important amplifying species. Virus has been isolated from several species of mosquitoes, including *Aedes taeniorhynchus, Ae. aegypti, Mansonia dubitans* and *Psorophora confinnis*. The incidence of encephalitis in clinically ill humans is generally less than 5% and the mortality less than 1%.

Transmission and Tissue Tropism

The primary mode of alphavirus transmission to birds and mammals is through the bite of an infected mosquito which inoculates virus extravascularly. Mosquitoes become infected by feeding on a viremic host, are able to transmit the virus 4–10 days later (external incubation) and remain persistently infected. Maintenance of this cycle requires an amplifying host that develops a viremia of sufficient magnitude to infect feeding mosquitoes. Other modes of transmission are occasionally important. EEE virus persists in the feather follicles of infected pheasants and secondary transmission among penned pheasants can occur through feather picking and cannibalism. VEE can be transmitted by the respiratory route between infected horses, to humans and in the laboratory. WEE and VEE viruses can be transmitted transplacentally.

In mammals, EEE virus replicates primarily in muscle and neurons and glia of the CNS, with occasional involvement of liver and lymphatic tissue. Skeletal and myocardial muscle and the CNS are infected in susceptible birds. In mosquitoes there is infection of the midgut, muscle and salivary glands without involvement of the nervous system. WEE virus replicates primarily in skeletal and cardiac muscle, brown fat and the choroid plexus, ependyma and neurons in the CNS in mammals. Little is known of its tissue tropism in birds and mosquitoes. Epizootic strains of VEE virus infect the upper respiratory tract, lymphatic and myeloid tissue, pancreas, liver and CNS to varying degrees in different mammals. Mosquito infection is initiated by penetration of the mesenteron, with dissemination to the nervous system, salivary glands and flight muscles.

Pathogenicity

EEE strains vary in their pathogenicity for horses (and presumably humans), with the South American strains being less virulent than the North American strains. The molecular basis of these differences has not yet been defined. The North American strains of EEE are amongst the most virulent of the alphaviruses, causing severe encephalitis in humans, horses, dogs, pigs, pigeons, emus, quail and pheasants. The case fatality rate in humans in 30–50%, up to 90% in horses and 50–70% in pheasants. Laboratory studies indicate a similar neurovirulence of EEE for monkeys, mice and hamsters. Hamsters also develop hepatitis and lymphatic organ infection. At 3–4 weeks of age mice become relatively resistant to peripheral, but not intracerebral, inoculation. Birds vary in their susceptibility, with some birds developing disease, while many others show no morbidity or mortality despite a prolonged viremia. Pheasant deaths are caused by encephalitis, whereas young chickens develop myocarditis. Mosquitoes develop persistent infection of

the fat body and salivary glands without obvious shortening of lifespan.

WEEV in the western USA causes epidemics of encephalitis in humans, horses and emus, but the case fatality rate of 10% for humans, 20–40% for horses and 10% for emus is lower than for EEE. Highlands J virus can cause fatal disease in horses and turkeys, but this is unusual. South American strains of WEE cause fatal encephalitis in horses, but little disease in humans. Epizootic strains are neurovirulent and neuroinvasive in adult mice, whereas enzootic strains are not. Highlands J is intermediate in virulence. With increasing age mice become relatively resistant to fatal infections, whereas hamsters remain susceptible.

Infection of horses with epizootic strains of VEE virus is frequently fatal. Disease is associated with leukopenia and a high-titered, prolonged viremia. In contrast to EEE and WEE, the disease is not consistently indicative of encephalitis and virus is shed in nasal, eye and mouth secretions as well as in urine and milk. Experimental infection of laboratory animals produces a variety of disease patterns. For guinea pigs and rabbits, VEE virus produces necrosis in lymph nodes, spleen, thymus, intestinal and conjunctival lymphoid tissue, liver and bone marrow. Hamsters develop encephalitis and pancreatitis in addition to widespread involvement of myeloid and lymphold tissue. Rats and mice have more limited destruction of lymphoid and myeloid tissue and death is usually due to encephalitis. Virus enters the CNS through the olfactory tract and causes neuronal infection and apoptosis. Neonatal mice show extensive replication in many tissues, including brain, myocardium and pancreas. Comparative studies of the virulent TRD and avirulent TC-83 strains of IA serotype VEE virus and construction of recombinant viruses have led to identification of the 5′ noncoding region and the E2 envelope glycoprotein as important determinants of virulence for mice and presumably for horses.

Clinical Features of Infection

EEE virus is the most virulent of the encephalitic alphaviruses, causing a high mortality due to encephalitis. Prodromal symptoms of fever, headache and myalgias are common. The onset of encephalitis tends to be fulminant and is associated with continued fever, increased headache, meningismus, obtundation and seizures. Mortality overall is 30% in recent studies, with higher rates in children and the elderly. Recovery is more likely in those individuals with a long (5–7 day) prodrome and without coma. Sequelae are common, with more that 80% of survivors having significant neurological residua including paralysis, seizures and mental retardation. The diagnosis is usually made by detection of antibody in serum or cerebrospinal fluid.

WEE virus causes encephalitis with signs and symptoms similar to those of EEE. There is a 3–5 day prodrome of fever and headache that may progress to irritability, nuchal rigidity, photophobia and altered mental status. Severe disease, seizures, fatal encephalitis and significant sequelae are more likely to occur in infants and in young children.

Infection with epizootic strains of VEE virus usually causes relatively mild disease in humans. Illness in adults usually manifests with fever, headache, myalgias and pharyngitis 2–5 days after exposure. Severe disease, including fulminant reticuloendothelial infection and encephalitis, may occur in young children. Children recovering from encephalitis may be left with neurological deficits. Fetal abnormalities and spontaneous abortions may occur with infection during pregnancy.

The cerebrospinal fluid during alphavirus encephalitis usually shows a moderate (up to 4000 cells/mm^3) pleocytosis with predominance of either polymorphonuclear or mononuclear cells. Protein is usually elevated and the glucose is normal. The electroencephalogram and magnetic resonance imaging scans are usually abnormal, but computed tomographic scans may be normal or indicative only of edema.

Pathology and Histopathology

Initial CNS infection with EEE virus in experimental animals is of the capillary endothelial or choroid plexus epithelial cells and spread within the CNS can be cell-to-cell or through cerebrospinal fluid. The targeted cell within the CNS is the neuron and damage to this cell may be severe and irreversible. Histopathology demonstrates a diffuse meningoencephalitis with widespread neuronal destruction, neuronophagia, gliosis and perivascular inflammation, with polymorphonuclear and mononuclear leukocytes early, and vasculitis and vessel occlusion late. Hamsters also exhibit necrosis of hepatocytes and lymphatic tissue accompanied by local infiltration of mononuclear leukocytes. Initial infection of mosquitoes is of midgut epithelial cells. Infection is facilitated when virus in the serum is concentrated next to these cells as the infected bloodmeal clots. Infected midgut epithelial cells subsequently degenerate and slough and this process may facilitate penetration of the virus into the hemocele and rapid dissemination of the infection.

Pathological examination of brains from fatal human cases of WEE demonstrates early perivascular extravasation of blood followed by endothelial hyperplasia, perivascular mononuclear and polymor-

phonuclear inflammation and parenchymal necrosis. Areas of neuronal degeneration, glial nodules and demyelination are found. Neonatal mice develop acute inflammation and necrosis in skeletal and smooth muscle, cartilage and bone marrow. In animals with encephalitis, the brain shows multifocal areas of necrosis and widespread lymphocytic infiltration of the leptomeninges and perivascular regions of the brain parenchyma. The heart shows a necrotizing, inflammatory myocarditis. Infiltration of mononuclear leukocytes into areas of lung, liver and brown fat also occurs.

The pathology of VEE in horses includes cellular depletion of bone marrow, spleen and lymph nodes, pancreatic necrosis and, in cases with encephalitis, swelling of vascular endothelial cells, edema and mononuclear infiltration in the brain. Encephalitis in rodents is characterized by neuronal cell death, severe mononuclear cell cuffing of cerebral vessels and meningitis, followed by the later appearance of demyelinating lesions. Small mammals with widespread involvement of the reticuloendothelial system may develop ileal necrosis.

Immune Response

Both cellular and humoral immune responses are induced by infection. Virus-specific IgM antibody is often detectable very early in the disease by enzyme immunoassay or neutralization in both serum and cerebrospinal fluid and provides a means for rapid diagnosis of infection. IgG antibody appears after 10–14 days and can be measured by enzyme immunoassay, HI or neutralization assays. Many lines of evidence suggest that recovery from infection is primarily dependent on the antibody response. The most extensive experimental studies to define the antibody specificity and the mechanisms of recovery and protection have been done using VEE virus and Sindbis virus, a member of the WEE complex. Neutralizing and nonneutralizing antibodies are protective. Multiple epitopes on the E1 and E2 glycoproteins induce neutralizing, HI and protective antibody. Sequencing of monoclonal antibody escape mutants has localized one neutralization domain to E2 residues 180–210 in VEE. Immunization with peptides has shown that an antibody response to residues 1–25 of the VEE E2 glycoprotein is also protective.

Cellular immunity has received more limited study, but virus-specific lymphoproliferative and cytotoxic responses have been documented. The importance of either for recovery or contribution to fatal disease has not been established. Prior infection with VEEV increases subsequent antibody responses to unrelated antigens. VEEV-specific T cells can inhibit virus replication by an unidentified mechanism. Antithymocyte globulin extends time to death in mice infected with VEE virus, suggesting a T cell-mediated immunopathogenic component to fatal disease.

Prevention and Control

Prevention of infection relies on efforts to control mosquito populations by spraying and reduction of breeding places. Individual use of protective measures such as mosquito repellents and protective clothing are important. Vaccines against EEE, WEE and VEE are available for horses and against EEE for birds. Experimental human vaccines against EEE, WEE and VEE are also available for laboratory workers exposed to these agents. Most of these vaccines consist of formalin-inactivated virus, but TC-83 is a successful live attenuated vaccine against VEE for horses. TC-83 is also an effective vaccine for humans, although minor side effects are common after immunization. No antiviral agents are of proven usefulness in treatment of these infections.

Future Perspectives

The ability to construct full-length cDNA alphavirus clones that can be transcribed into infectious RNA provides tremendous potential for understanding the functions of various genes and their importance for replication and virulence in the multiple hosts necessary for maintenance of these viruses in their natural cycles. An understanding of the folding and three-dimensional structures of each of the structural proteins is needed for interpretation of much of the sequence and virulence data that has been acquired. Further sequence information on virulent and avirulent strains and functional analysis of the nonstructural proteins is likely to provide the next level of understanding of virus–host relationships.

See also: **Japanese encephalitis virus (*Flaviviridae*); Nervous system viruses; Sindbis and Semliki Forest viruses (*Togaviridae*); Ross River virus and Barmah Forest virus (*Togaviridae*).**

Further Reading

Calisher CH (1994) Medically important arboviruses of the United States and Canada. *Clin. Microbiol. Rev.* 7: 89.

Johnston RE and Peters CJ (1996) Alphaviruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 843. Lippincott-Raven: Philadelphia.

Weaver SC (1997) Vector biology in viral pathogenesis. In: Nathanson N, Ahmed R, Gonzalez-Scaranes F *et al* (eds) *Viral Pathogenesis*, p. 329. Lippincott-Raven: Philadelphia.

EQUINE HERPESVIRUSES (*HERPESVIRIDAE*)

Dennis J O'Callaghan, Department of Microbiology and Immunology, Louisiana State University School of Medicine, Shreveport, Louisiana, USA

Nikolaus Osterrieder, Federal Research Centre for Virus Diseases of Animals, Friedrich-Loeffler-Institutes, Insel Riems, Germany

History

The first disease attributed to an equine herpesvirus (EHV-1; equine abortion virus; equine rhinopneumonitis virus) was documented at the University of Kentucky Agricultural Experiment Station in Lexington, which is home to the highest concentration of thoroughbreds in the world. HV-1 was first shown to be associated with spontaneous abortions in pregnant mares in 1932. Several years later (1941) equine abortions were found to be associated with mild respiratory disease with symptoms similar to those associated with infections due to equine influenza virus. EHV-1 infection was analyzed in more detail in 1954, and evidence from these studies demonstrated that EHV-1 was the etiological agent of epizootic respiratory disease in young horses. EHV-1 was then regarded mainly as a respiratory pathogen since the lesions in horses and aborted fetuses were concentrated in the respiratory tract. The disease was termed viral rhinopneumonitis, and the agent was called equine rhinopneumonitis virus. In the 1980s, however, the two disease manifestations, viral abortion and viral rhinopneumonitis, were shown to be caused by two closely related but clearly distinct viruses. These findings resulted in a reclassification into EHV-1 (equine abortion virus) and EHV-4 (equine rhinopneumonitis virus) in 1986.

Equine herpesvirus type 2 (EHV-2) was first isolated from horses in 1963. The cytopathology of this virus closely resembled that of cytomegalovirus infections which were first described in 1921 (in humans). Sequence analyses, however, were able to classify EHV-2, together with EHV-5, as slowly replicating viruses which are related to the human pathogen Epstein–Barr virus which causes B-cell lymphomas and nasopharyngeal carcinomas. EHV-2 is a ubiquitous slow-growing virus that infects horses at a very young age (1–2 years) and establishes a lifelong chronic infection such that the horse becomes a continuous shedder of the virus. To date, no major disease has been attributed to EHV-2; however, some workers claim an association of EHV-2 with chronic throat infections (the 'lumpy bumpies'). Also, EHV-2 may be a cofactor in EHV-1 and/or EHV-4 infections in that EHV-2 is obviously able to modulate EHV-1 and EHV-4 replication by an immunosuppression causing general malaise. Also, EHV-2 transactivators are able to modulate gene expression of EHV-1.

Equine herpesvirus type 3 (EHV-3; equine coital exanthema virus, ECE virus) was first isolated independently in 1968 in Canada, Australia and the US. EHV-3 is the etiological agent of equine coital exanthema, which is a generally mild genital infection of both mares and stallions that is transmitted venereally.

Equine herpesvirus type 4 (EHV-4), initially designated subtype 2 of EHV-1, is associated mainly with respiratory disease, but EHV-4 has also been associated occasionally with equine abortions. EHV-1 (equine abortion virus) on the other hand, is associated primarily with equine abortions, but frequently causes respiratory disease in young animals, neurological disease, fulminating neonatal pneumonitis and very rarely, an exanthematous condition involving the external genitalia of the mare.

Equine herpesvirus type 5 (EHV-5) is closely related to EHV-2 and no information on possible disease(s) caused by this virus is available.

Taxonomy and Classification

EHV-1, EHV-2, EHV-3, EHV-4 and EHV-5 are all members of the *Herpesviridae* but belong to several subfamilies in this large virus family. The morphology of all four members is typical of the herpesviruses in that they are enveloped, contain an icosadeltahedral capsid, and have a proteinaceous region called the tegument surrounding the nucleocapsid. The equine herpesviruses are composed of five distinct species: (1) EHV-1 is the major equine pathogen causing fetal abortions, respiratory illness and neurological disease, (2) EHV-2 (and EHV-5?) establish asymptomatic, long-term persistent infections; (3) EHV-3 (ECE virus) is the causative agent of mild genital exanthema; and (4) EHV-4 is a major respiratory pathogen that differs significantly from

Table 1 Characteristics of the genomes of EHVs

	Density (g cm^{-3})	*G+C ratios*	*Sedimentation coefficient*	*Genomic isomers*
EHV-1	1.716	57%	49-55S	2
HV-2	1.7165	58%	61.8S	1
EHV-3	1.725	66%	55.4S	2
EHV-4	ND	51%	ND	2
EHV-5	ND	52%	ND	1

ND = not determined.

EHV-1 at the DNA level, and is associated occasionally with equine abortions.

EHV-1, 3 and 4 are members of the *Alphaherpesvirinae* subfamily. EHV-1 and -4 belong to the genus Varicellovirus. EHV-3 is an unassigned species. EHV-2 and EHV-5 have recently been shown to be members of the *Gammaherpesvirinae* subfamily.

Geographic and Seasonal Distribution

EHV-1 and EHV-4 are distributed worldwide, and infections can occur year-round. Over the past 20 years in the United States, EHV-1 'abortion storms' have occurred in many areas. Major outbreaks have also been reported within the past several years in Australia and England. Due to the nature of the disease manifestation of EHV-1, viral abortion, there is a seasonal dependence of EHV-1 infections whereas EHV-4 infections (rhinopneumonitis) are observed mainly on racing tracks and after crowding of large numbers of animals.

EHV-2 has also been isolated in many countries, including England, Switzerland, Germany, USA and South Africa. The existence of the closely related EHV-5 was described for England and Australia. More thorough studies, however, have led to the assumption that both EHV-2 and also EHV-5 are distributed worldwide.

To date, EHV-3 has been isolated in five countries: Germany, USA, Australia, Canada and England.

Host Range and Virus Propagation

Although the horse is the natural host of the equine herpesviruses, a variety of animals and tissue culture systems can be used to propagate the viruses. Regarding the major equine pathogen, EHV-1, experimental animals include Syrian hamsters and baby hamsters, chick embryos, baby mice and adult mice, and kittens. Primary tissue culture systems used to propagate EHV-1 include cells from a variety of equine tissues such as fetal lung, dermis, spleen and kidney, as well as cells from domestic cats, dogs, hamsters, rabbits, mice, sheep and swine. In the laboratory, permanent tissue culture systems commonly used to cultivate EHV-1 include HeLa, Vero, CV-1 cells, rabbit kidney (RK), mouse L-M and equine Edmin337 cells.

The host range for EHV-2, EHV-3, EHV-4 and EHV-5 is more restrictive than that for EHV-1. Except for rabbit kidney cells and primary cat cells, EHV-2 and EHV-5 growth appears to be restricted to cells of equine origin. The host range for EHV-3 and EHV-4 is limited to cells of equine origin, although Vero cell culture adapted EHV-4 strains have been described.

Genetics

All five equine herpesviruses contain a linear, double-stranded DNA genome ranging between 140 and 184 kbp. The reported molecular masses of the genome of the equine herpesviruses are: EHV-1, 150 kbp, EHV-2, 184 kbp, EHV-3, 144 kbp, EHV-4, 146 kbp and EHV-5, 179 kbp. The genomes of EHV-1, EHV-3 and EHV-4 exist in two isomeric forms, since the short region can invert relative to the fixed orientation of the long region. The short region is composed of a central segment of unique sequences (US) bracketed by a pair of inverted sequences. In the case of the Kentucky A, tissue culture strain of EHV-1, each inverted repeat sequence is 12.8 kbp. In contrast, the genomes of EHV-2 and EHV-5 exist as one isomer and are composed of a large (149 kbp) central segment of unique sequences that is bracketed by a pair of direct repeat sequences. Each of the terminal direct repeat segments is 18 kbp, and the total genome size is 179–184 kbp. Other characteristics of the genomes of EHVs are shown in **Table 1**.

There is some homology at the DNA level among the five equine herpesviruses. The sequences shared by EHV-1, EHV-3 and EHV-4 appear to be arranged colinearly and are dispersed throughout the genome. EHV-1 and EHV-4 exhibit 55 to 84% homology at the DNA level and are antigenically related since antibodies can crossneutralize. EHV-1 and EHV-2 show only 2% homology, as does EHV-2 and EHV-3. EHV-

1 and EHV-3 exhibit approximately 10% homology at the DNA level. Lastly, EHV-2 and EHV-5 show approximately 60% identity both on the DNA and protein level.

Genome Structure

The entire genome of EHV-1, strain Ab4, was sequenced and shown to be 150 223 bp in size and to contain 80 potential open reading frames (ORFs). The 63 ORFs of the unique long region are arranged colinearly with those in the genomes of herpes simplex virus and varicella zoster virus. Several genes mapping within the inverted repeat and unique short segments of the short region differ in arrangement from those of other alphaherpesviruses. In addition, EHV-1 contains a limited number of unique genes, i.e. genes that are present neither in HSV-1 nor in VZV and which might represent the virus' gene repertoire determining host specificity.

Each identical, inverted repeat (IR) sequence of the genomic short region of the Kentucky A, tissue culture strain of EHV-1 is composed of 12 777 nucleotides. Six genes and the origin (ORI) of replication have been localized to the IR sequences of EHV-1:

1 the *IR1* gene is an immediate–early (IE) gene encoding a 6.0 kb mRNA and a major phosphoprotein (1487 amino acids) with an apparent molecular mass of 203 kDa;
2 the *IR2* gene is an early gene that is embedded within the IR1 gene, and its 4.4 kb mRNA appears to encode a 130 kDa polypeptide (1165 amino acids) – the protein product of the *IR2* gene represents a truncated form of the IE polypeptide;
3 the *IR3* gene is a delayed–early gene encoding a 0.9 kb mRNA that overlaps the IE promoter region on the opposite DNA strand;
4 the EHV-1 ORI maps downstream of *IR3* and exhibits 60% homology to the ORI(S) of HSV-1 and HSV-2;
5 the *IR4* gene is a homolog of the *ICP22* gene of HSV-1 and is differentially regulated to encode a 1.4 kb early mRNA and a 1.7 kb late mRNA;
6 the *IR5* gene is a homolog of the *US10* gene of HSV-1;
7 *IR6* is a 'very early' gene encoding a 1.2 kb mRNA and a 31/33 kDa phosphoprotein which has been shown to be a capsid constituent and a major determinant of virulence in some EHV-1 strains.

Additional genes of the Kentucky A, tissue culture strain of EHV-1 have also been identified and mapped to the unique short (US) sequences located between the inverted repeats. These include HSV-1 homologs of the *US2* gene, the protein kinase gene, glycoprotein (g) G, gD and the *US9* gene. In addition, a unique gene (*EUS4*, gene 71) was mapped in the US region. This gene encodes a highly O-glycosylated protein referred to as gp1/2. Some size variations in *EUS4* were documented for EHV-1 strains KyA and Ab4, and a gp1/2 null mutant was apathogenic in a murine model of EHV-1 infection. In contrast to strain KyA, homologues of HSV-1 glycoproteins I and E were also mapped to the US segment of the genomes of other EHV-1 strains. Thus, gI and gE are not essential for EHV-1 replication in cell culture, but appear to play a role in virulence. Restoration of the gI and gE sequences to a gI/gE deletion mutant virus with an attenuated phenotype restores pathogenicity for horses.

Functional analyses of the genes encoded in the unique long (UL) genome region have also been performed. Among the investigated genes is the viral thymidine kinase (TK), the genes and gene products of glycoprotein (g) B, C, and M which show high homology to the genes of HSV-1 and VZV. Functional homology between HSV-1 and EHV-1 genes could be demonstrated for EHV-1 gM and gB.

Several genes mapping within the long and short regions of the EHV-4 genome have been identified including: the genes encoding glycoproteins B, C, E and H; thymidine kinase gene; and genes that are homologues of *US1*, *US9* and *US10* of HSV-1. The genomes of EHV-1, EHV-2 and EHV-4 are now entirely characterized at the nucleotide sequence level, but intensive research on gene functions has not been performed. Nothing is known about EHV-3 gene functions.

Replication

The genes of the Kentucky A, a tissue culture strain of EHV-1 are regulated at the transcriptional and translational levels in a temporal fashion, and three kinetic classes of genes designated immediate–early (IE), early (E) and late (L) have been described. EHV-1 possesses a single IE gene which is present in both inverted repeats and gives rise to a spliced 6.0 kbp mRNA. Multiple IE polypeptide species have been observed, and the major IE protein (IE1, 203 kDa) is a nuclear-localized phosphoprotein that is capable of *trans*-activating other viral genes and autoregulating its own expression. The *trans*activation domain (residues 3–89), the DNA-binding domain (residues 422–597), and the nuclear localization domain (PPAPKRRV; residues 963–970) of the IE protein have been mapped. Following IE polypeptide synthesis, approximately 45 early transcripts can be detected. Three of these early proteins serve as regulatory proteins and are designated EICP22, EICP27

and EICP0. The EICP22 protein serves to enhance the DNA-binding of the IE protein to its target sequence (ATCGT) present within the promoters of EHV-1 genes characterized to date. Early gene expression is followed by viral DNA replication and the production of approximately 29 late transcripts. Although these 75 transcripts have been positioned on the viral genome, only a small number of protein products have been identified and characterized (see above).

Viral DNA replication occurs at approximately 4 h postinfection and requires the virus-encoded DNA polymerase which has been purified. DNA replication is thought to occur by the rolling circle mechanism whereby long concatemers of the viral genome are generated, cleaved and then packaged into the maturing virions. The UL15 homologue of HSV-1, one of the two spliced genes of EHV-1 known to date, appears to be essentially involved in the generation of unit-length genomes and their packaging into mature capsids. Sequences at the L-terminus of the EHV-1 genome are composed of direct repeats (DR1 = 18-mer and DR4 = 16-mer) as well as unique sequences (Uc = 60-mer), whereas sequences at the terminus of the short region contain a 54-mer designated Ub. Thus, the sequence arrangement that is formed at the concatemeric junction following the replication of the EHV-1 genome is Ub-DR1-Uc-DR4, which represents a functional cleavage/packaging signal, similar, but not identical to that of HSV-1.

Three different capsid species were identified in hepatocyte nuclei of Syrian hamsters and tissue culture cells infected with EHV-1 and probably correspond to the capsid forms found in HSV-1 which are designated type A, B and C capsids. The EHV-1 capsid species were designated: (1) L capsids, which appear to be empty capsids; (2) I capsids which possess an electron-lucent, immature core structure in the shape of a cross; and (3) H capsids, which contain an electron-dense, mature core. All three capsid species appear at approximately 6 h postinfection and remain at a ratio of 10:87:3 (L:I:H) throughout the infection. The I capsids are believed to be a major precursor in the formation of mature capsids. The main capsid protein has an apparent size of 148 kDa, whereas other major structural proteins of 59, 46, 37, 30 and 18 kDa have also been identified. As reported for other herpesviruses, EHV-1 maturation occurs by interaction of mature nucleocapsids with the inner portion of the nuclear membrane resulting in the formation of enveloped particles. Two theories for the pathway of herpesvirus release from infected cells after budding at the inner nuclear membrane are discussed. One theory claims that capsids are de-enveloped at the ER membrane and re-enveloped at Golgi-derived vesicles by which mature virions are finally transported to the cytoplasmic membrane. The other theory claims an '*in situ*' processing of enveloped particles, i.e. after budding of virions at the inner nuclear membrane, infectious particles are released via the secretory pathway including the ER and Golgi network.

Infectious EHV-1 particles contain an envelope that is mainly composed of glycoproteins. To date, 12 EHV-1 glycoproteins have been identified and an association of gB, gC, gD, gH, gL, gM, gp1/2 (EUS4), and the tegument protein VP13/14 with purified virions has been demonstrated. The glycoproteins were shown in other alphaherpesviral systems to be involved in virus binding, virus penetration, and cell-to-cell spread of infection. In the case of EHV-1, these functions were confirmed for gB, gM, and gp1/2, and the latter two proteins were shown to be non-essential for virus growth. In contrast, gB-negative EHV-1 mutants are unable to grow *in vitro*. The defect in replication of gB-negative viruses is primarily caused by an inability to spread from infected to uninfected cells. Detailed functional analyses for the other glycoproteins have not been performed, but are now facilitated by the use of engineered virus mutants that are produced by targeted gene deletion. Even less is known for proteins that make up the third component of the mature virion, the tegument. To date, none of the proteins that – by analysis of homology – are related to HSV-1 tegument proteins has been analyzed in detail. Recent studies have shown that the product of ORF12 is a homologue of HSV-1 αTIF. This 60 kDa protein (ETIF) is a late gene product, antigenically crossreacts with HSV-1 αTIF, and participates in an infected cell complex with the EHV-1 IE promoter TAATGARAT motif.

DI Particles and Persistent Infection

Altered outcomes of equine herpesvirus infections have been reported and include oncogenic transformation (EHV-1, EHV-2, EHV-3, and EHV-5) and persistent infection (EHV-1, EHV-2, and EHV-5). EHV-1 persistent infection and oncogenic transformation *in vitro* were shown to be mediated by virus preparations enriched for EHV-1 DI particles (DIPs). EHV-1 DI particles have been generated *in vivo* in the Syrian hamster model, and therefore DIPS may be generated in EHV-1 infection of the natural host. The DIPs are replication defective and require the standard virus as a helper. The majority of EHV-1 DNA sequences have been deleted from the genome of EHV-1 DIPs, such that sequences from three regions of the EHV-1 genome are conserved in the DIP genome. These three segments are: (1) the L-terminus, including genes *UL1*, *UL2* and the 3′ portion of *UL3*;

(2) the junction of the UL and internal IR sequence; and (3) the central portion of the IR, including the 5′ portion of gene *IR4*. The DIP genome also contains a perfectly conserved origin of replication and cleavage/packaging signal. The sequences at the L-terminus and IR are joined by a homologous recombination event mediated by a conserved 8-mer sequence present at both the L-terminus and within the IR4 gene. The joining of these sequences results in the generation of a unique ORF present only in the genome of DIPs. This ORF is expressed as a 31 kDa 'hybrid protein' composed of the amino-terminal 196 amino acids of the EICP22 protein (a homologue of ICP22 of HSV-1) linked in-frame to the carboxy-terminal 68 amino acids of the EICP27 protein (homologue of HSV-1 ICP27). Unique to EHV-1 persistently infected cells is a 2.2 kb transcript (not detected in EHV-1 cytolytic infection) that maps to the UL/IR junction and is antisense to the IE mRNA. Interestingly, this transcript exhibits significant homology to the 'latency associated transcripts' (LATs) of HSV-1, which appear to be associated with HSV-1 reactivation rather than establishment of latency.

Lastly, in EHV-1 persistently infected cells, transcription of certain viral genes appears to be delayed as compared with viral transcription in cytolytically infected cells. Recent findings reveal that expression of the 31 kDa EICP22/EICP27 hybrid protein down-regulates expression of specific EHV-1 promoters. Moreover, altered forms of the EHV-1 IE polypeptides have been observed only in the persistently infected cells. Taken together, these studies indicate that altered or aberrant viral regulatory mechanisms may be involved in establishing or maintaining persistent infection.

Evolution

The five members of the equine herpesviruses are related, but are biologically distinct. Initial DNA sequence analyses have revealed that genes identified to date are colinearly arranged in the genomes of EHV-1, EHV-3 and EHV-4, all of which possess a two-isomer genomic structure. The evolutionary relationships have become more apparent now that EHV-2 is sequenced in its entirety and data on EHV-5 sequences and genomic organization are available. It is clear that EHV-1 and EHV-4 are closely related and may have arisen from the same ancestor. The same is true for EHV-2 and EHV-5. However, it is not possible to determine exactly the EHV ancestor since additional sequence data (especially on EHV-3) are not available.

Serological Relationship and Variability

The four EHV serotypes do share certain antigens, but are considered antigenically distinct. EHV-1 and EHV-4 are closely related antigenically, such that crossneutralizing antibodies are generated following multiple exposures to either EHV-1 or EHV-4. Also, EHV-2 and EHV-5 are closely related. Almost no data are available on the relationship of EHV-3 to other members of the equine herpesviruses, however, all of the EHVs are believed to share complement-fixing antigens.

Epidemiology

Rhinopneumonitis caused by EHV-1 and EHV-4 is spread by direct or indirect contact (ingestion and inhalation). The virus is most commonly shed in nasal droplets for at least 14 days and is present in large amounts in aborted fetuses and the secundinae; however, the virus does not appear to be spread through the genital tract.

EHV-1 infection can also result in spontaneous abortions in pregnant mares. Horses are most susceptible to EHV-1 infection between the eighth and eleventh months of pregnancy. The peak incidence is in the ninth and tenth months at which time approximately 70% of abortions occur.

EHV-2 and EHV-5 have been isolated from the respiratory tract, kidneys, spleen, testicles, genital tract and rectum. Once infected, the horse is a lifelong carrier and excreter of the virus. The exact modes of spread of EHV-2 and EHV-5 are unknown.

EHV-3 causes a mild coital exanthema that is spread by genital contact and, rarely, the respiratory route. An EHV-3 infection is usually cleared after 14 days and is not associated with equine abortions.

Transmission and Tissue Tropism

EHV-1 and EHV-4 are spread mainly by nasal discharge. EHV-2 and EHV-5 establish a chronic infection and may be spread by the respiratory route. EHV-3 is spread by genital contact.

EHV-1 has a wide host range as described earlier, whereas EHV-4 is more limited. EHV-2, EHV-3 and EHV-5 are restricted mainly to cells of equine origin.

Pathogenicity

EHV-1 and EHV-4 cause a mild rhinopneumonitis characterized by a profuse nasal discharge. EHV-1 is also associated with spontaneous abortions as well as neurological disease. Although EHV-1 and EHV-4 respiratory infections are clinically indistinguishable, their pathogenicity is quite different. EHV-1 infection

results in a systemic viremia that can lead to neurological disease. Alternatively, EHV-4 infection is restricted to tissues of the respiratory tract. EHV-2 and also EHV-5 infections are acquired horizontally early in life usually by inhalation. EHV-2 and EHV-5 establish a chronic life-long infection. EHV-3 causes an acute coital exanthema in both the mare and the stallion.

Clinical Features of Infection

EHV-1 and EHV-4 cause outbreaks of upper respiratory disease ('common cold') in young horses (EHV-4 also in adult horses) with no previous exposure to the viruses. Infection is characterized by fever (39–41°C) lasting 2–5 days, and the animal suffers from serous nasal discharge and congestion of the nasal mucosa and conjunctiva. Less frequently, one can detect a transitory period of anorexia, enlargement of the submandibular lymph nodes and edematous swelling of the subcutis and tendon sheaths of the legs. An initial leukopenia is followed by leukocytosis before the temperature falls. Recovery is usually uneventful and occurs within one week. Older horses show few or no clinical signs, although increased sensitivity is seen in stressed horses. Death from naturally acquired infection resulting in neurological disease is not uncommon.

Equine abortions induced by EHV-1 usually occur late in gestation (8–11 months). The foals are usually born dead, but if alive, often succumb to pneumonia within the first few days. Clinical signs of neurological disease caused by EHV-1 are highly variable and include paresis, ataxia and paralysis. EHV-1 and EHV-4 have been isolated from the central nervous system of infected horses; however, isolation of the viruses from the CNS is uncommon. The virus strains do not invade the extravascular nervous tissue and do not appear to be neurotropic. The virus spread from the respiratory tract to the CNS probably occurs via infected leukocytes. Some horses have fully recovered from neurological disease with no permanent neurological sequelae.

The role of EHV-2 and EHV-5 in clinical disease is virtually unknown. However, the viruses have been associated occasionally with chronic throat infections and have been isolated from the respiratory tract of horses with respiratory disease.

An incubation period as long as 10 days can be observed after natural infection with EHV-3. The initial lesions are small (1–2 mm), raised and reddened papules. The lesions then progress rapidly to the pustular form, and there is a general reddening of the vaginal mucosa in the mare. The number of lesions increases in the first few days, and by day 6, many of the lesions form ulcers up to 20 mm in diameter and 5 mm deep. Lesions can also be seen on the vulva and perineal skin, as well as the penis and prepuce of the stallion. The disease is usually mild such that the temperature, pulse, appetite and respiration remain close to normal. The severity of the disease can be increased by secondary bacterial infections; however, uneventful cases are usually cleared within 2 weeks. Lastly, EHV-3 is not abortigenic and does not lead to infertility.

Pathology and Histopathology

Respiratory disease caused by EHV-1 and EHV-4 results in inflammation, congestion and sometimes necrosis of the upper respiratory tract. Extensive swelling of the nasal mucosa may occur, and in later stages, the lungs may become involved. One can find typical herpesvirus inclusion bodies in the nuclei of the respiratory epithelium. The respiratory infection can become more serious if followed by a secondary bacterial infection which may lead to bacterial pneumonia.

Pregnant mares that abort due to EHV-1 infection usually abort only once. The aborted fetuses present with widespread hemorrhages and edema, as well as a yellowish discoloration of the fetal conjunctiva and splenomegaly. The classic histopathology includes the presence of eosinophilic intranuclear inclusion bodies in various organs of the aborted fetuses. However, the gross pathological and histopathological alterations are sometimes not as obvious and only sensitive methods (virus isolation, polymerase chain reaction) are able to confirm the EHV-1 abortion. Classically, features associated with EHV-1 induced abortions differ in those fetuses aborted during the first 6 months of gestation as compared to those aborted after 6 months. Those aborted before 6 months present with widespread cell necrosis and inclusion bodies in the liver and lung. Those aborted after 6 months exhibit jaundice, subcutaneous edema, excessive pleural fluid, pulmonary edema, splenomegaly and necrosis of the liver.

In experimental EHV-1 infections, a severe hepatitis is observed following intraperitoneal infection of the Syrian hamster, and a model of respiratory infection is also available in the hamster. Intranasal infection of mice with EHV-1 results in respiratory disease and subsequent cell-associated viremia, thus serving as a model that closely mimics the disease in equines. The murine model of EHV-1 infection is widely used in virulence and immunogenicity studies.

EHV-2 and also EHV-5 infection becomes widespread throughout the body, and the viruses have been isolated from a variety of tissues. The infected animal becomes a life-long carrier, and the viruses remain highly cell-associated.

Tissues affected following an infection with EHV-3 include the vaginal and vestibular mucosa, penis, prepuce and the skin of the perineal region. One of the characteristics of an infection with EHV-3 is the sloughing of the surface epithelial cells. On occasion the skin of the lips and mucus membranes of the respiratory tract may become involved, but the exanthema is usually mild.

Immune Response

Neutralizing antibodies are detected in the serum soon after an EHV-1 or EHV-4 infection. The neutralizing antibodies can first be detected 7–10 days after infection and are most abundant after several weeks. However, the immunity is short-lived, in that horses can be re-infected and exhibit respiratory symptoms in just 3 months after the initial infection. Multiple exposures to either EHV-1 or EHV-4 will result in the development of neutralizing, cross-reactive antibodies. However, cell-mediated immune responses are thought to be primarily responsible for induction of a sustainable immunity.

Immunity to EHV-2, EHV-3 and EHV-5 is poorly understood; however, virtually all horses have antibodies to EHV-2 and EHV-5, confirming that these viruses are widespread.

Prevention and Control

A number of vaccine approaches are followed to combat EHV-1 infections, among them modified live vaccines (Rhinomune™ or Prevaccinol™), inactivated vaccines which mostly contain both EHV-1 and EHV-4 (Pneumabort K™, Resequin™, Prestige™ and Duvaxyn™) and also subunit vaccines, also covering both viruses (Cavalon IR™). Unfortunately, some EHV vaccines cause undesirable side effects, and most do not afford acceptable levels of protection. All vaccines must be given repeatedly to pregnant mares usually in the third, seventh and ninth months of pregnancy, and to ensure protection, a good level of population immunity is imperative. To protect against viral rhinopneumonitis outbreaks, the vaccine is usually given to all horses every 3–6 months.

Clinical management often involves the use of antibiotics to prevent severe bacterial complications following the viral rhinopneumonitis. Control of EHV-1 and EHV-4 infections involves isolation and quarantine of infected horses (at least 3 weeks) and sound hygiene for prevention of viral infection, since the viruses are highly contagious. Quarantine procedures are required more often with EHV-1 infections, since EHV-1 can lead to more serious disease of the CNS. Since most of the EHV vaccines provide unacceptable levels of protection, the first step in the prevention and control of EHV-1 and EHV-4 infections involves specific management practices and adequate day-to-day care of the animals. The viruses can be spread easily in contaminated feed and water. In addition, minimizing stress and close contact of large groups of horses can prevent the spread of disease.

Lastly, several investigators have examined the effects of some antiviral drugs on the replication of equine herpesviruses. EHV-1 was shown to be resistant to hydroxyurea, but was sensitive to arabinosylthymidine (Ara-T) which specifically inhibited the phosphorylation of deoxycytidine and deoxythymidine by the virus-encoded thymidine kinase, but not by host kinases. Other deoxythymidine analogs were also shown to be somewhat effective against viral replication.

Future Perspectives

Considerable progress in unraveling the nucleotide sequences of EHV-1, EHV-2, EHV-4 and EHV-5 has been made during the last years, and the genomes of EHV-1, EHV-2, and EHV-4 have been entirely sequenced. With this information in hand, it will be possible to pursue studies on gene expression and on those proteins that are involved in virulence of individual EHV-1 strains. These studies will in turn open the possibility for a rational design of anti-EHV vaccines, especially against the most important pathogens, EHV-1 and EHV-4. These goals may be achieved by the use of viral deletion mutants that carry targeted gene deletions. In addition, the suggested interaction of EHV-2 with EHV-1 can be addressed using molecular techniques. Several researchers concentrate on the site of EHV-1 latency which is probably – unlike other alphaherpesviruses – not the neuronal tissues. Using these approaches, a better understanding of EHV biology might be possible in the near future and will open new perspectives in the understanding of diseases caused by EHV.

See also: **Cytomegaloviruses (*Herpesviridae*): General features (human), Molecular biology (human), Animal cytomegaloviruses, Murine cytomegaloviruses; Defective interfering viruses; Latency; Persistent viral infection; Vaccines and immune response.**

Further Reading

Allen GP and Bryans JT (1986) Molecular epizootiology, pathogenesis, and prophylaxis of equine herpesvirus-1 infections. In: Pandey R (ed.) *Progress in Veterinary Microbiology and Immunology*, vol. 2, p. 78. Basel: S. Karger.

Bowles DE, Holden VR, Zhao Y and O'Callaghan DJ (1997) The ICP0 protein of equine herpesvirus 1 is an early protein that independently transactivates expression of all classes of viral promoters. *J. Virol.* 71: 4904.

Harty RN, Colle CF and O'Callaghan DJ (1991) Equine herpesvirus type 1 gene regulation: characterization of transcription from the immediate-early gene region in a productive infection. In: Wagner EK (ed.) *Herpesvirus Transcription and its Regulation*, p. 319. Boca Raton: CRC Press.

O'Callaghan DJ, Gentry GA and Randall CC (1983) The equine herpesviruses. In: Roizman B (ed.) *The Herpesviruses*, vol. 2. *Comprehensive Virology* (series 2), p. 215. New York: Plenum Press.

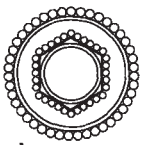
Telford EAR, Watson MS, McBride K and Davison AJ (1992) The DNA sequence of equine herpesvirus-1. *Virology* 189: 304.

Telford EAR, Studdert MJ, Agius CT *et al* (1993) Equine herpesviruses 2 and 5 are γ-herpesviruses. *Virology* 195, 492.

Telford EAR, Watson MS, Aird HC, Perry J and Davison AJ (1995) The DNA sequence of equine herpesvirus 2. *J. Mol. Biol.* 249, 520.

Telford EAR, Watson MS, Perry J *et al* (1998) The DNA sequence of equine herpesvirus-4. *J. Gen. Virol.* 79: 1197.

EQUINE INFECTIOUS ANEMIA VIRUS (*RETROVIRIDAE*)



Ronald C Montelaro, Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, USA

History

Equine infectious anemia (EIA), colloquially known as swamp fever, has been documented in numerous diverse geographical areas and is currently considered a worldwide disease that occurs only in members of the family Equidae. EIA was first identified as an infectious disease of horses by veterinarians in France in 1843. In 1904, the infectious organism that caused EIA was identified as a 'filterable agent', making EIA the first animal disease to be assigned a viral etiology.

Despite this early identification of the equine infectious anemia virus (EIAV), the characterization of this virus was extremely slow because of the difficulties experienced in the isolation and propagation of the virus in cell cultures. Thus, the major focus on the control of EIA has been the development of regulatory policies that involve the identification and elimination of EIAV-infected horses. More recently, advances in animal vaccine strategies and the demand for animal models for acquired immune deficiency syndrome (AIDS) vaccine development have provided renewed impetus to the development of an EIAV vaccine to prevent virus infection. EIAV also offers an important model for the role of antigenic variation in a persistent retrovirus infection.

Classification

EIAV is classified as a member of the lentivirus subfamily of retroviruses based on criteria of virion morphology, serological properties and genomic sequence homologies. There has been no formal further subdivision of EIAV isolates into subtypes.

Properties of the Virion

The EIAV particle has the general morphology of a lentivirus, including an oblong core enclosed in a viral envelope with surface projections (**Fig. 1**). The oblong core observed in EIAV is characteristic of lentiviruses, in contrast to the icosahedral cores found in most oncoviruses. This distinctive structural feature was the initial indication that human immunodeficiency virus 1 (HIV-1) was related to EIAV and was a member of the lentivirus, rather than the oncovirus, subfamily of retroviruses. The virus particles appear roughly spherical in the electron microscope, although there are various degrees of polymorphism, depending on the sample preparation. The overall diameter of the virion is about 100 nm. The surface projections extend about 7 nm and

Further Reading

Allen GP and Bryans JT (1986) Molecular epizootiology, pathogenesis, and prophylaxis of equine herpesvirus-1 infections. In: Pandey R (ed.) *Progress in Veterinary Microbiology and Immunology*, vol. 2, p. 78. Basel: S. Karger.

Bowles DE, Holden VR, Zhao Y and O'Callaghan DJ (1997) The ICP0 protein of equine herpesvirus 1 is an early protein that independently transactivates expression of all classes of viral promoters. *J. Virol.* 71: 4904.

Harty RN, Colle CF and O'Callaghan DJ (1991) Equine herpesvirus type 1 gene regulation: characterization of transcription from the immediate-early gene region in a productive infection. In: Wagner EK (ed.) *Herpesvirus Transcription and its Regulation*, p. 319. Boca Raton: CRC Press.

O'Callaghan DJ, Gentry GA and Randall CC (1983) The equine herpesviruses. In: Roizman B (ed.) *The Herpesviruses*, vol. 2. *Comprehensive Virology* (series 2), p. 215. New York: Plenum Press.

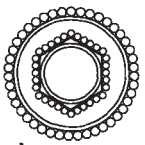
Telford EAR, Watson MS, McBride K and Davison AJ (1992) The DNA sequence of equine herpesvirus-1. *Virology* 189: 304.

Telford EAR, Studdert MJ, Agius CT *et al* (1993) Equine herpesviruses 2 and 5 are γ-herpesviruses. *Virology* 195, 492.

Telford EAR, Watson MS, Aird HC, Perry J and Davison AJ (1995) The DNA sequence of equine herpesvirus 2. *J. Mol. Biol.* 249, 520.

Telford EAR, Watson MS, Perry J *et al* (1998) The DNA sequence of equine herpesvirus-4. *J. Gen. Virol.* 79: 1197.

EQUINE INFECTIOUS ANEMIA VIRUS (*RETROVIRIDAE*)



Ronald C Montelaro, Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, USA

History

Equine infectious anemia (EIA), colloquially known as swamp fever, has been documented in numerous diverse geographical areas and is currently considered a worldwide disease that occurs only in members of the family Equidae. EIA was first identified as an infectious disease of horses by veterinarians in France in 1843. In 1904, the infectious organism that caused EIA was identified as a 'filterable agent', making EIA the first animal disease to be assigned a viral etiology.

Despite this early identification of the equine infectious anemia virus (EIAV), the characterization of this virus was extremely slow because of the difficulties experienced in the isolation and propagation of the virus in cell cultures. Thus, the major focus on the control of EIA has been the development of regulatory policies that involve the identification and elimination of EIAV-infected horses. More recently, advances in animal vaccine strategies and the demand for animal models for acquired immune deficiency syndrome (AIDS) vaccine development have provided renewed impetus to the development of an EIAV vaccine to prevent virus infection. EIAV also offers an important model for the role of antigenic variation in a persistent retrovirus infection.

Classification

EIAV is classified as a member of the lentivirus subfamily of retroviruses based on criteria of virion morphology, serological properties and genomic sequence homologies. There has been no formal further subdivision of EIAV isolates into subtypes.

Properties of the Virion

The EIAV particle has the general morphology of a lentivirus, including an oblong core enclosed in a viral envelope with surface projections (**Fig. 1**). The oblong core observed in EIAV is characteristic of lentiviruses, in contrast to the icosahedral cores found in most oncoviruses. This distinctive structural feature was the initial indication that human immunodeficiency virus 1 (HIV-1) was related to EIAV and was a member of the lentivirus, rather than the oncovirus, subfamily of retroviruses. The virus particles appear roughly spherical in the electron microscope, although there are various degrees of polymorphism, depending on the sample preparation. The overall diameter of the virion is about 100 nm. The surface projections extend about 7 nm and

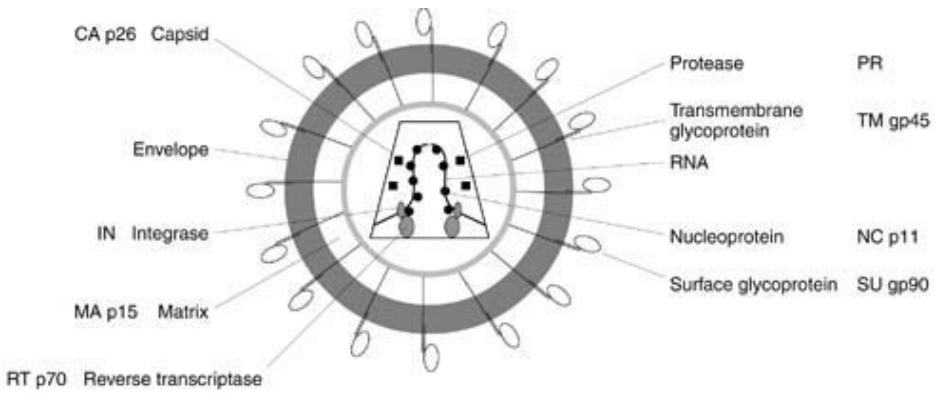


Figure 1 Virion morphology and protein organization of equine infectious anemia virus.

appear to be distributed on the viral surface in a symmetrical pattern.

Properties of the Genome

The EIAV genome consists of a duplex of single-stranded, positive-sense RNA. The genomic organization of EIAV is characteristic of a complex retrovirus, but is the simplest and smallest of characterized human and animal lentiviruses (**Fig. 2**). The viral RNA contains about 8200 bases and contains three major genes (*gag*, *pol* and *env*), encoding viral structural proteins, and three minor genes (*tat*, *S2* and *rev*), encoding nonstructural proteins that regulate various aspects of virus replication. The order of the EIAV genome is 5′-R–U5–*gag*–*pol*–*env*–U3–R-3′. The *tat* and *S2* genes are encoded as distinct alternate reading frames within the *pol-env* intergenic region, while the *rev* gene is encoded by alternate reading frames contained at the beginning of the *env* gene sequences and following the 3′ end of the *env* gene. The relatively small size of the EIAV genome compared to other lentiviruses is primarily due to the much smaller size of the *pol-env* intergenic region.

The EIAV *gag* gene encodes the four viral core proteins in the order of 5′-p15–p26–p11–p9-3′, while the *env* gene encodes the two envelope glycoproteins of the virus in the order of 5′-gp90–gp45-3′. The *pol* gene encodes a complex of enzymes with an organization of 5′-protease–reverse transcriptase–RNAse H–dUTPase–integrase-3′. As with other lentiviruses, the EIAV *tat* and *rev* genes encode important regulatory proteins that either transactivate virus transcription (Tat) or control viral transcription patterns (Rev) after infection of host cells. The function of the 8 kDa protein encoded by the *S2* gene remains to be defined, although mutation studies of this gene indicate that it is not essential for virus replication. The long terminal repeat (LTR) sequences of EIAV contain the usual complex of transcriptional regulatory domains distinctive of lentiviruses.

Properties of the Viral Proteins

The proteins encoded by the *gag* and *env* genes of EIAV constitute the major structural proteins of the virus (cf. **Fig. 1**). The gp90 protein is a highly glycosylated, hydrophilic surface (SU) protein that forms the outermost knobs of the envelope projections, while the gp45 is a sparsely glycosylated, hydrophobic transmembrane (TM) protein that forms the membrane-spanning spike of the envelope projection. There are about 300 copies each of the envelope glycoproteins per virion, and the surface projections appear to be composed of trimers of gp90 and gp45. The final component of the EIAV envelope

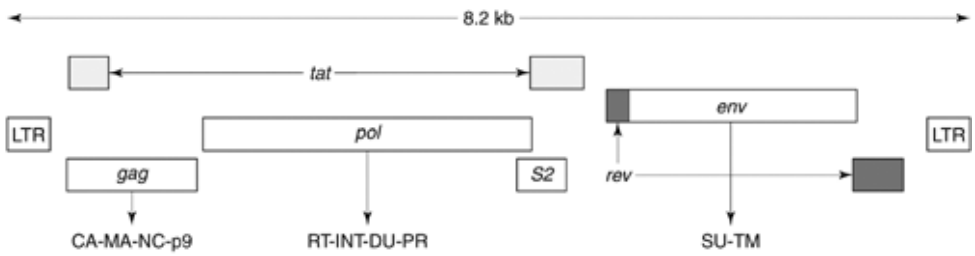


Figure 2 Organization of the EIAV genome indicating viral genes (italic) and the respective proteins encoded by these genes.

structure is the fatty acylated p15 that forms a continuous matrix (MA) immediately beneath the lipid bilayer of the virus particle. The virion core shell or capsid (CA), composed predominantly of p26 molecules, encloses a helical ribonucleoprotein complex containing the basic nucleoprotein (NP), p11, and various polymerases (reverse transcriptase (RT), integrase (IN) and dUTPase (DU)) in close association with the viral RNA genome. The location of the final core protein, p9, is not certain, but it has been proposed as a linker protein between the core shell and envelope matrix. Recent experiments have indicated that the EIAV p9 protein mediates late stages of viral budding.

The *gag*-encoded proteins of the virus are present in molar amounts that are at least 10-fold greater than the envelope glycoproteins, in the range of 3000–5000 copies per virus particle. In contrast, the *pol*-encoded enzymes appear to be present in the virus at levels of about 10 molecules per virion.

Replication Strategy

The EIAV replication cycle is characteristic of retroviruses in general and lentiviruses in particular. Viral recognition, attachment and penetration of target cells is believed to be mediated by specific interaction of the viral envelope glycoproteins and cellular receptor proteins contained in the plasma membrane. These putative receptors for EIAV have not been identified. Once inside the cytoplasm of the target cell the virus reverse transcriptase copies the single-stranded RNA genome into double-stranded DNA provirus that is then transported to the nucleus of the cell. There is no evidence for translation of the incoming EIAV genome at this stage of infection. Once inside the cell nucleus, the EIAV integrase mediates apparently random incorporation of the provirus DNA into cellular chromosomes, although some extrachromosomal viral DNA is always present in productively infected cells. There is typically a total of about 10 copies of proviral DNA per infected cell. Cytopathic infections by EIAV have been correlated with higher ratios of integrated to unintegrated proviral DNA.

Transcription of the proviral DNA by cellular polymerases produces a complex pattern of viral messenger RNA species whose relative proportions may differ, depending on the virus strain and target cell. In all instances, the predominant EIAV transcripts are an 8.2 kb transcript representing the full-length genomic RNA and translated to produce the *gag* and *pol* gene products, and a 3.5 kb mRNA that is a singly-spliced transcript translated to produce the viral envelope proteins. Lentivirus-infected cells usually contain, in addition to these major viral transcripts, a heterogeneous population of small multiply-spliced RNA that are used to produce the various regulatory proteins. In the case of EIAV, however, infected cells reveal only minute quantities of these small, multiply-spliced RNA species. The relatively low abundance of small transcripts may in part reflect the relative genetic simplicity of EIAV and the lack of extensive splicing to insure production of all of the minor viral genes. On the other hand, it is intriguing that the 3.5 kb transcript is in fact a tricistronic messenger that can produce by *in vitro* translation the viral Tat and Rev proteins in addition to the more abundant envelope glycoproteins. The use of a tricistronic messenger may represent a novel mechanism of maximizing genetic efficiency in EIAV replication.

The viral Gag and Env proteins are initially produced as polyproteins that are cleaved into the mature virion components by a combination of viral and cellular proteases. The only modification documented in the core proteins is a fatty acylation at the N-terminus of the matrix protein, p15. The envelope proteins are modified by *N*-linked glycosylation. Although the gp90 and gp45 polypeptides contain about 400 amino acid residues, the gp90 contains an average of 17 potential *N*-linked glycosylation sites, while the gp45 contains only about five potential glycosylation sites. By comparison to HIV-1 gp120, it is assumed that all potential glycosylation sites are occupied by complex oligosaccharides.

Little is known about the precise mechanisms of EIAV assembly, although it is assumed that it follows the general model for retrovirus assembly. Accordingly, the viral envelope glycoproteins are initially inserted into the plasma membrane to create distinct sites of virus assembly at which the Gag polyprotein is accumulated beneath the membrane lipid and cleaved into the mature virion proteins as the particle buds from the cell surface and is released to produce progeny virus.

The mechanisms of EIAV cytopathicity during this replication process are also uncertain. Various studies have suggested that a major mechanism of EIAV cytopathogenesis is structural and functional perturbations of cellular plasma membranes by the viral envelope glycoproteins. In this regard, peptide domains with cytolytic, membrane fusion, calmodulin-binding functions have been identified in the EIAV transmembrane glycoprotein.

Geographic and Seasonal Distribution

EIAV has been diagnosed in many areas of the world and is considered a worldwide disease of horses.

Although localized outbreaks of disease can occur, the incidence of EIAV-infected horses is highest in tropical and subtropical climates, presumably due to the longer seasons and more abundant populations of insect vectors that may transmit EIAV between horses. In the USA, the overall infection rate is reported to be about 0.2% of horses that are tested for EIAV-specific antibodies in diagnostic assays approved by the US Department of Agriculture (USDA). These infection rates, however, are somewhat misleading as only less than 10% of the general horse population in the USA is ever tested for EIAV. In more general surveys of selected herds in the southeastern USA, infection rates of 15% were observed. EIAV infections are especially prevalent in Central and South America, where unregulated herds may have infection rates up to 50%. Although the probability of EIAV infection by insect vectors is greatest during seasons that are warm, infections can occur throughout the year via mechanical transfers of blood by hypodermic needles and other veterinary instruments. Sexual transmission of EIAV has not been demonstrated to date.

Host Range and Virus Propagation

EIAV appears to infect only members of the family Equidae. There is no evidence to support the concept of natural or experimental infections of humans or of other mammalian species. EIAV infection of horses results in high levels of virus replication, persistent infection and clinical disease. EIAV infection of donkeys produces only limited virus replication, presumably a persistent infection, but no signs of clinical disease.

Field strains of EIAV can only be propagated *in vitro* in cultures of equine monocyte or macrophage cells, where virus infection typically produces a cytopathic effect within several days. Large-scale production of EIAV is limited to cell culture-adapted strains of virus that can be grown in primary cultures of equine dermal cells or fetal equine kidney cells and in a limited selection of nonequine continuous cell lines, including canine fetal thymus (Cf2th) cells and the Fea and FEF feline cell lines. The cell-adapted strains of EIAV are noncytopathic to these permissive cell lines. Field strains of EIAV will retain their pathogenic properties when propagated in leukocyte cultures, but usually become avirulent when adapted to other types of cell culture. Cell-adapted strains of EIAV that retain their virulence have been produced by back passage of avirulent cell-adapted strains in ponies or horses. There is no evidence for infection of cultured human cells by EIAV.

EIAV production in cell cultures is most easily detected by the presence of viral antigens or reverse transcriptase activity in culture media.

Genetics

Like other retroviruses, EIAV replication is mediated by a virion reverse transcriptase (RT) that copies the viral RNA genome into proviral DNA that is found in the nucleus of infected cells randomly integrated into the cellular chromosome and as extrachromosomal molecules. There are typically only about 10 copies of EIAV DNA per infected cell. EIAV replication in horses is characterized by relatively rapid and diverse genomic mutations that produce an apparently wide variety of variant virus strains. Analyses of sequential antigenic variants of EIAV from experimentally infected horses suggests that the rate of mutation in the envelope gene of the virus is greater than 10^{-2} base substitutions per site per year. The fidelity of DNA synthesis by purified EIAV RT has been measured *in vitro*, and an average error rate of 1/900 bp has been estimated. This value is similar to the *in vitro* error rate calculated for HIV (1/700 bp), but significantly lower than the rate observed for oncovirus RT, such as avian myeloblastosis virus (1/3000 bp). The error-prone nature of EIAV RT produces significant biological diversity that is important in EIAV persistence and pathogenesis. As demonstrated for HIV and simian immunodeficiency virus (SIV), recombination between variant EIAV genomes in infected cells may also contribute to genetic diversification during persistent infection.

Evolution

Phylogenetic analyses based on the nucleotide sequences of various retroviruses indicate that EIAV is most closely related to the ungulate lentiviruses (visna-maedi virus, caprine arthritis encephalitis virus and bovine immunodeficiency virus) and equally divergent from the human and simian immunodeficiency viruses.

Serologic Relationships and Variability

Field and laboratory isolates of EIAV display a remarkable variability in antigenic properties. The core proteins of the virus contain conserved antigenic determinants that are the basis of current serological diagnostic assays. The viral glycoproteins also contain a limited number of conserved antigenic determinants, but predominantly present an array of variable antigenic sites that can be distinguished by their reactivity with monoclonal antibodies or by neutralization properties with polyclonal immune

serum from infected horses. The range of variation observed among EIAV isolates has precluded any classification of virus strains on the basis of serological properties.

Immune serum from EIAV-infected horses is reactive with the respective major core protein of most animal and human lentiviruses, but not with any of the major core proteins of oncoviruses. Immune serum taken from other species infected with a lentivirus do not generally reveal a crossreactivity with EIAV or with other lentiviruses. This one-way serological reactivity suggests that horses infected with EIAV uniquely recognize a conserved lentivirus-specific antigenic determinant.

Epidemiology

The prevalence of EIAV infections is greatest in warm climate geographical areas with a high insect population. During the 1980s and 1990s, the EIAV infection rate reported by the USDA dropped from about 4% to less than 0.2%. However, these testing results do not reflect the general horse population: less than 10% of the horses in the USA are tested for EIA, usually because of requirements for transportation across state lines or for participation in organized shows or races. General surveys of unregulated herds in the Southeast demonstrate infection rates of up to 15%. In limited surveys of Latin American countries, infection rates in unregulated herds frequently approach 50%, indicating that EIAV infection is epidemic in these areas.

Transmission and Tissue Tropism

Blood from persistently infected horses is the most important source of EIAV for transmission; *in utero* transmissions of EIAV from mare to foal are evidently very rare. This blood transfer can be affected by human or blood-feeding vectors. EIAV has been shown to remain infectious on hypodermic needles for up to 96 h, emphasizing the potential for transmission via routine animal husbandry or veterinary medical practices. However, the mechanical transmission of EIAV by arthropods, especially horse flies, is generally accepted as the major natural means of transmission in the field. Transmission of EIAV by a single horse fly carrying only about 10 nl of virus-infected blood has been documented under experimental conditions.

The target cells during persistent EIAV infections appear to be exclusively cells of the monocyte/macrophage lineage; there is no evidence for infection of lymphocytes, as observed with some other lentiviruses. The virus burden in infected horses is predominantly in tissue macrophages found in liver, kidney and spleen, with much lower levels of virus found in lymph nodes, bone marrow or in circulating monocytes. Thus, the relatively high levels of viremia (10^{4-6} $TCID_{50}$) observed during episodes of chronic EIA evidently result primarily from the production and release of virus from infected tissue macrophages, rather than an extensive infection of blood monocytes.

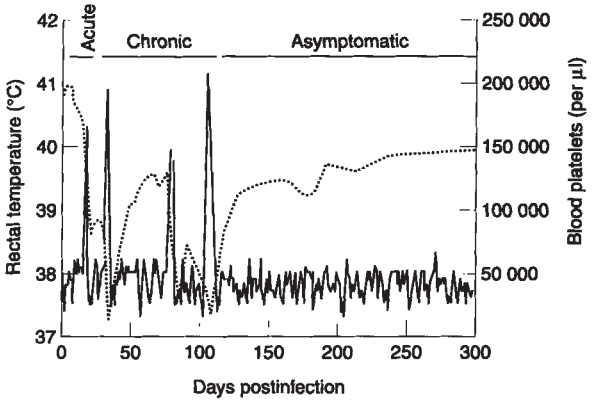


Figure 3 Clinical profile associated with EIAV infection of horses indicating the characteristic stages of EIA. Febrile episodes are defined as rectal temperatures (—) above 39°C (103°F), and thrombocytopenia is defined as platelet levels (----) below 105 000 per microliter of blood.

Pathogenicity

Field isolates and laboratory strains of EIAV differ markedly in their pathogenicity, ranging from avirulent to lethal strains of the virus. Little is known about the viral determinants or host factors that influence the course of virus replication and pathogenesis. In other lentiviruses, differences in viral pathogenesis have been mapped to specific changes in viral envelope genes or to changes in gene regulatory sequences in the viral genome. It is likely that variation in EIAV pathogenicity will follow a similar pattern.

Clinical Features of Infection

The clinical response of horses following artificial inoculation or natural exposure to EIAV is variable and depends in part on host resistance factors, viral virulence factors and environmental factors (e.g. weather, work load, etc.). In general, EIAV infections can be apparent, with distinctive clinical symptoms, or inapparent, without any clinical signs of EIA. The clinical disease is typically described as acute, chronic or asymptomatic (**Fig. 3**).

Acute EIA is most often associated with the first exposure to the virus, with fever and hemorrhages evident from 7–30 days after exposure. Acute disease is thought to be associated with massive virus replication in, and destruction of, infected macrophages. Horses in the initial phase of acute EIA will be seronegative because the immune system has had insufficient time to respond to the viral antigens. During the peak of the febrile response in acute EIA, viremia of greater than 10^6 horse infectious doses per milliliter of whole blood is often observed. The initial acute phase of EIA infection may not be seen by the veterinarian unless there is an epizootic of the infection in a group of horses. Even then, the horses must be under close supervision for the initial fever and anorexia to be detected. Neither anemia nor edema are seen at this stage of disease.

The more classic clinical signs of EIA, such as loss of weight, anemia, diarrhea and edema, are seen later during recurring cycles of the illness, which appear at irregular intervals ranging from a couple of weeks to several months. The frequency and severity of clinical episodes in horses with chronic EIA usually decline with time, about 90% occurring within 1 year of infection. Horses with chronic EIA are seropositive and have variable viremia levels which are highest during the periodic febrile episodes. Although a small percentage of chronic EIA cases may result in death, the predominant clinical course of infection is a cessation of detectable clinical symptoms by the end of the first year postinfection and the establishment of a lifelong inapparent carrier stage.

The highest percentage of EIAV-infected horses in the field are in fact inapparent carriers. These horses have no clinical illness associated with the viral infection and viremia is usually undetectable. However, these inapparent carriers maintain high levels of EIAV-specific antibodies, suggesting a chronic low level of virus replication. Inapparent carriers can be shown to harbor infectious EIAV by transfusions of whole blood (200 ml) to recipient horses, who will become seropositive to EIAV within 3–6 weeks postinoculation. In addition, treatment of certain inapparent carriers with immunosuppressive drugs or exposure to extreme stress can cause the recrudescence of chronic EIA, even in some horses that have been free of clinical symptoms for years.

Pathology and Histopathology

Gross and histopathological lesions in EIAV-infected horses are variable and closely associated with levels of viral replication. In the acute stage of EIA, gross pathological lesions consist of swelling of the parenchymatous organs, and hemorrhages can be observed in most tissues. The most pronounced histopathological lesions are hepatic and lymphoid necrosis in association with large numbers of activated macrophages and Kupffer cells. Hepatic necrosis is most severe near the central vein, evidently resulting from degenerative changes in the parenchymal cells. Lesions in the spleen are characterized by degenerative erythrocytes, and small focal hemorrhages are found in the splenic capsule and adjacent tissue. Lymphocytic infiltrations can be observed in several organs, including liver, spleen, lymph node, kidney, heart and lung. The majority of these lesions are thought to be the combined effect of immune-mediated lysis of virus-infected cells and an immune complex-mediated inflammatory response.

The pathological changes in the chronic form of EIA include a developing immunological control of virus replication. Gross pathological lesions include splenomegaly, lymphadenopathy and hepatomegaly. Microscopic changes are characterized by infiltration of lymphoid cells in almost all organs and tissues. Anemia has long been considered the hallmark of EIA. The two major causes of anemia, hemolysis and bone marrow depression, are closely associated with replicating virus. Hemolysis is immunologically mediated. Erythrocytes are coated with the viral surface glycoprotein which, in the presence of specific antibodies and bound C3, induces erythrophagocytosis and complement-mediated hemolysis. Bone marrow suppression is less well characterized but appears to be associated with iron deficiency. Thrombocytopenia is frequently the earliest pathology observed during chronic EIA and can precede the detection of virus-specific antibodies. The mechanism for the marked reduction in blood platelets is unknown but has recently been associated with cytokine dysregulation.

Immune Responses

Horses infected with EIAV typically become seropositive in standard serological assays within 21 days postinfection. The humoral immune responses are predominantly against the viral envelope glycoproteins, gp90 and gp45, and the major core protein, p26. All currently approved diagnostic assays for EIAV infection are based on the detection of antibody to the major core antigen, although the antibody response to the envelope glycoproteins is at least 10-fold greater than the antibody titer measured against the p26 protein. Significantly lower levels of antibody can be detected against virtually all of the other structural and nonstructural proteins encoded by EIAV. Interestingly, EIAV-specific antibody levels remain rela-

tively constant throughout the course of chronic EIA and even in the asymptomatic stage of infection.

The immune responses generated during chronic EIA initially mediate significant pathogenesis in the presence of sufficient levels of EIAV antigenemia, but progressively evolve to establish a strict immunologic control over virus replication. Thus, the EIAV system is unique among lentiviruses in that the host immune responses routinely accomplish an effective control of aggressive virus replication and recurring clinical disease to maintain an indefinite inapparent stage of infection. The immune correlates of this protection remain to be defined. However, it has been shown that the neutralizing capacity of serum antibodies elicited to EIAV during the chronic stage of disease progressively increases, indicating an evolution of immune responses to the sequential generation of antigenic variants of virus. In addition, recent studies have demonstrated a lengthy and complex evolution of antibody and cellular immune responses to experimental EIAV infections of horses, demonstrating a dynamic maturation process that apparently correlates with the development of protective immunity. The specific humoral or cellular immune correlates of protection have not yet been identified. The ability of the horse immune responses to overcome the array of persistence and escape mechanisms employed by EIAV suggests that a successful vaccine is feasible.

Prevention and Control

The transmission of EIAV infection has been controlled by improving animal husbandry techniques to prevent the spread of infected blood, by reducing the horse fly population in the vicinity of herds, and primarily by identifying and segregating or sacrificing horses that are seropositive for the virus. In the USA the most common diagnostic assays are an agar gel immunodiffusion test, the Coggins assay, which was developed in the early 1970s, and enzyme-linked immunosorbent assay (ELISA), which was approved by the USDA as an EIA diagnostic assay in the 1980s and 1990s. Both diagnostic assay procedures are based on the detection of serum antibodies to the major viral core protein, p26. The diagnostic ELISA has been engineered to produce a sensitivity that is equivalent to the previously established Coggins test. More sensitive diagnostic assays based on the detection of antibodies to the EIAV envelope glycoproteins have been developed, but have not yet been approved for commercial use. The most sensitive and specific assay for detecting EIAV infection is horse inoculation tests with 200 ml of whole blood from the horse being tested. The horse inoculation test is used only in rare cases where the standard serological assays may give ambiguous results.

There is currently no effective vaccine for the prevention of EIAV infection and disease. The primary challenge in developing an effective EIAV vaccine is overcoming the antigenic diversity intrinsic to this virus. An important practical requirement in the development of any EIAV vaccine will be compatibility with established regulatory policies and diagnostic assays. The ability of EIAV-infected horses to routinely establish immunologic control over virus replication and disease suggests that an effective vaccine can indeed be developed, if the critical natural immune correlates of protection can be elicited by a candidate vaccine. An attenuated live EIAV vaccine with a reported protection efficacy of about 70% has been used in China and Cuba since the early 1980s, but the effectiveness of this vaccine remains to be confirmed outside these two countries. Evaluation of other candidate EIAV vaccines (inactivated whole virus, subunit vaccines, synthetic peptides, etc.) under experimental conditions has revealed a spectrum of vaccine efficacy that ranges from 'sterile protection' (prevention of infection upon inoculation with EIAV) to severe elevation of EIAV replication and exacerbation of disease. These results indicate that immune responses to EIAV are a double-edged sword that can either mediate protection or vaccine enhancement. Vaccine enhancement has previously been reported for other viral infections (dengue virus, respiratory syncytial virus, feline infectious peritonitis virus) and is of special concern with macrophage-tropic viruses. Similar examples of vaccine protection and enhancement have been reported in studies of experimental vaccines for other lentiviruses, including feline immunodeficiency virus, caprine arthritis encephalitis virus, and visna-maedi virus. These observations in several diverse animal lentivirus systems suggests that the potential for immune enhancement may be a general property of lentiviruses, including HIV-1. Current efforts in the production of a commercial EIAV vaccine are focused on the development of a vaccine that can achieve sufficient maturation of immune responses to provide protection from virus infection, but allow the serological differentiation between vaccinated and infected horses. In this regard, DNA vaccine strategies appear to be well suited to accomplish these criteria for a commercial EIAV vaccine.

Future Research

EIAV provides a dynamic system for examining the interaction between virus populations and host immune responses that are evolving in response to

each other. In addition, EIAV offers a remarkable model for studying the delicate balance between immune responses to a persistent virus infection that result in disease and those that have beneficial results. A characterization of the nature of protective and enhancing immune responses can provide important information about the mechanisms of lentivirus disease and the type of immune responses to be elicited or avoided by a vaccine. The results of these studies in the EIAV system should be applicable to other lentiviruses, including HIV-1.

See also: **Vaccines and immune response; Human immunodeficiency viruses (*Retroviridae*): General features; Bovine immunodeficiency virus (*Retroviridae*); Feline immunodeficiency virus (*Retroviridae*); Persistent viral infection; Pathogenesis: Animal viruses.**

Further Reading

Cook RF, Issel CJ and Montelaro RC (1996) Equine infectious anemia virus. In: Studdert R (ed.) *Viral Diseases of Equines*. Amsterdam: Elsevier.

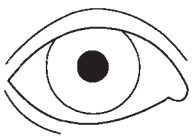
Cordes TA and Issel CJ (1996) *Equine Infectious Anemia: A Status Report on its Control*. USDA Animal and Plant Health Inspection Service publication no. APHIS 91-55-032.

Montelaro RC and Bolognesi DP (1995) Vaccines against retroviruses. In: Levy J (ed.) *The Retroviridae*, vol. 2, New York: Plenum Press.

Montelaro RC, Ball JM and Rushlow KE (1992) Equine retroviruses. In: Levy J (ed.) *The Retroviridae*, vol. 2, New York: Plenum Press.

Sellon DC, Fuller FJ and McGuire TC (1994) The immunopathogenesis of equine infectious anemia virus. *Virus Res.* **32**: 111.

EYE INFECTIONS



James Chodosh, Molecular Pathogenesis of Eye Infection Research Center, Dean A. McGee Eye Institute and Department of Ophthalmology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, USA

Introduction

The eye contains diverse tissues intricately linked to subserve visual function (**Fig. 1**). The ocular adnexae – periorbita, eyelids and lashes, lacrimal and meibomian glands – produce, spread and drain the preocular tear film, physically protect the sensitive ocular mucosa, and cushion the globe. The redundant conjunctiva with its low-viscosity tear film allows rapid multidirectional eye movements. Lymphoid tissues within the conjunctiva and lacrimal glands furnish acquired immune defense. The cornea and its tear film fashion the major refractive surface of the eye. The sclera forms the wall of the globe and scaffolds the intraocular tissues. The eye's lens provides additional refractive power and filters ultraviolet light. The iris diaphragm dynamically regulates the amount of light incident on the retina, and together with the choroid and optic nerve head provides immune effector cells to the interior of the eye. The retina transduces light energy into neural signals; retinal function is requisite for vision. The vascular choroid nourishes the outer layers of the retina. The anterior (aqueous) and posterior (vitreous) humors provide internal pressure sufficient for maintenance of normal anatomic relationships, nourish the interior ocular tissues, provide immunosuppressive factors necessary to the maintenance of immune deviation, and during infection act as conduits for the distribution of inflammatory cells derived from the iris, ciliary body and optic nerve head.

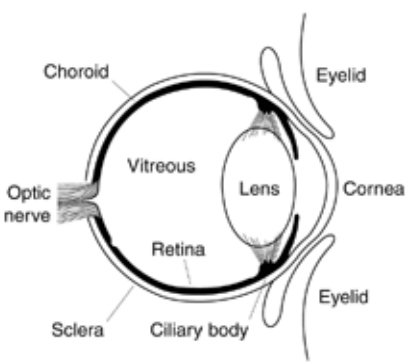


Figure 1 Cross-section of the human eye.

Eye infection by viruses most often follows direct contact with virus externally, either from infected

each other. In addition, EIAV offers a remarkable model for studying the delicate balance between immune responses to a persistent virus infection that result in disease and those that have beneficial results. A characterization of the nature of protective and enhancing immune responses can provide important information about the mechanisms of lentivirus disease and the type of immune responses to be elicited or avoided by a vaccine. The results of these studies in the EIAV system should be applicable to other lentiviruses, including HIV-1.

See also: **Vaccines and immune response; Human immunodeficiency viruses (*Retroviridae*): General features; Bovine immunodeficiency virus (*Retroviridae*); Feline immunodeficiency virus (*Retroviridae*); Persistent viral infection; Pathogenesis: Animal viruses.**

Further Reading

Cook RF, Issel CJ and Montelaro RC (1996) Equine infectious anemia virus. In: Studdert R (ed.) *Viral Diseases of Equines*. Amsterdam: Elsevier.

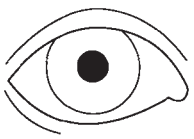
Cordes TA and Issel CJ (1996) *Equine Infectious Anemia: A Status Report on its Control*. USDA Animal and Plant Health Inspection Service publication no. APHIS 91-55-032.

Montelaro RC and Bolognesi DP (1995) Vaccines against retroviruses. In: Levy J (ed.) *The Retroviridae*, vol. 2, New York: Plenum Press.

Montelaro RC, Ball JM and Rushlow KE (1992) Equine retroviruses. In: Levy J (ed.) *The Retroviridae*, vol. 2, New York: Plenum Press.

Sellon DC, Fuller FJ and McGuire TC (1994) The immunopathogenesis of equine infectious anemia virus. *Virus Res.* **32**: 111.

EYE INFECTIONS



James Chodosh, Molecular Pathogenesis of Eye Infection Research Center, Dean A. McGee Eye Institute and Department of Ophthalmology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, USA

Introduction

The eye contains diverse tissues intricately linked to subserve visual function (**Fig. 1**). The ocular adnexae – periorbita, eyelids and lashes, lacrimal and meibomian glands – produce, spread and drain the preocular tear film, physically protect the sensitive ocular mucosa, and cushion the globe. The redundant conjunctiva with its low-viscosity tear film allows rapid multidirectional eye movements. Lymphoid tissues within the conjunctiva and lacrimal glands furnish acquired immune defense. The cornea and its tear film fashion the major refractive surface of the eye. The sclera forms the wall of the globe and scaffolds the intraocular tissues. The eye's lens provides additional refractive power and filters ultraviolet light. The iris diaphragm dynamically regulates the amount of light incident on the retina, and together with the choroid and optic nerve head provides immune effector cells to the interior of the eye. The retina transduces light energy into neural signals; retinal function is requisite for vision. The vascular choroid nourishes the outer layers of the retina. The anterior (aqueous) and posterior (vitreous) humors provide internal pressure sufficient for maintenance of normal anatomic relationships, nourish the interior ocular tissues, provide immunosuppressive factors necessary to the maintenance of immune deviation, and during infection act as conduits for the distribution of inflammatory cells derived from the iris, ciliary body and optic nerve head.

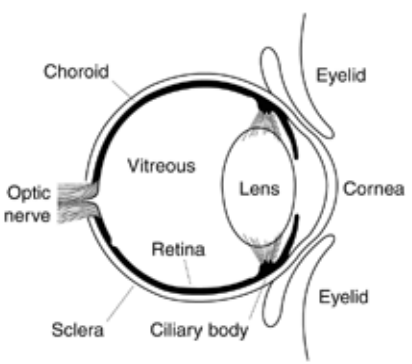


Figure 1 Cross-section of the human eye.

Eye infection by viruses most often follows direct contact with virus externally, either from infected

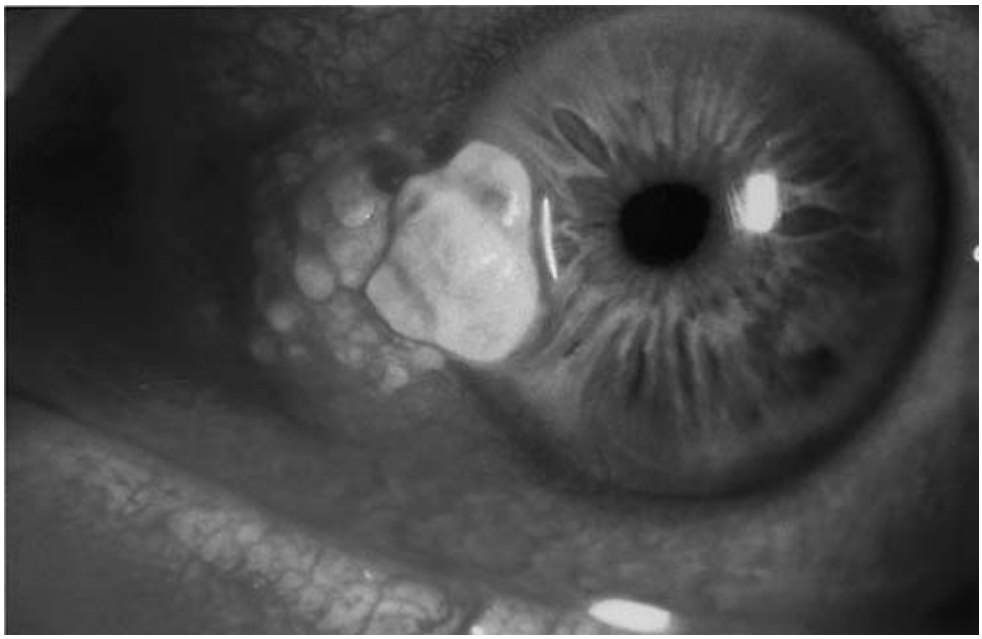


Figure 2 Squamous cell carcinoma of the corneal limbus is associated with infection by human papilloma virus types 16 and 18. **(For color references see Color Plate 2.)**

secretions in the birth canal (herpes simplex virus, human papillomavirus), on fomites (adenovirus), or air-borne particles (rhinovirus), or is acquired during viremia (human cytomegalovirus, measles virus). Other mechanisms of ocular viral infection include extension from contiguous adnexal disease (herpes simplex virus), spread from the upper respiratory tract via the nasolacrimal duct (rhinovirus), and transplacental passage of infectious virus (rubella virus). Rarely, ocular infection may disseminate elsewhere (enterovirus 70).

Acute viral infection produces stereotypic changes in ocular target tissues. Infection of the eyelid skin induces the formation of vesicles and ulcers. Viral infection of the conjunctiva results in vasodilatation, serous discharge, hyperplasia of conjunctival lymphoid follicles, and enlargement of the corresponding draining lymph nodes. Severe conjunctival infection can cause permanent scarring of the globe to the eyelids and turning in of the eyelashes against the eye. Viral infection of the corneal epithelium induces punctate epithelial cytopathic effect evident biomicroscopically as isolated swollen epithelial cells (punctate epithelial keratitis) and loss of individual epithelial cells (punctate epithelial erosions). When extensive, the punctate erosions may coalesce to form confluent epithelial ulcers with dendritic, dendritiform or geographic morphology. With herpetic infection, corneal anesthesia can ensue, and in the absence of epitheliotropic neural growth factors, corneal epithelial integrity is impaired. Reduced corneal clarity and progressive sterile ulceration may result. Corneal stromal infection induces white blood cell recruitment; subsequent corneal scarring, vascularization and lipid deposition may permanently reduce vision. Intraocular infection manifests in inflammatory cell deposits on the posterior surface of the cornea and on the vitreous scaffold, and in free-floating leucocytes and biomicroscopically visible protein spillage into the normally cell-free and protein-poor aqueous humor. Iridocorneal and iridolenticular adhesions may develop and lead to glaucoma and cataract. Retinal infection concludes with necrosis and lost function. Viral encephalitis and meningitis can result in cranial nerve inflammation and secondary dysfunction of vision and extraocular motility.

Classical viral pathogenic mechanisms of latency, reactivation, and carcinogenesis all can be demonstrated in the eye. Herpes simplex virus causes recurrent lytic epithelial keratitis when viral reactivation within sensory ganglia of the first division of the fifth cranial nerve gives rise to virus in the preocular tear film. Necrotizing herpes stromal keratitis follows viral reactivation within the cornea stroma. Intraepithelial neoplasia and invasive squamous cell carcinoma of the conjunctiva and cornea (**Fig. 2**) have been associated with human papilloma virus types 16 and 18. When infected with oncogenic human papillomaviruses, corneal limbal stem cells can

provide a persistent source of dysplastic ocular surface epithelium. Molecular mimicry has also been demonstrated as an immunopathogenic mechanism in ocular disease. Systemic infection with hepatitis C virus is associated with autoimmunity against a corneal stromal antigen and peripheral ulcerative keratitis. In a murine model of herpes simplex infection, nonnecrotizing stromal keratitis accompanies T cell reactivity against a corneal protein antigenically similar to a herpes simplex coat protein.

Ocular Immunology of Relevance to Viral Infection

Tissue diversity within the eye and adnexa compel varied means of innate immune defense. The external surfaces of the eye (conjunctiva and cornea) encounter viruses by both airborne and contact routes. The eyelids, an intermittent barrier, periodically wipe the eye's surface free of debris and spread and drain the preocular tear film. The ability of the tear film to nonspecifically impede primary infection by viruses is unknown, although such mechanisms are well established for bacterial pathogens. An inhibitory effect of goblet cell-derived and intrinsic mucins and meibomian gland-derived lipids on viral adsorption to the ocular surface is speculative. Early in infection, aqueous tears from the main and accessory lacrimal glands furnish proinflammatory cytokines, and the conjunctival blood vessels provide both soluble and cellular components of innate immunity. After viral infection is established, aqueous tears carry lacrimal gland-derived monospecific secretory immunoglobulin A.

The constitutive defense armaments of the cornea and conjunctiva differ. The normal cornea is considered an immune-privileged site due to the high success rate of corneal transplantation; it lacks blood vessels, lymphatics, resident lymphoid cells, and Langerhans cells, expresses Fas ligand on its surface epithelium, and demonstrates reduced delayed hypersensitivity responses. Because corneal inflammation and subsequent scar reduce vision, corneal function is best served by its reduced immunologic responsiveness, also known as immune deviation. Necrotizing inflammation presupposes infection beneath the surface epithelium, and follows chemokine synthesis by infected corneal stromal fibroblasts. In contrast to the cornea, the conjunctiva is well endowed with blood and lymphatic channels, lymphoid cells and Langerhans cells, and demonstrates classical delayed hypersensitivity responses. The immunology of the interior eye is less well established, but immune deviation appears to extend beyond the cornea to the aqueous and vitreous humors and to the central retina.

Ocular Disease Caused by RNA Viruses

Conjunctivitis is probably the most common viral ocular syndrome, and typically accompanies upper respiratory infections due to RNA viruses (**Table 1**). Rhinovirus, influenza virus, respiratory syncitial virus, and parainfluenza virus conjunctivitis typically are mild and self-limited, and most patients do not seek medical attention. More serious are the keratitis, uveitis and retinitis caused by some RNA viruses. For example, influenza virus infection of the respiratory tract, usually associated with a mild and short-lived conjunctivitis, less commonly causes inflammation in the lacrimal gland, cornea, iris, retina, optic and other cranial nerves.

Like influenza virus, other RNA viruses can infect virtually every ocular tissue. For instance, rubella virus when acquired *in utero* may have devastating consequences for the eye. Characteristic features include microphthalmos, corneal haze, cataracts, iris hypoplasia, iridocyclitis, glaucoma and 'salt and pepper' pigmentary retinopathy. Rubella virus can be cultured from the lens of infected neonates at the time of cataract extraction. Congenital ocular abnormalities due to rubella, like those in other organ systems, are much worse when maternal infection ensues earliest in pregnancy.

In contrast to rubella virus, measles (rubeola) virus infection *in utero* rarely causes significant ocular disease. The classic triad of postnatally acquired measles – cough, coryza, and follicular conjunctivitis – can be accompanied by Koplik spots on the conjunctiva and a mild epithelial keratitis. Less common are optic neuritis, retinal vascular occlusion and pigmentary retinopathy. Measles keratopathy, a major source of blindness in the nonindustrialized world, typically presents as corneal ulceration in a malnourished child. A rare and fatal complication of measles virus infection, subacute sclerosing panencephalitis (SSPE), occurs in about 1 per 100 000 cases, and often years after clinically apparent measles. Along with devastating central nervous system damage, ocular abnormalities occur commonly in SSPE, including central retinal (macular) hyperpigmentation and inflammation, optic nerve atrophy, peripheral retinitis, and ocular motility disorders. Cortical blindness can occur in the absence of ocular involvement.

The most common ocular complication of mumps virus infection is dacryoadenitis, and this may occur concurrently with parotid gland involvement. Aseptic meningitis, associated oculomotor palsy, and optic neuritis also occurs. Follicular conjunctivitis, epithelial and stromal keratitis, iritis, trabeculitis, and scleritis have all been reported within the first 2 weeks after onset of parotitis.

Table 1 Ocular targets of human RNA viruses

Virus	*Family*	*Subfamily/genus*	*Nucleic acid*	*Env.*	*Ocular target*
Rift valley fever virus	*Bunyaviridae*	*Bunyavirus*	ss (−)	+	Retina
Human coronavirus	*Coronaviridae*	*Coronavirus*	ss (+)	+	Conjunctiva
Influenzavirus	*Orthomyxoviridae*	*Influenza virus* (A, B, C)	ss (−)	+	Lacrimal gland Conjunctiva Episclera Cornea Uvea Retina Optic nerve Cranial nerves
Measles (rubeola) virus	*Paramyxoviridae*	*Morbillivirus*	ss (−)	+	Conjunctiva Cornea Uvea Retina Optic nerve Cranial nerves
Mumps virus	*Paramyxoviridae*	*Paramyxovirus*	ss (−)	+	Lacrimal gland Conjunctiva Sclera Cornea Trabecular meshwork Uvea Optic nerve Cranial nerves
Newcastle disease virus	*Paramyxoviridae*	*Paramyxovirus*	ss (−)	+	Conjunctiva Cornea
Parainfluenzavirus(es)	*Paramyxoviridae*	*Paramyxovirus*	ss (−)	+	Conjunctiva
Respiratory syncitial virus	*Paramyxoviridae*	*Pneumovirus*	ss (−)	+	Conjunctiva
Enterovirus(es) (includes Poliovirus, Coxsackievirus, Echovirus, Enterovirus)	*Picornaviridae*	*Enterovirus*	ss (+)	−	Conjunctiva Cornea
Rhinovirus	*Picornaviridae*	*Rhinovirus*	ss (+)	−	Conjunctiva
Colorado tick fever virus	*Reoviridae*	*Coltivirus*	ds (+/−)	−	(?: reported to cause photophobia, retroocular pain)
Human immunodeficiency virus	*Retroviridae*	*Lentivirus*	ss (+)	+	Lacrimal gland Retina
Rabies virus	*Rhabdoviridae*	*Lyssavirus*	ss (−)	+	(Transmission via corneal button)
Alphavirus/Flavivirus (encephalitis, encephalomyelitis, yellow fever, dengue viruses)	*Togaviridae*	*Rubrivirus*	ss (+)	+	Conjunctiva
Rubella virus	*Togaviridae*	*Rubrivirus*	ss (+)	+	Cornea Uvea Lens Trabecular meshwork Retina Globe

+ Enveloped; −, nonenveloped; ss, single stranded; ds, double stranded; (+) positive sense RNA genome; (−) negative sense RNA genome.

Acute hemorrhagic conjunctivitis (AHC), caused predominantly by enterovirus type 70 and coxsackievirus A24 variant, but also adenovirus type 11, is one of the most dramatic ocular viral syndromes. Sudden onset of follicular conjunctivitis associated with multiple petechial conjunctival hemorrhages characterizes AHC. The hemorrhages may become confluent and appear post-traumatic. In approximately 1/10 000 cases due to enterovirus type 70, a polio-like paralysis can ensue. Neurologic deficits are permanent in up to one-third of affected individuals.

Human immunodeficiency virus (HIV) is the etiologic agent of the acquired immunodeficiency syndrome (AIDS). Although HIV can be cultured from the retinas of individuals with AIDS, and has been shown to be present in the donated corneas of deceased AIDS patients, a direct relationship between local viral infection and ocular disease remains to be established. One example is the dry eye so common in AIDS patients. It is not known whether primary HIV infection of the lacrimal gland, immune deficit-induced potentiation of another virus such as Epstein–Barr virus within the lacrimal gland, or a putative HIV-induced neuroimmune–endocrine defect can account for AIDS-related dry eye. However, the severe immunosuppression of AIDS results in a host of other ocular diseases (discussed below).

Ocular Disease Caused by DNA Viruses

DNA viruses (**Table 2**) are responsible for most significant ocular viral infections in the industrialized world. Even the protean ocular manifestations of the HIV, an RNA virus, result largely from reduced immunity to DNA viruses.

Adenovirus is probably the most common DNA virus to cause eye disease. Three common ocular syndromes have been identified. Simple follicular conjunctivitis occurs with infection by many adenovirus types and may be subclinical. Pharyngoconjunctival fever typically follows infection with adenovirus types 3, 4, and 7. As the name implies, patients have pharyngitis, conjunctivitis and fever, and may be misdiagnosed as having influenza. Epidemic keratoconjunctivitis, most often caused by adenovirus types 8, 19 and 37, is a highly contagious syndrome with significant morbidity. The conjunctivitis can be severe (**Fig. 3**); associated inflammatory conjunctival membranes can permanently scar the eyelids to the globe. Corneal involvement begins as a punctate epithelial keratitis and may proceed to a large central epithelial ulcer. Stromal keratitis presents about 2 weeks after the conjunctivitis as multifocal subepithelial corneal infiltrates, and causes both foreign body sensation and reduced vision. The stromal infiltrates may resolve spontaneously, but can become chronic, require long-term treatment with corticosteroids, and cause persistent visual morbidity. A fourth ocular syndrome occasionally associated with adenovirus infection, AHC (discussed above) may be caused by adenovirus type 11. Interestingly, adenovirus type 11 also causes acute hemorrhagic cystitis. Follicular conjunctivitis clinically indistinguishable from adenovirus conjunctivitis can also be caused by Newcastle disease virus, an RNA virus that gives rise to fatal epidemics in poultry and infects the birds' human handlers.

The human herpes viruses are preeminent among DNA viruses in eye disease with at least seven of the eight known human herpes viruses associated with ocular disorders. Herpes simplex virus type 1 (HSV-1) is the most common herpes virus to cause eye disease and herpes simplex keratitis is the most common cause of infectious blindness in the industrialized world. HSV-1 causes self-limited and relatively benign infections of the eyelids, the conjunctiva and the corneal epithelium, but infections of the corneal stroma, uvea and retina may result in chronic or recurrent blinding stromal keratitis, uveitis and retinal necrosis, respectively. Elevation of intraocular pressure due to involvement of the trabecular meshwork is not uncommon and may help to differentiate herpetic uveitis from noninfectious causes. Postnatally acquired HSV-2 ocular infection, less common than HSV-1, causes disease similar in most respects to HSV-1. Neonatal herpes simplex infection, acquired during transit through the birth canal and usually due to HSV-2, commonly causes vesicular blepharitis and conjunctivitis, but can also cause permanent visual loss due to keratitis, chorioretinitis, optic neuritis and encephalitis of the visual cortex.

Varicella zoster virus, the etiologic agent of chickenpox and shingles, rarely causes keratouveitis with primary infection (chickenpox). However, vision-threatening keratitis, uveitis, and less commonly, retinal necrosis, are complications of varicella zoster virus reactivation in the distribution of the fifth cranial nerve (zoster ophthalmicus). Lid ulceration with frank tissue loss or lid malposition leads to corneal exposure and ulceration. Optic neuritis and cranial nerve paresis can accompany onset of the zoster rash. Sectoral iris atrophy is pathognomonic for zoster ophthalmicus. Postinfectious corneal anesthesia and secondary sterile corneal ulceration may follow herpes simplex types 1 and 2, but are most severe in zoster ophthalmicus. Chronic scleritis, keratitis, uveitis and glaucoma may ultimately limit the visual acuity.

Acute systemic infection with Epstein–Barr virus may cause conjunctivitis and epithelial keratitis. Stromal keratitis occurs but is difficult to differentiate

Table 2 Ocular targets of human DNA viruses

Virus	*Family*	*Subfamily/genus*	*Nucleic acid*	*Env.*	*Ocular target*
Adenovirus	*Adenoviridae*	*Mastadenovirus*	ds	−	Conjunctiva Cornea
Herpes simplex virus, type 1 (HHV1)	*Herpesviridae*	*Alphaherpesvirinae/ Simplexvirus*	ds	+	Eyelid Conjunctiva Cornea Trabecular meshwork Uvea Retina
Herpes simplex virus, type 2 (HHV2)	*Herpesviridae*	*Alphaherpesvirinae/ Simplexvirus*	ds	+	Eyelid Conjunctiva Cornea Trabecular meshwork Uvea Retina
Varicella zoster virus (HHV3)	*Herpesviridae*	*Alphaherpesvirinae/ Varicellovirus*	ds	+	Eyelid Conjunctiva Cornea Trabecular meshwork Uvea Retina Optic nerve
Epstein–Barr virus (HHV4)	*Herpesviridae*	*Gammaherpesvirinae/ Lymphocryptovirus*	ds	+	Lacrimal gland Conjunctiva Cornea Uvea Retina Optic nerve
Human cytomegalovirus (HHV5)	*Herpesviridae*	*Betaherpesvirinae/ Cytomegalovirus*	ds	+	Retina Optic nerve
Human herpes virus 6 (HHV6)	*Herpesviridae*	*Betaherpesvirinae/ Roseolovirus*	ds	+	Retina
Human herpes virus 8 (HHV8)	*Herpesviridae*	*Gammaherpesvirinae*	ds	+	Conjunctiva (Kaposi sarcoma)
Human papillomavirus	*Papovaviridae*	*Papillomavirus*	ds	−	Eyelid Conjunctiva Cornea
Molluscum contagiosum virus	*Poxviridae*	*Molluscipoxvirus*	ds	+	Eyelid Conjunctiva Cornea
Orf virus	*Poxviridae*	*Parapoxvirus*	ds	+	Eyelid
Smallpox (variola) virus	*Poxviridae*	*Orthopoxvirus*	ds	+	Eyelid Conjunctiva Cornea Uvea Optic nerve
Vaccinia virus	*Poxviridae*	*Orthopoxvirus*	ds	+	Eyelid Conjunctiva Cornea

ds, double stranded; +, enveloped; −, nonenveloped; HHV, human herpes virus.

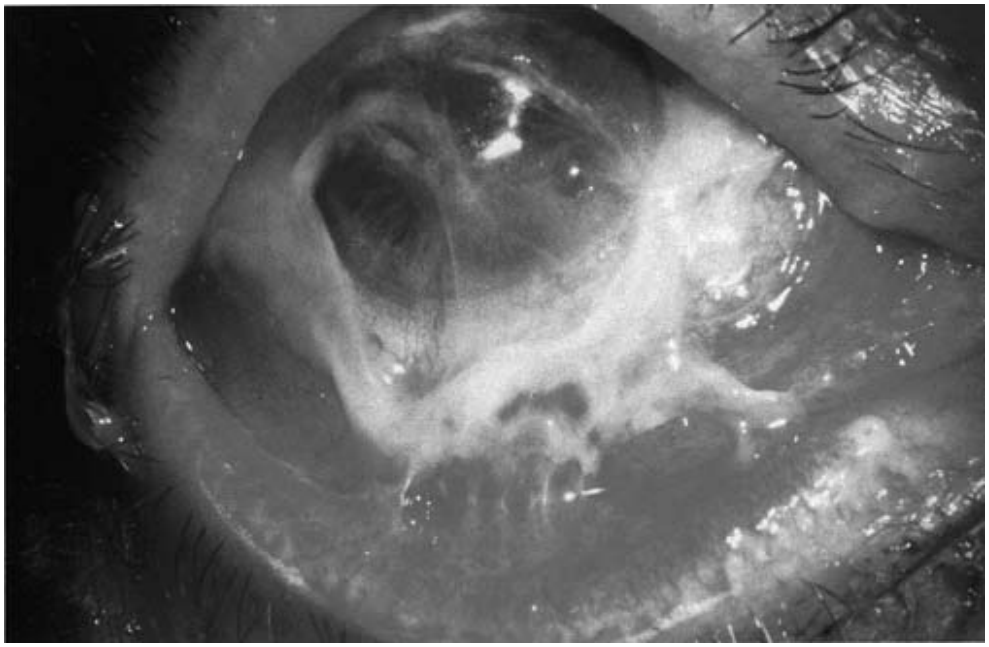


Figure 3 Epidemic keratoconjunctivitis. Infection with adenovirus serotype 19 has resulted in severe ocular surface inflammation. **(For color references see Color Plate 3.)**

clinically from herpes simplex keratitis, and the true incidence of Epstein–Barr viral keratitis is unknown. Reports of uveitis and retinochoroiditis are unconfirmed. Delayed-onset optic neuritis following infectious mononucleosis is not uncommon.

Human cytomegalovirus (CMV) typically causes infectious retinitis (Fig. 4) in immunocompromised patients with CD4+ T cell counts of less than 50 cells μl^{-}. Although not the most common ocular complication of AIDS, CMV retinitis is the most common cause of blindness in AIDS patients. CMV retinitis in AIDS patients can be controlled but not cured. In contrast, congenital cytomegalovirus infection in an otherwise normal fetus results in various degrees of retinochoroiditis, but is not progressive postnatally.

Human papillomavirus (HPV) causes a range of conjunctival tumors ranging from venereally acquired benign papillomas (HPV types 6 and 11) to invasive squamous cell carcinoma (HPV types 16 and 18). Venereal papillomas are clinically similar to those of the larynx and anogenital tract. Conjunctival intraepithelial neoplasia and invasive squamous cell carcinoma are most similar to dysplastic intraepithelial and invasive squamous lesions of the uterine cervix. Papillomatous eyelid neoplasms due to HPV also occur, and can be benign or malignant.

Molluscum contagiosum virus is a poxvirus that may infect the eyelid skin or less commonly the conjunctiva. Skin lesions typically appear as elevated nodules with umbilicated centers, and may be multiple and quite large in HIV-infected patients. Molluscum lesions of the eyelid are fairly common in children, and can be associated with a follicular conjunctivitis that resolves with incisional or excisional biopsy of the lid lesion.

Prior to eradication, smallpox virus infection was associated with pustular blepharoconjunctivitis, secondary lid scarring and stromal keratitis. In nonindustrialized nations, secondary bacterial infection of smallpox keratitis was a major source of blindness. Vaccination against smallpox virus with vaccinia virus was occasionally complicated by inadvertent autoinoculation of vaccinia into the eye, with potential for a severe blepharoconjunctivitis, keratitis and globe perforation.

Ocular Complications of AIDS

Tay-Kearney and Jabs (1996) classified the ocular complications of HIV infection into five broad categories: (1) HIV retinopathy; (2) opportunistic ocular infections; (3) ocular adnexal neoplasms; (4) neuroophthalmic lesions; and (5) drug-induced manifestations.

HIV retinopathy is seen in over half of AIDS patients; cotton-wool patches, or multifocal infarcts of the retinal nerve fiber layer, are the most common ocular sign of AIDS. Intraretinal hemorrhages occur less often. HIV can be cultured from the retina of

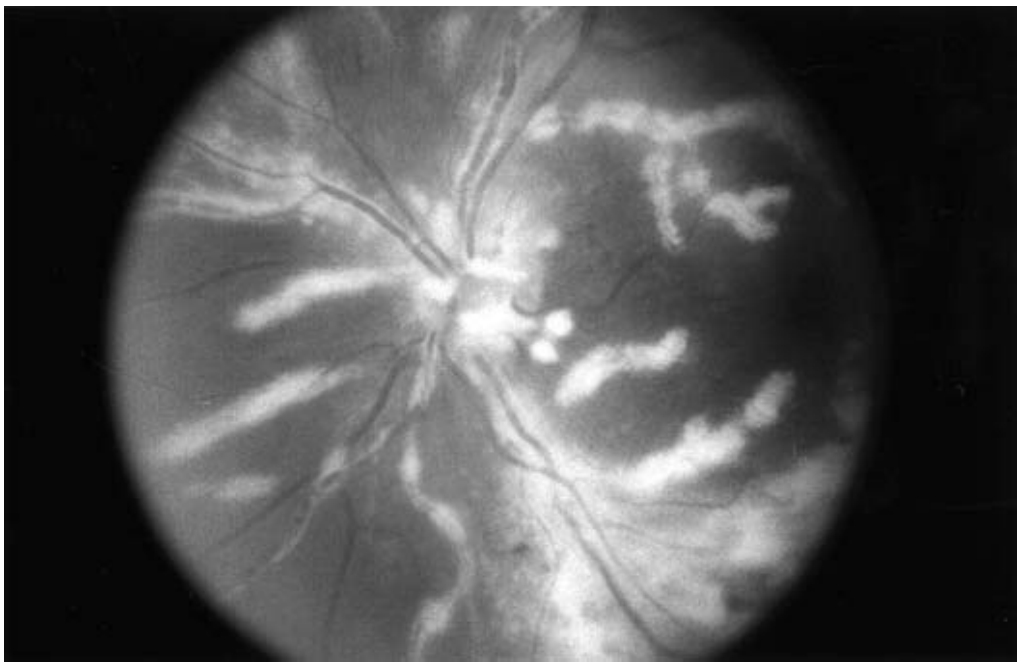


Figure 4 Cytomegalovirus retinitis. Discrete areas of perivascular necrosis and hemorrhage are typical. **(For color references see Color Plate 4.)**

AIDS patients, but a direct relationship between retinal infection and AIDS retinopathy has not been established.

Some ocular infections, including CMV retinitis (**Fig. 4**), *Pneumocystis carinii*, fungal and mycobacterial choroiditis, and microsporidial keratoconjunctivitis are seen almost exclusively in AIDS. Cytomegalovirus retinitis is a major cause of morbidity in AIDS patients. Other infections, such as toxoplasmosis retinochoroiditis, ocular syphilis, herpes zoster ophthalmicus, and molluscum contagiosum of the eyelids are seen in immunocompetent as well as immunosuppressed individuals, but may be more severe and leave more profound deficits in HIV-infected patients. Herpes zoster ophthalmicus in young patients may be the first clinical clue to HIV infection. Acute retinal necrosis due to herpes simplex virus types 1 or 2, or varicella zoster virus, occurs more commonly in HIV-infected than in otherwise normal patients and can result in unilateral or bilateral blindness despite antiviral therapy.

Kaposi sarcoma of the eyelids or conjunctiva, associated with human herpes virus 8 infection, is exceedingly uncommon in immunocompetent individuals, but is probably the most common adnexal tumor in AIDS patients. Non-Hodgkin's lymphomas of the orbit, although rare overall, occur more frequently in AIDS patients than in the general population. Recently, squamous cell carcinoma of the conjunctiva and cornea has been suggested as a marker for AIDS, but whether HIV infection potentiates HPV-induced carcinogenesis in the eye remains speculative.

Neuro-ophthalmic lesions in AIDS may occur directly due to HIV infection of the central nervous system, but most commonly are caused by cryptococcal meningitis or other opportunistic infections. Retinitis and uveitis due to anti-HIV medications can be confused with opportunistic intraocular infections.

Conclusion

Diverse ocular tissues act in concert to create vision. All of the tissues and structures within the eye are susceptible to viral infection, with consequences ranging from mild discomfort to severe pain and blindness, and almost all known human viruses cause ocular disease. Often, the same virus can infect widely disparate tissues within an eye. Classical viral pathogenic mechanisms are readily demonstrated in the eye, but the fine functions of ocular tissues within the visual axis (cornea, anterior chamber, lens, vitreous, and macula) compel altered immune responsiveness. The eye is uniquely affected by viral infection and provides an exceptional model for studies of viral pathogenesis and immunity.

Further Reading

Chodosh J and Stroop WG (1998) Introduction to viruses in ocular disease. In: Tasman W and Jaeger EA (eds) *Duane's Foundations of Clinical Ophthalmology*, Phi-

ladelphia: Lippincott Williams & Wilkins, ch. 85, pp. 1–10.

Darrell RW (ed.) (1985) *Viral Diseases of the Eye*. Philadelphia: Lea & Febiger.

Pepose JS, Holland GN, Wilhelmus KR (eds) (1995) *Ocular Infection & Immunity*. New York: Mosby.

Streilein JW (1996) Ocular immune privilege and the Faustian dilemma. *Invest. Ophthalmol. Vis. Sci.* 37: 1940.

Tay-Kearney ML and Jabs DA (1996) Ophthalmic complications of HIV infection. *Med. Clin. North Am.* 80: 1471.

F

FABAVIRUSES (*COMOVIRIDAE*)

JI Cooper, Institute of Virology and Environmental Microbiology, Oxford, UK

History

In 1947, Stubbs attributed vascular wilt symptoms in broad bean (*Vicia faba*) to a virus that he named after the syndrome. The genus *Fabavirus* derives from the Linnean species name of that host.

Broad bean wilt disease had been noticed more than a decade earlier in the state of Victoria, Australia, and the pathogen (broad bean wilt virus, BBWV) has continued to be a cause of significant local plant death and periodic epiphytotics. Soon thereafter, Smith noticed a disease in broad beans growing near Cambridge, UK. An agent transmitted from these plants caused necrotic lesions in inoculated leaves of *Nicotiana glutinosa* and subsequently the 'same' virus was identified in nasturtium (*Tropaeolum majus*). Surprisingly, Smith named his isolates after the common name of this host (nasturtium) but incorporated a symptom descriptor from the tobacco indicator he had used. Hence the name nasturtium ringspot virus (NRSV) entered the literature. From time to time over the intervening years, a range of similar viruses were isolated from carrot (*Daucus carota*), french bean (*Phaseolus vulgaris*), globe artichoke (*Cynara scolymus*), lilac (*Syringa vulgaris*), parsley (*Petroselenium hortense*), spinach (*Spinacea oleracea*), *Catalpa, Digitalis, Galvezia, Helleborus, Petunia, Phytolacca* and plantain (*Plantago lanceolata*) in Europe, and from dogwood (*Cornus florida*), lettuce (*Lactuca sativa*), peas (*Pisum sativum*), spinach, *Amaranthus retroflexus, Pl. lanceolata*, and *Sonchus asper* in the USA. This short list indicates the diversity of potential hosts; BBWV occurs worldwide in many of the crops (notably pulses) which are traded internationally. There is some evidence that BBWV *sensu lato* occurs in South America and spreads in Japan, where narcissus (*Narcissus tazetta*), patchouli (*Pogostemon patchouli*) and eggplant (*Solanum melongena*) are infected, in Africa, where grape (*Vitis vinifera*) and pepper (*Capsicum annuum*) are recorded hosts, in the Peoples Republic of China (infecting many plant species) and in the Republic of Singapore where *Megaskepasma erythrochlamys* is infected. There are no records of BBWV isolation from plants growing in the Indian subcontinent but fabaviruses are most unlikely to be absent from that region.

Thus, as a consequence of work in several crops and continents, a variety of similar virus isolates with an array of common names were described and not all of these have been fully characterized. In and before 1975, Doel concluded that NRSV is a strain of BBWV (which has historical presidence). In 1982, Lisa *et al* characterized an isolate of Lamium mild mosaic virus (LMMV), which was first recorded in 1953 from wild labiates *Lamium album, L. purpureum, Marrubium peregrinum* and *M. vulgare* showing mild mosaic or no symptoms at all when growing near Cambridge, UK, and showed why LMMV should be considered as a fabavirus distinct from BBWV.

Taxonomy and Classification

Fabaviruses are in the genus *Fabavirus* of the family *Comoviridae*. Species in the genus include BBWV-1, BBWV-2 and LMMV. Other genera in the family are *Comovirus* and *Nepovirus*. Fabaviruses are very similer to comoviruses in their capsid composition/morphology, in the sizes of RNA-1/RNA-2 and in the associated cytopathology. Comoviruses, nepoviruses and fabaviruses have been segregated largely on the basis of their vectors and on the fact that they are not known to share any antigenic determinants.

Virus Structure and Composition

Virions of fabaviruses tend to aggregate and no isolate is convenient to purify in large amounts. The particles, which do not have envelopes and are icosahedral (having diameters that have been reported to be in the range 22–32 nm) are very stable and sediment as three components with $s_{20,w}$ of 56–63,

93–100 and 113–126 for T, M and B respectively. M particles contain about 25% RNA and B particles about 35% RNA. The topmost component (T) in rate density gradients is virtually free of RNA. The A_{260}/A_{280} ratio of unfractionated virions was 1.65 and the separate components have values of 1.69 (M), 1.79 (B) for BBWV or 1.00 (T), 1.71 (M) and 1.79 (B) for NRSV. Neither lipid nor carbohydrate has been associated with purified particles. The buoyant densities of M and B components in cesium chloride – Igepon T73 have been estimated as 1.40 and 1.44 g ml^{-1}, respectively. A significant feature of the virions is that each fabavirus which has been analyzed using SDS-PAGE contained two polypeptides in its capsid. The peptides of NRSV and BBWV comigrate and have M_r of 43 000 and 27 000 but neither of these species comigrated with the peptides from capsids of LMMV which, when judged in this way, had M_r of 45 000 and 28 500.

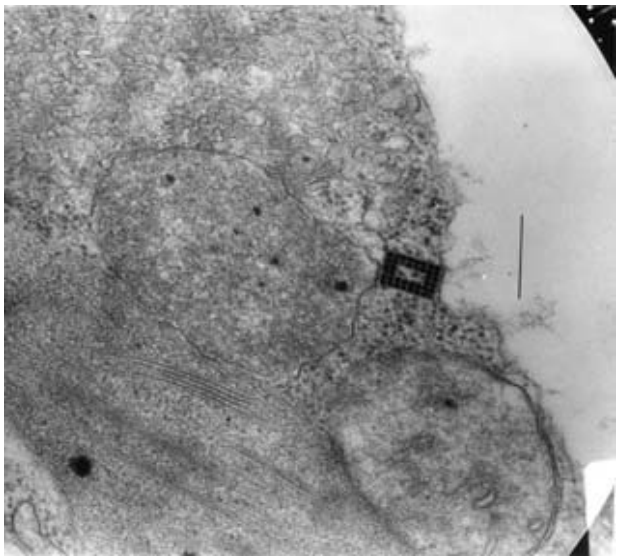


Figure 1 Perpendicular cross-section across a four-sided tubule formed by virions of BBWV. Scale bar = 300 nm. Photograph courtesy of Jorge Vega, Department of Plant Physiology, Institute of Biology, Campinas State University, Brasil.

Genome Structure

The genomes of NRSV and BBWV are linear single-stranded RNA (ssRNA) in two parts that are encapsidated separately. When denatured with formaldehyde, RNA-1 has a molecular weight estimated as 2.0×10^6 Da and RNA-2 a value of 1.5×10^6 Da. When not denatured, RNAs from virions of LMMV, BBWV and NRSV have different molecular sizes; the equivalent molecular weights deduced for LMMV are 2.23×10^6 and 1.59×10^6 Da whereas the values for NRSV are 2.02×10^6 and 1.47×10^6 Da, respectively, with data for BBWV being intermediate. There is some scatter in the data concerning the RNA base ratios of fabaviruses but they are characterized by a high A + U content; another of many features which they share with comoviruses and nepoviruses. Proteinase K did not substantially alter the infectivity of three fabaviruses suggesting that a genome-linked virus-coded protein (VPg), if present, is not required for biological function, a feature which fabaviruses share with some comoviruses but not all nepoviruses.

Cytopathology

Virions of all the fabaviruses occur in the cell cytoplasm of foliar mesophyll and epidermis. In some isolates, virions, virus-like shells and probably also capsid protein assemble into a variety of ordered arrays (tubules, rings, square patterns) that can be recognized by electron microscopy (**Figs. 1–3**). Amorphous X bodies (spindle-shaped fibrous or crystalline inclusions visible using light microscopy) may have some diagnostic value. In this respect, the cytopathology of fabaviruses resembles that of some tymoviruses and is unlike that of comoviruses or nepoviruses. Fabaviruses resemble nepoviruses and comoviruses in that single rows of virus-like particles (VLPs) occur in tubules in cells of infected plants. However, the tubules in nepovirus-infected cells tend to be double-walled, whereas those in comovirus-infected tissue tend to be single-walled. Interestingly, cells supporting the NRSV isolate of BBWV contain single-walled tubules. When the same fabavirus isolate was examined in more than one host, the cytopathology has been consistent, implying that the inclusion bodies are features directed by the viruses rather than the hosts. Too few fabavirus isolates have been examined in sufficient detail to form a reliable basis for judging the value of cytopathology for differentiating virus isolates.

Host Range and Symptomatology

Fabaviruses kill lettuce, bean and spinach plants and cause necrotic streaks in foliage of some pea cultivars. Symptoms in other crops are usually transient and have little commercial significance, except in the People's Republic of China where complex intercropping patterns favor damaging epiphytotics also involving celery (*Apium graveolensis*) and pakchoi (*Brassica chinensis*). Along the fertile Yangtse river, soybean (*Glycine max*) and *A. retroflexus* and *Pseudostellaria heterophylla* (an important medicinal plant) are significant summer hosts of BBWV and

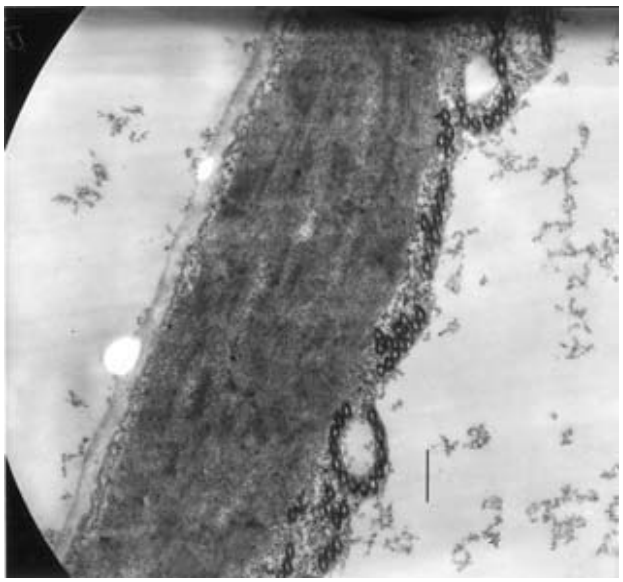


Figure 2 Near-perpendicular section across cylindrical tubules lining the tonoplast. Scale bar = 500 nm. Photograph courtesy of Jorge Vega, Department of Plant Physiology, Institute of Biology, Campinas State University, Brasil.

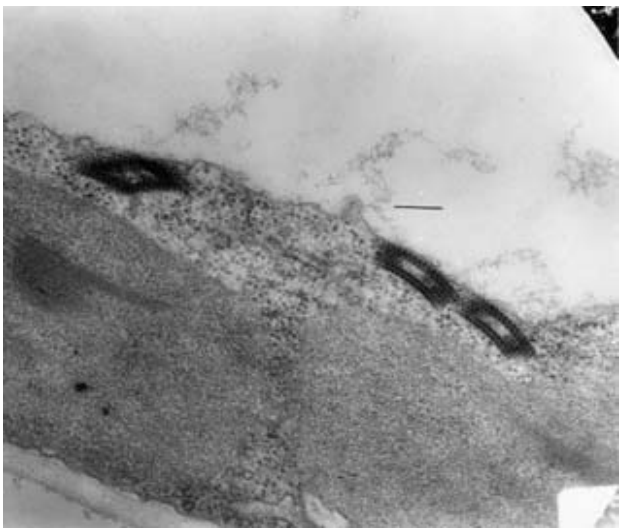


Figure 3 A group of three four-sided tubules sectioned in a plane oblique to their long axis and in contact with the tonoplast. Scale bar = 300 nm. Photograph courtesy of Jorge Vega, Department of Plant Physiology, Institute of Biology, Campinas State University, Brasil.

vector aphids. In other countries, natural hosts of fabaviruses are numerous and are thought to act as amplifying reservoirs from which the viruses spread to cultivated crop species. No systematic surveys have been done to determine the prevalence of fabaviruses in the uncultivated flora but there are many recorded instances of natural infections in trees or herbaceous nonfood plants. BBWV is recorded as seed-borne in fababeans (0.4–0.6%) but not in nasturtium; there are no comparable data on LMMV.

The experimental host range of fabaviruses is wide. Edwardson and Christie (1986) listed 328 species of 186 genera in 44 families as infectable by BBWV; of these, 40 species in 16 genera were in the family *Leguminosae*. LMMV has been less extensively tested but differs from BBWV/NRSV in not infecting, for example, cowpea (*Vigna unguiculata*) or tomato (*Lycopersicon officinale* cv. Mamande). Furthermore, LMMV produces only local lesions in inoculated leaves of *V. faba* or *P. vulgaris*. Published data suggest that symptoms associated with the fabaviruses have only limited diagnostic value, not only because they are inconsistent but also because they vary seasonally and tend to be transient or partial, if indeed present at all.

As glasshouse indicators, *V. unguiculata, Chenopodium amaranticolor* and *Chenopodium quinoa* are usually reliable bioassay hosts. For maintenance or propagation, *V. faba, C. quinoa* or *Nicotiana clevelandii* are convenient for BBWV, whereas *N. clevelandii* is the most suitable host for LMMV.

Serology

The virions are strongly immunogenic, several polyclonal (rabbit) sera with moderate titers (1/2000) are available. Traditionally, gel-diffusion tests have been used to confirm diagnoses and to assess relationships among putative fabaviruses. When testing for fabaviruses in some hosts (e.g. *V. faba*) satisfactory precipitation requires the presence of a reducing agent in the diluent and in the gel. Immunodiffusion tests with LMMV and, to a lesser extent, the other fabaviruses that have been appropriately tested, are unusual in that their precipitation is facilitated more by citrate than, for example, phosphate-buffered saline.

Fabaviruses, as presently constituted, can be divided into two distantly related serological groups with LLMV representing a third, more distinct, type. NRSV, petunia ringspot and the parsley isolates are serologically indistinguishable from the type (Australian) isolate of BBWV which has been designated serotype I. This serotype seems to predominate in Europe.

A virus obtained from *Helleborus vescarius* growing in a commercial nursery in the UK gave a reaction of identity with a plantain isolate of BBWV from Argentina. These isolates, plus another from *Sisymbrium irio* grown in Argentina, one from dogwood in the USA and one from periwinkle (*Catharanthus roseus*) in Australia failed to react visibly in gels with sera specific for viruses in serotype I and were therefore designated serotype II. All the BBWV

isolates (about 20) so far characterized in the People's Republic of China and the one isolate obtained in Singapore are attributed to serotype II. Isolates differing from BBWV serotype I, BBWV serotype II and LMMV have been recorded in *Pl. lanceolata* growing in Italy, *Ajuga reptans* in Australia, patchouli in Japan and in globe artichoke from several countries but too few tests have been done to decide whether or not these are distinct fabaviruses or additional serotypes of BBWV. However, one virus isolated in Northern Italy from *Phytolacca americana* differs serologically from both BBWV serotypes I and II and all other suspected fabaviruses.

As judged by electron microscopy (antiserum-coated grids/decoration), virions representing the serotypes I or II of BBWV have common antigens. Furthermore, use of cross-absorbed sera indicated that a few antigens present in the type isolate of BBWV are not present in NRSV although both viruses are assigned to serotype I. Viruses attributable to BBWV serotypes I or II cannot be reliably differentiated on the basis of their pathological properties. However, one fabavirus isolate, from *Cynara scolymus* and named artichoke French latent virus, has been assigned by some authors to serotype III of BBWV and was reported as causing distinctive symptoms in *C. amaranticolor, Vigna radiata or Nicotiana tabacum* cv Xanthi-nc thereby differentiating it from LMMV or representative isolates of the other BBWV serotypes.

In electron microscope serology, LMMV was clumped but not decorated by undiluted sera prepared against viruses representing either serotypes I or II of BBWV. In gel-diffusion tests LMMV did not react visibly with antisera against BBWV serotype II. However, in gels, a serum with an homologous titer of 1/2048 (determined in mixed liquids) against a parsley isolate of BBWV (serotype I) reacted at 1/4 with LMMV. These fabaviruses have few if any of their antigens in common with comoviruses, tymoviruses or with nepoviruses including strawberry latent ringspot nepovirus (SLRV).

Virus Transmission

In contrast to nepoviruses such as SLRV which are transmitted by *Xiphinema* nematodes and comoviruses or tymoviruses that have beetle vectors (or no known vector), fabaviruses are transmitted by aphids in the nonpersistent manner (although it is worth noting that the vector potential of nematodes or beetles for fabaviruses does not seem to have been assessed). Whereas nepoviruses and comoviruses are transmitted from seed to seedling progeny, there is only one report showing that BBWV is seed transmitted and there are many other experiences which show that BBWV is not seed transmitted. These properties of fabaviruses can be exploited in that the analysis of virions purified from plants inoculated using aphids rather than by manual means helps to eliminate the risk of nepovirus or comovirus contamination in isolates obtained from 'the field'.

The list of known vector aphids extends to some 20 species including *Acyrthosiphon pisum, Aphis craccivora, A. nasturtii* and *Macrosiphum euphorbiae*. However, there are unpredictable and unexplained differences (some relative and others absolute) between fabavirus isolates in the efficiencies of their transmission by different aphid species. Thus, LMMV is transmitted efficiently by *Cryptomyzus alboapicalis* whereas *Myzus persicae*, which is an inefficient vector of LMMV, is the most efficient vector for the type isolate of BBWV (serotype I). Furthermore, *Aphis fabae* transmitted the NRSV isolate of BBWV but not a parsley isolate (PV3) also attributable to serotype I. Similarly, whereas *Myzus persicae* did not transmit artichoke French latent fabavirus between *Physalis floridana*, isolates from globe artichoke were transmitted from and to physalis by *Capitophorus horni* aphids.

Surprisingly, having regard to the abundance and stability of virions in systemically invaded plants, nasturtium isolates of BBWV were not transmitted by the vascular parasitic plant dodder (*Cuscuta californica, C. campestris* or *C. subinclusa*) except when source plants also contained the unrelated cucumber mosaic cucumovirus. This phenomenon of assisted transmission has not been investigated in detail. The transmission of fabaviruses by aphids is not known to require the presence in source plants of another 'helper' virus.

See also: **Comoviruses (*Comoviridae*); Nepoviruses (*Comoviridae*); Tymoviruses.**

Further Reading

Doel TR (1975) Comparative properties of type, nasturtium ringspot and petunia ringspot strains of broad bean wilt virus. *J. Gen. Virol.* 26: 95.

Edwardson JR and Christie RG (1986) Viruses infecting forage legumes. Vol. I. *Florida Agric. Exp. Station Monogr.* 14: 95.

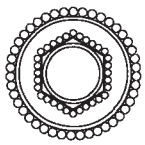
Lisa V and Boccardo G (1996) Fabaviruses: broad bean wilt and allied viruses. In: Harrison BD and Murant AF (eds), *The Plant Viruses*, vol. 5, pp. 229–250. New York: Plenum Press.

Lisa V, Luisoni E, Boccardo G, Milne RG and Lovisolo O (1982) Lamium mild mosaic virus: a virus distantly related to broad bean wilt. *Ann. Appl. Biol.* 100: 467.

Uyemoto JK and Provvidenti R (1974) Isolation and identification of two serotypes of broad bean wilt virus. *Phytopathology* 64: 15.

Feline Calicivirus *see* **Caliciviruses**

FELINE IMMUNODEFICIENCY VIRUS (*RETROVIRIDAE*)



Janet K Yamamoto, University of Florida, College of Veterinary Medicine, Gainsville, Florida, USA

History

Since the isolation of the human immunodeficiency virus (HIV) in 1983, the search for its counterpart in domestic and laboratory animals has been initiated in a number of laboratories. Prior to the discovery of HIV, retroviruses in the subfamily *Onconovirinae* were better known for their immunosuppressive potential than were the ungulate retroviruses belonging to the same lentivirus subfamily as HIV. Furthermore, none of the animal lentiviruses known till then had antigenic characteristics or clinical manifestations resembling those of acquired immunodeficiency syndrome (AIDS). Animal lentivirus models with clinical disease more closely resembling AIDS were deemed important for research, leading to prophylaxis and therapy of HIV infection and disease. Clinic and laboratory animals ranging from mice to non-human primates with signs of immunodeficiency were evaluated for potential lentiviral infection. Isolation of a lentivirus from a colony of pet cats with clinical symptoms of immunodeficiency-like syndrome in California was reported in 1987. The lentiviral origin of the new feline retrovirus was based initially on the biochemical, antigenic and morphologic characteristics. However, in 1989, sequence analysis of its genome further confirmed its lentiviral lineage and the virus was renamed feline immunodeficiency virus (FIV) in compliance with international nomenclature.

The discovery of FIV in domestic cats has also led to the reassessment of immunodeficiency cases that were previously placed under the category of unknown etiology. FIV causes a naturally occurring infection in domestic cats that is clinically similar to HIV in its immunopathogenesis. FIV infection causes chronic lifelong infections which are followed by a progressively degenerative immune disorder in cats. As with HIV, FIV infection has been found worldwide and has a major impact in veterinary medicine.

Taxonomy and Classification

Based on morphology, genomic organization and nucleotide sequence analysis, FIV belongs to the genus *Lentivirus* in the family *Retroviridae*. Phylogenetic tree analyses of amino acid sequence indicates that FIV is more closely related to the ungulate lentiviruses, equine infectious anemia virus and visna virus, than to the primate lentiviruses, HIV and simian immunodeficiency virus (SIV). FIV has been classified into five clades or subtypes (A–E) based on nucleotide sequence homology at viral *env* and *gag*. Serological survey has demonstrated that wild cats, including lions, pumas, leopards, ocelots and jaguars, have antibodies that react with FIV. Based on phylogenetic analysis, wild cats are infected with lentivirus distantly related to the domestic FIV isolates. Although over 80% of the Serengheti lion population may be infected with lion lentivirus (FIV-Ple), Asian lions appear to be free of such infection. Currently FIV-Ple is classified into three subtypes based on the reverse transcriptase coding sequences of the *pol* gene.

Geographic Distribution

Based on serological surveys, and more recently on sequence analyses, FIV infection of domestic cats occurs worldwide. In general, FIV subtypes distribute according to the geographic cluster. Subtype A is found predominantly in California and Europe, subtype B in central Japan and the USA (excluding California), subtype C in British Columbia, subtype D in southern Japan, and subtype E in Brazil.

Virion and Genome Structure

The FIV virions are 100–125 nm in size and consists of an outer envelope surrounding a nucleocapsid core

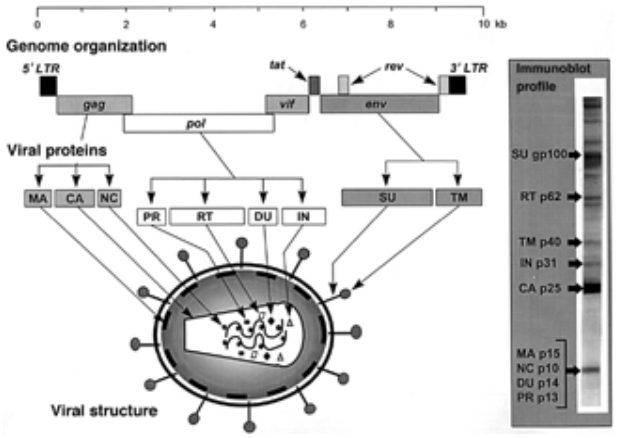


Figure 1 FIV genome organization, viral structural proteins and enzymes, general structure of the virion and the profile of an FIV immunoblot strip reacted with antisera from an FIV-infected cat.

(**Fig. 1**). The nucleocapsid core has the physical appearance of a cone, typical of all nonungulate lentiviruses. The FIV genome consists of two identical single-stranded RNA molecules of approximately 9.5 kb. Associated with the viral genome is nucleoprotein (NC, p10), which may be involved in viral assembly. The viral genome, nucleocapsid protein and viral enzymes are all packaged within the nucleocapsid core. Viral enzymes consist of the reverse transcriptase (RT, p62), integrase (IN, p31), deoxyuridine triphosphatase (dUTPase or DU, p14), and protease (PR, p13). The dUTPase is unique to the nonprimate lentiviruses and its function in FIV replication remains unknown. However, speculation is that dUTPase is important in FIV replication in nondividing macrophages or resting T lymphocytes. The capsid core, composed of capsid protein (CA, p25), is surrounded by a lining of myristoylated matrix protein (MA, p15) associated with the viral envelope. Myristoylation of MA protein is unique to HIV and SIV and only nonmyristoylated MA proteins are found in ungulate lentiviruses. The viral envelope consists of surface (SU, gp100) and transmembrane (TM, gp40) glycoproteins, projecting from the lipid bilayer which is derived from cellular membrane.

The FIV genome, in common with other lentiviral genomes, is more complex in organization than other retroviruses. In addition to the general genomic organization of retroviruses, *5'-LTR-gag-pol-env-LTR-3'*, regulatory genes are found in the open reading frames (ORF 1–4) overlapping or flanking *5'* and *3'* ends of the *env* gene. Based on alignment analysis and physiochemical properties of the predicted polypeptide, *orf 1* is speculated to be analogous to *Q orf* of visna virus and *vif* gene of HIV-1. *Orf 2* is predicted to be the first exon of *tat* gene found in primate lentiviruses. *Orf 3* and *orf 4* are analogous to the exons of *L* gene of visna virus and *rev* gene of HIV-1, HIV-2 and SIV, both of which encode for a splice gene product important in production and transport of viral RNA transcripts.

Host Range and Virus Propagation

FIV has limited host range in that productive infection is found only in felid species. Domestic FIV has been found in the wild cat population, Tsushima cat (*Felis bengalensis euptilura*), and may present problems to the conservation of this endangered species. In domestic cats, FIV infects macrophages, endothelial cells, B cells, and CD4+ T cells as well as CD8+ T cells. In addition, FIV has been shown *in vitro* to infect feline astrocytes and fibroblastic Crandell feline kidney cells (CrFK). Defective infection of a human cell line has been reported; however, there has been no evidence of zoonotic transmission and infection of humans with FIV. As with HIV, propagation of FIV in culture will rapidly adapt the virus and appears to alter the tissue tropism. Thus, the use of laboratory strains for vaccine challenge has been criticized and challenge inocula from *in vivo* sources have been recommended.

Genetics and Evolution

Owing to the large spectrum of genetic variability between worldwide FIV isolates, FIV is currently classified into five subtypes (A–E). Studies on mutation rates in FIV *env* and *gag* have shown positive selective pressure for *env* mutations, consistent with reports that HIV *env* has a high mutation rate compared with *gag* and *pol*. Polymerase chain reaction analyses of FIV variants isolated within individual cats over a 3 year period indicate that sequence variation in *env* increases over time, with later isolates showing divergence of 0.5–1.5%. Hence, the divergence of the variants within individual cats appears to be less than in those variants from an individual infected with HIV.

Phylogenetic analyses of lentiviral *pol* reveal that domestic FIV isolates are more closely related to equine infectious anemia virus and visna virus than to the primate lentiviruses, SIV and HIV. Similar analysis reveals that the domestic FIV (FIV-Fca) is more closely related to lion (FIV-Ple) and puma (FIV-Pco) lentiviruses. FIV-Ple can cause lytic infection in domestic cat T lymphoma cells (3201), suggesting

cross-species transmission. Interestingly, a large number of African lions are infected with FIV-Ple and yet they appear to coexist without apparent pathology. These findings have led to the speculation that the domestic FIV originated from a lineage associated with the African lion lentivirus.

Serologic Relationships and Variability

Antisera from cats infected with different FIV strains react to prototype FIV isolate (FIV_{Pet}). The antibody crossreactivity of the infected sera has been the basis for the diagnostic kit commercially available for FIV. Similarly, antisera from wild cats infected with nondomestic lentivirus reacted with domestic FIV isolates, and vice versa. Rabbit polyclonal antibodies to ungulate lentiviruses, Visna-Maedi virus, and caprine arthritis encephalitis virus, also reacted to domestic FIV at major and minor core proteins (p25 and p15), demonstrating the conserved epitopes of these structural proteins.

The crossreactivity of the FIV antibodies appeared to be limited to the conserved epitopes on the FIV structural proteins and was less frequently detected at the virus-neutralizing domains. In general, FIV-neutralizing antibodies can neutralize highly related strains but not divergent strains. FIV subtyping is based on genotype and little is known about the correlation between the genotype and the cross-neutralizing antibodies elicited by strains from the same and different subtypes. Since neutralizing antibodies generally react to domains in the envelope region, correlation between neutralizing antibodies and *env* sequences may provide insight as to whether antibodies to FIV strains within a subtype can more efficiently crossneutralize than those between subtypes. Preliminary studies suggest that a loose correlation exists between the genotype based on *env* sequence and the virus-neutralizing antibodies elicited by infected cats.

Epidemiology

The worldwide prevalence of FIV infection ranges from 3 to 43.9% in high-risk groups (consisting of symptomatic cats) and 1 to 12.4% in asymptomatic pet cat populations. Serological surveys show that male cats are 2–3 times more likely to be infected than female cats and that FIV infection is found more frequently in cats over 5 years of age than in younger cats, especially those under 1 year old. Furthermore, free-roaming cats have a higher incidence of infection than with indoor cats. Since male cats are territorially aggressive and have higher incidence of wounds and bite abscesses, the high prevalence of FIV infection in male free-roaming cats is consistent with the major mode of FIV transmission via biting.

Transmission and Tissue Tropism

The identity of FIV receptor(s) is still unknown. Although significant loss of CD4+ cells occurs during FIV infection, findings from transinfection studies suggest that the feline CD4 molecule may not be the receptor for FIV. In initial studies, an adhesion molecule, CD9, present on feline B cells, monocytes and T cells was thought to be the FIV receptor. However, recent findings suggest that CD9 play a role at the postentry stage of the viral life cycle and is not the receptor for FIV. It has been reported that chemokine receptor, feline CXCR4, acts as a co-receptor for FIV infection in the way human CXCR4 serves as coreceptor for HIV in human T cells. In recent studies, feline CXCR4 has been shown also to serve as coreceptor for HIV. Although β-chemokine receptor CCR5 has been shown to be a coreceptor for HIV in monocytes, there are no studies demonstrating feline CCR5 as a FIV coreceptor.

Based on experimental transmission studies, the major route of FIV transmission is through bites from infected cats. This route of transmission is consistent with the epidemiological studies as well as with the fact that cats shed significant amount of virus in saliva. FIV transmission by ingestion of virus via grooming, as well as presumably when licking bleeding wounds of an infected cat, cannot be excluded, as oral administration of infected blood can result in experimental FIV infection. Sexual transmission has yet to be reported as a natural route of transmission; however, FIV infection has been demonstrated by experimental vaginal and rectal inoculation and FIV has been isolated from vaginal swabs and semen of experimentally infected cats. Another route of infection is through virus found in the colostrum/milk of infected queens. FIV has been shown to be transmitted to newborn kittens nursed by queens experimentally infected shortly before giving birth. Findings from experimental studies have suggested the possibility of transplacental infection as well as transmission during birth through a contaminated birth canal. In conflict with these experimental observations is the low incidence of FIV infection in kittens born to chronically-infected queens, suggesting that vertical transmission of FIV is rare in nature. This observation is further supported by the epidemiological findings that cats under 1 year of age have the lowest incidence of FIV infection. Thus, it is still uncertain whether transplacental and perinatal routes of transmission play a major role in natural transmission.

Clinical Features and Infection

FIV infection is a natural infection of domestic felid populations and causes a chronic and progressive acquired immunodeficiency syndrome resembling HIV infection of humans. The immunological hallmark of FIV infection is depletion of CD4+ peripheral T cells and reduced CD4:CD8 ratios, leading to B and T cell dysfunctions and hypergammaglobulinemia. T cell dysfunctions include decreased proliferative responses and decreased interleukin 2 (IL-2) production. B cell dysfunctions are less dramatic than T cell dysfunctions, and seem to predominate in primary antibody responses to T cell-dependent antigens, especially during early stages of FIV infection. Interestingly, increased serum IgG levels are also observed in infected cats, indicative of FIV-induced virus-specific B cell hyperactivity akin to that observed in HIV.

Experimental infection studies demonstrate that the clinical stages of FIV infection are similar to human AIDS in several ways. The acute stage of FIV infection is characterized by immunological abnormalities followed by depression, fever, diarrhea, neutropenia and persistent generalized lymphadenopathy. FIV is primarily detected in lymphoid tissues (lymph nodes, spleen, thymus, tonsils and bone marrow), followed by dissemination of the virus into nonlymphoid organs (kidney, lung and liver). In experimental infection studies, both the antibodies against FIV and the virus recovery from peripheral blood lymphocytes persist throughout infection. However, the levels of virus in the blood appear to be lower at the asymptomatic or clinical latency stage than the acute stage. Nevertheless, virus can be readily isolated from lymphoid tissues at the asymptomatic stage. Although there is no major clinical manifestation, the CD4+ T cell counts and CD4:CD8 ratios steadily decline to the extent that, by the late symptomatic stage, the animals are severely immunosuppressed and refractory to supportive treatment. The late stage of FIV infection is similar to that of human AIDS and may be characterized by wasting syndrome, neurologic disorders and a variety of persistent secondary opportunistic infections. The virus load is extremely high at this stage, so that in addition to lymphoid tissues, FIV can be readily isolated from nonlymphoid tissues and organs, such as kidney, saliva and the central nervous system (CNS). In contrast to natural infection, experimentally infected cats housed in a specific pathogen-free environment generally succumb to B cell lymphomas. These lymphomas have FIV proviral integration, suggesting that somewhat innocuous B cell infection can develop into B cell lymphoma of more unusual extranodal forms (found predominantly in the neck and head). The incidence of FIV-induced lymphomas appears to be low in nature. It has been speculated that naturally infected cats succumb to diseases more commonly associated with the immunodeficient state, such as opportunistic infections, before the development of lymphomas.

The major clinical manifestations observed in naturally infected cats are chronic oral diseases, chronic upper respiratory tract disease, chronic enteritis and chronic conjunctivitis. Anorexia, weight loss, lethargy, vomiting, fever of unspecified origin and recurrent cystitis were other signs observed in these cats. Abnormal behavioral problems, lymphosarcoma and myeloproliferative disease were also seen in a small proportion of affected cats. Abnormalities in kidney function and pathology have been reported both in naturally and experimentally infected cats. Glomerulosclerosis, fibrosis, tubular degenerative changes and diffuse interstitial infiltration by phagocytes and plasma cells have been observed in naturally infected cats, while glomerular mesangial cell proliferation has been seen in experimentally infected cats. Overall, natural FIV infection causes progressively degenerative immune disorder, neurologic disorders, wasting syndrome and a variety of persistent secondary opportunistic infections.

Pathology and Histopathology

Major histopathological changes during the acute stage of experimental FIV infection are observed in the lymphoid tissues (thymus, lymph nodes, spleen, tonsils and gut-associated lymphoid tissues). In the first three weeks postinfection, lymphoid hyperplasia is observed in the lymph nodes, tonsils and spleen. The majority of the FIV-infected cells are found in the germinal centers of lymphoid follicles of these tissues. However, some infected cells have been observed in the paracortex and medullary cords of lymph nodes, and in periarterial lymphoid sheaths and the red pulp of the spleen. Shortly after, cats develop myeloid hyperplasia in bone marrow and cortical involution, thymitis and follicular hyperplasia of the medulla in the thymus. Meanwhile, the CD4:CD8 ratio begins to decrease and complete inversion can occur in the acute stage of infection. Both T cells and monocytes/macrophages are infected with FIV at the early phase of the acute stage, followed by infection of B cells. Throughout the asymptomatic stage, a steady loss of the CD4+ T cell population may be the only abnormality detected in infected cats. At the early symptomatic stage, the lymph nodes display a combination of histological lesions, including follicular hyperplasia, involution and lymphoid depletion.

By the late symptomatic stage, destruction of nodal architecture with involution and depletion of lymphoid follicles has been reported.

In comparison to HIV and SIV infection of the CNS, multinucleated cells in the CNS of FIV-infected cats are rare. Furthermore, the number and severity of CNS lesions are much less in experimentally and naturally FIV-infected cats than those observed in HIV-infected individuals or SIV-infected macaques. However, there are many features of the CNS lesions of FIV-infected cats that resemble those induced by HIV and SIV. These include perivascular mononuclear cell infiltrates, glial nodules and diffuse gliosis of gray and white matter, and neuronal loss. Similar to HIV infection, neurotropic FIV strains infect microglia, astrocytes and brain microvascular endothelial cells. Current findings suggest that neuronal cells cannot be infected by FIV. Both anti-FIV antibodies and FIV virions have been isolated from cerebral spinal fluid (CSF) of experimentally and naturally FIV-infected cats, in addition to the elevated IgG index detected in the CSF. Like HIV, the level of FIV infection in the CNS cannot account for the cognitive/motor function abnormalities observed in these cats, suggesting that cytokines induced during CNS infection may play a key role in FIV neuropathogenesis. Neurologic abnormalities included limb paresis, delayed righting and pupillary reflexes, behavioral changes, delayed visual and auditory evoked potentials, decreased spinal and peripheral nerve conduction velocities, and sleep abnormalities similar to sleep disturbances described in AIDS patients. In fact, cats infected with neurotropic FIV-MD strain spent 50% more time awake than the sham-inoculated controls, and approximately 30% of these cats had rapid eye movement (REM) sleep reduction when compared with controls. Neurophysiological abnormalities and sleeping disorders of FIV-infected cats have been more extensively investigated than any other AIDS model.

Immune Response

Both humoral and cellular immune responses to FIV are generated in the infected cats. The nature of immunity elicited appears to be dependent on both the exposure dose and the strain infecting the cats. Some strains do not elicit high or even significant virus-neutralizing antibodies even though high levels of antibodies to their structural proteins, including surface envelope, are produced. Early on during the infection, infected cats produce antibodies to FIV, as well as FIV-specific cellular immunity. The cellular immune responses of these cats include FIV-specific cytotoxic T lymphocyte (CTL) activity and FIV-specific T-helper cytokine production. In spite of the presence of virus-neutralizing antibodies and CTL responses, a steady loss of T cells occurs, resulting in an immunodeficient state. The immunological events that ensue upon FIV infection can be assessed more readily in experimentally infected cats. Upon experimental infection with a moderate dose of FIV, anti-FIV antibodies can be detected as early as 3 weeks postinfection, by FIV ELISA and immunoblot assays, followed by virus-neutralizing antibodies starting about 6 weeks postinfection. A decrease in primary proliferative response to foreign antigen (keyhole-limpet hemocyanin, KLH) has been observed at 5 weeks postinfection, the earliest time point tested. Since the proliferative response to T cell mitogen and the antigen-specific recall response were still unaffected at this time, it has been speculated that selective defects in primary antigen-specific response of naive CD4+ T-helper cells represent the early signs of T cell dysfunction. However, CTL response to FIV Gag peptide can be detected in peripheral blood mononuclear cells at 7–9 weeks postinfection, and to FIV Env peptide by 16 weeks postinfection. Since the earlier time points were not tested for these cellular responses, it is possible that the onset of these cellular changes may be earlier than those described.

A defect in B cell mitogen response has been observed as early as 8 weeks postinfection, before a major decrease in CD4:CD8 ratio occurs. The time of CD4:CD8 inversion depended greatly on the FIV strain used in the study. Overall, the earliest time of CD4:CD8 inversion detected with a highly virulent strain has been 6 weeks postinfection with high dose and 12 weeks with moderate dose. In general, the loss of CD4+ cell numbers accounted for the CD4:CD8 inversion with most FIV strains, but increase in CD8+ cell counts also contributed to this inversion in a number of FIV strains. A defect in proliferative response of memory T cells to recall antigen has been observed at about 19 weeks postinfection. Decreased T cell mitogen responses appear to occur late in the infection when a considerable decrease in CD4:CD8 ratios prevails. In addition to T cell and B cell dysfunctions, functional abnormalities in macrophages, neutrophils and natural killer cells have also been observed in these cats.

Elevated serum levels of proinflammatory cytokines (IL-1, IL-6 and tumor necrosis factor α (TNF-α)) have been reported in naturally infected, symptomatic cats. In contrast, experimentally infected asymptomatic cats maintain normal TNFα levels in the serum, resembling the serum TNFα levels of asymptomatic HIV-positive humans. In spite of the humoral and cellular responses mounted by the infected host, a steady loss in CD4+ T cells continues

throughout the asymptomatic stage, leading to an immunocompromised state which can potentially be fatal to the infected cats. Furthermore, the anti-FIV immunity generated in the infected host is not sufficient to prevent superinfection with other FIV subtype strains. In experimental studies, long-term infected cats (>1 year infection) appear to resist superinfection more than short-term infected cats, suggesting that antiviral immunity broadens with increased duration of infection. However, the presence of FIV recombinant isolates in nature (which are recombinants of two subtypes) also supports the view that immunity generated by primary FIV infection may not be sufficient to prevent superinfection.

Prevention and Control

Currently, no commercial FIV vaccine is available. Experimental FIV vaccine trials ranging from conventional inactivated virus vaccine to proviral FIV DNA vaccines are being tested. Protective efficacy of these vaccines to homologous FIV challenge has been observed. However, protection against highly divergent strains has been more difficult to achieve. Based on studies evaluating the immune correlates of vaccine protection, it has been speculated that an effective FIV vaccine needs to elicit both anti-FIV humoral (virus-neutralizing antibodies) and cellular (CTL) immunity for complete and long-lasting immunity against the virus. Such a vaccine is an achievable goal, as inactivated virus or infected-cell vaccines have been shown to elicit virus-neutralizing antibodies, FIV-specific T-helper proliferative responses and FIV-specific CTL responses. However, these single-subtype vaccines were unable to protect cats against highly divergent strains, especially from different subtypes. As a result, multisubtype FIV immunogens are currently being investigated as a means of broadening protective immunity against worldwide FIV isolates.

A number of antiretroviral drugs have been tested in clinic and experimental cats. These include azidothymidine (AZT), 9-(2-phosphonylmethoxyethyl)adenine (PMEA), dideoxycytidine 5′-triphosphate, dideoxycytidine, lamivudine (3TC), cyclosporine A and interferon α. AZT has been studied most extensively as therapy against FIV. AZT monotreatment suppresses FIV infection in culture; however, its antiviral efficacy in cats has been controversial. In general, high-dose AZT therapies initiated shortly before or after FIV infections caused a significant loss or delay in detection of plasma viral core antigen levels, some delay in development of viral antibodies, and milder FIV-related clinical episodes. However, adverse effects were noted at these high doses, including hemoglobinemia, anemia and depressed packed cell volume. Furthermore, a resurgence of the virus has been observed in cats upon withdrawal of the drug in a majority of these studies. The AZT-resistant FIV mutants have been shown to develop in culture by drug selection. In a more recent study, prophylactic therapy with a high dose of AZT and 3TC combination, started 3 days before experimental FIV inoculation, resulted in complete protection of cats from FIV infection. In contrast to this observation, low-dose therapy with AZT and 3TC combination did not decrease virus load or improve CD4:CD8 ratios and counts of chronically FIV-infected asymptomatic cats. Although the prophylaxis with nucleoside analogues appears to be remarkable, the therapeutic use of these drugs has been somewhat disappointing. However, a promising light has been shed on FIV therapy by a recent finding that some of the drugs initially developed as HIV protease inhibitors have potent anti-FIV activity in culture and enhance inhibition of *in vitro* FIV replication when combined with AZT and/or 3TC. Thus, FIV therapy, like the triple drug combination currently used in HIV-positive individuals, will require multiple drug combination with each drug inhibiting different stages of lentiviral life cycle.

Future

Advances in FIV vaccine development and therapy is important, not only for veterinary medicine but as an animal model for human AIDS. Although our understanding about FIV pathogenesis has greatly increased in recent years, the development of an effective prophylaxis and therapy against FIV has been slow. The lack of immunological reagents for feline species has been one of the initial setbacks in the progress of FIV research. However, within the last decade, more feline reagents are now available as a result of concerted efforts made by laboratories throughout the world. Hence, the FIV–cat system can now serve as a more effective small animal model for AIDS. Innovative therapy, such as immune reconstitution therapy using stem cell growth factor, can be assessed using the FIV model. New generation antiretroviral drugs can be combined with established antiretroviral drugs or with novel immunotherapy and tested in the FIV model. Novel vaccine approaches can be evaluated more rapidly and economically in the FIV model than many of the nonhuman primate lentivirus models. These vaccine studies can increase our understanding of the types of immunity required for vaccine protection. Similarly, better understanding of the mechanisms underlying the FIV-induced immuno-

suppressive diseases may provide new insights into HIV immunopathogenesis, also important in identifying novel prophylactic and therapeutic strategies. Overall, future progress in FIV research should make a major impact in the feline medicine of pet cats and enhance our understanding of the control and prevention of lentiviral infections and diseases.

See also: **Equine infectious anemia virus (*Retroviridae*); Caprine arthritis encephalitis virus (*Retroviridae*); Human immunodeficiency viruses (*Retroviridae*): Molecular biology, Anti-retroviral agents, General features; Visna-Maedi viruses (*Retroviridae*).**

Further Reading

Bachmann MH, Mathiason-Dubard C and Learn GH *et al* (1997) Genetic diversity of feline immunodeficiency virus: dual infection, recombination, and distinct evolutionary rates among envelope sequence clades. *J. Virol.* 71: 4241.

Bendinelli M, Pistello M, Lombardi S *et al* (1995) Feline immunodeficiency virus: an interesting model for AIDS studies and an important cat pathogen. *Clin. Microbiol. Rev.* 8: 87.

Bloom FE and Elder JH (1995) Feline immunodeficiency virus as a model for study of lentivirus infection of the central nervous system. *Curr. Top. Microbiol. Immunol.* 202: 167.

Elyar JS, Tellier MC, Soos JM and Yamamoto JK (1997) Perspectives on FIV vaccine development. *Vaccine* 15: 1437.

Henriksen SJ, Prospero-Garcia O, Phillips TR *et al* (1995) Feline immunodeficiency virus as a model for study of lentivirus infection of the central nervous system. *Curr. Top. Microbiol. Immunol.* 202: 167.

Willett BJ, Flynn JN and Hosie MJ (1997) FIV infection of the domestic cat: an animal model for AIDS. *Immunol. Today* 18: 182.

FELINE LEUKEMIA AND SARCOMA VIRUSES (*RETROVIRIDAE*)

James Neil and **David Onions**, University of Glasgow Veterinary School, Glasgow, Scotland, UK

History

The description of murine leukemia viruses by Moloney and others stimulated an intensive search for similar viruses in other species. William Jarrett made the perceptive observation that lymphomas (lymphosarcomas) in cats often occurred at particularly high incidence in certain households, and in 1964 he showed that typical type C retroviruses could be demonstrated in the tumor cells by electron microscopy. He went on to show that these feline leukemia viruses (FeLVs) could be transmitted to cats where they induced lymphosarcomas and a range of degenerative diseases, including anemias and thymic atrophy.

Following these studies, Snyder and Theilen isolated a retrovirus from a feline fibrosarcoma that rapidly reproduced this tumor on inoculation into experimental cats. It is now recognized that feline sarcoma viruses (FeSVs) arise by recombination between FeLV and cellular proto-oncogenes and that, in contrast to FeLV, these viruses are not transmitted from cat to cat.

In clinical veterinary medicine, FeLV is one of the most important viruses affecting the cat and considerable attention has been paid to controlling this infection. However, FeLV has a wider importance in the study of viral oncogenesis. Research on FeLV has led to the discovery of novel cellular transforming genes and elucidated the complex interactions between this class of retroviruses and their host species.

Taxonomy and Classification

Feline leukemia viruses are RNA viruses and belong to the family *Retroviridae*, genus Epsilonvirus.

Virion and Genome Structure

The virion particles are around 100 nm in diameter and consist of an outer membrane derived from the host cell surrounding an icosahedral core particle. The core encapsidates the viral genome which, as in other members of this viral family, is present as two linear single-stranded (ss) RNA molecules linked as dimers. The virion RNA is positive-stranded and

suppressive diseases may provide new insights into HIV immunopathogenesis, also important in identifying novel prophylactic and therapeutic strategies. Overall, future progress in FIV research should make a major impact in the feline medicine of pet cats and enhance our understanding of the control and prevention of lentiviral infections and diseases.

See also: **Equine infectious anemia virus (*Retroviridae*); Caprine arthritis encephalitis virus (*Retroviridae*); Human immunodeficiency viruses (*Retroviridae*): Molecular biology, Anti-retroviral agents, General features; Visna-Maedi viruses (*Retroviridae*).**

Further Reading

Bachmann MH, Mathiason-Dubard C and Learn GH *et al* (1997) Genetic diversity of feline immunodeficiency virus: dual infection, recombination, and distinct evolutionary rates among envelope sequence clades. *J. Virol.* 71: 4241.

Bendinelli M, Pistello M, Lombardi S *et al* (1995) Feline immunodeficiency virus: an interesting model for AIDS studies and an important cat pathogen. *Clin. Microbiol. Rev.* 8: 87.

Bloom FE and Elder JH (1995) Feline immunodeficiency virus as a model for study of lentivirus infection of the central nervous system. *Curr. Top. Microbiol. Immunol.* 202: 167.

Elyar JS, Tellier MC, Soos JM and Yamamoto JK (1997) Perspectives on FIV vaccine development. *Vaccine* 15: 1437.

Henriksen SJ, Prospero-Garcia O, Phillips TR *et al* (1995) Feline immunodeficiency virus as a model for study of lentivirus infection of the central nervous system. *Curr. Top. Microbiol. Immunol.* 202: 167.

Willett BJ, Flynn JN and Hosie MJ (1997) FIV infection of the domestic cat: an animal model for AIDS. *Immunol. Today* 18: 182.

FELINE LEUKEMIA AND SARCOMA VIRUSES (*RETROVIRIDAE*)

James Neil and **David Onions**, University of Glasgow Veterinary School, Glasgow, Scotland, UK

History

The description of murine leukemia viruses by Moloney and others stimulated an intensive search for similar viruses in other species. William Jarrett made the perceptive observation that lymphomas (lymphosarcomas) in cats often occurred at particularly high incidence in certain households, and in 1964 he showed that typical type C retroviruses could be demonstrated in the tumor cells by electron microscopy. He went on to show that these feline leukemia viruses (FeLVs) could be transmitted to cats where they induced lymphosarcomas and a range of degenerative diseases, including anemias and thymic atrophy.

Following these studies, Snyder and Theilen isolated a retrovirus from a feline fibrosarcoma that rapidly reproduced this tumor on inoculation into experimental cats. It is now recognized that feline sarcoma viruses (FeSVs) arise by recombination between FeLV and cellular proto-oncogenes and that, in contrast to FeLV, these viruses are not transmitted from cat to cat.

In clinical veterinary medicine, FeLV is one of the most important viruses affecting the cat and considerable attention has been paid to controlling this infection. However, FeLV has a wider importance in the study of viral oncogenesis. Research on FeLV has led to the discovery of novel cellular transforming genes and elucidated the complex interactions between this class of retroviruses and their host species.

Taxonomy and Classification

Feline leukemia viruses are RNA viruses and belong to the family *Retroviridae*, genus Epsilonvirus.

Virion and Genome Structure

The virion particles are around 100 nm in diameter and consist of an outer membrane derived from the host cell surrounding an icosahedral core particle. The core encapsidates the viral genome which, as in other members of this viral family, is present as two linear single-stranded (ss) RNA molecules linked as dimers. The virion RNA is positive-stranded and

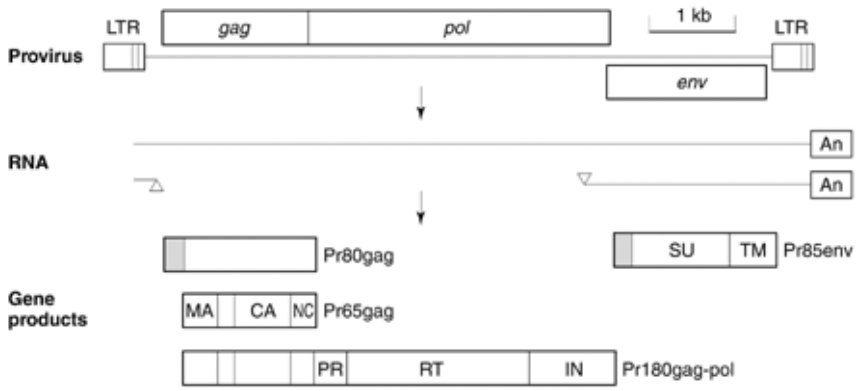


Figure 1 Proviral structure, RNA transcripts and gene products of feline leukemia virus. The provirus is flanked by long terminal repeats (LTR) and includes two long open reading frames (gag-pol and env). Two major polyadenylated (An) transcripts are produced, one encompassing the entire genome and the other spliced to join the 5′ leader sequence to env coding sequences. The longer RNA species is translated to produce the precursor for the internal virion proteins (Pr65gag) which is cleaved by the viral protease to yield mature products including the p15 matrix protein (MA), p27 capsid protein (CA) and the p10 nucleocapsid (CA). The envelope protein precursor (Pr85env) is cleaved by cellular enzymes to yield the mature gp70 virion surface glycoprotein (SU) and the transmembrane anchor protein p15E^{TM}. The virion enzymes (protease, PR; reverse transcriptase, RT; integrase, IN) are produced from the Pr180gag-pol precursor which is expressed by virtue of suppression of the gag termination codon. Also, an upstream in-frame AUG gives rise to an alternative Gag precursor (Pr85gag) which is glycosylated and expressed at the infected cell surface.

resembles cellular RNA having a 5′ cap and a 3′ poly(A) tract. As deduced from sequencing of proviral forms, the FeLV genome is around 8 kb long with a 67 base terminal redundancy and the gene order *gag-pol-env*.

The particles have surface spikes composed of multimers of the two env-coded proteins, the gp70 surface glycoprotein (SU) and the p15E transmembrane anchor protein (TM). Inside the envelope is the structural *gag* gene product which forms an icosahedral core particle composed of p27, the major capsid protein (CA), with an outer layer formed by the p15 matrix protein (MA). Another gag product, the p10 nucleocapsid (NC) is associated with the virion RNA. Other minor virion proteins encoded by the *pol* gene comprise the protease (PR), reverse transcriptase (RT) and integrase (IN) enzymes.

Replication

Virus replication proceeds, after binding to specific host cell surface receptors, internalization and uncoating. The virion RNA is converted into a double-stranded DNA form by the virion reverse transcriptase which used a proline tRNA primer and carries a ribonuclease H function that degrades the virion RNA. After nuclear translocation, viral integration is catalyzed by the integrase protein and involves the creation of a staggered cut in cellular DNA with a consequent 4 bp duplication of host DNA at the insertion site.

As illustrated in **Fig. 1**, the proviral form is flanked by long terminal repeats (LTRs) of 480–560 bp. These are generated during reverse transcription by duplication of unique sequences at the 3′ (U3) and 5′ (U5) ends of the RNA genome. The LTRs contain promoters and enhancers that drive transcription of viral RNA and also processing signals for cleavage and polyadenylation of the RNA transcripts. The 5′ LTR functions to initiate transcription whereas the 3′ LTR acts primarily as an RNA processing signal.

The virion RNA can function as a messenger RNA for Gag and Pol products, whereas a spliced subgenomic mRNA of around 3 kb encodes the Env products. Most full-length RNA translation products terminate at the 3′ end of *gag* to produce the Pr65gag precursor, whereas a small percentage readthrough into *pol* by misreading of UAG termination codon, generating the Pr180Gag-pol precursor. During and after the budding process, the virion aspartyl protease catalyzes the cleavage of both precursor proteins to their mature forms. The envelope gene products are synthesized as a Pr80env precursor and processed by cellular proteases to the mature, disulfide-linked gp70 and p15E envelope proteins. The *gag* gene is also abundantly expressed in an alternative, glycosylated form via an upstream AUG codon. This product is expressed on the cell surface and shed after cleavage by cellular proteases. Its role *in vivo* is unknown. It is dispensable for *in vitro* virus replication but highly conserved among FeLV strains.

Assembly of virus particles occurs at the cell surface by extrusion of cores which form at the budding

site, concomitantly acquiring a host cell-derived outer membrane with virus-coded surface spikes. Virus replication and release is often noncytopathic.

Geographic Distribution

FeLV occurs worldwide in domestic cat populations and it has also been isolated from the European wild cat (*Felis sylvestris*). Endogenous retroviral sequences closely matched to FeLV are found in the same species and in related small felids such as the sand cat (*F. margarita*) and the jungle cat (*F. chaus*). Although not a direct source of disease, these endogenous sequences can participate in recombination with exogenous FeLV to generate variant viruses with altered host range.

Epidemiology

The outcomes of FeLV infection can be divided into three categories. The majority of cats undergo a transient infection lasting up to 3 months, during which they are viremic and shed virus. They then develop neutralizing antibody and concomitantly eliminate the virus and a little later, virus antigen from the bloodstream. It is now recognized that these recovered cats may harbor latent virus in the bone marrow for some years following recovery but reactivation from latency is not a frequent event in the absence of iatrogenic immunosuppression. The vast majority of these cats do not develop an FeLV-related disease.

In a few cases, virus production from the hematopoietic tissue is halted, but productive virus infection is sequestered in an epithelial compartment like the mammary gland. These cats are not usually viremic but they may have circulating viral antigen.

The final group remain actively infected, shedding virus from epithelial surfaces and possessing an associated high titer plasma viremia. Such cats may remain healthy for 2–3 years before succumbing to an FeLV-related disease. A recent survey in the UK showed prevalence of active FeLV infection in sick cats at 18%, but only 6% in healthy individuals.

The proportion of cases falling into these groups differs between multicat households and those households containing one or two free-ranging cats. In the former case, the introduction of an FeLV carrier results in repeated exposure of susceptible cats, often at a young age so that up to 30–40% become persistently viremic and at risk of disease. Approximately 50% of free-ranging urban and suburban cats have serological evidence of exposure to FeLV but only 1–5% of these cats are actively infected and the disease incidence is correspondingly lower.

Dual infection with FeLV and feline immunodeficiency virus (FIV) occurs and is associated with rapid disease onset, particularly if cats with pre-existing FeLV infection encounter FIV. Rapid death of dually infected animals may reduce the apparent overlap of these agents in the field, but the populations at risk of infection also differ. FIV infection rates increase directly with age whereas persistent FeLV infection has a peak incidence in young cats.

FeLV Subgroups

FeLV isolates are classified as subgroup A, B or C according to their viral interference properties in feline fibroblast cells *in vitro*, that is, viruses of a given subgroup prevent superinfection (interfere) with other viruses of the same subgroup (**Table 1**). This property appears to be based on the use of three different host cell surface receptors by FeLV-A, B and C, and the blockade of these receptors in persistently infected cells by viral Env glycoproteins. Natural isolates contain either subgroup A alone, or mixtures of subgroups A + B, A + C or A + B + C.

Host Range and Virus Propagation

FeLV isolates of subgroup A are generally restricted to growth in feline cells, whereas subgroups B and C have a greatly expanded host range, infecting cat, human, mink and canine cells. Their host ranges are further distinguishable since FeLV-C also infects guinea-pig cells, whereas FeLV-B appears to use the same human cell receptor as gibbon ape leukemia

Table 1 Properties of feline leukemia virus subgroups

Subgroup	*Origin*	*Host range*	*Receptor*	*Pathogenesis*
A	Exogenous	Ecotropic	?	Minimally pathogenic to acute immunosuppression
B	Recombination FeLV-A × endogenous FeLV	Amphotropic	Pit-1, Pit-2	More common in leukemic cats Some isolate-specific diseases e.g. FeLV-GM1 myeloid leukemia
C	Mutation of FeLV-A (Env vr1)	Amphotropic	?	Erythroid hypoplasia

virus. FeLV infection is generally noncytopathic and persistent and the virus is commonly propagated in long-term cultures of embryo-derived fibroblasts.

Clinical Features and Pathology

Of those cats which become persistently viremic following FeLV exposure, over 80% will die within 3.5 years. Most young cats infected with FeLV die from degenerative diseases rather than from tumors. Profound immunosuppression associated with thymic atrophy is a common finding in kittens. Immunosuppression is also a feature of infection in older cats but its pathogenesis is complex and not fully understood. Highly immunosuppressive (feline autoimmune deficiency syndrome, FAIDS) variants have been isolated. These are defective for replication and genomes accumulate as unintegrated forms in lymphoid tissue.

Other diseases seen in FeLV-infected cats include enteritis, immune complex glomerulonephritis, pancytopenia and hemolytic anemia. Erythroid hypoplasia, an acute disease involving failure of red cell development past the erythroid burst-forming unit (BFU)-E-stage, is specifically associated with FeLV subgroup C.

The most common neoplasm induced by FeLV is lymphosarcoma of T cell origin, usually restricted to the thymus or sometimes occurring as a multicentric tumor in lymph nodes. The tumors often develop between 1 and 3 years after infection and the first signs may be chronic wasting and anemia. At presentation, the normal architecture of the lymphoid organ has usually been destroyed by a monomorphic infiltrate of lymphoblastic cells. The thymic tumors frequently display a rearrangement of the T cell antigen receptor β-chain gene and are often CD4+ and CD8+. FeLV is also found in association with 35% of alimentary tumors, primarily of B cell origin, but the virus is not always clonally integrated in these tumors and the role of the virus is, therefore, unclear.

FeLV is also associated with a variety of other neoplastic diseases, including primary myeloid and lymphoblastic leukemias and multicentric fibrosarcoma.

Envelope Gene Variation and Pathogenicity

The common infectious form of FeLV is FeLV-A which is a remarkably highly conserved virus as shown by sequence analysis of several strains and serotypic analysis of a large number. FeLV variants frequently arise from FeLV-A by mutation and recombination, and such variants are often implicated in the acute diseases which develop in persistently infected cats. The variant viruses generated from FeLV-A are generally dependent on the continued presence of the prototype for their propagation *in vivo*.

The most commonly isolated FeLV recombinants are subgroup B viruses (**Table 1**). These are derived by recombination between FeLV-A and endogenous FeLV-related proviruses which are found in the genome of the domestic cat and related small feline species. Although the endogenous FeLV-related proviruses all appear to be replication-defective, their envelope genes can be rescued by the recombination process leading to the generation of FeLV-B viruses. FeLV-B can infect cells refractory to, or already containing, FeLV-A by virtue of their distinctive receptor specificity.

The anemia-inducing FeLV-C isolates are rarer and appear to be derived from FeLV-A by mutation of a single variable domain (VRA) of the *env* gene. Minor *env* mutations also appear to give rise to the acutely immunosuppressive FeLV–FAIDS variants. The prevalence of acutely immunosuppressive viruses in nature is unknown, but immunosuppressive disease is a common manifestation of FeLV infection.

The relationship of subgroup variation to oncogenesis is complex. FeLV-B recombinants are more common in tumor-bearing than in infected asymptomatic cats. This higher frequency might reflect merely longer-standing infection, but some FeLV-B-containing isolates have an altered spectrum of neoplastic disease. For example, FeLV-GM1, which contains a replication-defective FeLV-B component, induces mainly myeloid leukaemia.

FeLV Oncogenesis: Virus Evolution and Mutagenesis of Cellular Oncogenes

Two modes of virus-induced host gene mutation have been described in FeLV-associated cancers. The first is *transduction,* where recombination leads to the generation of an acutely oncogenic variant. Such viruses are found in nature in association with a replication-component FeLV helper virus. Although they may explain the genesis of the primary neoplasm in which they occur, the recombinant viruses are not transmissible and will die out with their host.

Multicentric fibrosarcomas of young cats are relatively rare, but are generally FeLV positive and frequently involve a novel sarcoma virus. Similarly, transduction of c-*myc* has been observed in up to 20% of naturally occurring thymic lymphosarcomas in FeLV-positive cats. In all, nine different host cell genes have been shown to be transduced (**Table 2**). The transducing viruses induce tumors with short

Table 2 FeLV gene transduction in neoplasia

Gene	*Normal function of host gene product*	*No. of examples*	*Associated tumor*	*Examples*[a]
abl	Plasmamembrane protein kinase	1	Fibrosarcoma	FeSV-HZ2
fes	Plasmamembrane protein kinase	3	Fibrosarcoma	FeSV-GA, -HZ1, -ST
fgr	Plasmamembrane protein kinase	1	Fibrosarcoma	FeLV-GR, -TP1
fms	Receptor protein kinase (CSF-1 receptor)	2	Fibrosarcoma	FeSV-SM, -HZ5
kit	Receptor protein kinase (SCF receptor)	1	Fibrosarcoma	FeSV-HZ4
myc	Transcription factor	10	T-cell lymphoma	FeLV (T3, T17, FTT)
Notch2	Transmembrane receptor	2	T-cell lymphoma	(Inoculum FeLV-61E)
sis	Growth factor (B chain PDGF)	1	Fibrosarcoma	FeSV-PI
tcr	T-cell antigen receptor (β-chain)	1	T-cell lymphoma	FeLV-T17

[a] Isolated from naturally occurring tumors apart from the indicated exception.

latency in cats and in the case of feline sarcoma viruses may transform cells in tissue culture.

Alternatively, host genes can be affected by proviral *insertional mutagenesis* (*cis*-activation). Three known oncogenes and two novel integration loci have been identified as common tumor-specific insertion sites for FeLV in thymic lymphosarcomas (**Table 3**).

Changes within the LTR are also a feature of tumor-associated FeLV. In thymic lymphosarcomas, sequence duplications of the core enhancer domain are frequently found, even when the infecting virus is a molecularly cloned FeLV isolate lacking such features. By analogy with the murine oncoretroviruses, the duplications are likely to increase the oncogenicity of the virus and reduce the latent period for tumor formation, possibly by increasing the potency of viral enhancer activity on nearby cellular promoters. In proviruses integrated at the *flvi*-1 locus in non-T, non-B splenic lymphomas, triplication of an alternative motif 3′ to the core enhancer has been observed, suggesting that these adaptive changes to the LTR operate tissue-specifically.

Immune Response

Unlike infection with the human leukemia virus, HTLV, FeLV infection of cats may lead to recovery. Recovery is associated with the production of neutralizing antibody and passive transfer of this antibody can also protect kittens from infection. The role of cell-mediated immunity in recovery has not been extensively investigated.

In the early literature on FeLV a distinction was made between antiviral immunity and antitumor immunity. Cats with antitumor (FOCMA) antibody were thought to be protected from tumor development. This antibody response is now believed to be directed to endogenous FeLV *env* proteins and its role in modulating tumor development is uncertain.

Transmission

Cats persistently infected with FeLV shed virus in their saliva, urine and feces but, as the virus is fragile, close contact is required for transmission. The most frequent routes involve saliva and transplacental spread. Kittens infected *in utero* become persistently infected, but the consequences of infection in older cats depend on a number of factors. There is an age-related resistance to infection such that cats up to 12 weeks of age are highly susceptible but above 16 weeks they are difficult to infect either naturally or experimentally.

FeLV subgroup A is always found in field isolates and about half also contain FeLV-B whereas FeLV-C is present in only 2% of isolates. Although FeLV-B

Table 3 Insertional mutagenesis and FeLV oncogenesis

Locus	*Gene function*	*Tumor*	*Frequency*[a]
fit-1	Unknown, linked to c-*myb*	T cell lymphoma	3/38
flvi-1	Unknown	non-T, non-B lymphoma	4/11
flvi-2(bmi-1)	Transcription factor	T cell lymphoma	9/38
c-*myc*	Transcription factor	T cell lymphoma	12/38
pim-1	Cytoplasmic protein kinase	T cell lymphoma	2/38

[a] From analyses of naturally occurring tumors.

Table 4 FeLV vaccines

Vaccine	*Protection*	*Commercial use*
Live attenuated FeLV	Yes	No (safety concerns)
Inactivated whole virus	Yes	Yes
Subunit vaccines		
SU from *Escherichia coli*	Yes	Yes
ISCOM-Env (native)	Yes	No
Lymphoma cell supernatant	Yes (poor in some studies)	Yes
Live vector vaccine		
Vaccinia-Env	No	No
Canarypox-Env-Gag	Yes	No
Feline herpesvirus-Env	Partial	No

can arise *de novo* by recombination, it may also be transmitted between cats. This occurrence is dependent on pseudotype formation in which the genome of the B virus becomes enclosed in an envelope containing glycoproteins of the A subgroup.

Prevention and Control

Successful control measures can be adopted in multicat households by removing or isolating persistently infected animals. Productively infected cats are detected by virus isolation from plasma or more usually by enzyme-linked immunosorbent assay (ELISA) for virus antigen in the blood. A few cats remain persistently antigenemic but nonviremic. These cats do not usually transmit the virus unless they are shedding virus in the milk or saliva. Assays are conducted twice, 3 months apart, to exclude cats that are transiently viremic.

Numerous vaccine strategies have been shown to offer protection against FeLV in laboratory conditions (**Table 4**) and FeLV is the only retrovirus for which commercial vaccines are in routine use. These vaccines offer a measure of protection against experimental challenge but do not appear to be 100% effective against exposure in the field.

Future Perspectives

There is continuing interest in control of FeLV infection due to its importance in veterinary medicine. In the future we can look forward to improvements in vaccine efficacy and further dissection of the host responses that confer protection. Although the focus of attention of cancer genetics has moved on to more easily manipulated models, FeLV remains as a useful touchstone for our understanding of oncovirus pathogenesis in an outbred, naturally infected host. Also, with the impetus of feline immunodeficiency virus as a model for human AIDS, the generation of reagents to probe the feline immune system offers new opportunities for comparative study of FeLV.

See also: **Feline immunodeficiency virus (*Retroviridae*); Murine leukemia viruses (*Retroviridae*); Retroviruses – type D (*Retroviridae*).**

Further Reading

Hardy WD Jr (1993) Feline oncoretroviruses In: Levy JA (ed.) *The Retroviridae* vol. 2, p. 109. New York: Plenum Press.

Neil JC, Fulton R, Rigby M and Stewart M (1991) Feline leukaemia virus; generation of pathogenic and oncogenic variants. *Curr. Top. Microbiol. Immunol.* 171: 67.

Rohn JL, Gwynn SR, Lauring AS, Linenberger ML and Overbaugh J (1996) Viral genetic variation, AIDS, and the multistep nature of carcinogenesis: the feline leukemia virus model. *Leukemia* 10: 1867.

Tsatsanis C, Fulton R, Nishigaki K *et al* (1994) Genetic determinants of feline leukemia virus-induced lymphoid tumors: patterns of proviral insertion and gene rearrangement. *J. Virol.* 68: 8296.

Feline Panleukopenia Virus *see* **Parvoviruses**

Feline Sarcoma Virus *see* **Feline Leukemia and Sarcoma Viruses**

FILAMENTOUS PHAGES (*INOVIRIDAE*)

Robert E. Webster, Department of Biochemistry, Duke University Medical Center, Durham, North Carolina, USA

Introduction

Filamentous bacteriophages are a group of structurally related viruses which contain a circular single-stranded DNA genome. Many use the tip of a conjugative pilus as a cell surface receptor and thus are specific for bacteria containing certain conjugative plasmids. They do not kill their host during productive infection. Filamentous bacteriophages with these characteristics have been classified in the family *Inoviridae*, genus *Inovirus*. Those which infect *Escherichia coli* containing the F plasmid are collectively referred to as Ff bacteriophages. The three most extensively studied Ff phages are f1, fd and M13, which were independently isolated in the early 1960s. They differ in their DNA sequence by only a small number of nucleotides and, consequently, the protein sequences of the gene products are nearly the same.

E. coli containing other conjugative plasmids can also be infected by specific filamentous phages. The most studied is IKe, which requires the presence of N pili and consequently the N plasmid for infection. Its DNA sequence is 55% homologous to the Ff bacteriophage and it appears to have the same genome organization. Filamentous phages that infect other bacterial species, such as *Pseudomonas* (Pf1, Pf3), *Salmonella* (If1, If2), *Vibrio* (CTXphi, fs1) and *Xanthomonas* (Cf, Lf, Xf), have also been isolated. CTXphi encodes the gene for cholera toxin, one of the virulence factors of *Vibrio cholerae*. Some of these phage are able to integrate their DNA into the genome of the host.

Structural analysis has been done primarily on Pf1 and the Ff group of filamentous bacteriophages. X-ray crystallographic and nuclear magnetic resonance techniques show differences in the packing of the protein and DNA between Ff and Pf1 viral particles. On the basis of these studies, the filamentous bacteriophages have been divided into two structural classes: class I containing the Ff bacteriophages and class II bacteriophages Pf1, Pf3 and Xf. The structure of Pf1 has been subjected to the most extensive study. Comparison of the structure of the major capsid protein in the membrane and in the Pf1 phage particle itself has suggested a mechanism by which the phage particle may be assembled. However, it is difficult to know how accurate such a hypothesis is, in view of the lack of knowledge about the biology of this particular filamentous bacteriophage. At the present time, the biology and life cycle of the Ff bacteriophages f1, fd and M13 are the most completely studied and will be the subject of the remaining discussion. However, increasing knowledge of the other filamentous phage suggests there exists great similarity between all filamentous bacteriophage.

Structure of the Bacteriophage

The bacteriophage is a flexible rod, about 900 nm long and 7 nm in diameter (**Fig. 1**). It has a mass of approximately 16.3 MDa, of which 13% is contributed by the DNA. The virus consists of a protein sheath enclosing a circular single-stranded DNA extending the entire length of the particle. The length of the sheath consists of approximately 2700 molecules of the major coat protein, a 50 amino acid protein encoded by the phage gene VIII (pVIII). It is highly α-helical in structure with its N-terminal acidic domain exposed to the outside and its basic C-terminal domain toward the inside of the protein tube. These two domains are connected by a very hydrophobic sequence which appears to interact to form the stable protein tube. The proteins are arranged in an overlapping shingle-like array and have a symmetry defined by a fivefold rotational axis with a twofold screw axis of pitch 3.2 nm.

About five copies each of the 33 amino acid gene VII protein and the 32 amino acid gene IX protein make up one end of the particle. The other end contains approximately five copies each of the gene III (406 residue) and gene VI (112 residue) proteins. This end can be recognized in the electron micrograph as containing small knob-like structures. These knobs consist of the N-terminal portion of the gene III protein and contain the information for F pilus recognition as well as recognition of the bacterial proteins involved in the translocation of the phage DNA into the bacteria. The C-terminal portion of the

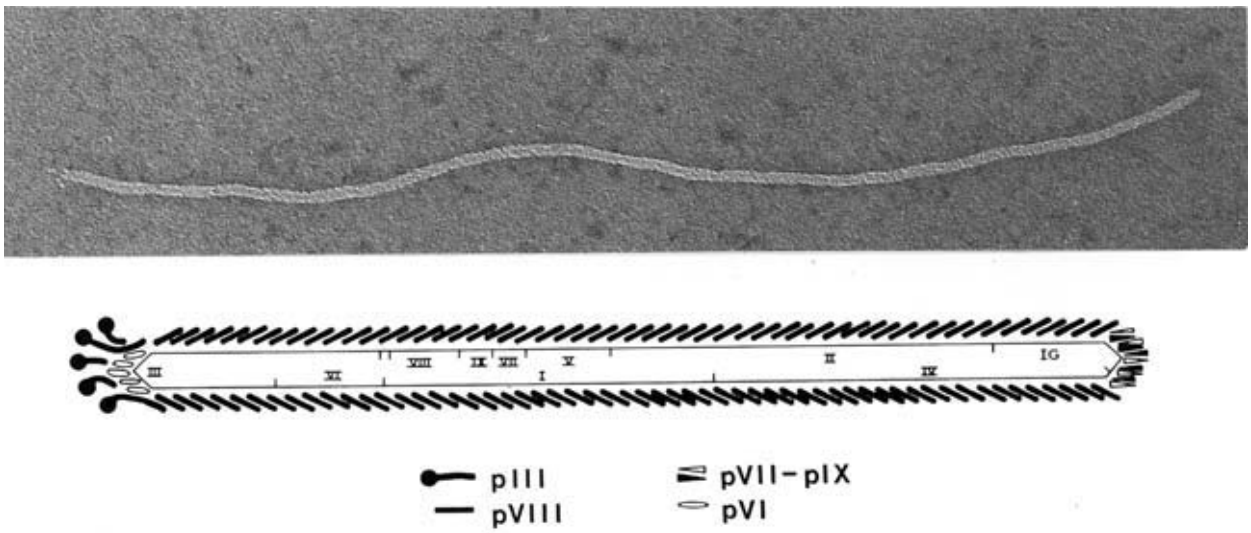


Figure 1 The f1 filamentous bacteriophage. The top is an electron micrograph of a negatively-stained particle with the pIII–pVI end located on the left side. The bottom is a schematic representation of the positions of the structural proteins and DNA in the bacteriophage. This figure is adapted from Webster RE and Lopez J (1985) In: Casjens S (ed.) *Virus Structure and Assembly*, p. 235. Boston: Jones and Bartlett.

gene III protein along with that of the gene VI protein are required to maintain the stability of the particle.

The capsid proteins form a tube with an internal diameter of approximately 3.4 nm, in which the viral DNA resides in essentially a non-base-paired anti-parallel form. The DNA is oriented within the virion such that a 78 nucleotide hairpin loop is always at the gene VII/IX protein end. This hairpin region is called the packaging signal (PS) and is required for efficient assembly of the bacteriophage (**Fig. 2**, hairpin A). The bases of the DNA are arranged in the center of the phage particle so that the negatively charged sugar–phosphate backbone of the DNA can interact with the positively charged C-terminal region of the pVIII protein exposed on the inside of the protein tube of the phage. It is the overall charge density in this region of the coat protein which governs the DNA packing. Removing one of the positive amino acids in the basic region of pVIII results in a longer phage particle containing more altered pVIII to maintain the charge density.

Viral Genome: Proteins and Expression

The genomes of the filamentous bacteriophages f1, fd and M13 have been completely sequenced and shown to code for 11 proteins (**Fig. 2**). There is an intergenic region of approximately 500 nucleotides which contains the origins of replication for the viral and complementary DNA as well as the packaging signal needed for efficient assembly. The genes are tightly packed and are arranged according to the function of their products: capsid proteins (pIII, pVI, pVII, pVIII and pIX), replication proteins (pII, pV and pX) and assembly proteins (pI, pIV and pXI).

All of the capsid proteins appear to reside in the inner membrane until assembled around the viral DNA. The major coat protein, pVIII, is synthesized as a precursor, containing a 23 residue signal sequence which is removed by signal peptidase following insertion into the membrane. Membrane insertion is independent of the *E. coli* encoded Sec proteins normally involved in the translocation of proteins into and across the bacterial membrane. Mature pVIII spans the membrane via its hydrophobic central domain with its N-terminal domain exposed to the periplasm and the C-terminus in the cytoplasm.

The product of gene III is also synthesized as a precursor with an 18 residue signal sequence which is removed following translocation into the membrane. Most of the pIII, including the mature N-terminus, is located in the periplasm, anchored to the bacterial inner membrane via a 23 amino acid hydrophobic sequence near its C-terminus. The other three capsid proteins, pVI, pVII and pIX, are not synthesized as precursor molecules but are inserted into the membrane, where they remain until assembly into a phage particle. Both the small pVII and pIX retain their initiating formylmethionine, suggesting that rapid insertion into the membrane prevents its removal.

Gene II encodes a 409 residue protein, pII, which is a site- and strand-specific endonuclease absolutely required for both the synthesis of the double-stranded replication intermediate and the single-stranded viral

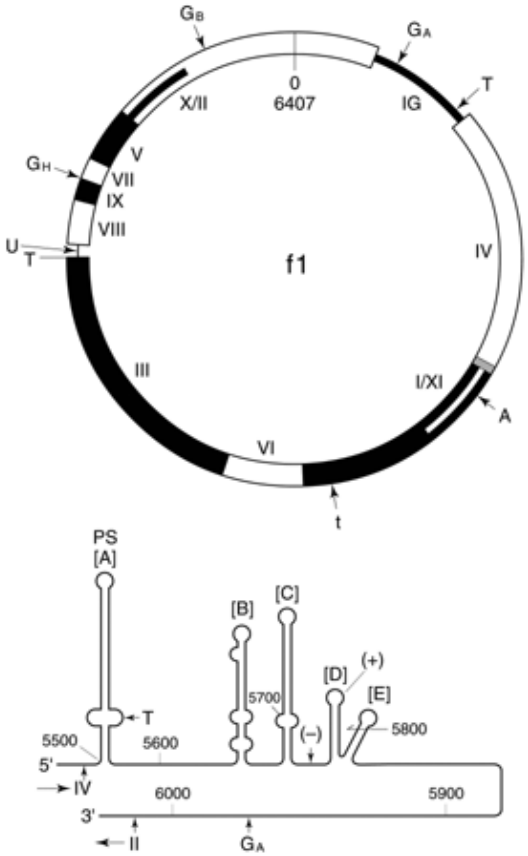


Figure 2 Genome of f1 filamentous bacteriophage. The top represents the whole genome showing the genes, promotors and terminators used *in vivo*. All transcription, along with the number of nucleotides, proceeds in a counterclockwise direction. IG refers to the intergenic region, T the two strong terminators, t the weak terminator in gene I, and G_A, G_B, G_H, U and A marking the beginning of transcripts in the frequently and infrequently transcribed regions. The bottom shows a schematic representation of the intergenic region. The direction of nucleotides has been reversed from the top. A represents the packaging signal hairpin (PS), B and C the proposed hairpins in the complementary strand origin and D and E the proposed hairpins in the viral strand origin. (−) is the beginning of the RNA primer for complementary strand synthesis, (+) the pII nicking site needed for viral strand synthesis. Also shown are the position of the strong rho-dependent terminator (T), the start of the gene II mRNA (G_A) and the beginning of gene II and the end of gene IV. This figure is adapted from Model P and Russel M (1988) In: Calandar R (ed.) *The Bacteriophages*, p. 375. New York: Plenum Press.

DNA. Gene X is encoded within and in phase with gene II and encodes a protein comprised of the C-terminal 111 amino acids of pII. It appears to be required for synthesis of the single-stranded viral DNA. Gene V encodes an 87 amino acid single-stranded binding protein for which the crystal structure has been solved. It exists as a dimer and cooperatively binds the newly synthesized viral DNA to form a linear antiparallel structure which appears to be an intermediate in the assembly of the bacteriophage. This structure contains approximately 1500 molecules of pV per viral single-stranded DNA.

Three phage encoded proteins (pI, pIV, pXI) are required for the membrane-associated assembly of the particle but are not part of the virion itself. The 405 residue gene IV protein is primarily found in the outer membrane of an infected bacteria. It is synthesized as a precursor with a 21 amino acid signal sequence. After translocation to the periplasm and removal of the signal sequence, it integrates into the outer membrane to form a cylinder composed of 14 subunits which spans the outer membrane. The pore of this pIV cylinder has a diameter of approximately 8 nm, large enough to accommodate an emerging filamentous phage. Production of pIV during phage infection induces the expression of the four genes in the *E. coli* stress-induced phage shock (*psp*) operon.

The product of gene I is a 348 residue protein that spans the bacterial inner membrane via an internal hydrophobic region such that its N-terminal 253 amino acids reside in the cytoplasm and its C-terminal 75 amino acids are exposed in the periplasm. Gene XI is encoded within gene I and directs the synthesis of the 108 residue pXI, which is homologous to the C-terminal portion of pI. It is inserted in the inner membrane with the same orientation as pI. It is proposed that the periplasmic regions of both pI and pXI interact with the pIV in the outer membrane to help form an assembly site through which the assembling bacteriophage particle is extruded. Genetic studies have suggested that the N-terminal region of pI, which is missing in pXI, interacts with thioredoxin, ATP and the packaging signal during the assembly process.

Transcription only uses the complementary strand as a template giving mRNAs having the sequence found in the viral strand. The direction of transcription is from gene II through gene IV (**Fig. 2**). There are two strong terminators for transcription, a rho-dependent terminator in the intergenic region and a rho-independent terminator in the region between genes VIII and III. These two terminators divide the genome into two transcription regions: the frequently transcribed region containing genes II through VIII and an infrequently transcribed region extending from gene III through the intergenic region.

The frequently transcribed region contains three strong promoters, G_A, G_B and G_H, leading to three primary transcripts terminated at the end of gene VIII (**Fig. 2**). The 5′ portions of the G_A and G_B transcripts are rapidly removed to leave a set of six smaller, more stable mRNA species. The shortest mRNA of this set is the stable primary transcript starting at G_H. All of

these processed transcripts have a common 3′ end and thus contain the information for pVIII, while four also encode pV. Both of these proteins are needed in large amounts: the pVIII to form the capsid of the 200–2000 virions produced per bacterial generation; and pV to interact with the 200 newly synthesized viral single-stranded DNA molecules in each bacterium. This processing or 'cascade' mechanism provides a sufficient amount of mRNA to code for the large amount of these proteins needed for phage production. However, very little of the products of genes VII and IX, located between genes V and VIII, are synthesized in the bacteria. This is because the expression of these two genes is translationally coupled to that of gene V. The translation initiation site for pVII is inherently inactive and the initiation site for pIX is masked by the secondary structure of the mRNA. Translational activity in the gene V portion of the mRNA is transmitted through the pV–VII junction with a low frequency.

The transcripts from the infrequently transcribed region begin at moderately efficient promoters just before genes III and IV (**Fig. 2**). There is a weak rho-dependent terminator in the region coding for the N-terminal portion of pI. Transcription from the promoter proximal to gene III results in two classes of mRNA: one encoding pIII and pVI, and a smaller amount of the longer transcript encoding pIII, pVI and pI/XI. This mechanism of downregulation for gene I expression appears necessary because large amounts of pI have been shown to be deleterious to the growth of the host bacteria. There is substantial expression of gene IV RNA from the promotor located at the end of gene I.

The synthesis of pII and pX also are regulated at the level of translation. In addition to its role in binding to the newly synthesized viral DNA, pV can specifically bind to the gene II and X mRNA and inhibit translation. In the case of gene II mRNA, the binding site has been localized to a 16 nucleotide region just upstream of the pII coding region. This inhibition occurs at high cellular concentrations of pV, when the amount of pV exceeds that needed to bind the newly synthesized viral DNA.

Life Cycle

Infection is minimally a two-step process requiring interactions with the F conjugative pilus and the bacterial TolQ, R and A proteins. These three Tol proteins are associated with the cytoplasmic membrane and appear to function in maintaining the integrity of the bacterial outer membrane. They also are required for the translocation of the group A colicins to their target following the interaction of these bacteriocins with their specific receptors. TolQ spans the inner membrane three times with the major portion of the protein in the cytoplasm. TolR and A are anchored to the inner membrane by a region near the N-terminus leaving the bulk of the protein, including the C-terminal end, in the periplasm. The C-terminal domain of TolA is connected to the membrane anchor by a long helical region, allowing the C-terminal domain to interact with the outer membrane.

It is the N-terminal portion of the pIII phage capsid protein which appears to interact with these bacterial proteins during infection. pIII is a three domain protein with each domain separated by a glycine-rich region. The C-terminal domain anchors the protein to the end of the phage particle. The N-terminal domain interacts with the Tol proteins, while the middle domain recognizes the tip of the F conjugative pilus. When pIII is present in the phage particle, the N-terminal and middle domains of pIII interact with each other.

Infection is initiated by the binding of the filamentous phage to the tip of the conjugative pilus via the middle domain of pIII, freeing up the N-terminal domain of pIII. The pilus is thought to retract by depolymerization of pilin into the inner membrane, thus bringing the pIII end of the phage particle to the membrane surface. There, the free N-terminal region of pIII interacts with the C-terminal domain of TolA. Then, while the pVIII and probably the pVII and pIX capsid proteins dissociate into the inner membrane, the DNA is translocated into the cytoplasm. The specific mechanism of this process is unknown, but the TolQ, R and A proteins are definitely required. Once in the membrane, the pVIII, pVII and pIX capsid proteins are able to join the pool of newly synthesized capsid proteins and be reutilized in the assembly of new phage particles. The fate of pIII and pVI is unclear.

The entering viral DNA is converted to a double-stranded form by the combined action of the bacterial RNA polymerase, DNA polymerase III and *E. coli* single-stranded binding protein (SSB). The RNA polymerase, in the presence of SSB, specifically recognizes the region containing the possible hairpin structures B and C in the intergenic region (complementary strand origin) (**Fig. 2**). The enzyme initiates the synthesis of a short RNA primer at nucleotide 5753 and DNA polymerase extends this primer the length of the viral strand. The action of DNA polymerase I and ligase generates the closed complementary strand. The resulting double-stranded DNA is supercoiled by gyrase to give the parental replicative form I (RFI). This molecule is a template for

transcription, allowing for the synthesis of the bacteriophage proteins.

Further replication of the DNA requires the presence of the bacteriophage gene II protein. This protein is able to specifically nick the viral strand of supercoiled RFI between nucleotides 5781 and 5782. The 3′ hydroxyl then acts as a primer for the synthesis of a new viral strand in a 'rolling-circle' mode of replication. The bacterial DNA polymerase III, SSB and Rep helicase are required for this reaction. After one round of replication, pII cleaves and circularizes the displaced viral single-strand. The double-stranded molecule, containing the newly synthesized viral strand, is closed, supercoiled and can act as a substrate for pII again. The displaced single-strand becomes a template for complementary strand synthesis, resulting in the formation of progeny RFI molecules.

The specific region recognized by pII (the viral strand origin) is difficult to define. Approximately 40 nucleotides around the nicking site are required for the nicking and termination functions of pII. This would include the region capable of forming the hairpin structures D and E in the intergenic region (**Fig. 2**). Efficient viral strand synthesis also requires up to 150 nucleotides downstream of the nick site (nucleotides 5781–5941 in the intergenic region). The nature of the enhancing effect of this region is not clear.

The RF molecules formed by these reactions can be used for transcription and allow the synthesis of increasing amounts of the bacteriophage proteins. The capsid and assembly proteins are inserted into the membrane, while pV accumulates in the cytoplasm. When the concentration of pV is great enough, it sequesters the newly displaced viral single-strand to form the pV–viral DNA complex. This prevents the viral single-strand from becoming a substrate for complementary strand synthesis and thus contributes to the switch in synthesis from RF to single-stranded DNA.

In addition to pV, pX is needed for the synthesis of viral single-stranded DNA when pII is present. In some sense, it appears that the activity of pII must be kept at a low but persistent level to allow synthesis of viral single-strand while maintaining the necessary amount of RF. This may be accomplished by pX being able to downmodulate the activity of pII. This control would be in addition to the ability of pV to repress the translation of gene II mRNA. Although this translational repression is a real effect, recent results suggest that it may be dispensable for M13 replication.

The assembly of the bacteriophage particle requires the five capsid proteins, the three assembly proteins, ATP and at least one bacterial protein, thioredoxin. This process occurs at membrane sites which resemble bacterial adhesion zones, where the inner and outer membrane appear in close contact. It is proposed that the formation of these assembly sites may require an interaction of the inner membrane-associated pI and pXI with the pIV oligomer in the outer membrane.

The substrate for the assembly reaction is the pV–viral DNA complex. It is proposed that separate segments of the viral DNA bind, in opposite orientations, in the cleft located on the outside of each pV dimer pair. This interaction brings two parts of the circular DNA close together. By binding approximately 750 pV dimers, the DNA becomes part of an elongated structure 1.1 μm long and 8 nm wide. From electron microscopy studies, the overall structure has a left-handed helical appearance with a variable pitch of 6–12 nm. The packaging signal, the 78 bp hairpin structure denoted A in **Fig. 2**, is located at one end of the pV–viral DNA complex, probably not bound by the pV protein.

Assembly appears to be initiated by an interaction of the packaging signal with the cytoplasmic domain of pI and the membrane-associated pVII, pIX and pVIII to form what might be described as an initiation complex. The fact that the packaging signal is located at the pVII/pIX end of the bacteriophage particle, the end of the particle that first emerges from the bacteria, is consistent with the formation of such a complex. The exact order of events involved in the formation of such an initiation complex is not known. However, genetic observations are consistent with the packaging signal interacting with pI, pVII and pIX during the initiating event.

Elongation then proceeds, a process whereby pV is displaced and the positively charged C-terminal domain of the membrane-associated pVIII interacts with the viral DNA. Structural changes probably occur in pVIII during this process, as it may exist as a dimer in the membrane and also appears to contain much less helical structure in the membrane than when it is part of the phage particle. Thioredoxin is proposed to function at this stage, interacting with pI and perhaps aiding it in displacing the pV from the DNA. This does not require the redox activity of the thioredoxin, but only that it be in its reduced conformation. ATP hydrolysis is also required for phage assembly, possibly via its interaction with pI, as the cytoplasmic portion of pI contains a well-conserved Walker nucleotide binding site which is required for phage assembly.

Termination of assembly involves the addition of pVI and pIII to the end of the elongating particle and its release into the media. This occurs when the end of

the DNA is reached and thus the length of the particle is determined by the size of the DNA. Elongation continues in the absence of either pIII or pVI, resulting in the production of extremely long particles which contain many unit length molecules of the viral single-stranded DNA. These polyphage are much less stable than normal virions, suggesting an important role for pVI and pIII in maintaining the structural integrity of the particle. This stabilizing effect contributed by pIII resides in its C-terminal region, as alteration in its N-terminal region still results in stable particles.

Uses of the Filamentous Bacteriophage

The filamentous bacteriophage have been used extensively as cloning vectors. Since there is no constraint on the size of the DNA packaged, theoretically any segment of DNA can be inserted into a noncoding region with no deleterious effect, although the yield of phage particles appears to diminish with inserts greater than 6 kb. Vectors have been constructed with cloning sites between hairpins A and B or distal to hairpin E in the intergenic region (**Fig. 2**). Although those in the latter site disrupt part of the region required for efficient viral strand replication, compensating mutations in the expression or structure of pII allow reasonable production of chimeric bacteriophage with such inserts. An advantage of using these vectors is that both single-stranded and double-stranded forms of the DNA can be obtained from the phage particle or from the intracellular RF DNA, respectively.

Other cloning vehicles, called phagemids, have been constructed; they combine the features of plasmids and the phage. These vectors contain the intergenic regions of the filamentous bacteriophage in addition to a plasmid origin of replication. They are capable of maintaining themselves as a plasmid until infection by a helper filamentous bacteriophage activates the phage origin of replication. The resultant plasmid single-stranded DNAs are encapsulated into phage-like transducing particles using the proteins provided by the helper bacteriophage.

The filamentous phage have been used as vehicles to enrich for peptides or proteins with specific properties, a process termed 'phage display'. Sequences coding small peptides, or epitopes, are commonly inserted into the N-terminal region of pIII. The resulting phage particles display these foreign peptides as part of the chimeric pIII at the tip of the bacteriophage. In this way, large 'epitope libraries' are made which can be screened for a range of binding activities. Peptides have also been displayed as fusion proteins at the N-terminus of pVIII and reportedly with pVI. Only small peptides can be displayed fused to either pIII or pVIII, presumably because larger proteins disrupt the normal function of pIII in infection or make pVIII too large to be assembled into phage particles. Large proteins can be displayed if enough wild-type capsid proteins are available during assembly. To accomplish this, foreign proteins fused to the appropriate portion of pIII or pVIII are expressed from a plasmid in an infected bacterium. The bacteriophage produced have a minor portion of their pIII or pVIII replaced by the chimeric protein.

See also: **Phages as cloning vehicles.**

Further Reading

Linderoth NA, Simon MN and Russel M (1997) The filamentous phage pIV multimer visualized by scanning transmission electron microscopy. *Science* 278: 1635.

Lubkowski J, Hennecke F, Pluckthun A and Wlodawer A (1998) The structural basis of phage display elucidated by the crystal structure of the N-terminal domains of g3p. *Nat. Struct. Biol.* 5: 140.

Riechmann L and Holliger P (1997) The C-terminal domain of TolA is the coreceptor for filamentous phage infection of *E. coli*. *Cell* 90: 351.

Russel M, Linderoth NA and Sali A (1997) Filamentous phage assembly: variation on a protein export theme. *Gene* 192: 23.

Waldor MK and Mekalanos JJ (1996) Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* 272: 1910.

Webster RE (1996) Biology of the filamentous bacteriophage. In: Kay BK, Winter J and McCafferty J (eds) *Phage Display of Peptides and Proteins*. p. 1. London: Academic Press.

FISH HERPESVIRUSES (*HERPESVIRIDAE*)

Andrew J Davison, MRC Virology Unit, Institute of Virology, Glasgow, Scotland

Introduction

Vertebrates from fish to humans are hosts to herpesviruses. Although herpesviruses have also been reported in invertebrates (oyster species), those that infect bony fish represent the earliest occurrences of herpesviruses in vertebrate evolution. Most currently recognized herpesviruses of fish infect species that are farmed relatively intensively. Presumably, the conditions used in aquaculture enhance the disease potential of these pathogens and increase the likelihood of their detection. It is likely, therefore, that many more fish herpesviruses await discovery. Herpesviruses of fish, like those of higher vertebrates, are highly species specific. This suggests that they have long been restricted to their hosts and have evolved in close association with them. Those that cause mortality do so only in young fish, and some are also agents of epidermal hyperplasia or neoplasia. Some have been investigated to such a limited extent that causal links with the disease whose occurrence led to their recognition have not yet been fully established.

Channel catfish virus (CCV) was the first fish herpesvirus to be isolated. It has a greater economic impact on farming of its host species and has been studied more extensively than other members of the group. It has also been subject to closest examination at the molecular level. Consequently, most of what follows is devoted to this virus, and the other fish herpesviruses are discussed only briefly.

Two salmonid herpesviruses have been shown to cause virulent disease either naturally or experimentally. Herpesvirus salmonis (ICTV proposed nomenclature salmonid herpesvirus 1; SalHV-1) was isolated on several occasions from a rainbow trout (*Oncorhynchus mykiss*) hatchery in the state of Washington. The virus causes disease when injected into young rainbow trout maintained at 6–9°C, but not in other salmonid species. A similar temperature optimum is characteristic of growth in the rainbow trout cell line RTG-2. *O. masou* virus (ICTV proposed nomenclature salmonid herpesvirus 2; SalHV-2) has a slightly wider host range, causing virulent disease in the young of several members of the genus *Oncorhynchus* (Pacific salmon) and the rainbow trout. It has a higher temperature optimum for growth in RTG-2 cells than salmonid herpesvirus 1 (15°C), and has the interesting property of causing epithelial tumors in survivors of experimental infection.

SalHV-1 and SalHV-2 have been shown to be distinct viruses on the bases of serological comparisons, DNA hybridization studies and limited DNA sequencing. Two additional herpesviruses, the NeVTA and Yamame tumor viruses, have been isolated from *Oncorhynchus* species. They appear to be related to each other, but it is not known whether they represent distinct salmonid herpesviruses or whether they are isolates of SalHV-2 or, as seems less likely, SalHV-1.

Several other fish herpesviruses have been described. Walleye herpesvirus (ICTV proposed nomenclature percid herpesvirus 1) is associated with epidermal hyperplasia in the walleye (*Stizostedion vitreum*), but causality has not been demonstrated. It has been cultured in cells of walleye origin, but not in cell lines from other fish species. Carp pox herpesvirus (ICTV proposed nomenclature cyprinid herpesvirus) is also implicated as the cause of a localized epithelial hyperplasia in the common carp (*Cyprinus carpio*). The condition manifests itself as benign smooth nodules on the skin, and may be transferred by applying material from the lesions to the abraded skin of other fish. The agent has not yet been successfully grown in cell culture, and so its direct involvement in disease has not been proven. Similarly, the role of turbot herpesvirus (ICTV proposed nomenclature pleuronectid herpesvirus) has not been elucidated in episodes of substantial mortality among farmed fry. Recent reports of new fish herpesviruses include those that infect the pilchard (*Sardinops sagax*), the Japanese eel (*Anguilla japonica*), the white sturgeon (*Acipenser transmontanus*) and the goldfish (*Carassius auratus*).

History and Classification

In 1968, Fijan reported the first isolation of CCV from young channel catfish (*Ictalurus punctatus*), in which it causes an acute disease of short duration and high mortality. The first detailed characterization of the virus was published 3 years later by Wolf and Darlington, and showed that the nucleocapsid is a 100 nm icosahedron with 162 capsomeres, and that assembly of capsids takes place in infected cell nuclei. These features are typical of a herpesvirus, and have led the ICTV to propose the nomenclature ictalurid herpesvirus 1, as the type species of the genus

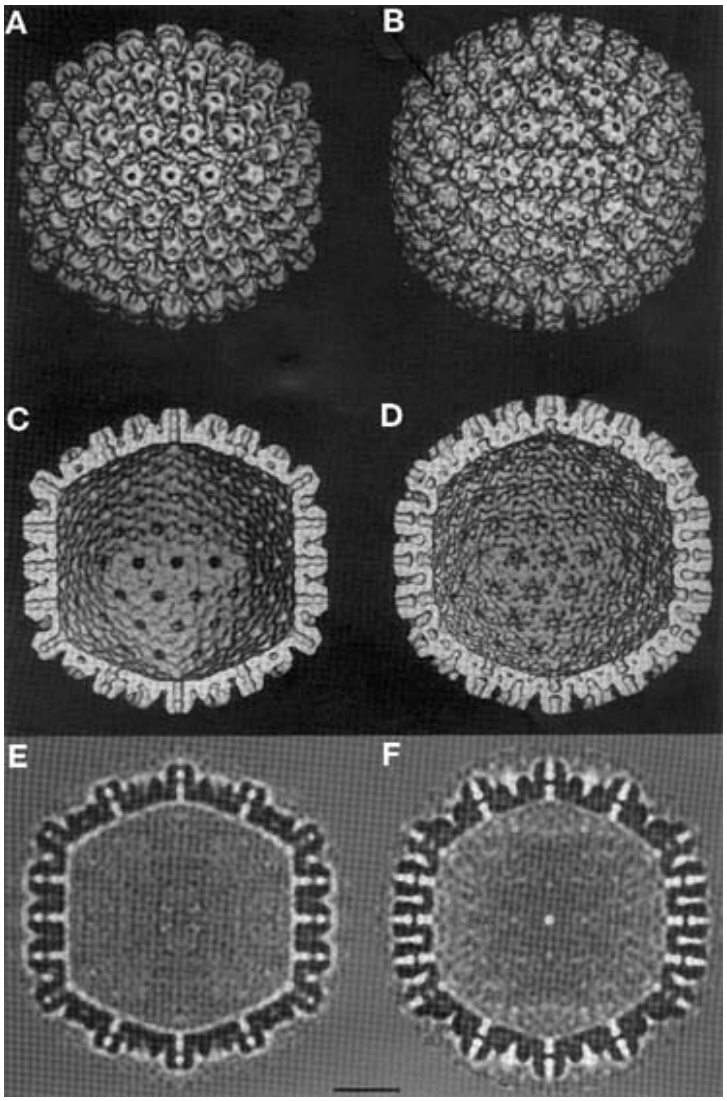


Figure 1 Comparison of the three-dimensional capsid structures of CCV (**A**, **C**, **E**) and HSV-1 (**B**, **D**, **F**). The capsids are viewed along a twofold symmetry axis. Outer surfaces, (**A**) and (**B**); inner surfaces, (**C**) and (**D**); central thin sections, (**E**) and (**F**). The distribution of capsomers (**A** and **B**) is distinctive for the triangulation number, $T = 16$, with lines of three hexons connecting pentons along each edge of the icosahedral surface lattice. Bar = 25 nm. (Reproduced from Booy *et al* (1996) *Virology* 215: 134–141, with permission of Academic Press.)

Ictalurovirus. This genus is as yet unassigned to a subfamily for reasons described below in the section on evolution. Three-dimensional reconstructions derived by cryoelectron microscopy have recently shown that, apart from minor dimensional differences, the CCV capsid is strikingly similar to that of herpes simplex virus type 1 (HSV-1) (**Fig. 1**).

Geographic and Seasonal Distribution

The channel catfish is a North American species. The frequency of occurrence of CCV disease has paralleled the intensification of channel catfish farming in the southern USA and some other warmer temperate regions. The lack of reported isolations of CCV from wild channel catfish strongly indicates that factors such as dense stocking and poor environmental conditions may predispose farmed fish stocks to outbreaks of disease. A key factor is temperature, as epizootics occur in the summer months and the incubation period has been shown to be considerably shorter at higher temperatures up to 30°C.

Host Range and Virus Propagation

CCV causes acute infection only in young channel catfish, usually up to about 6 months old – the degree of mortality depending on the strain of fish. Certain other closely related species, such as the blue catfish (*I. furcatus*), may be infected experimentally by injection, but other species are refractory to infection even by this route. Host cell requirements are a little less stringent in cell culture, but virus growth still only occurs in ictalurid and clariid fish cell lines. BB (*I. nebulosus* or brown bullhead) and CCO (channel catfish ovary) cell lines are commonly used; the latter

is the more susceptible. Optimal virus yield is obtained at 25–30°C, and about 50% of progeny virus is released into the culture medium. Virus infection causes the formation of syncytia, particularly at lower multiplicities of infection, followed eventually by disaggregation and lysis. Under optimal conditions, peak virus yield may be attained only 12 h after infection. In this respect, CCV is one of the fastest growing herpesviruses.

Genetics

Expression of CCV proteins during infection of cells in tissue culture appears to be coordinately regulated. The major immediate early protein, which is likely to regulate the expression of other virus genes, has an apparent molecular mass of 117 000 Da. Experimental determinations of the number of proteins expressed or induced in infected cells are likely to be underestimates, but about 50 have been identified. The capsid contains an abundant constituent with a molecular mass of 128 000 Da and at least two smaller proteins. The major capsid protein of higher vertebrate herpesviruses is invariably larger, having a molecular mass of about 150 000 Da.

Different isolates of CCV may be differentiated by restriction endonuclease digestion of their DNAs, but nevertheless are recognizably similar. The reference strain Auburn is deposited as VR-665 with the American Type Culture Collection. The overall structure of the linear double-stranded DNA genome consists of a unique region flanked at each end by a large direct repeat. CCV DNA replicates in the nuclei of infected cells via head-to-tail concatemers made up of a unit comprising the unique region linked to a single copy of the direct repeat.

The complete CCV DNA sequence has been determined. It contains 134 226 bp and has a nucleotide composition of 56.2% guanosine plus cytosine. The unique region and direct repeat are 97 114 bp and 18 556 bp in size respectively. Analysis of the DNA sequence indicates the presence of 63 genes in the unique region (one comprising three exons) and 14 genes in each direct repeat (**Fig. 2**). The CCV genome shares some general characteristics with other herpesvirus genomes, but these indications of an evolutionary relationship are not reflected at the genetic level. Several gene functions have been proposed from examination of encoded amino acid sequences and computer-aided comparisons of predicted CCV proteins with proteins from other organisms. They include two sets of related protein kinases (genes 14, 15 and 16; genes 73 and 74), a set of three related proteins containing a potential zinc-binding domain (genes 9, 11 and 12); another potential zinc-binding protein (gene 78), a helicase (gene 25), a DNA polymerase (gene 57/58, probably composed of two exons), a deoxyuridine triphosphatase (gene 49), a thymidine kinase (gene 5), a serine protease (gene 47), a DNA-packaging protein (gene 62/69/71, composed of three exons), an envelope glycoprotein (gene 46) and seven other membrane-associated proteins (genes 6, 7, 8, 10, 19, 51 and 59).

The principal constituent proteins of CCV virions have been identified. The capsid includes the major capsid protein (gene 39), a potential scaffold protein (gene 28) and two other proteins (genes 27 and 53). The tegument (the proteinaceous region between the capsid and the envelope) includes proteins encoded by genes 11, 15, 65, 72, 73 and 74, in addition to actin. The major envelope protein is specified by gene 59.

Evolution

Surprisingly, no CCV proteins appear to be related to proteins that are considered to be specific to higher vertebrate herpesviruses, such as those that make up the virion. This is in distinct contrast to herpesviruses of mammals and birds, which are clearly related to each other and probably evolved from an ancestral virus. Thus, the initial biological classification of CCV as a member of the *Alphaherpesvirinae*, a subfamily of the herpesviruses typified by HSV-1, is not supported by genetic data. Indeed, CCV does not fit into any of the three currently recognized subfamilies. It is probable that CCV has diverged from herpesviruses of mammals and birds to such a degree that no convincing genetic evidence remains of a common evolutionary origin.

The SalHV-1 genome is 174 kb in size and has counterparts to at least 18 CCV genes. Limited sequence data indicate that SalHV-1 and SalHV-2 are related to each other and, more distantly, to CCV. These observations indicate that fish herpesviruses form a group that is distinct from herpesviruses of mammals and birds. In that classification should reflect genetic phylogenies, radical modifications in the current taxonomy are necessary in order to accommodate fish herpesviruses in the family.

Pathogenesis

CCV is readily transmitted from fish to fish, probably entering through the gills. In more artificial settings, it can also be transmitted by injection or orally by ingestion of contaminated food. After experimental infection, CCV can be isolated first from the kidneys and then from other organs, in some of which impressively high titers of virus may be attained.

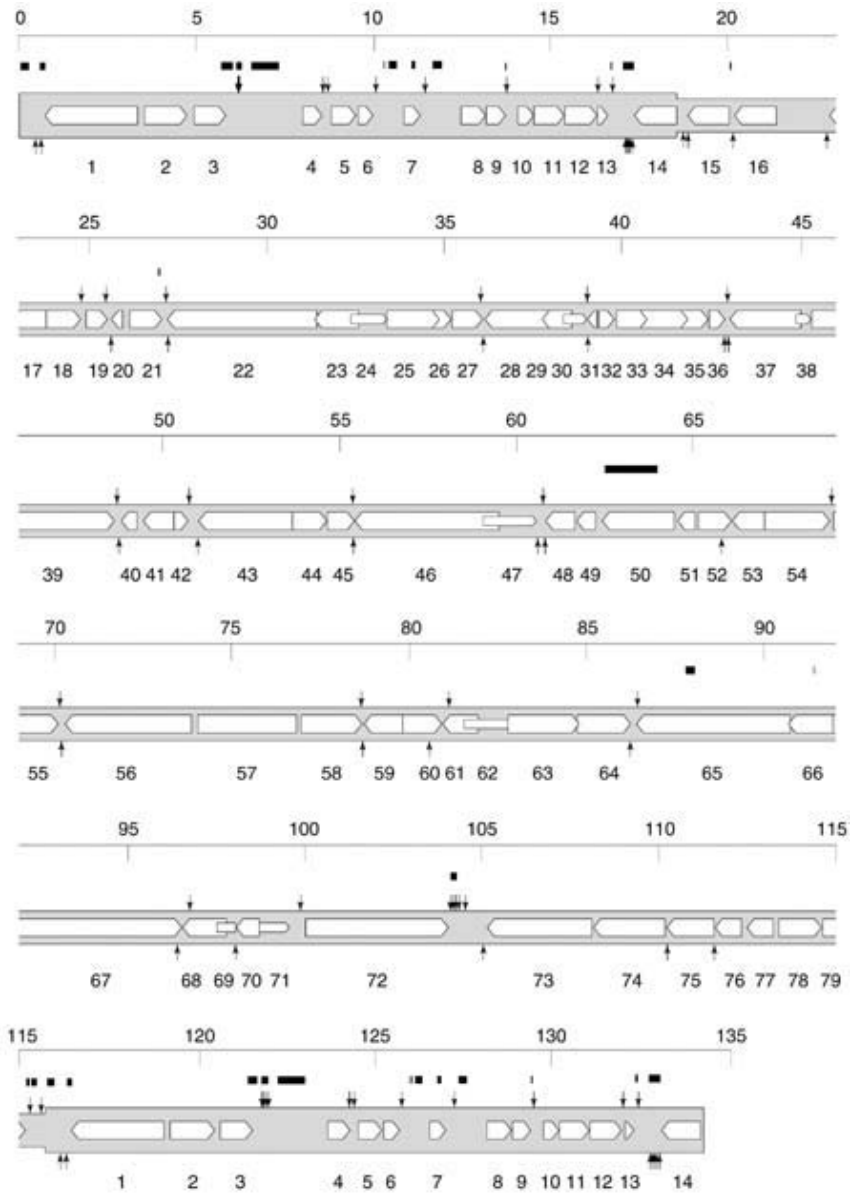


Figure 2 The arrangement of genes in the CCV genome predicted from the complete DNA sequence. The thinner and thicker portions of the genome denote the unique region and direct repeats respectively. The scale is in kilobase pairs. Protein-coding regions are shown as open arrows and numbered below the genome. Thinner arrows are used to reveal regions of overlap. Protein-coding regions 62, 69 and 71 are probably expressed by splicing; regions 57 and 58 may also be spliced. Potential polyadenylation sites are indicated by vertical arrows above and below the genome for rightward and leftward oriented genes respectively. Locations of tandem direct reiterations of short elements are denoted by filled rectangles above the genome. (Modified from Davison AJ (1992) *Virology* 186: 9–14, with permission of Academic Press.)

The primary route for virus shedding is probably via the urine.

Given the virulence of CCV in young catfish, it is quite reasonable to suppose that the virus might persist in an inapparent form in adult fish. This hypothesis is made all the more attractive by the existence of a characteristic latent phase of infection in the natural growth cycles of higher vertebrate herpesviruses. Viral mRNA and antigens have been detected in adult fish and, more recently, virus was recovered by cocultivation of tissues from wintering adult fish, some of which had been immunosuppressed. The site of latency has not, however, been identified. Reactivated CCV from adult fish could, of

course, be transmitted horizontally, but there is good circumstantial evidence that vertical transmission may also occur.

Clinical Features and Pathology

CCV can be remarkably virulent in susceptible populations of channel catfish. Under optimal conditions the incubation period can be as short as 3 days, and mortality can rise rapidly to 100%. Signs of distress may be accompanied by convulsive swimming, including a 'head-up' posture, and lethargy and death follow. Externally, affected fish may display exophthalmia, a distended abdomen and hemorrhages, largely on the ventral surface. Internally, viscera may be enlarged and hemorrhagic, but certain organs, such as kidneys, liver and spleen, may be pale. The digestive tract is empty of food, instead containing a mucoid secretion, and a fluid accumulation is present in the peritoneal cavity. Histopathological examination reveals widespread and profound changes. Initially, the kidneys show edema, hemorrhage and necrosis, and then these features develop in the liver and digestive tract. Electron microscopic studies reveal virus particles in affected organs.

Immune Response, Prevention and Control

Adult channel catfish produce peak neutralization titers on average 8 or 9 weeks after primary immunization with CCV, and a further moderate increase in titer of short duration is apparent after boosting. The importance of virus growth in eliciting the immune response is indicated by the observation that heat-inactivated virus is poorly immunogenic. Serum neutralization indices have been used to document a link between outbreaks of CCV disease and potential carriers of the virus. These studies, though not extensive, have given some indication that seroconverted adult fish are able to transmit CCV. Hopefully, other tools and techniques developed from genome studies will widen the scope and sensitivity of investigations into this aspect of pathogenesis.

Attempts to vaccinate channel catfish against CCV disease have shown promise, and there is increasing interest in this aspect of prophylaxis. CCV that has been attenuated by passage in a clariid fish cell line is able to provide substantial protection against the lethal effect of infection by virulent CCV, particularly when the initial immunization is subsequently boosted. This virus exhibits several genomic differences from wild-type virus, most notably a substantial deletion in gene 50, which potentially encodes a secreted glycoprotein. The use of avirulent strains of virus produced by deleting specific genes may lead in the long term to an efficacious vaccine which, in being unable to revert to virulence, would also be safe. In this context, deletion of gene 5, which encodes thymidine kinase, has been shown to cause attenuation of virulence in channel catfish fingerlings.

CCV is sensitive to nucleoside analogues which inhibit the growth of higher vertebrate herpesviruses. In general, these compounds are phosphorylated by the virus, but not the cellular, thymidine kinase. Further steps in phosphorylation probably involve cellular enzymes. The triphosphate form then inhibits DNA replication by direct interaction with the DNA polymerase or by incorporation into nascent DNA. These compounds include the widely used antiherpetic drug acyclovir. Phosphonoacetate, which inhibits the DNA polymerase by mimicking pyrophosphate, is also inhibitory. It is doubtful, however, whether antiviral therapy will ever be practical for the treatment of CCV disease. Avoidance and containment of outbreaks presently involve the use of ostensibly virus-free breeding stock, as clean environmental conditions as practical, destruction of affected populations and disinfection of ponds.

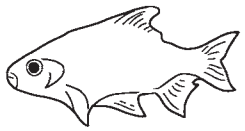
See also: **Antivirals; Fish viruses; Herpes simplex viruses (*Herpesviridae*): General features, Molecular biology; Latency; Vaccines and immune response.**

Further Reading

Plumb JA (1989) Channel catfish virus. In: Ahne W and Kurstak E (eds) *Viruses of Lower Vertebrates*, p. 198. Berlin: Springer.

Wolf K (1983) Biology and properties of fish and reptilian herpesviruses. In: Roizman B (ed.) *The Herpesviruses*, vol. 2, p. 319. New York: Plenum Press.

FISH VIRUSES



Carol H Kim and **Jo-Ann Leong**, Department of Microbiology and Center for Salmon Disease Research, Oregon State University, Corvallis, Oregon, USA

Introduction

Fish, like all other living organisms, can be infected by virus representatives from most of the virus families. Predominantly described as fish pathogens, these viruses include rhabdoviruses, birnaviruses, herpesviruses, iridoviruses, reoviruses, orthomyxoviruses and retroviruses. In this brief review, the most well characterized viruses from each family will be discussed and tables listing the general characteristics of these fish viral pathogens as well as other less well characterized viruses within the family are included (**Tables 1** and **2**). In addition, viral agents that have not been confirmed as the etiologic agent and are tentatively classified are listed separately in **Table 3.**

The history of fish virology parallels the development of fish aquaculture, which began more than 3000 years ago. The culture of cyprinid fishes in China was first described at that time and, since then, many more fish species have been cultured for food and the ornamental trade. Along with the development of fish husbandry have come the first descriptions of viral diseases of fish, including spring viremia of carp and carp pox, which was noted as early as 1563.

The basic replication mechanisms and infectious processes of the fish viruses appear to be no different from their counterparts in warm-blooded vertebrates. However, as the viral genomes have been characterized by sequence and expression analysis, several new genera in different virus families have been discovered. For example, the aquabirnaviruses are classified as a separate genus in the family *Birnaviridae* and are distinguished from the other genera by serological and host range parameters. The novirhabdoviruses, containing certain members of the fish rhabdoviruses, are distinguished by a unique viral gene located between the glycoprotein and polymerase gene. No other rhabdovirus contains such a gene encoding a nonvirion protein. Thus, the study of fish viruses has become a fruitful field of investigation that provides unique opportunities to study pathogenesis in significant numbers of animals.

A great deal of effort has been devoted to the development of control strategies to prevent the spread of virus and to control it once it appears at a hatchery. For aquaculture, as with care of other food animals, the disease control measures must be cost-effective, practical and easy to administer in the field. Avoidance continues to be the most effective and economical control measure for the fish pathogens described here. Presently, strict certification of eggs and fry as specific pathogen-free is required for the shipment of eggs and fish between states and countries. Outbreaks are controlled by the destruction of all eggs and fish at the facility that might be contaminated with virus. Stocks of specific pathogen-free eggs or fish are reared in noncontaminated water (well water). Vaccines have been developed for several of the fish pathogens, and include attenuated vaccines, killed vaccines, DNA vaccines and subunit vaccines prepared by recombinant DNA technology. The vaccines have varying degrees of efficacy, but none are presently licensed and commercially available.

The host species and the relative fecundity of these animals make it possible to develop models for transmission and to study environmental effects that may be too subtle to detect in groups of 10–20 animals. With fish studies, it is possible to work with groups of 500–1000 fish. It is also possible to study the effects of developmental stage on virus infection, which is not always possible with warm-blooded mammals, which undergo a large part of early development *in utero*. With egg-laying fish, the effect of different stages of development on viral pathogenesis can be examined very early in the life cycle of the animal.

Significant contributions to the basic understanding of biological processes in eukaryotic cells are being made through the study of viral diseases of fish. This, as well as the need to control diseases in aquaculture, has provided the impetus to discover and characterize more viruses of fish.

1. RNA Viruses

Rhabdoviridae

Infectious hematopoietic necrosis virus

Infectious hematopoietic necrosis virus (IHNV) is a member of the *Rhabdoviridae* family, genus 'IHNV-like viruses', and is the causative agent of an acute, systemic disease in young salmonid fish.

Table 1 Fish RNA viruses

Classification	*Virus*	*Size (nm)*	*Environment*	*Host*	*Disease*
Birnavirus	Infectious pancreatic necrosis virus	55–65	Marine and freshwater	Salmonids	Necrosis of internal organs and tissue
Orthomyxovirus	Infectious salmon anemia virus	80–120	Marine	Atlantic salmon	Anemia
Paramyxovirus	Chinook salmon paramyxovirus	125–250	Marine	Chinook salmon	No associated disease
Retrovirus	Walleye dermal sarcoma virus	100–130	Freshwater	Walleye pike	Tumors
	Golden shiner reovirus	75	Freshwater	Golden shiner, grass carp	Viremia and necrosis
Reovirus	Channel catfish reovirus	75	Freshwater	Channel catfish	Transient viremia
	Chum salmon reovirus	75	Marine and freshwater	Salmonids	Focal necrosis of liver
	Infectious hematopoietic necrosis virus	160–180 × 60	Marine and freshwater	Salmonids	Necrosis of internal organs and tissues
Rhabdovirus	Viral hemorrhagic septicemia virus	185 × 65	Marine and freshwater	Salmonids	Necrosis of liver, kidney, spleen
	Spring viremia of carp virus	180 × 70	Freshwater	Cyprinids	Viremia, swim bladder inflammation

Table 2 Fish DNA viruses

Classification	*Virus*	*Size (nm)*	*Environment*	*Host*	*Disease*
Iridovirus	Lymphocystis virus	150–300	Freshwater and marine	Freshwater and marine fish	Lymphocystis
	Erythrocytic necrosis virus	140–360	Marine	Marine and anadromous fish	Viral erythrocytic necrosis
Adenovirus	Atlantic cod adenovirus	77	Marine	Atlantic cod	Hyperplastic dermal lesions

Table 3 Fish viruses

Tentative classification[a]	*Virus*
Iridovirus	Carp gill necrosis virus, catfish iridovirus, chromide cichlid iridovirus, cod ulcus syndrome iridovirus, epizootic hematopoietic necrosis virus, goldfish iridovirus, icosahedron cytoplasmic deoxyribovirus, lymphocystis virus, sheatfish iridovirus, white sturgeon iridovirus
Adenovirus	Dab adenovirus
Birnavirus	Eel virus European, flounder ascites virus, Menhaden virus, sand goby virus, turbot birnavirus, yellowtail ascites virus
Calicivirus	Opaleye virus
Coronavirus	Carp coronavirus
Picornavirus	Atlantic samon virus, bluegill virus, cutthroat trout virus, rainbow trout smelt picornavirus
Paramyxovirus	Rainbow trout paramyxovirus
Reovirus	13p2 reovirus, Atlantic salmon reovirus, chinook salmon reovirus, eel reovirus, fancy carp reovirus
Rhabdovirus	Eel rhabdovirus, Hirame rhabdovirus, pike fry rhabdovirus, snakehead rhabdovirus
Unclassified viruses	Atlantic salmon papilloma virus, brown bullhead papilloma virus, erythrocytic inclusion body syndrome virus, intraerythrocytic virus, platyfish virus, winter flounder papilloma

[a] Tentative classification is based on virus morphology.

History The virus was first discovered in sockeye salmon (*Oncorhynchus nerka*) dying at hatcheries in Washington in 1953. Similar outbreaks among hatchery-reared chinook salmon in California were reported in 1960. The virus was spread to new sites by the use of unpasteurized sockeye salmon viscera as feed. Although this practice was soon stopped, the disease continued to spread in the 1970s by the shipment of infected eggs to Japan, the eastern USA and Europe. The first laboratory propagation of IHNV was carried out when salmonid fish cell lines became available.

Taxonomy and classification IHNV particles have a bullet-shaped or cone-shaped morphology. The virus belongs to a new genus which has six viral genes in the order 3′-N-P-M-G-NV-L-5′ on the negative-sense single-stranded RNA genome. The N is the nucleoprotein gene; P, the phosphoprotein gene; M, the matrix protein gene; G, the glycoprotein gene; NV, the nonvirion protein gene, and L the polymerase gene. Recently the International Committee on the Taxonomy of Viruses accepted the classification of IHNV with a new genus called *Novirhabdovirus* for the unique *Non-Vir*ion protein gene located between G and L on the viral genome.

Geographic and seasonal distribution Originally, the range of IHNV was thought to be confined to salmonid fish in the Pacific Northwest of North America. However, the shipment of infected eggs or fry has extended the range to include Japan, Korea, Taiwan, China, Spain, Belgium and France. Sporadic outbreaks of the disease have also been reported in Colorado, South Dakota, Virginia and Montana. Most infectious hematopoietic necrosis (IHN) outbreaks occur at temperatures of 12°C or lower among fry and fingerlings. Thus, the disease among steelhead trout fry normally appears in March or April in Washington.

Host range and virus propagation Initially, the host range was confined to the genus *Oncorhynchus* and included the sockeye, kokanee, chinook, cherry, biwa and chum salmon, and yamame, amago, steelhead, cutthroat and rainbow trout where the virus can kill at 3–18°C. The virus can also infect and kill Atlantic salmon (*Salmo salar*), brown trout, brook trout and Japanese char. Coho salmon, arctic grayling, pink salmon, lake trout and Arctic char are relatively resistant to IHNV. The virus can be propagated in a large number of cell lines including RTG-2 (rainbow trout gonad), CHSE-214 (chinook salmon embryo), and EPC (epithelioma papillosum cyprini) at temperatures ranging from 4 to 20°C. Growth is not supported at 23–25°C.

Transmission and tissue tropism Horizontal transmission of the virus from infected feed and in the water from infected fish has been amply demonstrated. However, the vertical transmission of the virus has been the subject of much discussion among scientists studying IHN in the field. Clearly, vertical transmission from infected parents occurs predominantly from surface-contaminated eggs and sperm. The decontamination of eggs by iodophore treatment prevents the vertical transmission of the virus in most cases.

In the infected animal, IHNV is found predominantly in the anterior kidney tissue, where the fish

hematopoietic cells are located, in the spleen, liver and pyloric caeca. There are isolates of the virus that target the brain tissue and selected variants can be found preferentially in liver tissue.

Clinical features and infection In acute disease, moribund fish are lethargic, with periods of sporadic whirling or hyperactivity. Affected fish show signs of the disease, with dark coloration and petechial hemorrhages at the base of fins as well as distended abdomen, exophthalmia, pale gills and mucoid, opaque fecal casts. Histopathologic examination reveals severe degenerative changes throughout the kidney, the hematopoietic organ of fish.

Immune response Fish that have recovered from a virus infection have been shown to have anti-IHNV antibodies by both virus neutralization assays and by ELISA. The presence of a cellular immune response has not been demonstrated satisfactorily because assays for cell-mediated immunity in rainbow trout have not been adequately developed. Nevertheless, laboratory studies with infected versus uninfected CSHE-214 cells indicate that the infected cells are killed at a much increased rate by cytotoxic cells from fish that have recovered from IHN than from control fish.

Viral hemorrhagic septicemia virus

Viral hemorrhagic septicemia virus (VHSV) is the etiologic agent for an acute to chronic disease of rainbow trout and other salmonid species. The disease, viral hemorrhagic septicemia (VHS), is caused by this virulent strain of the virus that is found only in Europe. A VHSV strain that is avirulent in salmonid fish has been found in cod and herring in marine waters of the Pacific Northwest and Alaska.

History The disease was first recognized in the small village of Egtved, Denmark, where trout were dying of an entero-hepatic-renal syndrome. The virus is thought to have existed in Europe for eons as a low level pathogen among the native salmonid fish species, i.e. brown trout and Atlantic salmon. However, when the North American rainbow trout was introduced in 1879 into France and in 1882 into Germany, the new host–pathogen relationship proved deadly. The virus is still a problem at trout farms in France, Belgium, Germany, Norway, Czech Republic, Switzerland and Italy.

Taxonomy and classification VHSV is a member of the *Rhabdoviridae* family with an unassigned genus. The entire sequence of the viral genome has been determined. The genome contains six viral genes arranged in the order, 3′-N-P-M-G-NV-L-5′, where N is the nucleoprotein gene, P is the phosphoprotein gene, M is the matrix protein gene, G is the glycoprotein gene, NV is a nonvirion protein gene, and L is the polymerase gene. The genome order, the presence of the NV gene, and phylogenetic analysis of the glycoprotein gene has placed VHSV in the *Rhabdoviridae* along with infectious hematopoietic necrosis virus, snakehead rhabdovirus and hirame rhabdovirus. The virion has the typical bullet-shaped morphology and measurements indicate that the size of the virion in negatively stained preparations is 70 nm × 180 nm.

Geographic and seasonal distribution To date, VHS is only known to occur in Europe. Outbreaks of the disease have not been found in other parts of the world where trout are raised in large numbers, such as Australia, Japan, North or South America. A North American strain of VHSV has been isolated and appears to be enzootic among certain species of marine fish in the North Pacific Ocean. This strain is not proven to be virulent in salmonid fish species.

Outbreaks of VHS typically involve young fish at the fingerling to yearling stage of development. The disease usually appears when water temperatures drop to 10°C or lower.

Host range and virus propagation The susceptible species include Atlantic salmon, brook trout, brown trout, golden trout, grayling, lake trout, pike, rainbow trout, sea bass, turbot and whitefish. The incubation time after virus infection is usually 2 weeks after inoculation and 3–4 weeks by waterborne exposure. Interestingly, common carp, chinook salmon, chum, coho salmon, goldfish, roach and tench are refractory to the lethal effects of virus infection. In cultured cells, the virus grows well in BF-2 (bluegill fibroblasts), CHSE-214, EPC, FHM (fathead minnow epithelial cells) and RTG-2 cells. The range of replication extends from 4 to 20°C, with optimal yields at 10–15°C. The virus loses infectivity at temperatures above 20°C. At 14°C, new virus first appears at 7–10 h postinfection, with exponential production to 6–10 h until production reaches a plateau at about 24–48 h. Virus production can reach high titers of 7.5×10^7 PFU ml^{-1}.

Transmission and tissue tropism Horizontal transmission is very temperature dependent. It occurs readily at temperatures between 1 and 12°C; temperatures above 15°C are nonpermissive to virus transmission. The source of this virus is believed to be the urine of infected fish. Vertical transmission is

considered extremely rare and probably due to the surface contamination of the eggs with virus in the ovarian fluid. However, unlike IHNV, VHSV has never been isolated from seminal fluid. The virus has been found in the anterior kidneys where the hematopoietic tissues are located and focal necrosis occurs in the liver, another prime target organ.

Clinical features and infection The disease outbreaks begin with individual fish that do not feed and become lethargic or hyperactive. The afflicted fish are noticeably darker, may have exophthalmia in one or both eyes, pale gills which may have petechiae, and hemorrhages at the fin bases. The mortality rates are high. Among the survivors, virus can be isolated for a short period after the peak of mortalities and then infectious virus cannot be isolated, much as has been described for IHNV.

Immune response Production of neutralizing antibody in fish is surprisingly low even after monthly infection of attenuated virus for a year. Specific lymphostimulation to inactivated VHSV was observed in fish that survived a VHS outbreak and in about half of the trout vaccinated by immersion. These studies indicate that fish can mount an immune response to VHSV, and efforts to develop a useful vaccine has been continuing since 1976.

Spring viremia of carp virus

Spring viremia of carp virus is a member of the *Rhabdoviridae* family and the causative agent of an acute infectious dropsy of carp

History In 1971, Fijan and colleagues reported the multiple isolation of a rhabdovirus from carp with signs of the disease, acute infectious dropsy of carp (IDC). They subsequently showed that the rhabdovirus, which they named *Rhabdovirus carpio*, was the causative agent of the viral infection, spring viremia of carp (SVC). The virus is now called spring viremia of carp virus (SVCV). Outbreaks of the disease have been confined to Europe, where reports of its isolation from infected carp have come from The Netherlands, France, Hungary and Germany.

Taxonomy and classification SVCV is a member of the *Rhabdoviridae* family and has been placed within the *Vesiculovirus* genus. The sequence of the viral genome has largely been determined and its gene order is 3′-N-P-M-G-L-5′, where N is the nucleoprotein; P, the phosphoprotein; M, the matrix protein; G, the glycoprotein; and L, the virion polymerase. Unlike the other fish rhabdoviruses described in this review, SVCV does not have an additional gene between the G and L genes. The virus is distinct serologically from IHNV, VHSV, several eel viruses, pike fry rhabdovirus and Rio Grande perch rhabdovirus. Two strains of SVCV have been recognized by serological analysis.

Geographic and seasonal distribution The range of SVCV is still considered to be confined to cyprinid fish species in Europe, Russia and China. The isolation of SVCV has never been reported in the USA despite the repeated introductions of common carp in North America. This may be due to the fact that carp are not highly regarded as game or food fish. Little effort has been expended in determining the virologic status of the carp population in the USA.

As the name implies, disease outbreaks occur in the spring. When temperatures warm in the spring from 11 to 16–20°C, fish become more easily infected; however, the virus kills more fish at the lower temperatures when the fish immune system is less active.

Host range and virus propagation SVCV kills both adult and young common carp. The virus has also been isolated from other cyprinid species, such as silver carp (*Hypophthalmichthys molitrix*), bighead carp (*Aristichthys nobilis*), crucian carp (*Carassius carassius*) and the sheatfish (*Silurus glanis*). It has been grown in guppies (*Lebistes reticulata*) and the fry of the northern pike (*Esox lucius*).

The virus can be propagated in tissue culture cells grown at 20°C. The susceptible lines which exhibit cytopathic effects induced by SVCV infection are FHM, EPC and BF-2 (Bluegill fibroblast) cells. At 20°C, cytopathic effects appear within 2 days.

Transmission and tissue tropism Horizontal transmission is thought to be the primary mode of virus transmission. There is circumstantial evidence that vertical transmission also takes place, as ovarian fluids but not seminal fluids of 5–10-year-old asymptomatic carp contained virus. The carp louse (*Argulus foliaceus*) may be a vector for the virus, as these parasites have been shown experimentally to ingest the virus from SVCV-infected carp and transmit the virus to healthy fish 6–7 days later.

The virus is normally found in the kidneys at 7–14 days after infection. By days 14–21 the liver and spleen have the highest concentration of virus. The virus can also be isolated from the blood, brain, gills, intestine and spleen.

Clinical features and infection The clinical diagnosis of SVC is aimless swimming, abdominal

distension, ascites and hemorrhagic condition of skin, gills and viscera.

Immune response The immune response to SVCV is influenced by temperature, route of immunization, and the age and condition of the host. Adult carp infected at 14°C produced neutralizing antibody slowly; when the fish were held at 25°C, antibody appeared more rapidly. In addition, the fish respond with interferon production within 24 hours of infection.

Birnaviridae

Infectious pancreatic necrosis virus

Infectious pancreatic necrosis virus (IPNV) is the etiologic agent for an acute systemic disease of young trout and Atlantic salmon. The disease is widespread and is a serious pathogen in fish farms in Europe.

History Although the disease had been described in newly feeding hatchery trout fry as early as 1941, the discovery of the viral agent was not made until 1957 by Wolf and Dunbar. They described the isolation of IPNV, the first fish virus to be grown *in vitro*. Since those early investigations, the virus genome has been characterized, cloned and sequenced for a variety of different IPNV strains. The disease process has been characterized and vaccines have been developed for IPNV infection in Atlantic salmon.

Taxonomy and classification IPNV is the prototype virus for the genus *Aquabirnavus*, family *Birnaviridae*. The virions are about 60 nm in diameter, single-shelled, nonenveloped icosahedrons and contain two segments of double-stranded RNA, each with a covalently linked protein at the 5′ end. The buoyant density of the virion in CsCl is 1.33 g ml^{-1}. The viral genome constitutes between 9 and 10% of the virus particle by weight. The size of the larger segment A for IPNV ranges in size from 2962 to 3092 bp and encodes a polyprotein containing NH_2-VP2-NS-VP3-COOH. The smaller segment B, 2731–2784 bp, encodes the viral protein, VP1.

The major virion polypeptides are: VP1 (94 kDa), which is the RNA-dependent RNA polymerase as well as the genome-linked protein; VP2 (54 kDa), the major capsid polypeptide and type-specific antigen; and VP3 (30 kDa), an internal capsid protein and group-specific antigen. A nonstructural protein, NS (29 kDa), is the virus-coded protease that autocatalytically cleaves the polyprotein to produce VP2, NS and VP3. An additional nonstructural polypeptide that is positively charged has been designated as the 17 kDa protein and is encoded in an overlapping open reading frame preceding the segment A encoded polyprotein.

Geographic and seasonal distribution Although the disease was first known in North America, outbreaks of infectious pancreatic necrosis (IPN) among fish have been reported in almost all parts of the world where fish are reared. The virus has been found in countries in Europe, Asia, and North and South America. It has not appeared in Australia and New Zealand, yet. The mortality rate for the virus is markedly influenced by temperature: at 14–15°C, it may be 80%; and at 10°C, it may be only 20%.

Host range and virus propagation The possible hosts for IPNV range from salmonid fish species to at least 20 piscine families, including tropical fish, eels, sea bass and lamprey. The virus is pathogenic for many of these species. IPN-like viruses have also been isolated from mollusks and crustaceans as well as trematode parasites of fishes. Virus propagation *in vitro* can be carried out in a wide variety of cell lines, although the CHSE-214 and RTG-2 cell lines are generally recommended for isolation. The temperature range of incubation is 4–26°C and cytopathic effects become apparent sooner at higher temperatures (9 h at 26°C versus several days at 4°C).

Transmission and tissue tropism Survivors of IPNV epizootics become carriers and shed large quantities of virus (10^3–10^5 $TCID_{50}$ g^{-1}) intermittently for long periods of time in their sex products and feces. Thus, horizontal transmission occurs by contact and ingestion of contaminated water. The virus enters through the digestive tract and gills. Vertical transmission is likely due to the virus presence in the seminal and ovarian fluids. There is also strong evidence that the salmonid egg from carrier fish can carry the virus at a site that is resistant to iodophore treatment. In acutely infected fish, virus can be isolated from the pancreas, intestine and kidneys.

Clinical features and infection In brook and rainbow trout, the disease is first detected by an erratic swimming behavior when the victim trout rotates about its long axis. This is usually a terminal sign but some fish do recover. There is overall darkening, mild exophthalmia and abdominal distension. The affected fish may trail long, thin, whitish cast-like excretions from the vent. In order fingerling trout, IPNV infection sometimes results in petechial hemorrhages through the viscera. Histopathologic examination of the pancreas reveals extensive necrosis.

Immune response Survivors and fish vaccinated with killed IPNV vaccine all produce neutralizing anti-IPNV antibody. Despite high concentrations of IPNV antibody in these fish, virus is also produced in the survivors (carrier) and the presence of antibody is not a good indicator that fish will be protected against the lethal effects of IPNV infection. Thus far, no IPNV vaccine has proven to be effective in field trials.

Reoviridae

Golden shiner reovirus

Golden shiner virus (GSV) disease is a reovirus infection that occurs during high temperatures, frequently in the late summer and fall, among commercially reared bait minnow *Notemigonus crysoleucas*.

History GSV was first discovered in the southern USA in 1977 by Schwedler and Plumb in populations of golden shiner bait fish. The virus only kills fish in large numbers when the fish are stocked at high density.

Taxonomy and classification The virus belongs to the genus *Aquareovirus* in the family *Reoviridae*. Its genome is composed of 11 segments of double-stranded RNA that occur in three size classes. The three large segments range in size from 2.6 to 2.1×10^6 Da; the three medium segments from 1.9 to 1.5×10^6 Da, and the five small segments, from 1.1 to 0.3×10^6 Da. The total genome length is 22 500–23 000 nt. The virus has five structural proteins: two large polypeptides of 135 and 125 kDa, one medium size polypeptide of 70 kDa, and two small polypeptides of 45 and 34 kDa.

The virion is nonenveloped and contains a double-layered icosahedral capsid of 75 nm in diameter. The diameter of the core structure in 50 nm.

Geographic and seasonal distribution The virus was first recovered from golden shiners in several southern states, where they were raised and shipped to other areas of the USA. Since then, the virus has been found in California golden shiners and in grass carp.

Host range and virus propagation Only the golden shiner and grass carp have been identified as susceptible to virus infection. In the case of grass carp, the infected fish appear normal. Virus can be propagated in grass carp cells and EPC.

Transmission and tissue tropism Horizontal transmission has been documented and accounts for natural infections. The incubation time is estimated to be 1–2 weeks. Although specific histologic studies have not been carried out, the visceral organs of suspect fish are sampled for virus infectivity assays.

Clinical features and infection Infection in golden shiner is first noted when the fish swim near the surface and appear listless. Dying fish will swim in a spiral. There is marked hemorrhage on the dorsal surface and petechial hemorrhages on the ventral surfaces, the cornea, the intestinal mucosa and visceral fat.

Orthomyxoviridae

Infectious salmon anemia virus

Infectious salmon anemia (ISA) is a disease of farmed Atlantic salmon (*Salmo salar* L.) in Norway that affects both erythrocytic and leukocytic cells to produce a severe anemia.

History ISA has been the cause of massive economic losses in the Atlantic salmon farming industry in Norway, where it was first recorded at the end of 1984. The disease incidence peaked in 1991 and is now considered the most dangerous fish disease in the European Union. The etiological agent has been identified as an orthomyxovirus.

Taxonomy and classification The genome of infectious salmon anemia virus (ISAV) is composed of eight segments of single-stranded RNA that range in size from 1.0 to 2.3 kb, with a total molecular size of approximately 14.5 kb. Investigators in Norway have cloned and sequenced the smallest genome segment. Although analysis of this segment does not reveal any significant homology with the available sequences for orthomyxoviruses, the morphological and replication properties indicate strongly that ISAV is a member of the *Orthomyxoviridae*.

Geographic and seasonal distribution Outbreaks of ISA were once confined to Norway at Atlantic salmon farms; it has since been reported in New Brunswick, Canada, at Atlantic salmon farms in the area.

Host range and virus propagation The virus has only been reported in farmed Atlantic salmon. It has been propagated in tissue culture cell lines, including CHSE-214, SHK-1 (salmon head kidney) and AS (Atlantic salmon) cells. Cytopathic effects are observed 12–14 days after inoculation with ISA-infected

tissue material. The virus propagation is severely reduced when the growth temperature exceeds 18°C.

Transmission and tissue tropism Transmission trials demonstrated that Atlantic salmon parr developed ISA after intraperitoneal injection. The macrophages in the liver were the first cells to show infection and then hemorrhagic necroses appeared by day 21. The erythrocytic cells are targets for virus infection.

Clinical features and infection The infected fish (postsmolt) have ascites, congestion and enlargement of liver and spleen, congestion of the foregut and petechiae in the peritoneum. The hematocrit values drop from 25 to less than 10 near the end of the disease.

Retroviridae

Walleye epidermal hyperplasia retrovirus, walleye dermal sarcoma retrovirus

Four virus-associated skin lesions have been observed in walleyes, *Stizostedion vitreum vitreum:* lymphocystis, diffuse epidermal hyperplasia, dermal sarcoma and discrete epidermal hyperplasia. Lymphocystis is caused by an iridovirus. Diffuse epidermal hyperplasia appears to be caused by a herpesvirus. The other two diseases are caused by closely related retroviruses. These diseases can be found singly or in combination.

History The presence of translucent and raised nodular lesions on the skin of walleye in New York was first reported in 1969 by Walker. Similar neoplastic lesions were reported in walleyes from two provinces in central Canada and further studies showed that the 'tumors' contained C-type virus particles. More recently, Bowser and Casey at Cornell University have conducted further studies regarding the epizootiology and virus structure of the retroviral agents of walleye discrete epidermal hyperplasia and provided evidence for two different retroviral etiologic agents.

Taxonomy and classification The viruses, walleye dermal sarcoma virus (WDSV) and walleye discrete epidermal hyperplasia virus (WEHV) types 1 and 2 (WEHV1 and WEHV2) are closely related by sequence analysis. The polymerase regions are related by 77% identity for WEHV1 and WEHV2, and 64% identity for WDSV. Although the viruses appear by phylogenetic analysis of the reverse transcriptase domain to cluster with the mammalian type C retroviruses, their classification in the family *Retroviridae* remains unspecified. The WDSV virus has been cloned from tumor tissue and found to be 12.7 kb in length with three open reading frames in addition to gag, pol and env.

Geographic and seasonal distribution The disease, walleye discrete epidermal hyperplasia (WEH), has been observed on approximately 10% of adult breeding walleyes in Oneida Lake, NY, and up to 20% of the walleyes in some lakes in Canada. WEH is present in sexually mature fish during the fall through spring and disappear during the summer months. Apparently, the lesions appear and regress on a seasonal basis. In the case of walleye dermal sarcoma (WDS), the disease can affect up to 27% of walleyes in North American lakes. These lesions, which are distinct from epidermal hyperplasia, also develop in the fall, regress in the spring, and disappear in the summer.

Host range and virus propagation Both viruses, WEHV and WDSV, are found in walleye. Cell free filtrates of tumor tissue when injected into 9-week-old walleye fingerlings produce tumors in 87% of the animals in a 14 week period. The introduction of the virus into other fish species has not been reported. There are no reports of the propagation of WEHV or WDSV in tissue culture cells. All reports on sequence of different viral genes are on clones derived from tumor tissue-prepared virus.

Transmission and tissue tropism Horizontal transmission from infected fish is presumed. Whether or not vertical transmission occurs has not been determined and alternate hosts or reservoirs have not been identified. Both diseases appear to be confined to the surface areas of the fish dermis and epidermis.

Clinical features and infection WEH appears as a hyperproliferative skin disease with plaques of thickened epidermis that can be found on any part of the body. WDS is a disease of benign cutaneous neoplasms that arise multicentrically from the superficial surface of the scales. These lesions often appear to be malignant histologically but they regress in the summer and neither local invasion nor metastasis has been observed in feral fish.

Immune response The immune response to WEHV and WDSV has not been determined. Investigators are determining whether fish whose tumors have regressed during the summer will develop a new crop of tumors in the fall.

2. DNA Viruses

Iridoviridae

Lymphocystis virus

Lymphocystis virus is the etiologic agent for a benign, chronic infection of lymphocytic cells that causes a gross hypertrophy but has little impact on the host. A viral infection is generally superficial and the condition is much like warts.

History Lymphocystis has been documented in the literature for the last century and was first thought to be the result of a parasitic infection of the fish. In 1914 it was first proposed that the disease was caused by a virus, and in 1951 this claim was substantiated by demonstrating transmission of the disease with cell-free filtrates. The virus was transmitted to healthy bluegill fish with bacteria-free homogenates in 1962, and in 1966 the virus was isolated in tissue culture which was subsequently used to infect naive fish.

Taxonomy and classification Lymphocystis virus has an icosahedral symmetry with a large double-stranded genome. It is in the *Lymphocystivirus* genus of the *Iridoviridae* family. The particles contain a dense core within two noncellular membrane-derived envelopes and have filaments extending from the vertices. The size of the virus particles varies with the host that is infected and range from 150 to 300 nm.

Geographic and seasonal distribution This virus has a worldwide distribution and, although it infects fresh as well as marine fishes, it is primarily found in the more evolved fish species, with the exception of salmonid fish. Lymphocystis virus displays a host specificity, and may crossinfect at the genus level, but not family. Temperature plays a role in the development of lesions and there may therefore be seasonal variation in infection rates.

Host range and virus propagation At least 97 species of fish have been infected with the virus, which can also infect invertebrates. There is a host specific for each isolated virus, indicating that there may be many serotypes. The virus replicates in fibroblastic cell lines and is best propagated in cells derived from the host or closely related species. These cultures are maintained in conditions that would provide the best environment for the cells. Cytopathic effects caused by the virus in culture include the formation of giant cells, cytoplasmic inclusion bodies and hyaline capsules.

Transmission and tissue tropism The disease is maintained in host fish and is passed horizontally by fish that have damaged or abraded lesions. Contact is the principal means of transmission, but external parasites have also been implicated in the spread of disease. The virus enters through external surfaces, including the gills. Onset and duration of clinical disease is dependent on the infected host species and the host environment, with fish in colder temperatures experiencing longer incubation periods and lesions persisting for up to a year, while fish in warmer temperatures may resolve the infection and ensuing lesions within a few weeks.

Clinical features and infection The virus causes a lymphocystis in fish that is chronic but benign. Lesions on the skin and fins are composed of grossly hypertrophied cells. The hypertrophy can result in a 50 000–100 000-fold increase in cell volume. Usually there are no mortalities associated with infection and the lesions heal spontaneously. The infection is first apparent with the development of small, pearl-like tumefactions on skin of body, fins, tail and gills. The lesions increase in size and become bibulous, white to opalescent and may become pink in larger lesions. As the lesions mature, they may become hemorrhagic in appearance and look more wart-like. Lesions have also been found in the peritoneum or pericardium, pharynx, intestinal walls, ovaries, spleen and liver. At maturity, the lesions are 100 μm in diameter and can sometimes exceed 1 mm.

Erythrocytic necrosis virus

Formerly called piscine erythrocytic necrosis virus (PENV), viral erythrocytic necrosis virus (VENV) is the etiologic agent that causes serious anemia in at least 20 species of marine and anadromous fish.

History Viral erythrocytic necrosis (VEN) was first reported in 1969 and described as a pathological condition affecting the erythrocytes of three marine species of fish. A hallmark of this condition was the appearance of inclusion bodies in the cytoplasm of infected cells; in 1971, electron microscopy studies showed that virus particles were associated with the inclusion bodies.

Taxonomy and virion structure VEN, in the *Iridoviridae* family, are icosahedral particles with a hexagonal profile. The particles are 140–360 nm in diameter, depending on the species of fish that is infected. Structural details also vary according to species infected. For example, viral particles isolated from herring and salmon have an electron dense coat

8 nm thick, a 22 nm clear zone and a 130 nm nucleoid core with a less electron dense center. Particles from cod have a larger outer hexagonal capsid of 300–360 nm, which appears trilamellar, and an electron dense central core.

Geographic distribution and host range VEN is most probably found worldwide in marine and anadromous fish. The virus has been reported from the Pacific and Atlantic coasts of North America, the UK, Japan, Norway and Chile. Hosts include cyclostomes, elasmobranchs and 14 families of marine or anadromous fish. Chum, pink, coho and chinook salmon and steelhead trout are all susceptible. Older inapparent carrier fish are assumed to be the reservoirs of infection for young fish. Infection of fish has been demonstrated by intraperitoneal and waterborne transmission studies. In addition, transmission of virus infection from herring to salmon under experimental conditions indicates a marine source of virus in aquaculture. To date this virus has never been successfully grown in cell culture.

Clinical features and infection Younger fish are more susceptible to VEN infection and the disease is more devastating to juvenile fish. Infected erythrocytes can be readily identified by appearance and are more easily lysed than normal cells. Infected fish are more susceptible to secondary bacterial infection and environmental stresses. Blood smears show inclusion bodies in the cytoplasm of erythrocytes. Giemsa-stained smears display inclusions that are acidophilic, 1–5 nm in diameter, and intracytoplasmic. Infected erythrocytes show nuclear degeneration, margination of chromatin, pyknosis, karyorhexis and karyolysis. An increased number of immature erythrocytes occurs in peripheral blood, and hematocrit values are depressed. In addition, blood from fish with VEN clots abnormally slowly or not at all. Two types of inclusion bodies have been identified from herring erythrocytes by electron microscopy. One type is associated with virions and is coarsely granular, round and electron dense. The other type of inclusion body is membrane bound with few, if any, virions. The primary diagnostic tool is stained blood smears, with definitive diagnosis by the examination of particles by electron microscopy.

Pathology and histopathology There are no external signs of disease, except for pale gills due to severe anemia, and no mortalities directly associated with VEN infection. Herring may display red external surfaces if heavily infected with virus and organs may be pale as a result of anemia.

Adenoviridae

Atlantic cod adenovirus

Atlantic Cod adenovirus was first described in 1980 and was the first adenovirus-associated disease described in fish. Fish with raised epidermal hyperplastic lesions were examined during a study of ulcus syndrome in cod (*Gadus morhua*) off the coast of Denmark, and virus was seen in the nuclei of cells of these lesions. Epithelial neoplasms have also been found in dab (*Limada limanda*) from the adjacent North Sea, and a common viral etilogy has been proposed.

Taxonomy and classification The virus is in the *Adenoviridae* family and unassigned to a genus. The particles have an icosahedral morphology and are 77 nm in diameter. A 10 nm capsid surrounds an electron dense nucleoid. Although this virus is considered the etiologic agent of the disease, there are few virus particles associated with infected tissue and they are found only in the outermost cells of the epidermis.

Clinical features and infection The skin lesions are flat, raised and transparent plaques that can occur anywhere on the body of the fish, but are most often seen on the caudal regions. The lesions range from 3 to 20 nm in diameter. The infected epidermis is four times thicker than normal tissue and has no or few mucous glands. In addition, there is an increase in the vasculature under the plaques.

Sturgeon adenovirus

Sturgeon adenovirus particles have an icosahedral morphology with a double encapsidation of the nucleoid core. The virus is associated with hypertrophied nuclei of infected epithelial cells of the gastrointestinal tract and causes mortalities of white sturgeons (*Acepenser transmontanus*).

History The sturgeon adenovirus was the first virus to be identified in a sturgeon. In 1984, white sturgeon fry from the Sacramento River in California were experiencing devastating mortalities. It was first thought to be of bacterial etiology but was later found to be caused by an adenovirus.

Virion structure The viral particles have an icosahedral morphology with an inner capsid of 47 nm diameter and outer capsid of 74 nm in diameter.

Virus propagation Propagation of the sturgeon adenovirus has been attempted in several fish cell culture systems, including sturgeon heart (SH-1) and

spleen (SS-2) cells, without success. Primary cultures from infected sturgeon tissues have also been unsuccessful in propagating the virus.

Fish were experimentally infected with the sturgeon adenovirus by intraperitoneal inoculation with tissue homogenates from infected fish. The fish were maintained for 4 weeks and then histologically examined. The gastrointestinal epithelial cells displayed hypertrophy of the nuclei similar to, but in a less intense presentation, the natural infection.

Clinical features and infection White sturgeon infected with the adenovirus were lethargic, anorexic and emaciated, with no food found in the alimentary tract, and the liver tissue was pale. Histological examination showed hypertrophied nuclei of mucosal cells of the gastrointestinal tract.

See also: **Fish herpesviruses (*Herpesviridae*).**

Further Reading

Hetrick FM and Hedrick JP (1993) New viruses described in fin fish from 1988–1992. *Annu. Rev. Fish Dis.* 4: 187.

Wolf K (1988) *Fish viruses and fish viral diseases.* Ithaca: Cornell Press.

FOOT-AND-MOUTH DISEASE VIRUSES (*PICORNAVIRIDAE*)

David J Rowlands, Molecular Science Department, The Wellcome Research Laboratories, Beckenham, Kent, UK

History

The earliest recorded account that clearly describes foot-and-mouth disease was made by Fracastorius in 1546. The importance of this highly contagious disease of cloven hoofed animals increased with the accelerating productivity of the livestock industry and the control of more lethal infections of cattle such as rinderpest towards the end of the nineteenth century. It is now amongst the most important and feared diseases of domestic livestock.

It was the first animal disease demonstrated to be caused by a filterable agent in 1897 by Loeffler and Frosch, who also demonstrated the presence of neutralizing antibody in serum. It is also the first virus for which serotype differences were recognized. Research on the virus was greatly facilitated when Waldemann and Pope showed in 1921 that guinea pigs could be infected, and in 1951 when Skinner showed that it caused a lethal infection in suckling mice. Subsequently the demonstration that the virus could be cultured in tissue culture cells and particularly in the BHK-21 cell line has enabled both studies of viral replication and the large-scale production of vaccines.

Taxonomy and Classification

Foot-and-mouth disease viruses (FMDVs) form the *Aphthovirus* genus of the *Picornaviridae* family. The nature and organization of the genome, the mode of replication and the structure of the virion are, in general, similar to other viruses in the family. The original subdivision of the *Picornaviridae* into the four genera, *Enterovirus*, *Rhinovirus*, *Cardiovirus* and *Aphthovirus*, was based on physicochemical properties such as susceptibility to acid inactivation, buoyant density of CsCl solution and the nucleotide composition of the genomic RNA. More recently, analysis of evolutionary relationships by nucleotide sequence comparisons have largely endorsed the original classifications. However, hepatitis A virus is now classified within a separate genus, *Hepatovirus*, and Equine Rhinovirus Type 1 is included within the genus *Aphthovirus*. Properties which distinguish the aphthoviruses are: (1) extreme sensitivity to acid inactivation (<pH 6.8 in low ionic strength buffer); (2) high buoyant density in CsCl (1.43–1.50 g ml^{-1}); (3) possession of a poly(C) tract in the 5′ untranslated region (UTR) of the RNA (a property shared with some cardioviruses); (4) three separately encoded VPg proteins; (5) the use of two alternative in-frame protein translation initiation sites; and (6) a leader protease protein located in the N-terminal region of the polyprotein.

Properties of the Virion

As for other picornaviruses, FMDV particles are naked icosahedrons comprising 60 copies each of four

spleen (SS-2) cells, without success. Primary cultures from infected sturgeon tissues have also been unsuccessful in propagating the virus.

Fish were experimentally infected with the sturgeon adenovirus by intraperitoneal inoculation with tissue homogenates from infected fish. The fish were maintained for 4 weeks and then histologically examined. The gastrointestinal epithelial cells displayed hypertrophy of the nuclei similar to, but in a less intense presentation, the natural infection.

Clinical features and infection White sturgeon infected with the adenovirus were lethargic, anorexic and emaciated, with no food found in the alimentary tract, and the liver tissue was pale. Histological examination showed hypertrophied nuclei of mucosal cells of the gastrointestinal tract.

See also: **Fish herpesviruses (*Herpesviridae*).**

Further Reading

Hetrick FM and Hedrick JP (1993) New viruses described in fin fish from 1988–1992. *Annu. Rev. Fish Dis.* 4: 187.

Wolf K (1988) *Fish viruses and fish viral diseases.* Ithaca: Cornell Press.

FOOT-AND-MOUTH DISEASE VIRUSES (*PICORNAVIRIDAE*)

David J Rowlands, Molecular Science Department, The Wellcome Research Laboratories, Beckenham, Kent, UK

History

The earliest recorded account that clearly describes foot-and-mouth disease was made by Fracastorius in 1546. The importance of this highly contagious disease of cloven hoofed animals increased with the accelerating productivity of the livestock industry and the control of more lethal infections of cattle such as rinderpest towards the end of the nineteenth century. It is now amongst the most important and feared diseases of domestic livestock.

It was the first animal disease demonstrated to be caused by a filterable agent in 1897 by Loeffler and Frosch, who also demonstrated the presence of neutralizing antibody in serum. It is also the first virus for which serotype differences were recognized. Research on the virus was greatly facilitated when Waldemann and Pope showed in 1921 that guinea pigs could be infected, and in 1951 when Skinner showed that it caused a lethal infection in suckling mice. Subsequently the demonstration that the virus could be cultured in tissue culture cells and particularly in the BHK-21 cell line has enabled both studies of viral replication and the large-scale production of vaccines.

Taxonomy and Classification

Foot-and-mouth disease viruses (FMDVs) form the *Aphthovirus* genus of the *Picornaviridae* family. The nature and organization of the genome, the mode of replication and the structure of the virion are, in general, similar to other viruses in the family. The original subdivision of the *Picornaviridae* into the four genera, *Enterovirus*, *Rhinovirus*, *Cardiovirus* and *Aphthovirus*, was based on physicochemical properties such as susceptibility to acid inactivation, buoyant density of CsCl solution and the nucleotide composition of the genomic RNA. More recently, analysis of evolutionary relationships by nucleotide sequence comparisons have largely endorsed the original classifications. However, hepatitis A virus is now classified within a separate genus, *Hepatovirus*, and Equine Rhinovirus Type 1 is included within the genus *Aphthovirus*. Properties which distinguish the aphthoviruses are: (1) extreme sensitivity to acid inactivation (<pH 6.8 in low ionic strength buffer); (2) high buoyant density in CsCl (1.43–1.50 g ml^{-1}); (3) possession of a poly(C) tract in the 5′ untranslated region (UTR) of the RNA (a property shared with some cardioviruses); (4) three separately encoded VPg proteins; (5) the use of two alternative in-frame protein translation initiation sites; and (6) a leader protease protein located in the N-terminal region of the polyprotein.

Properties of the Virion

As for other picornaviruses, FMDV particles are naked icosahedrons comprising 60 copies each of four

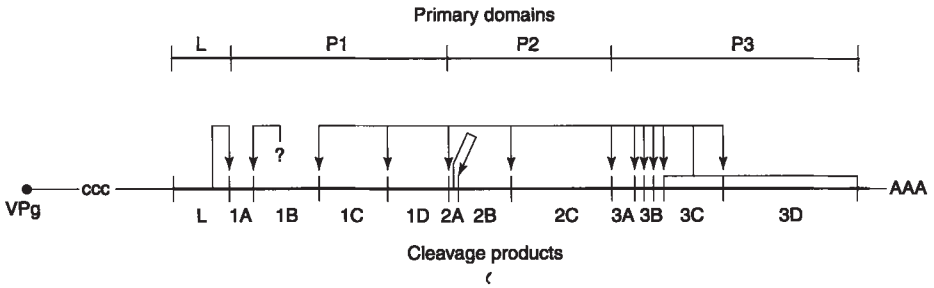


Figure 1 Organization of the FMDV genome. (Modified from the original by LS Titey and AMQ King.)

structural proteins, VP1–4, encapsidating a single copy of the single-stranded positive-sense genomic RNA. The crystallographic structure of the virus has shown that the three larger proteins (VP1–3) have the same eight-stranded antiparallel β-barrel folding motif seen in other picornaviruses and some plant viruses. VP4 is disposed on the inner surface of the particle. In common with other picornaviruses, VP1 molecules are located around the axis of fivefold symmetry, whereas VP2 and VP3 alternate around the two- and threefold symmetry axes. Heat or acid degradation of the particles results in the separation of pentameric subunits, consisting of five copies each of VP1–3, with release of the RNA and an insoluble aggregate of VP4.

Several features of the structure are unique to FMDV. The protein shell is generally thinner and the external surface is smoother than in other picornaviruses. This results from the comparatively smaller sizes of VP1–3 (VP1 213, VP2 218, VP3 220 amino acids for virus O1) compared with other picornaviruses. The reductions in size have generally been in the loop regions, linking the core elements of the β barrels, which in other picornaviruses form prominent features at the outer surface. Another consequence is that the deep grooves or canyons encircling the fivefold axes of enteroviruses and rhinoviruses or the series of pits in the cardioviruses are not present in FMDV. The position of FMDV particles equivalent to these invaginations is occupied by the C-terminal portion of VP1 which arises at the surface at about residue 200 and traverses in a clockwise direction from one protomer to the next. The lack of depressions in the surface at positions equivalent to the canyon or pits of other picornaviruses is of particular significance because these have been implicated as the sites at which those viruses interact with their receptors at cell surfaces.

An important exception to the generally smooth contours of the surface of FMDV is provided by the G–H loop of VP1. This large loop extends from about residue 130 to 160, of which circa 135–158 are too disordered to be visible in electron density maps. In serotype O1 viruses the disorder of the VP1 G–H loop is induced by a disulfide bond between the cystine residues VP2 130 and VP1 134. Under reducing conditions this bond is broken and the G–H loop collapses on to the surface of the virus in an ordered configuration. This feature includes an immunodominant antigenic site to which a high proportion of virus-neutralizing antibodies are directed. Also, synthetic peptides representing sequences from this region are highly effective immunogens and can induce protective immunity. The sequence of this region is hypervariable both in composition and length between different viruses, with the exception of a highly conserved triplet, Arg, Gly, Asp at residues 145–147.

Another structural consequence of the truncation of VP1 is the exposure of a pore at the fivefold axis. This is large enough (*c.* 6 Å) to explain the unique permeability of FMDV to photoinactivating dyes, such as proflavine, and may account for the high buoyant density of the virus in cesium salts.

Properties of the Genome

The genome consists of a single molecule of single-stranded positive-sense RNA, which is infectious. The order of the gene products on the genome is basically similar to other picornaviruses but there are some unique features (**Fig. 1**). The genomic RNA terminates at the 5′ untranslated end in a small protein, VPg, linked by a phosphodiester bond through a Tyr. There is a variable length of poly(A) tract at the 3′ end. Instead of the usual single VPg, the FMDV genome encodes three distinct versions of the molecule, which are used with equal efficiency. The reasons for this gene triplication are unknown. In fact, virus derived from an infectious cDNA clone from which one or two of the Vpg copies had been deleted was still infectious, although RNA synthesis was reduced. The 5′ UTR is exceptionally long (*c.* 1300 nucleotides) even by picornavirus standards. There is an uninterrupted poly(C) tract of 100–200 residues, depending on the virus isolate, located *c.* 400

nucleotides from the 5′ end. The function of the poly(C) tract is unknown, but studies with an infectious clone of the genome have shown that a minimum of 35 C residues is required for virus viability (cf. cardioviruses which are viable in the absence of a poly(C) tract), and circumstantial evidence suggests that the length of the poly(C) tract may be related to virus virulence.

The sequence between the 5′ end and the poly(C) tract has a high degree of secondary structure and is predicted to fold into an almost complete hairpin. Its function is unknown. To the 3′ side of the poly(C) tract there is a variable number (three or four, depending on virus strain) of repeat domains which are predicted to fold as pseudoknot structures. The 3′ 435 nucleotides of the 5′ UTR fold into a series of stem loop structures similar to those present in the equivalent region of cardiovirus UTR.

Protein Products

The protein coding region is a continuous open reading frame of 6999 or 6915 nucleotides for A^{10}, depending on which of two functional in-frame initiation codons is used. The order of the gene products is shown in **Fig. 1.**

1. Leader protein. The leader protein(s), Lab and Lb, which precede the structural proteins, have a proteolytic function which results in cleavage at the L–P1 junction and also affects the cell translation machinery (see Translation). The significance of the presence of two forms of L, dependent on which translation initiation site is used, is unknown. The L proteins are not essential for virus viability, as virus derived from an infectious clone from which their coding region had been deleted was still infectious. However, this virus has an attenuated phenotype and has been proposed as a candidate live vaccine.
2. P1 region. The P1 region consists of the structural proteins. P1A, B, C and D are equivalent to VP4, 2, 3 and 1 respectively.
3. P2 region. The P2A protein is vestigial in size compared to other picornaviruses, being only 18 amino acids long, but it enables the nascent cleavage of the polyprotein at the P2A–2B junction. The function of P2B is unknown. P2C is involved in RNA replication, as mutations affecting sensitivity to guanidine inhibition occur in this protein, but its precise role is unclear.
4. P3 region. P3A does not have the hydrophobic character of the equivalent protein in poliovirus. P3B or VPg occurs as three tandem copies on the genome. The three proteins are 23, 24 and 24 amino acids in length. Although differing from each other in sequence, they share the properties of being rich in Pro, Arg and Lys residues and each has a single Tyr at position three. Despite differing from each other, each of the VPg molecules is highly conserved between the A, O and C serotype viruses. The VPg molecules probably play a role in the initiation of RNA synthesis since they are found at the 5′ end of nascent RNA molecules of both positive and negative polarity. All encapsidated RNA molecules terminate with a VPg molecule and each of the three forms is found in equal abundance. A proportion of intracellular viral RNA, including actively translating polysomal RNA, lacks VPg, and cell extracts contain an enzyme that specifically removes the protein. This enzyme cleaves the phosphodiester bond to produce RNA terminating with a 5′-monophosphate. The observation that no VPg-lacking RNA molecules are found in viral particles suggests that it may have a role in the selection of molecules for encapsidation. Mutagenesis experiments with an infectious cDNA clone have provided some support for this conclusion. P3C is a protease that is responsible for the majority of the processing cleavages. In common with the 3C protease of other picornaviruses, sequence analysis suggests that the catalytic site of the enzyme is related to trypsin, a serine protease, but with the replacement of the nucleophilic serine residue with cysteine. P3D is an RNA-dependent RNA polymerase. Purified preparations of the enzyme are active *in vitro* when provided with natural (viral RNA) or synthetic substrates and a complementary primer.

RNA Replication

RNA of infecting virus acts as a template for the synthesis of a negative-sense complementary strand(s) which is capped at the 5′ end with a VPg molecule. Complementary strand RNA serves in turn as a template for the synthesis of positive-sense strands, identical to the original infecting molecule. Positive-strands are synthesized in a complex structure (replicative intermediate (RI)) consisting of a single negative-strand template and several (*c.* six) nascent positive-strands. RNA synthesis is asymmetrical in favour of positive strands. The details of the features that differentiate positive- and negative-strand synthesis are unclear. A proportion of the negative-strand templates occur as full-length double-stranded hybrids (replicative form (RF)) and appear to take no

further part in RNA synthesis. RF molecules accumulate in the cell during viral replication.

A single molecule of viral RNA is sufficient to initiate infection, which implies that it can function sequentially as a template for translation, to produce the polymerase enzyme(s), and as a template for RNA replication. Single-stranded viral RNA is infectious in the presence of inhibitors of host cell DNA-dependent RNA polymerase. Double-stranded viral RNA is also infectious but not in the presence of inhibitors of the cellular polymerases.

Translation

FMDV RNA is efficiently translated in a cell-free system (rabbit reticulocyte lysates) to produce protein products similar to those found in infected cells. Truncated viral RNA lacking *c.* 600 nucleotides from the 5′ end, including the poly(C) tract, is translated as efficiently as intact RNA. The secondary structural folding of the 435 nucleotides upstream of the first AUG initiation codon for the polyprotein is similar to that of the equivalent region of cardiovirus RNAs. This sequence acts as an internal ribosome-binding site (internal ribosome entry site, IRES), allowing the initiation of translation in the absence of the host cell CAP-binding complex. Because of these properties, the IRES sequence can efficiently direct the translation of the second cistron when inserted between protein-coding sequences in a bicistronic mRNA construct. The rate of total protein synthesis by virus-infected cells does not change until its decline at the later stages of the growth cycle, when cytopathic effects are apparent. There is, however, a marked change in the profile of proteins produced. At times when viral replication is maximal, virtually no host cell proteins are produced. This 'swap over' of translation from host to viral products is similar to the situation in cardiovirus-infected cells and differs from the kinetics of translation following infection of cells with entero- or rhinoviruses. In the latter, infection results in a rapid shutdown of host cell protein translation, which is followed later by a resumption of protein synthesis due to the increasing production of viral proteins. The shutdown induced by entero- and rhinoviruses is largely, if not entirely, due to a virus-induced cleavage of a host protein, p220 or eIF4G, an important component of the CAP-binding complex required for the initiation of translation of host mRNAs, which are generally capped at the 5′ end. In these viruses, eIF4G cleavage is indirectly induced by P2A protease. Cardioviruses do not induce eIF4G cleavage and appear to simply outcompete host mRNAs for utilization of the translation machinery. Although the kinetics of protein translation in FMDV-infected cells resemble those of cardiovirus-infected cells, eIF4G is cleaved. In contrast to entero- and rhinoviruses, FMDV cleavage of eIF4G is not induced by P2A but by the leader protein(s).

Post-translation Processing

The polyprotein translation product of FMDV RNA is proteolytically processed by three of four virus-encoded enzyme activities. Three of the cleavages occur nascently on the growing polypeptide chain. The first separates the leader protein(s) from P1, the structural protein precursor, and is carried out by leader protein. The cleavage occurs between a Lys-Gly dipeptide. Cleavage probably occurs in *cis* but can also occur in *trans*. L-P1 cleavage is the only processing step which is inhibited by the tripeptide D-Val-Phe-Lys-CH_2Cl. Both Lb and Lab are proteolytically active.

The second nascent or primary cleavage occurs at the junction of P2A and P2B and is catalyzed by the 18 amino acid P2A sequence. This short sequence promotes cleavage at its C-terminus when inserted within a variety of protein sequences by DNA recombinant techniques. However, it contains no obvious catalytic motifs and the precise mechanism of cleavage is unclear. Cleavage only appears to occur nascently during the process of translation, as synthetic peptides incorporating the sequence do not self-process. It has been suggested that the mechanism may involve a novel process of interrupted translation rather than proteolytic cleavage. FMDV P2A has sequence homology with a region near the C′-terminus of cardiovirus P2A.

The third nascent cleavage is between P2C and P3A and is catalyzed by P3C protease. This protease is responsible for all other processing cleavages, apart from that which generates VP2 (P1B) and VP4 (P1A) from the precursor, VP0 (P1AB). Many cleavages catalyzed by P3C protease occur at Glu-Gly junctions but many other dipeptides are recognized and 3C of FMDV is the most promiscuous of the picornavirus proteases. The cleavages of P1 to generate P1AB, P1C and P1D can occur with 3C alone but are more efficiently carried out by 3CD.

The cleavage event to produce VP2 and 4 from the precursor, VP0, occurs in the final stages of virus maturation. The mechanism of this cleavage is not known.

Virus Assembly and Release

A variety of assembly intermediates containing equimolar amounts of VP1, 2 and 0 are detected in infected cells. These correspond to monomer and

pentamer subunits of the icosahedral capsid and 75S empty particles, which lack viral RNA but possess antigenic properties similar to mature viral particles. Following pulse-labeling experiments, empty particles can be 'chased' into viral particles, but it has not been shown that they are on the direct morphogenetic pathway. Only about 10% of the viral RNA present in cells is incorporated into virions. All encapsidated RNA terminates with VPg, suggesting that this plays a role in selection. Mutated VPg molecules within an infectious cDNA clone are less efficiently encapsidated.

Paracrystalline arrays of virus are visible in infected cells and are released by lysis of the cell. There is evidence that some viral particles are secreted prior to cell disruption.

Attachment to Cell Receptor(s)

Protease treatment of viral particles can destroy their ability to attach efficiently to cells. Only VP1 is cleaved under these conditions. Cleavage within the G–H loop or in the C-terminal region prevents cell attachment, suggesting that both are important for receptor binding.

Synthetic peptides including Arg, Gly, Asp, the highly conserved triplet found in the G–H loop, can compete with virus for attachment to cells and inhibit infectivity. The conformation of the Arg, Gly, Asp triplet in the VP1 G–H loop of reduced O1 virus, in which the loop is ordered, is similar to that in γ-II crystallin, an integrin-binding protein. These observations have led to the suggestion that the cell receptor(s) for the virus belongs to the integrin family. Competition binding experiments suggest that different viral strains bind to different receptors. Integrins $\alpha v\beta 3$ and $\alpha 5\beta 1$ have both been implicated as receptors for the virus. Viruses in which residues within the Arg, Gly, Asp motif are mutated have been selected *in vitro*. Heparin sulphate has been shown to act as a 'second receptor' for the virus in tissue culture cells. The mechanism of virus uncoating is unknown but the conserved property of acid lability may be related to the process.

Geographic Distribution

Foot-and-mouth disease occurs widely and is endemic in many countries, especially in tropical regions (**Fig. 2**). North America, Australia, New Zealand and Japan are free of the disease and maintain this status by rigorous application of import controls and quarantine. Mass vaccination campaigns have virtually eliminated the virus from some areas, e.g. Europe, but have been less effective in others, owing largely to logistical problems of vaccine distribution and the techniques of animal husbandry employed. Of the seven serotypes of the virus, serotypes A, O and C are the most widely distributed while serotypes SAT1, 2 and 3 are confined to the African continent and Asia; 1 is only found in Asia.

Host Range and Viral Propagation

The virus typically infects cloven hoofed species, with domestic cattle being among the most susceptible. Domestic pigs are also important hosts and are particularly effective in propagating the disease, as they secrete large quantities of virus in the form of aerosols. In sheep and goats, the clinical manifestations of infection are usually less severe than those seen in cattle and pigs. Natural infection of Indian elephants and of camels has been reported. Many wild species of deer and antelope are susceptible to infection; in African buffalo infection is asymptomatic. Persistent infection with prolonged shedding of virus for months or years has been reported in wild and domestic species. A wide range of animals, including Australian marsupials and birds, have been infected under laboratory conditions, and, very rarely, infection of humans has been demonstrated.

The most important small animals for laboratory investigations are the guinea pig and the suckling mouse. In the former, injection of virus intradermally into plantar pads results in the formation of vesicular lesions both at the site of injection and in the mouth and the remaining feet, and so resembles the lesion distribution in naturally infected susceptible species. Intraperitoneal infection of suckling mice results in rapid death and is useful for the titration of virus. The viruses can be propagated in primary cells and cell lines of bovine or porcine origin. Cells derived from the BHK-21 line are most widely used for research or vaccine production purposes. The virus can be titrated by plaque assay in cultured cell monolayers or by cytopathic endpoint dilution assay in microtitre plates.

Genetics and Evolution

In common with other RNA viruses, the mutation rate is extremely high, so that, on average, no two genomes are identical. Antigenic sites on the viral particle are tolerant of sequence variation, and antigenic diversity is a significant property of the virus.

In addition to evolution by the accumulation of point mutation, genomic recombination occurs at a high rate *in vitro*. The frequency and genomic

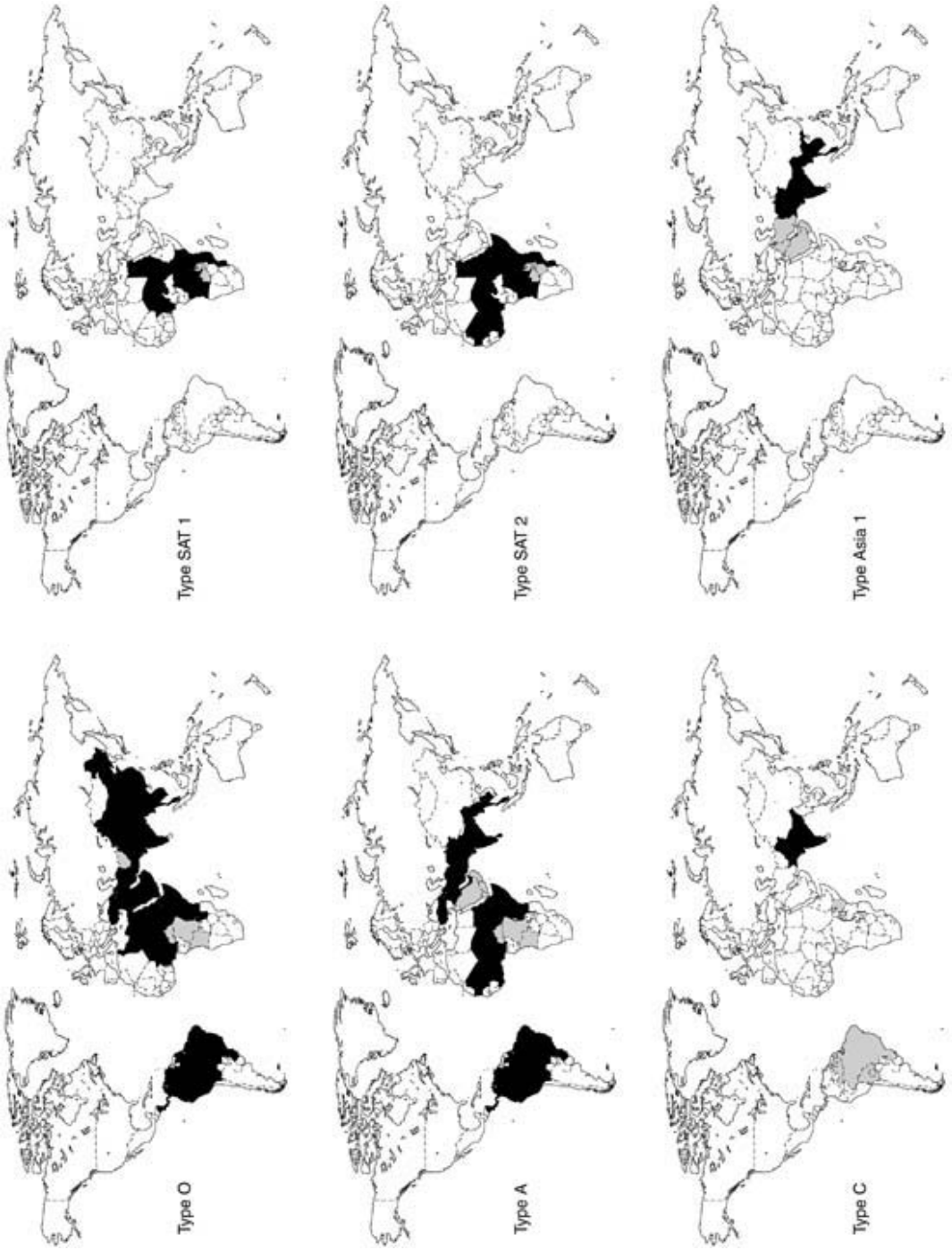


Figure 2 World distribution of FMDV by type. Virus type SAT3 has been recorded only in Botswana, Zimbabwe and the Republic of South Africa.

location of recombinatorial events mirrors the genetic relatedness of the parental viruses.

Serological Relationships and Variability

Seven serotypes of FMDV are recognized, the distinction of serotypes being that an animal convalescent from infection by virus of one serotype is fully susceptible to viruses of any of the remaining six. In addition to the major serotype differences, there is considerable antigenic variation between viruses within serotypes. Serologically distinguishable viruses within serotypes were originally classified as subtypes, but these distinctions became blurred into a continuous antigenic spectrum, and the system has now been abandoned.

For practical purposes in epidemiological studies and vaccine strain selection the serological relationships between field virus isolates or laboratory strains are expressed as *r* values, i.e. the ratio of the neutralizing titers of immune sera against heterologous and homologous viruses. The serological relationships between virus isolates are frequently nonreciprocal, showing that closely related viruses may induce broadly crossreactive or narrowly specific immune responses. Complement fixation assay and enzyme-linked immunosorbent assay (ELISA) using polyclonal sera or monoclonal antibodies are also used for epidemiological studies.

Epidemiology

In regions endemic for foot-and-mouth disease, the virus is most likely maintained in persistently infected animals. It has been shown experimentally that infected bovines can secrete virus for long periods after the initial episode of disease. In some areas the wild animal population may act as a reservoir for infection (e.g. Cape buffalo in Africa). In nonendemic areas infection may be introduced from a variety of sources, such as the importation of infected livestock, contaminated animal products such as carcasses containing bone (in contrast to meat, the postmortem acidification of bone marrow and other nonmuscular tissues is insufficient to inactivate the virus), or contaminated materials. More locally, transmission of infection is by direct transport of contaminated animals or materials or by wind-borne carriage of infectious aerosols. It is also suspected that the virus can be passively transmitted by migrating birds.

Transmission and Tissue Tropism

The principal route of infection appears to be via aerosol impinging on the pharynx and respiratory tract. Aerosols may be produced locally during feeding on contaminated foodstuff or may be transmitted over considerable distances under appropriate meteorological conditions. Pigs secrete particularly high levels of virus-contaminated aerosols. In addition to mucosal secretion, high levels of virus are found in milk. Pasture may be contaminated with virus from urine and feces.

Vesicular lesions appear in the mouth, on the tongue, gums and cheeks, and later on interdigital mucosa and coronary bands of the feet. Virus can be isolated from many tissues in the body. The onset of clinical disease is usually very rapid and lesions can develop as early as 1–2 days after infection, depending on the virus strain and level of exposure.

Pathogenicity

The pathogenicity of FMDV varies according to the virus strain, host species and age. The factors that govern the virulence of FMDVs are poorly understood but there is some evidence from studies of laboratory attenuated strains that the length of the poly(C) tract may influence this property. Domestic cattle are usually the most susceptible species and morbidity is usually approximately 100% in nonvaccinated animals. Wild bovines, such as African buffalo, may produce no clinical manifestations. Although the disease is rarely fatal in adult domestic animals (<5%), significant mortality may occur in young animals (*c.* 50%).

Clinical Features of Infection

Infection typically produces a rapidly progressing febrile illness and the development of often massive vesicular lesions in the mouth and on the feet. The lesions rupture, with considerable loss of epithelial tissue. The resulting discomfort discourages feeding until the lesions heal by the infiltration of fibrous tissue. The severity of the disease results in long-term loss of productivity in terms of meat and milk yield, and lameness may be a serious consequence for draught animals. Abortion and chronic subfertility are also common. Other organs infected include mammary glands, pancreas and heart.

Immune Response

Infection elicits a vigorous humoral antibody response and, after recovery, immunity to reinfection with viruses of the same serotype is prolonged. The role of cytolytic T cells in recovery is unclear.

Five antigenic sites recognized by antibodies capable of neutralizing the virus have been described. One of these is an immunodominant linear sequence located between residues 130 and 160 of VP1, and

synthetic peptides representing this tract are effective in inducing high levels of virus-neutralizing antibody and can protect animals from infection.

Nonprotective antibodies are directed against antigenic sites present on the virus itself, on the 12S pentameric virus subunits and on nonstructural viral proteins, especially the RNA polymerase (virus-infection-associated (VIA) antigen). The presence of antibodies to VIA antigen can serve to distinguish animals that have been infected from those that have been vaccinated.

Prevention and Control

In regions that are free of the disease, natural protection afforded by geographical barriers is rigorously reinforced by strict control measures on the import of susceptible animals and potentially contaminated materials. Where outbreaks occur sporadically, due to occasional introduction from external sources, embargoes on animal movement and slaughter of infected herds have been successful in maintaining a disease-free national herd. In endemic areas, control is by mass vaccination. Ring or barrier vaccination is also used to limit the spread of infection.

The first vaccines against foot-and-mouth disease were produced by formalin inactivation of lymph drawn from lesions on the tongues of infected cattle. This source of immunizing virus was replaced by the Frenkel method of culture in fragments of epithelium stripped from the tongues of slaughtered cattle. Most vaccine in use today is produced by growing the virus in suspensions of BHK-21 cells in fermentation vessels of up to 10 000 litres capacity. Approximately 2×10^9 monovalent doses of vaccine are administered annually. Aziridines have largely replaced formalin as the inactivant, as the inactivation kinetics of the latter are nonlinear and residual live virus in vaccines has occasionally been the source of outbreaks of disease. The serotype and strain composition of vaccines have to be tailored for local requirements. Inactivated virus is usually adjuvanted by adsorption on to aluminum hydroxide gel, and saponin may also be added to enhance potency. In pigs, such vaccines elicit only immunoglobulin M responses and for this species vaccines are formulated with oil adjuvants.

Solid protection requires high levels of neutralizing antibodies, and, to achieve this with inactivated vaccines, immunization is repeated 2–3 times a year. The development of live attenuated vaccines has largely been abandoned, owing mainly to the complexity of antigenic diversity and the fear of reversion to a virulent phenotype.

Future Perspectives

There is scope for developments in foot-and-mouth disease vaccines to improve stability, crossprotective efficacy and duration of immunity. Peptide vaccines or rationally designed nonreverting attenuated viruses are potential routes by which these goals may be achieved. The production of nonbiodegradable peptides and the creation of an attenuated L protein deletion mutant may be important developments.

As to the molecular properties of the virus, both the determination of the crystallographic structure of the particle and the cloning and manipulation of full-length infectious cDNA molecules are important steps towards understanding the unique features of the virus.

See also: **Cardioviruses (*Picornaviridae*); Immune response: Cell mediated immune response, General features; Vaccines and immune response; Virus structure: Atomic structure, Principles of virus structure; Foreword – 100 Years of Foot and Mouth Disease virus.**

Further Reading

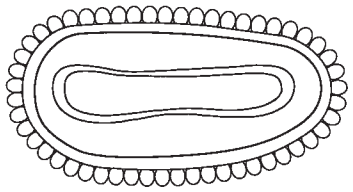
Bartling SJ and Vreeswijk J (1991) Developments in foot and mouth disease vaccines. *Vaccine* 9: 75.

Brooksby JB (1982) Portraits of viruses: foot and mouth disease virus. *Intervirology* 18: 1.

Brown F (1989) The development of chemically synthesised vaccines. *Adv. Vet. Sci. Comp. Med.* 33: 173.

Fry E, Logan D, Acharya R *et al* (1990) Architecture and topography of an aphthovirus. *Semin. Virol.* 1: 439.

FOWLPOX VIRUS (*POXVIRIDAE*)



Deoki N Tripathy and **William M Schnitzlein**, Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Illinois, Urbana, Illinois, USA

History

Poxvirus infection in avian species resembles its counterparts occurring in mammals and it may have coevolved with its hosts, as it has been observed from time immemorial; indeed, during the seventeenth and eighteenth centuries the infection was described in doves, hawks and turkeys.

The term 'fowlpox' initially included all poxvirus infection of birds, although now this designation usually refers only to disease of chickens. Various synonyms that have been used in the past include chicken pox, avian pox, bird pox, contagious epithelioma, sorehead, avian molluscum, avian diphtheria, *Geflugelpocken* (German), *viruela aviare* (Spanish), *difteria aviar* (Spanish), *Bouba* (Portuguese) and *variole aviaire* (French).

Taxonomy and Classification

Fowlpox virus is the type species of the genus *Avipoxvirus*, in the subfamily *Chordopoxvirinae* of the *Poxviridae* family. Other members of this genus are canarypox, juncopox, mynahpox, pigeonpox, psittacinepox, quailpox, peacockpox, penguinpox, sparrowpox, starlingpox and turkeypox. All of these viruses have a restricted host range, infecting only avian species. Since fowlpox virus is the most studied member of this genus, most of this entry focuses on this prototype virus but information on related viruses is also provided.

Chemical Composition of Fowlpox Virus

During infection of a cell, the majority of the virus is retained in inclusion bodies. The average weight of an inclusion body is 6.1×10^{-7} mg, of which approximately 50% is extractable lipid. The average amount of protein and DNA per inclusion is 7.69×10^{-8} mg and 6.64×10^{-9} mg, respectively. The average weight of a fowlpox virus is 2.04×10^{-11} mg. A virion contains 7.5×10^{-12} mg of protein (36.8% w/w), 4.03×10^{-13} mg DNA (2% w/w) and 5.54×10^{-12} mg of lipid (27.2% w/w). Predominant lipids in fowlpox virus are cholesterol and phospholipids.

Geographic and Seasonal Distribution

Avian poxviruses are distributed worldwide in poultry, pet birds and wild birds. The incidence of fowlpox in domestic poultry is variable in different geographical regions because of differences in management practices, hygienic conditions and the use of prophylactic immunization. Avian poxviruses have been considered to be population limiting factors in some Hawaiian forest birds. In some areas the disease is usually more common during summer months when the mosquito population is high. However, in large poultry operations, especially in multiple age complexes, it may occur at any time of the year.

Host Range and Virus Propagation

Most avian species are susceptible to one or more poxvirus strains. On the basis of host specificity, strains can be mono-, bi- or tripathogenic. Usually, avian poxvirus strains are pathogenic for several species. Naturally occurring poxvirus infections have been reported in some 60 species of wild birds comprising 20 families. In domestic poultry, fowlpox is an economically important disease in chickens and turkeys. In pet birds, canarypox is of grave concern because of the high mortality associated with this disease. Poxviruses of birds are host-restricted and do not productively infect mammals.

Avian poxviruses can be propagated on the chorioallantoic membranes (CAMs) of 9–12-day-old developing chicken embryos. Virus inoculated embryos are incubated at 37°C in a humidified atmosphere for 5–7 days, when the CAMs are examined for lesions. Additionally, fowlpox, canarypox, pigeonpox, Hawaiian crowpox, mynahpox, psittacinepox and quailpox viruses have been grown in either primary cell cultures obtained from chicken embryos or chickens (fibroblasts, liver, kidney and skin) or secondary cell lines derived from chicken liver (LMH), quail fibroblasts (QT-35) and quail liver (IQ1A).

Genetics

The general properties of fowlpox virus include: a large complex virion containing enzymes capable of synthesizing mRNA; a single linear A- and T-rich

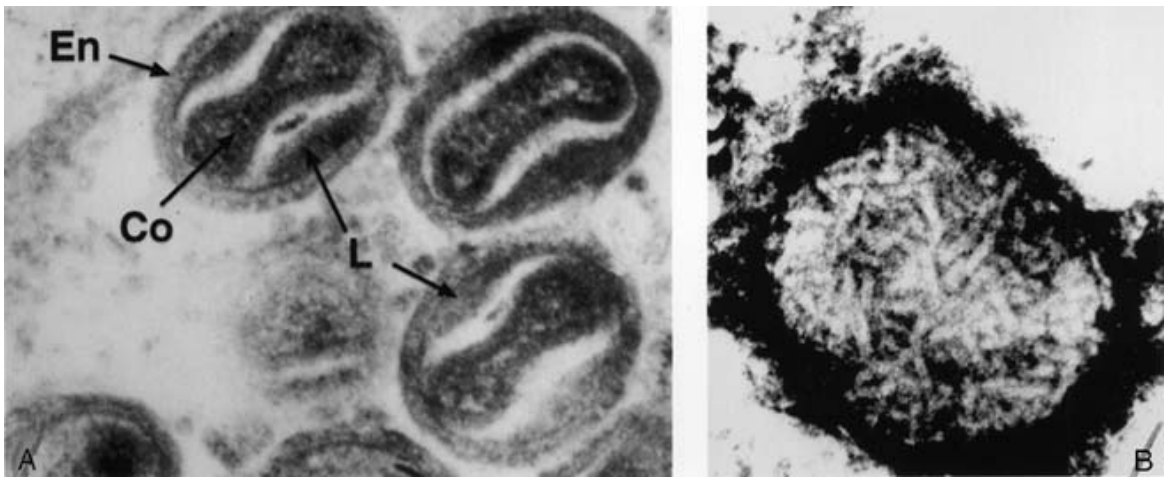


Figure 1 (**A**) Ultrathin section of fowlpox viruses from a diphtheritic lesion. Co, core; L, lateral bodies; En, envelope. (**B**) Negatively stained fowlpox virus particle showing random distribution of surface tubules. For additional illustrations of fowlpox pathology.

double-stranded DNA genome of approximately 300 kb with a hairpin loop at each end; and a replication site primarily in the cytoplasm of infected cells. While poxviruses in general are larger than other animal viruses, fowlpox is the largest of all poxviruses. Indeed, the viral particles, referred to as elementary bodies, can be seen in stained preparations under light microscopy. Individual virions are brick-shaped, with dimensions of about 330 nm × 280 nm × 200 nm. They consist of an electron-dense, centrally located biconcave dumbbell-shaped core or nucleoid, containing the viral genome, and two lateral bodies in each concavity, which are enveloped by one or more membranes (**Fig. 1A**). Infectivity is usually ether-resistant.

Like other poxviruses, the fowlpox virus genome consists of a conserved central region and variable termini. The ends of fowlpox virus DNA contain inverted terminal repeats which are not identical in size at each terminus. Thus, variations in physical maps near the ends of the genome of different strains of fowlpox virus may be observed. Changes have also been found near to or at the ends of fowlpox virus DNA after multiple tissue culture passages of the virus.

Genomic characterization of virus isolates is usually based on restriction fragment length polymorphisms (RFLPs), in which the DNA profiles are compared after restriction enzyme digestion of the virus genome and subsequent agarose gel electrophoresis of the resulting fragments. Based on this type of analysis, the genomes of fowlpox, pigeonpox and juncopox viruses show a marked degree of similarity. On the other hand, the DNAs of quail, canary and mynah poxviruses are distinct from each other and those of fowlpox virus. Likewise, the genomes of two poxvirus isolates from Hawaiian forest birds are not only different from fowlpox virus but also from each other. Like vaccinia virus, fowlpox virus must encode several enzymes involved in the synthesis of deoxyribonucleotides in order to enhance DNA replication in the cells. However, unlike vaccinia virus, only part of the fowlpox virus genome has been sequenced and not all of its genes have been identified. Among those which have been recognized in fowlpox virus are ones encoding for DNA polymerase, NTPaseI, uracil glycosylase, and thymidine kinase (TK). Despite the nonessentiality of TK for virus multiplication in cell cultures, deletion mutants become significantly attenuated *in vivo*. These biological properties have been exploited for genetic modification of the virus.

Replication

DNA replication and packaging within the infectious virus particle occur exclusively in the cytoplasm of infected cells. These cytoplasmic replicative events have been determined through the use of light, fluorescent and electron microscopy. Ultrastructural studies reveal that, after adsorption to and penetration of the cell membrane by fowlpox virus (1 h for dermal epithelium and 2 h for CAMs of developing chicken embryos), there is an uncoating of the virus prior to the synthesis of progeny virus from the precursor material. Areas of viroplasm with incomplete membranes around them can be seen in the cytoplasm by 48 h postinfection. Inclusion bodies are observed after an additional 24 h (dermal epithelium) to 48 h (CAM). The virus emerges from cells by a

budding process, with acquisition of an additional outer membrane obtained from the cell.

The infection of dermal epithelium of the chicken or of the ectodermal CAM is characterized by hyperplasia, ballooning of the cells and presence of cytoplasmic inclusion bodies. The elementary bodies or virus particles are contained within the inclusion bodies.

Evolution

In recent years outbreaks of fowlpox have occurred in previously vaccinated chicken flocks. Most outbreaks are characterized by the diphtheritic form of infection resulting in high mortality. Some of these isolates show minor genetic and antigenic as well as biologic differences from the vaccine strains. Instances of persistent dual infection by fowlpox and herpes viruses, e.g. Marek's disease virus and infectious laryngotracheitis virus, have been reported. During sequencing of a segment of Marek's disease virus, a fowlpox virus open reading frame (ORF) was detected. In spite of differences in the mode of replication of these viruses, these observations suggest a possible exchange of genetic material from one type of DNA virus to another.

Recent evidence of the genomes of fowlpox viruses carrying integrated sequences of reticuloendotheliosis virus indicate the possibility of re-emerging strains of fowlpox virus. Integration of a retrovirus into the DNA of a large DNA virus provides an important mechanism for virus evolution, resulting in genetic, antigenic and biologic alterations. Moreover, reticuloendotheliosis virus is known to induce immunosuppression – a property conducive to extending the viruses' persistence in the host.

Serologic Relationships and Variability

Avian poxviruses are antigenically and immunologically distinguishable from each other to an extent, but various degrees of crossrelationships do exist. In this regard, a nucleoprotein precipitinogen is common to all poxviruses. Limited crossprotection may occur between some viruses. The various strains of avian poxviruses are thought to be variants originating from a common ancestor. Some strains have remained similar or closely related antigenically to each other, while others have become immunologically distinct. For example, the similarity between fowlpox and pigeonpox viruses has enabled the use of the latter as a vaccine against fowlpox infection. Minor antigenic differences in strains of fowlpox virus can be detected by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting, although the majority of their antigens are common. In contrast, the antigenic profiles of quailpox virus are remarkably different from those of fowlpox virus, and thus these viruses do not provide crossprotection against infection by each other.

Epidemiology

In commercial poultry, fowlpox and turkeypox infections are of economic significance. Prophylactic vaccination using live modified virus vaccine of fowlpox or pigeonpox virus origin has been effective for many years. Consequently, vaccination was indicated only in areas where the disease was endemic. Improved management, biosecurity, hygienic conditions and timely vaccinations have been effective in controlling the disease in many poultry operations. Thus in such operations vaccination was unnecessary unless there was a threat of an infection spreading from neighboring flocks or wild birds. However, in recent years the epidemiology of disease in many areas has changed because of an increasing concentration of poultry in large complexes, retention of layer flocks for a second cycle of production, and maintenance of multiple age birds. As a result, frequent outbreaks of fowlpox have been reported from different geographical regions in the USA and elsewhere, especially where multiple age chickens are raised in one complex. In addition, outbreaks of fowlpox have occurred in previously vaccinated birds. Moreover, in some flocks a low level persistent infection can erupt into a highly pathogenic one, especially in birds subjected to the stresses of invasion by other microbes and molting.

Transmission and Tissue Tropism

Overall, poxviruses can withstand extreme environmental conditions and remain viable in dried scabs for extended periods. For example, the viability of fowlpox virus is undiminished by emersion in 1% phenol or 0.1% formalin for 9 days. Trypsin has no effect on the DNA or whole virus. The virus is, however, inactivated by 1% caustic potash when separated from its matrix or by heating at 122°F (50°C) for 30 min or 140°F (60°C) for 8 min. It can be readily preserved by lyophilization. The virus can survive in dried scabs for months or even years. Scabs shed by the recovered birds may contaminate soil, food and water. The virus can be transmitted either directly by contact between infected and susceptible birds or mechanically by arthropod vectors. Small abrasions in the skin or mucous membranes are necessary to allow the entry of the virus, as it is unable to penetrate unbroken tissue. Skin lacerations as a

result of cannibalism, fighting or preening may assist in the entry of the virus. Insects such as mosquitoes act as mechanical vectors to transfer the virus from infected to susceptible birds. In addition, oral and respiratory infections may occur by exposure to aerosols present in contaminated environments, especially in concentrated housing. In this regard, inhalation of virus-laden dust, which may contain particles of feathers, skin or scabs, provides an important route for virus exposure. Transmission is facilitated by the housing of a large number of birds in close quarters. In some flocks of chickens, fowlpox virus may persist for a long time. Since the disease spreads slowly, the virus may circulate in the susceptible population for a considerable time. This is a common occurrence where multiple age chicken flocks are maintained.

Pathogenicity

Generally the disease occurs in two forms, cutaneous and diphtheritic, although a systemic form of infection caused by canarypox virus is observed in canaries. The cutaneous form is characterized by the development of lesions on the comb, wattles, angle of the beak, feet, vent and other areas of the skin (**Fig. 2A**). In the diphtheritic form, lesions may occur on the mucous membrane of mouth, nares, esophagus, larynx, pharynx and trachea (**Fig. 2B**). Both cutaneous and diphtheritic forms of the disease may be present in a single bird. When the diphtheritic form of the disease is manifested by mouth or tracheal lesions, the afflicted birds experience difficulty in breathing or feeding and thus may die by suffocation or starvation. Birds affected with the cutaneous form of the disease are more likely to recover than those that have the diphtheritic form involving the oral or respiratory tract. Flock mortality due to cutaneous infections alone is usually low and affected flocks generally return to normal productivity upon recovery. Depending upon the virulence of the virus, mortality is significant, however, in flocks with the diphtheritic or generalized form of the disease. Among pet birds, infections often occur in large aviaries of canaries and the disease is likely to be enzootic. Canarypox virus infection is, therefore, of great concern to aviculturists, as the virus is highly lethal for susceptible canaries. Mortality may be as high as 80–100%. Severe outbreaks of quailpox in pen-raised quails have also been reported.

Clinical Features and Infection

A mild cutaneous form of the disease involving unfeathered areas of skin may often remain unnoticed due to the lack of any significant effect on the host, and may be self-limiting. When only the cutaneous form is present in the flock, the birds recover readily. However, those lesions on the eyelids (**Fig. 2A**) or around the beak may interfere with vision and feeding. In such birds productivity is reduced. There is poor feathering in young birds and a transient drop in egg production may also occur in layers. In the diphtheritic form of the disease clinical signs will vary, depending upon the location and severity of the lesions (**Fig. 2B**). Lesions in the trachea, pharynx and sinuses interfere with breathing. High mortality occurs due to suffocation resulting from blockage by tracheal lesions. Clinically, respiratory tract lesions simulate signs caused by other respiratory pathogens, especially infectious laryngotracheitis virus. Lesions in the mouth interfere with feeding, resulting in lowered productivity and increased mortality.

Pathology, Histopathology and Diagnosis

Localized proliferation of epithelial cells, characterized by hyperplasia and hypertrophy, is an important feature of avain poxvirus infections. The basal germinal layer of cells in the epithelium shows an increased rate of multiplication. Pocks are formed by the piling up of the infected epithelial cells. The time frame for the development and regression of the lesions is variable, depending upon the host and pathogenicity of the virus. Ultimately, desiccation and scab formation is followed by sloughing of the lesion. Diphtheritic lesions on the mucous membranes of respiratory and oral epithelium develop as white, opaque, slightly elevated nodules, which increase in size and coalesce to form yellowish, cheesy, necrotic material, appearing as a pseudomembrane. Solid, tumor-like masses involving the pharynx and anterior part of the esophagus may often be seen.

Microscopically, the affected dermal and mucous membrane epithelium of the chicken and the infected ectodermal CAM show marked hyperplasia with enlargement of the cells and associated inflammatory changes. The infected cells show eosinophilic cytoplasmic inclusion bodies when stained with hematoxylin and eosin (**Fig. 2D**). These inclusion bodies are often referred to as Bollinger bodies and contain the viral particles or elementary bodies also known as Borel bodies. These viruses produce both A- and B-type inclusion bodies, which contain a considerable amount of lipid.

Fowlpox is an economically important disease of commercial poultry and it has been responsible for significant economic losses, especially when a diphtheritic form of the disease with acute respiratory

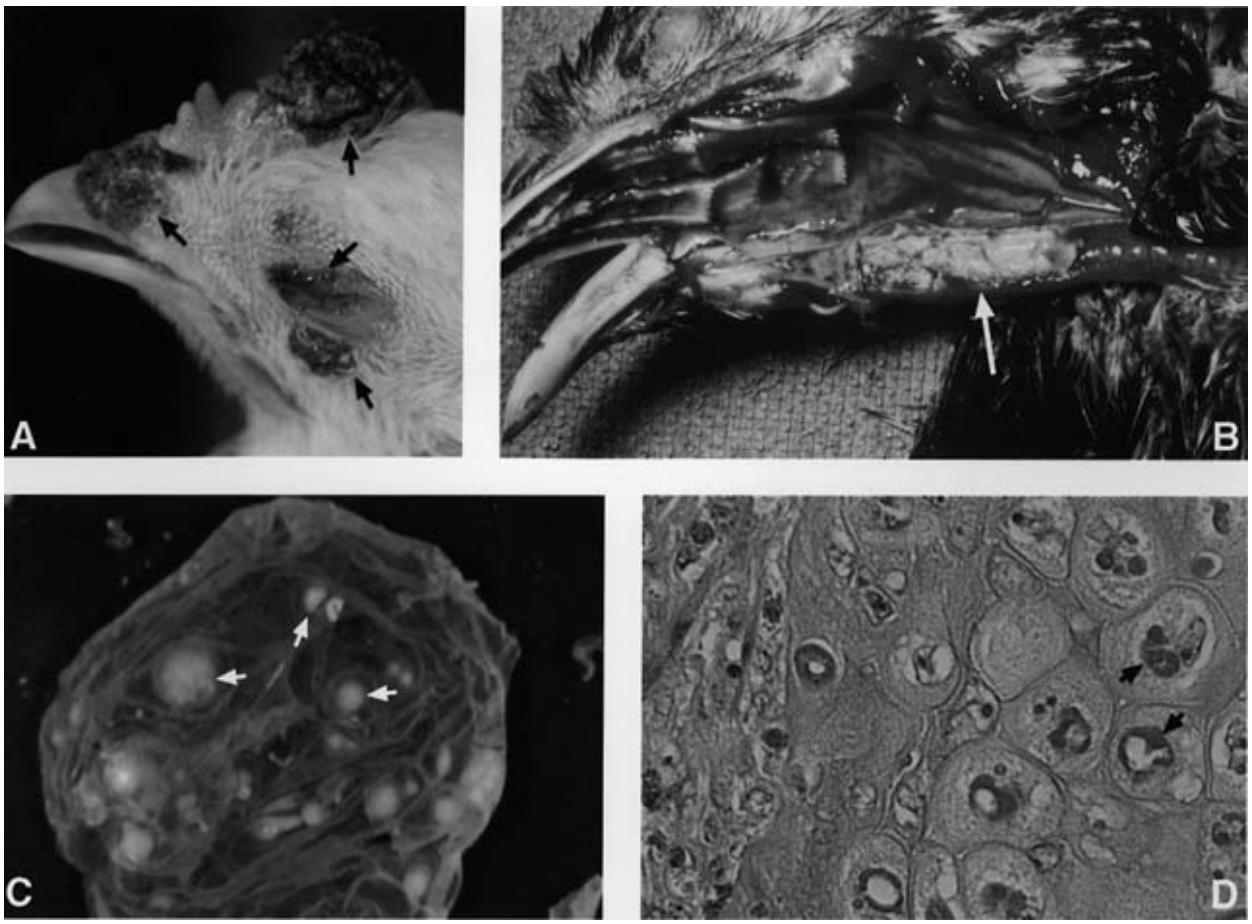


Figure 2 (**A**) Cutaneous lesions of fowlpox. (**B**) Diphtheritic fowlpox – tracheal plug. (**C**) Fowlpox virus lesions (pocks) in the chorioallantoic membrane of a developing chicken embryo. (**D**) Cells infected with fowlpox virus are enlarged and contain cytoplasmic inclusion bodies (arrows). Parts B and D are part of Fowlpox Slide Study Set prepared by Dr Tripathy for American Association of Avian Pathologists. **(For color references see Color Plate 7.)**

signs is prevalent. Rapid diagnosis of infection is therefore important for the initiation of appropriate preventive measures. The most commonly used method is the histopathologic examination of the lesion for the presence of cytoplasmic inclusion bodies. Since similar clinical signs involving the respiratory tract in chickens can be caused by infectious laryngotracheitis virus, rapid differential diagnosis of the disease becomes very important. Infection by this herpesvirus is characterized by the histologic demonstration of intranuclear inclusion bodies (**Fig. 2D**). On the other hand, poxvirus infections are diagnosed by histopathologic examination of the lesions for the presence of cytoplasmic inclusion bodies. These inclusions, which contain elementary bodies, can be observed under oil-immersion in stained smears of the lesions. In addition, viral particles exhibiting typical poxvirus morphology can be detected by electron microscopic examination of negatively stained lesion suspensions or in ultrathin sections of the lesions (**Fig. 1A, B**). The virus is isolated by inoculation of the CAM of 9–12-day-old developing chicken embryos. Pocks develop on the CAM in 5–7 days postinfection (**Fig. 2C**). The etiology is confirmed by histopathological examination of the CAM lesions for cytoplasmic inclusions or viral particles of poxvirus morphology in ultrathin sections or in negatively stained preparations (**Fig. 1A, B**). Isolated viruses can be evaluated for pathogenicity in susceptible hosts, e.g. chickens (development of primary and secondary lesions and the duration of their persistence), or for susceptibility of avian cell culture (cytopathic effect and plaque formation). Although cloned genomic fragments as probes and primers based upon published sequences for amplifying a specific portion of the genome can be used for diagnosis, these procedures are not routinely performed.

Immune Response

Crossprotection among avian poxviruses is variable, although these viruses exhibit an extensive degree of serologic crossreactivity. Attenuated fowlpox virus vaccines of chicken embryo or cell culture origin have been used extensively for prevention of fowlpox in chickens and turkeys. The vaccine is administered by wing-web stab or by rubbing the vaccine on the thigh after pulling a few feathers. Similarly, modified live vaccines of pigeonpox and turkeypox origin are also available commercially. Canarypox virus vaccine is used exclusively in canaries, and quailpox virus vaccine is required for quails. Birds recovered from natural pox infection are immune to reinfection with that strain. A natural infection or vaccination is followed by both cell-mediated and humoral antibody responses. Cell-mediated immune responses are detected earlier than humoral responses. Although not commonly practiced, serologic detection of infection may be important in experimental studies and for measuring the immune responses following vaccination. Antibody response can be measured by agar gel precipitation (AGP), passive hemagglutination, immunoperoxidase (IP) and enzyme-linked immunosorbant assays (ELISA). ELISA has become the most common method for evaluation of immune responses. Some tests, e.g. IP, indirect immunofluorescence (IFA) and AGP, can also be used to detect viral antigen in the lesion(s). Antigenic differences among isolates can be determined by immunoblotting, crossprotection tests and virus neutralization assays.

Prevention and Control

Actively acquired immunity against avian poxviruses results after recovery from natural infection or vaccination. Live modified vaccines of fowlpox and pigeonpox virus origin are used for the immunization of commercial poultry. The viruses are propagated either in CAMs or primary chicken embryonic or secondary avian cell cultures. The vaccine should contain the respective poxviruses at a minimum concentration of 10^5 EID_{50} ml^{-1} to establish satisfactory vaccination in parallel with good protective immunity. If the vaccine is properly applied to susceptible birds, immunity will normally develop 10–14 days after vaccination. The absence of elicited protective immunity in a bird could be the result of improper application of the vaccine or the use of a vaccine of inadequate potency. It is important that, in chickens exhibiting passive immunity, vaccination is delayed until the passive antibody titer has declined. Since spread of the disease is slow, all susceptible birds in a commercial poultry flock should be vaccinated as soon as the disease is diagnosed. Vaccination is also indicated for young stock and birds introduced on premises where an infection was diagnosed during the previous year. In areas where pox is prevalent, vaccination may be required to protect birds from virus present in outside sources such as neighboring flocks.

In the past this disease has been prevented by regular use of attenuated strains of fowlpox or pigeonpox virus vaccines. In recent years, however, several outbreaks of fowlpox, especially of the diphtheritic form, have occurred in large commercial poultry operations, despite vaccination. The isolates obtained from these outbreaks are highly pathogenic for susceptible chickens and are somewhat different from the vaccine strains, both biologically and physically.

Future Perspectives

Fowlpox virus as a vector for poultry and other vaccines

In recent years considerable interest has been generated in using fowlpox virus as a vector for the expression of foreign genes from both avian and mammalian pathogens. The main reasons for this are: (1) modified live fowlpox virus vaccines have been used for more than 70 years by the poultry industry for the prevention of fowlpox in commercial poultry; (2) fowlpox virus has a highly restricted host range affecting only avian species; (3) the virus has one of the largest genomes and can accommodate a substantial amount of foreign genetic material without becoming defective in replication; and (4) it has been shown to cause an abortive infection in mammalian hosts. Despite its replicative deficiency in mammals, foreign genes are expressed by recombinant fowlpox virus in these hosts and, more importantly, the resulting proteins can elicit immune responses. Structural and functional studies reveal that regulatory sequences (promoters) are conserved between poxvirus genera, eliminating the need for homologous promoters in the generation of recombinants. Therefore, several promoters from vaccinia virus, a member of the genus *Orthopoxvirus*, have been used rather extensively for transcriptional regulation of foreign genes by avian and other poxviruses. While genetic information on other avian poxviruses is lacking, these can also be utilized as vaccine vectors. In this regard, another member of this genus, canarypox virus, has also been used for expression of mammalian genes.

During the 1990s a number of fowlpox virus-vectored vaccines expressing proteins encoding pro-

tective antigens from several poultry pathogens have been developed. For example, recombinants expressing the hemagglutinin of avian influenza and Newcastle disease virus have been shown to provide protection in vaccinated birds against the respective virulent viruses. In fact, a fowlpox virus-vectored Newcastle disease virus recombinant vaccine is commercially available but its use has been limited because of its high cost compared with that of the conventional vaccine. Thus the future of such recombinants is limited unless a polyvalent vaccine expressing antigens from several pathogens becomes available and is more cost-effective than the current vaccines.

See also: **Pathogenesis: Animal viruses; Poxviruses (*Poxviridae*): Capripoxviruses, Leporipoviruses and suipoxviruses; Smallpox and monkeypox viruses (*Poxviridae*); Vectors: Animal viruses; Yabapox and Tanapox viruses (*Poxviridae*); Mousepox and rabbitpox viruses (*Poxviridae*); Parapoxviruses (*Poxviridae*); Vaccines and immune response; Vaccinia virus (*Poxviridae*).**

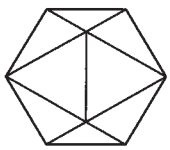
Further Reading

Buller MR and Palumbo GJ (1991) Poxvirus pathogenesis. *Microbiol. Rev.* 55: 80.

Moss B (1996) *Poxviridae:* the viruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology* 3rd edn, p. 2637. Philadelphia: Lippincott-Raven.

Tripathy DN and Reed WM (1997) Pox. In: Calnek BW, Barnes HJ, Beard CW, McDougald LR and Saif YM (eds) *Diseases of Poultry* 10th edn, p. 643. Ames: University of Iowa Press.

FROG VIRUS 3 (*IRIDOVIRIDAE*)



Rakesh M Goorha and **Allan Granoff**, St Jude Children's Research Hospital, Memphis, Tennessee, USA

History

Frog virus 3 (FV3) was isolated in 1966 by Granoff from a renal adenocarcinoma of the leopard frog *Rana pipiens*. Initially, this virus was suspected of causing the renal adenocarcinomas of *R. pipiens* (Lucké tumor); however, it turned out that FV3 has no relevance to formation of the tumor. Subsequently, additional isolates similar to FV3 were made from cultured normal adult frog kidney cells and liver homogenates of normal or tumor-bearing frogs. Further isolates were recovered from homogenates of normal newt tissue and from viscera homogenates of both normal and edematous tadpoles. Nucleic acid hybridization and restriction endonuclease analyses of DNA from several of these viruses as well as serological comparisons suggest that they probably represent different strains of the same prototype virus.

Taxonomy and Classification

Frog virus 3 is in the genus *Ranavirus* of the family *Iridoviridae* which comprises several viruses from vertebrate and invertebrate hosts. Three other genera *Iridovirus, Chloraridovirus* and *Lymphocystivirus* are included in the family. The characteristic features of an iridovirus are: (1) icosahedral symmetry; (2) a large (≈170 kbp), linear, double-stranded DNA genome; and (3) assembly of virus particles exclusively in the cytoplasm. Members of the *Iridoviridae*, so far examined, have a circularly permuted and terminally redundant genome – a unique characteristic among animal viruses. DNA hybridization studies have shown no sequence homology between FV3 and iridoviruses from other hosts such as fish and insect.

Properties of the Virion

Unenveloped FV3 virions measure 120 nm × 130 nm whereas virions with plasma membrane-derived envelopes measure 160–200 nm in diameter. Enveloped virions have a buoyant density of 1.28 g cm^{-3} and unenveloped particles 1.32 g cm^{-3}. Beneath the icosahedral lattice there is an inner membrane composed of lipids and proteins. The virus core below the membrane is composed of DNA and proteins and appears as a long filament. The unenveloped virion contains 9% lipid, and infectivity of the virus is destroyed by ether or phospholipase A. Even though the FV3 envelope is derived from the host membrane,

tective antigens from several poultry pathogens have been developed. For example, recombinants expressing the hemagglutinin of avian influenza and Newcastle disease virus have been shown to provide protection in vaccinated birds against the respective virulent viruses. In fact, a fowlpox virus-vectored Newcastle disease virus recombinant vaccine is commercially available but its use has been limited because of its high cost compared with that of the conventional vaccine. Thus the future of such recombinants is limited unless a polyvalent vaccine expressing antigens from several pathogens becomes available and is more cost-effective than the current vaccines.

See also: **Pathogenesis: Animal viruses; Poxviruses (*Poxviridae*): Capripoxviruses, Leporipoviruses and suipoxviruses; Smallpox and monkeypox viruses (*Poxviridae*); Vectors: Animal viruses; Yabapox and Tanapox viruses (*Poxviridae*); Mousepox and rabbitpox viruses (*Poxviridae*); Parapoxviruses (*Poxviridae*); Vaccines and immune response; Vaccinia virus (*Poxviridae*).**

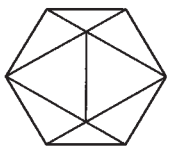
Further Reading

Buller MR and Palumbo GJ (1991) Poxvirus pathogenesis. *Microbiol. Rev.* 55: 80.

Moss B (1996) *Poxviridae:* the viruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology* 3rd edn, p. 2637. Philadelphia: Lippincott-Raven.

Tripathy DN and Reed WM (1997) Pox. In: Calnek BW, Barnes HJ, Beard CW, McDougald LR and Saif YM (eds) *Diseases of Poultry* 10th edn, p. 643. Ames: University of Iowa Press.

FROG VIRUS 3 (*IRIDOVIRIDAE*)



Rakesh M Goorha and **Allan Granoff**, St Jude Children's Research Hospital, Memphis, Tennessee, USA

History

Frog virus 3 (FV3) was isolated in 1966 by Granoff from a renal adenocarcinoma of the leopard frog *Rana pipiens*. Initially, this virus was suspected of causing the renal adenocarcinomas of *R. pipiens* (Lucké tumor); however, it turned out that FV3 has no relevance to formation of the tumor. Subsequently, additional isolates similar to FV3 were made from cultured normal adult frog kidney cells and liver homogenates of normal or tumor-bearing frogs. Further isolates were recovered from homogenates of normal newt tissue and from viscera homogenates of both normal and edematous tadpoles. Nucleic acid hybridization and restriction endonuclease analyses of DNA from several of these viruses as well as serological comparisons suggest that they probably represent different strains of the same prototype virus.

Taxonomy and Classification

Frog virus 3 is in the genus *Ranavirus* of the family *Iridoviridae* which comprises several viruses from vertebrate and invertebrate hosts. Three other genera *Iridovirus, Chloraridovirus* and *Lymphocystivirus* are included in the family. The characteristic features of an iridovirus are: (1) icosahedral symmetry; (2) a large (≈170 kbp), linear, double-stranded DNA genome; and (3) assembly of virus particles exclusively in the cytoplasm. Members of the *Iridoviridae*, so far examined, have a circularly permuted and terminally redundant genome – a unique characteristic among animal viruses. DNA hybridization studies have shown no sequence homology between FV3 and iridoviruses from other hosts such as fish and insect.

Properties of the Virion

Unenveloped FV3 virions measure 120 nm × 130 nm whereas virions with plasma membrane-derived envelopes measure 160–200 nm in diameter. Enveloped virions have a buoyant density of 1.28 g cm^{-3} and unenveloped particles 1.32 g cm^{-3}. Beneath the icosahedral lattice there is an inner membrane composed of lipids and proteins. The virus core below the membrane is composed of DNA and proteins and appears as a long filament. The unenveloped virion contains 9% lipid, and infectivity of the virus is destroyed by ether or phospholipase A. Even though the FV3 envelope is derived from the host membrane,

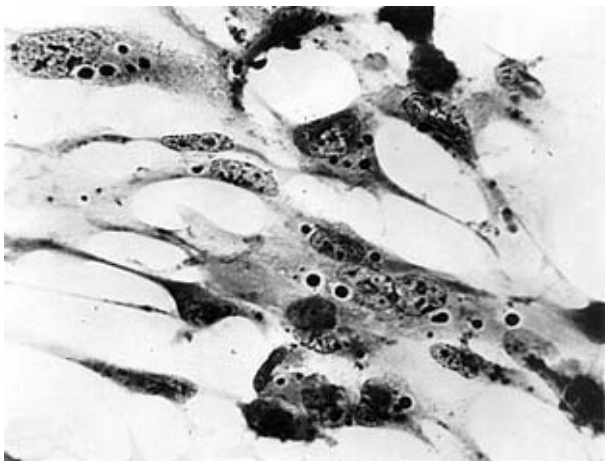


Figure 1 Feulgen-stained FV3-infected baby hamster kidney (BHK 21/13) cells illustrating Feulgen-positive inclusion bodies. Approximately ×210.

its lipid composition differs from that of the host membrane. Apparently, the host membrane is modified after virus infection.

Properties of the Virus Proteins

Two-dimensional gel electrophoresis has revealed approximately 50 viral structural proteins. FV3 proteins do not undergo detectable post-translational processing. No evidence for glycosylation, sulfation, or cleavage from precursor proteins has been obtained. Purified FV3 virions contain at least six enzymatic activities. Two of these, nucleotide phosphohydrolase and pH 5 endodeoxyribonuclease, are found in viral cores, whereas protein kinase, endoribonuclease and pH 7.5 endodeoxyribonuclease are external, i.e. they are solubilized by 0.5% Nonidet P-40 (NP-40). The sixth activity, a protein phosphatase has not been localized.

Properties of the Genome

FV3 virions contain a single, linear, double-stranded genome with a GC content of 53%. The genome is approximately 170 kbp in size and is terminally redundant and circularly permuted. The extent of circular permutation is restricted to 30% of the genome length. An unusual property of FV3 DNA is its high degree of methylation. About 25% of deoxycytosine residues are methylated at C-5. Genetic and biochemical evidence suggests that the FV3 genome is methylated by a virus specified DNA methyltransferase.

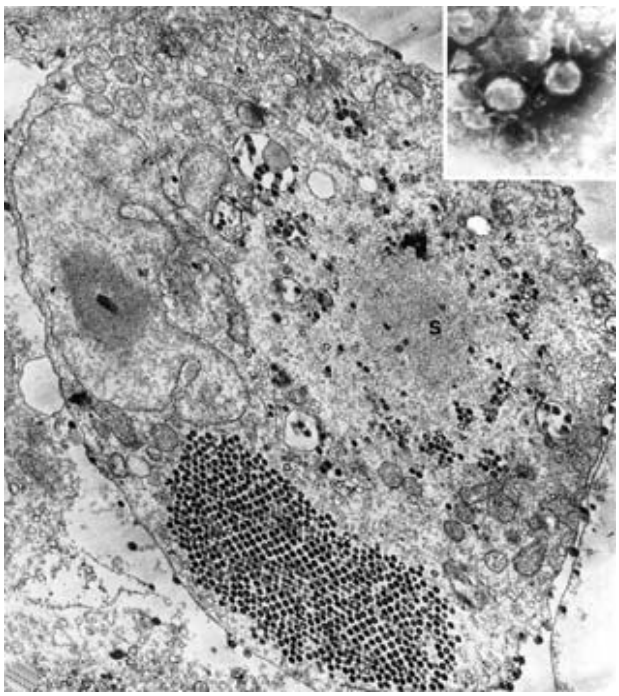


Figure 2 Electron micrograph of a thin section of a FV3-infected fish (FHM) cell at 8 h postinfection. Virions in various stages of assembly are present in assembly site (S). The cytoplasm contains mature virions in a crystalline array and some of the mature virions can be seen budding through the cell membrane. ×8750. The inset shows negatively stained virions. ×52 500.

Replication

General characteristics

FV3 grows in a wide variety of tissue culture cells of piscine, amphibian, avian and mammalian origin at temperatures between 12 and 32°C. At the light microscopy level, virus assembly sites are seen as Feulgen-positive inclusion bodies in the cytoplasm of infected cells (**Fig. 1**) that contain viral DNA and proteins. Electron micrographs of FV3-infected cells show assembly sites as regions free of cellular structures (e.g. organelles, ribosomes, cytoskeletal filaments) that contain virus particles in various stages of assembly (**Fig. 2**). Like all other viruses, the time course of infectious virus production is variable, depending on the type of cells, temperature of incubation, multiplicity of infection, conditions of growth and the strain of the virus. An unusual feature of FV3 replication is that it uses both the nucleus and cytoplasm for its nucleic acid synthesis but virus assembly takes place exclusively in the cytoplasm. At 33°C, a nonpermissive temperature for virus growth, early virus-specific transcription takes place but no

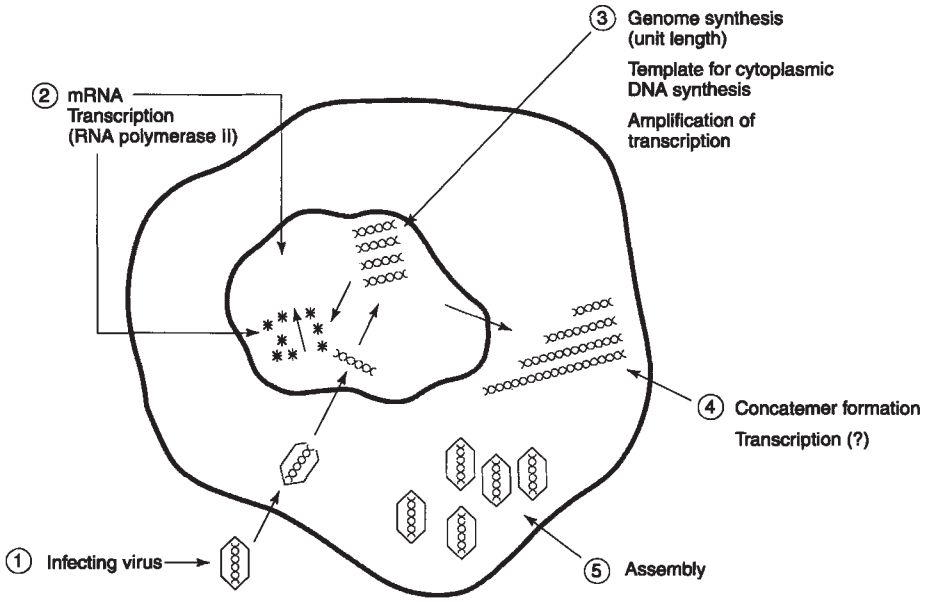


Figure 3 Proposed replication cycle of frog virus-3 (FV3). The genomes of parent FV3 particles reach the nucleus where they are transcribed during the early stages of infection. Cellular RNA polymerase II, modified by the structural protein(s) of the virus particles, is probably used for virus transcription at this stage. The parent genome in the nucleus also serves as the template for stage 1 of DNA replication. Replicative molecules are then transported to the cytoplasm where they participate in stage 2 of DNA replication. The large replicative complex produced in stage 2 is cleaved to produce mature viral DNA. Virus morphogenesis and assembly also occur in the cytoplasm.

viral DNA or infectious virions are produced. Apparently, one or more of the viral proteins are temperature sensitive and nonfunctional at this temperature. FV3 infection results in the 'shut-off' of host cell macromolecular syntheses (DNA, RNA, protein) allowing ready detection of viral DNA, RNA and protein.

DNA replication

An unusual feature of FV3 DNA replication is that it occurs in two stages that are physically compartmentalized in the nucleus and cytoplasm (**Fig. 3**). In the first stage, FV3 DNA replication is initiated at one or more preferred origins, probably on the linear DNA molecule in the nucleus with newly synthesized progeny DNA of genome or less than genome length. Newly synthesized viral DNA in the nucleus is not methylated. Arginine starvation of FV3-infected cells or infection of cells with a temperature sensitive mutant (*ts*12488) at nonpermissive temperature results in the arrest of FV3 DNA replication in the first stage, suggesting that transition from the first to second stage of DNA replication is mediated by a viral protein(s). During infection, progeny DNA synthesized in the nucleus is transported to the cytoplasm where it participates in the second stage of DNA replication. Second-stage DNA replication occurs only in the cytoplasm late in infection (after 3 h) and the replicating DNA is present as a large concatemer (more than 10 times the genome size). Evidently, progeny DNA during the second stage of DNA replication consists of multiple genome length molecules (concatemers). A virus-specified DNA methyltransferase methylates the DNA after its replication in the cytoplasm. During the second stage, FV3 DNA replication is intimately associated with recombination. Progeny molecules at this stage extensively recombine which creates many more replication forks leading to a rapid increase in the rate of DNA synthesis. Since FV3 DNA is circularly permuted and terminally redundant, it greatly facilitates recombination and most likely promotes formation of a complex, branched network of replicating DNA. Inhibition of recombination greatly reduces DNA synthesis, suggesting that recombination and replication at this stage are interdependent. Pulse-chase experiments showed that concatemeric DNA serves as the precursor for production of mature viral DNA molecules.

Functions of FV3 concatemeric DNA

The production of concatemers is required for regeneration of the end of the DNA molecules in viruses that possess linear genomes. The most

thoroughly studied example of such a function is phage T4, in which concatemeric DNA is cleaved and then packaged into virions via a 'headful' mechanism. DNA packaged into phage heads through this mechanism becomes circularly permuted and terminally redundant. As mentioned earlier, FV3 concatemeric DNA is used for production of mature viral DNA. It is, therefore, reasonable to assume that FV3 may also use the 'headful' packaging mechanism to generate circularly permuted and terminally redundant progeny molecules that are found in the infectious virions. However, experimental evidence for the 'headful' or any other mechanism of DNA packaging remains to be established.

Transcription

The genome of an incoming FV3 particle reaches the nucleus where it is transcribed during the early stages of infection. Cellular RNA polymerase II is used for transcription at this stage with a virion-associated protein required for FV3 transcription. It is not known whether this protein modifies RNA polymerase II or the FV3 DNA template for transcription.

FV3 transcripts lack polyadenylation tracts at their 3′-end. At the 5′-end, FV3 mRNAs are capped and methylated. Early viral mRNA molecules are also methylated at the internal position in adenosine residue. In contrast, although late mRNAs are terminally blocked and methylated, internal methylation in this mRNA class is not detectable. Since early FV3 transcripts are synthesized in the nucleus, whereas late transcripts are synthesized in the cytoplasm, these differences in methylation of early versus late transcripts may be due to the different site of their synthesis.

Because of the complete switch-off of host cell mRNA synthesis, and lack of variable-length poly(A) tracts on FV3 mRNA, the FV3 transcript from infected cells can be resolved into 47 bands on denatured gels. Analysis of these bands indicated that FV3 transcription is temporally regulated and can be subdivided into three classes: immediate-early (IE), delayed-early (DE) and late (L). The IE class consists of those RNAs synthesized in the absence of protein synthesis and represents approximately one-third of the single-stranded genome equivalent. Depending on the cell line, IE synthesis takes place 1–2 h postinfection. An additional third of the genome is transcribed in the presence of amino acid analogue fluorophenylalanine or by a temperature sensitive (*ts*) mutant, *ts*9467, that is defective in late transcription at nonpermissive temperature. DE mRNA is normally synthesized 2–3 h postinfection. By 3–4 h postinfection, a complete single-stranded equivalent of the genome is transcribed.

Transcriptional mapping and sequencing of 10 FV3 genes have shown that various classes of FV3 mRNAs, as well as mRNAs within classes, are transcribed from separate promoters. FV3 genes contain no intervening sequences and no polyadenylation sites; apparently there is no post-transcriptional processing of the FV3 transcript.

Site-specific DNA methylation has a strong silencing effect on genes transcribed by eukaryotic RNA polymerase II, yet FV3 uses this enzyme to transcribe FV3 RNA from its highly methylated genome. Experimental evidence indicates that FV3 infection results in the transcription of exogenously supplied, normally silent, methylated genes in infected cells. Thus, FV3 has evolved a mechanism to permit cellular RNA polymerase II to transcribe normally silent methylated genes.

Protein synthesis

In conjunction with FV3 transcription, viral protein synthesis occurs in three phases. First, IE proteins are synthesized at the beginning of infection, then DE proteins after 2 h postinfection, and L proteins by 3–4 h postinfection. Several viral regulatory proteins are required for transition from IE to DE to L protein synthesis. In contrast to other DNA viruses, DNA replication is not necessary for L protein synthesis. However, DNA replication increases the amount of L proteins synthesized in FV3-infected cells. FV3 protein synthesis is also regulated at the translational level. Although transcription of IE and DE genes continues concomitantly with that of L genes, their translation is greatly reduced late in infection. Experimental evidence suggests that at least one viral protein is required to inhibit the translation of IE and DE mRNA late in infection. Late FV3 mRNAs are poorly translated *in vitro*; addition of a factor present in infected cells is required for efficient translation of these messages. Thus, efficient translation of late mRNAs is also mediated by viral regulatory protein(s).

Assembly and release of virions

FV3 virus particles are assembled in specialized structures termed the assembly sites found exclusively in the cytoplasm of infected cells. These sites are less dense than the surrounding cytoplasm, devoid of cellular structures (e.g. organelles, cytoskeletal filaments, ribosomes), and contain assembling virions. Assembly sites are formed in two steps; in the first step, viral DNA accumulates in the cytoplasm as a spheroid mass but devoid of viral proteins (pre-

assembly site). During the second step, reorganized intermediate filaments surround preassembly sites and viral proteins accumulate into it. Cells infected with a temperature sensitive mutant (*ts*9467) at nonpermissive temperature form preassembly sites but not mature assembly sites. The virus assembly sites are the only region of the cytoplasm at which the viral proteins and viral genomes are present in great abundance. Data suggest that viral proteins synthesized elsewhere in the cytoplasm are transported into the assembly sites. In summary, the virus assembly site is a specialized area of the cytoplasm composed of a three-dimensional filamentous matrix in which the viral proteins, viral genomes and assembling virions are suspended. It is possible that the matrix may serve as a substratum on which virus proteins interact in an ordered fashion to form icosahedral particles.

Late in infection, the surfaces of FV3-infected cells exhibit many projections through which the virus buds. These projections have been termed microvillus-like projections because they resemble normal cellular microvilli in general morphology. At a time when the virus is released in abundance, the number of projections greatly increase and each projection possesses a series of bulges, each of which contains a single virus particle. The microvillus-like projections contain microfilaments and virus particles in the process of budding. Microfilaments play an active role in FV3 budding since microfilament-depolymerizing drugs such as cytochalasine B and D prevent virus release. FV3 acquires its envelope during the process of budding. However, acquisition of the envelope is not necessary for virus infectivity. Intracellular virus particles, often seen as crystalline arrays in electron microscopic pictures, are infectious. Similarly, inhibition of virus budding by cytochalasine D has no effect on virus infectivity.

FV3-induced cytoskeletal changes

Soon after infection, FV3 induces dramatic organizational changes in all three classes of cytoskeletal filaments: the microtubules, the intermediate filaments and the microfilaments. In FV3-infected cells, the microtubules appear to decrease progressively with the course of FV3 infection. Concomitant with a decrease in microtubule numbers, there is a reduction (80% reduction by 4 h postinfection) in tubulin synthesis – the constituent protein of microtubules.

In FV3-infected cells, intermediate filaments retract from the cell periphery and reorganize to surround the preassembly sites. There is increased phosphorylation of vimentin (constituent protein of intermediate filaments in fibroblasts) in FV3-infected cells before they reorganize around the preassembly sites. In *ts*9467-infected cells at nonpermissive temperature, there is neither increased phosphorylation of vimentin nor reorganization of intermediate filaments around the preassembly sites. These results suggest that phosphorylation of vimentin is a prerequisite for reorganization of intermediate filaments.

In FV3-infected cells, the bundles of microfilaments (stress fibers) disappear but microfilaments underlying the microvillus-like projections are seen late in infection. The significance of the destruction of stress fibers is not known but it may be related to changes in the cell shape that occur during virus infection. Reorganization of actin into cell surface projections, however, appears to be important in virus release. Biochemical studies have shown that actin, the constituent protein of microfilaments, becomes more acidic than its counterpart in uninfected cells. It is not known whether the observed shift in actin is due to post-translational modification or whether the phenomenon represents the synthesis of a new isoform of actin. In either case, the process must occur during virus replication, since actin synthesized before infection did not show increased acidity. It is noteworthy that the changes in actin precede the formation of microvillus-like structures used in virus release.

Genetics

An ordered genetic map of FV3 is not yet available, but the genetic analysis of 12 FV3 temperature sensitive (*ts*) mutants have revealed 10 complementation groups. Many more complementation groups, i.e. genes, must exist since the FV3 genome is quite large. Recombination frequencies between the temperature-sensitive mutants ranged from 9 to 63%. These high frequencies of recombination are consistent with the mode of FV3 replication, indicating that DNA replication and recombination are interdependent during the second stage of FV3 DNA replication. In spite of high recombination frequencies among *ts* mutants, marker rescue experiments have not yet been successful. Presumably, the exogenously supplied viral DNA does not recombine with the endogenously replicating DNA. Reasons for the failure of marker rescue experiments are not known.

Pathogenicity

FV3 is not known to cause any disease in naturally occurring populations of frogs. However, FV3 causes death of frog embryos and larvae within 15 days of inoculation of the virus. In developing tadpoles, it causes edema of the tail. Intravenous inoculation of purified virion proteins in mice causes acute hepatitis

Table 1 Shared features of frog virus 3 and bacteriophages

Features	*Phage example*
Circularly permuted and terminally redundant genome	T4, P22
High degree of DNA methylation	T4
Two stages in DNA replication	T4
Lack of intervening sequences in viral genes	λ, P22
Lack of polyadenylation in viral RNA	λ, P22
Presence of hyphenated dyad symmetry near transcription termination sites	λ

but the inoculation of the virus in healthy adult frogs causes no apparent effects.

Evolution

Eukaryotic DNA viruses are classified as cytoplasmic or nuclear depending on whether the nucleus or the cytoplasm is the site of nucleic acid synthesis and virus assembly. FV3 replication represents a striking departure from replication strategies of other nuclear or cytoplasmic DNA viruses. Phylogenetically, FV3, because it uses both the nucleus and the cytoplasm for its replication, may be considered as an intermediate form in the evolution of nuclear and cytoplasmic DNA viruses. Another interesting aspect of FV3 replication is its similarity to that of the lambdoid and T bacteriophages. Indeed, FV3 with its highly methylated DNA, circularly permuted and terminally redundant genome, mRNAs lacking poly(A) and two-stage DNA replication most closely resembles a bacteriophage rather than an animal virus. **Table 1** lists the features that are common to FV3 and bacteriophages. Whether the similarity represents an evolutionary relationship between the two organisms or is a coincidence remains unknown.

Future Perspectives

The life style of FV3 is only partly understood. Unknown features include mechanisms of DNA replication (through a circular or linear intermediate), concatemer formation and assembly (headful?). It is known that transcription of early viral mRNAs depends on RNA polymerase II in the host nucleus but the sites and machinery (whether viral or host) of late transcription remain to be identified. Although some clues to the role of highly methylated DNA in FV3 replication (e.g. morphogenesis) and assembly or in restriction modification are available, they require more rigorous experimental support. Investigations into these aspects of FV3 replication, while contributing to our knowledge of DNA structure and replication, may also elucidate the evolutionary relationship of FV3 to other cytoplasmic and nuclear DNA viruses and to bacteriophages.

See also: **Coliphage lambda (*Siphoviridae*); Host-controlled modification and restriction; *Iridoviridae* – Invertebrate; Lymphocystis disease virus (*Iridoviridae*); Phage Homologous Recombination.**

Further Reading

Goorha RM (1989) Frog virus 3 DNA replication. In: Ahne W and Kurstak E (eds), *Viruses of Lower Vertebrates*, p. 30. New York: Springer-Verlag.

Murti KG, Goorha R and Granoff A (1985) An unusual replication strategy of an animal iridovirus. *Adv. Virus Res.* 30: 1.

FUROVIRUSES

Yukio Shirako, University of Tokyo, Bunkyo-ku, Tokyo, Japan

T Michael A Wilson, Scottish Crop Research Institute, Invergowrie, Dundee, UK

Taxonomy and Classification

Furovirus was the group name first approved for *fu*ngus-transmitted, *ro*d-shaped plant viruses by the International Committee on the Taxonomy of Viruses in 1987. Criteria for membership of the group were a divided genome composed of two or more components of single-stranded RNA, hollow rigid particles, and transmission through moist soil by motile zoospores of fungi which belong to the poorly characterized taxon, Plasmodiophoromycetes (**Table 1**). Among ten fungus-transmitted, rod-shaped

Table 1 Shared features of frog virus 3 and bacteriophages

Features	*Phage example*
Circularly permuted and terminally redundant genome	T4, P22
High degree of DNA methylation	T4
Two stages in DNA replication	T4
Lack of intervening sequences in viral genes	λ, P22
Lack of polyadenylation in viral RNA	λ, P22
Presence of hyphenated dyad symmetry near transcription termination sites	λ

but the inoculation of the virus in healthy adult frogs causes no apparent effects.

Evolution

Eukaryotic DNA viruses are classified as cytoplasmic or nuclear depending on whether the nucleus or the cytoplasm is the site of nucleic acid synthesis and virus assembly. FV3 replication represents a striking departure from replication strategies of other nuclear or cytoplasmic DNA viruses. Phylogenetically, FV3, because it uses both the nucleus and the cytoplasm for its replication, may be considered as an intermediate form in the evolution of nuclear and cytoplasmic DNA viruses. Another interesting aspect of FV3 replication is its similarity to that of the lambdoid and T bacteriophages. Indeed, FV3 with its highly methylated DNA, circularly permuted and terminally redundant genome, mRNAs lacking poly(A) and two-stage DNA replication most closely resembles a bacteriophage rather than an animal virus. **Table 1** lists the features that are common to FV3 and bacteriophages. Whether the similarity represents an evolutionary relationship between the two organisms or is a coincidence remains unknown.

Future Perspectives

The life style of FV3 is only partly understood. Unknown features include mechanisms of DNA replication (through a circular or linear intermediate), concatemer formation and assembly (headful?). It is known that transcription of early viral mRNAs depends on RNA polymerase II in the host nucleus but the sites and machinery (whether viral or host) of late transcription remain to be identified. Although some clues to the role of highly methylated DNA in FV3 replication (e.g. morphogenesis) and assembly or in restriction modification are available, they require more rigorous experimental support. Investigations into these aspects of FV3 replication, while contributing to our knowledge of DNA structure and replication, may also elucidate the evolutionary relationship of FV3 to other cytoplasmic and nuclear DNA viruses and to bacteriophages.

See also: **Coliphage lambda (*Siphoviridae*); Host-controlled modification and restriction; *Iridoviridae* – Invertebrate; Lymphocystis disease virus (*Iridoviridae*); Phage Homologous Recombination.**

Further Reading

Goorha RM (1989) Frog virus 3 DNA replication. In: Ahne W and Kurstak E (eds), *Viruses of Lower Vertebrates*, p. 30. New York: Springer-Verlag.

Murti KG, Goorha R and Granoff A (1985) An unusual replication strategy of an animal iridovirus. *Adv. Virus Res.* 30: 1.

FUROVIRUSES

Yukio Shirako, University of Tokyo, Bunkyo-ku, Tokyo, Japan

T Michael A Wilson, Scottish Crop Research Institute, Invergowrie, Dundee, UK

Taxonomy and Classification

Furovirus was the group name first approved for *fu*ngus-transmitted, *ro*d-shaped plant viruses by the International Committee on the Taxonomy of Viruses in 1987. Criteria for membership of the group were a divided genome composed of two or more components of single-stranded RNA, hollow rigid particles, and transmission through moist soil by motile zoospores of fungi which belong to the poorly characterized taxon, Plasmodiophoromycetes (**Table 1**). Among ten fungus-transmitted, rod-shaped

Table 1 Host range and geographic distribution of fungus-transmitted, rod-shaped plant viruses

Crop type	*Virus*	*Main hosts*	*Fungal vector*	*Geographic distribution*
Cereals	OGSV	*Avena sativa* (oat)	*Polymyxa graminis*	UK, France, USA
	RSNV	*Oryza sativa* (rice)	*Polymyxa graminis*	Ivory Coast
	SBWMV	*Secale cereale* (rye) *Triticum aestivum* (wheat) *Hordeum vulgare* (barley)	*Polymyxa graminis*	USA, Italy, France, Japan, China, Brazil
	SCSV	*Sorghum bicolor* (sorghum)	Unknown	USA
Roots/tubers	BNYVV	*Beta vulgaris* (sugarbeet) *Spinacia oleracea* (Swiss chard)	*Polymyxa betae*	Europe[a], China, USA, Russia, Japan
	BSBV	*Beta vulgaris* (sugarbeet)	*Polymyxa betae*	UK, Sweden, Finland, Belgium, Germany
	BSBMV	*Beta vulgaris* (sugarbeet)	*Polymyxa betae*	USA
	PMTV	*Solanum tuberosum* (potato)	*Spongospora subterranea*	Peru, Japan, Western Europe, Sweden, Finland
Legumes	BBNV	*Vicia faba* (broadbean)	Unknown	Japan
	PCV	*Arachis hypogaea* (peanut) *Sorghum arundinaceum* *Sorghum bicolor (sorghum)* *Setaria italica*	*Polymyxa graminis*	West Africa, India

[a] Reported in Italy, France, Germany, Yugoslavia, Czech Republic, Austria, Switzerland, Netherlands and Greece.

viruses, four infect cereals (soil-borne wheat mosaic virus, SBWMV; oat golden stripe virus, OGSV; sorghum chlorotic spot virus, SCSV; rice stripe necrosis virus, RSNV), four infect roots and tubers (beet necrotic yellow vein virus, BNYVV; beet soil-borne virus, BSBV; beet soil-borne mosaic virus, BSBMV; potato mop-top virus, PMTV) and two infect legumes (peanut clump virus, PCV; broadbean necrosis virus; BBNV). There are also two weed-infecting viruses, *Hypochoeris* mosaic virus (HMV) and *Nicotiana velutina* mosaic virus (NVMV), which have not been well-characterized but might have been included in this group.

Recently, the complete nucleotide sequences of four fungus-transmitted, rod-shaped viruses (SBWMV, PCV, BSBV and BNYVV) were determined, enabling a more meaningful classification based on genome structure and organization, gene expression strategy and amino acid sequence similarities, rather than a taxonomy based on virion morphology, the number of RNA segments and mode of vector transmission. Taken together with partial nucleotide sequences of several other viruses in the group, the previous grouping was reinvestigated and, except for RSNV, nine viruses have been reclassified into four genera, the genus *Furovirus* (SBWMV, the type species; OGSV and SCSV, the members), the genus *Pomovirus* (PMTV, the type species; BSBV and BBNV, the members), the genus *Pecluvirus* (PCV, the type species) and the genus *Benevirus* (BNYVV, the type species, and BSBMV, a possible member) (**Table 2**). The four genera differ from each other in the numbers of RNA segments, the types of cell-to-cell movement proteins, RNA structures in the 3′-terminal region (tRNA-like structure or poly(A) tail) and phylogeny based on RNA polymerase domains (**Tables 2** and **3** and **Fig. 1**) and have not been assigned a family. Members of the *Furovirus, Pomovirus* and *Pecluvirus* genera are closely related to each other and to tobamoviruses, tobraviruses and hordeiviruses in the amino acid sequence of the polymerase domain and the presence of a tRNA-like structure in the 3′-terminal region of the viral RNA. Only beneviruses have a poly(A) tail at the 3′ terminus and a distant phylogenetic relationship in the polymerase domain to viruses in the other three genera. Nevertheless, they all belong to the Alphavirus-like superfamily based on genome organization, expression strategy and amino acid sequence conservation. Thus, the previous genus *Furovirus* included all ten fungus-transmitted, rod-shaped viruses, whereas the new genus *Furovirus* includes only SBWMV, OGSV and SCSV.

Virus Structure and Composition

Most fungus-transmitted, rod-shaped viruses consist of hollow, rigid nucleoprotein helices around 20 nm in diameter, with two or more dominant particle lengths (270 and 150 nm). In BNYVV and RSNV, the long and intermediate particle lengths are 390 and 270 nm, respectively. NVMV has been claimed to have particles up to 700 nm in length, but the range is

Table 2 Particle sizes and genome compositions of fungus-transmitted, rod-shaped plant viruses

Genus	Virus	Diameter (nm)	Length (nm)	Coat protein (kDa)	RNA (nt)[a] 1	2	3	4
Furovirus	SBWMV	20	300, 160	19	7099	3593		
	OGSV	20	300, 150	19	(6.2 kb)	(3.5 kb)		
	SCSV	20	260, 140	19	(6.9 kb)	(3.6 kb)		
Pecluvirus	PCV	20	240, 170	23	5897	4504		
Pomovirus	PMTV	17	290, 150, 100	20	(6.4 kb)	2962	2315	
	BSBV	19	300, 150, 65	19	5834	3454	3006	
	BBNV	25	250, 150	—	—	—	—	
Benevirus	BNYVV	20	390, 265, 105, 65	21	6746	4612	1775	1431
	BSBMV	19	400–50	21	(6.7 kb)	4616	—	—
Unclassified	RSNV	20	380, 270, 160, 110	—	—	—	—	—
	HMV	21	240, 120	—	—	—		
	NVMV	18	700?, 125–150	21	(8.0 kb)	(3.0 kb)		

[a] GenBank/EMBL/DDBJ accession numbers: SBWMV RNA1, L07937; SBWMV RNA2, L07938; PCV RNA1, X78602; PCV RNA2, L07269; PMTV RNA2, D30753; PMTV RNA3, D16193; BSBV RNA1, Z97873; BSBV RNA2, U64512; BSBV RNA3, Z66493; BNYVV RNA1, X05147; BNYVV RNA2, X04197; BNYVV RNA3, M36894; BNYVV RNA4, M36896; BSBMV RNA2, AF06189.

extremely wide with the mode around 125–150 nm. BNYVV, BSBV, PCV and RSNV also possess short particles of 160 nm or less (**Table 2**). Coat protein subunits (approximately 19–24 kDa) form right-handed, single-start helices (pitch 2.6–2.9 nm, with 12.25 subunits per turn for BNYVV) which encapsidate each single-stranded genomic RNA component separately. In all cases, RNA comprises about 5% of the particle weight. Most likely, four ribonucleotides interact with each coat protein subunit. No data are available on the possible presence of divalent cations or polyamine content.

Serology

Studies with conventional rabbit polyclonal antibodies against purified virions indicate that SBWMV, PMTV, OGSV and BBNV are distantly related serologically. BSBV antiserum does not react with BNYVV, while BNYVV and BSBMV are distantly related serologically. Ivory Coast and several Indian isolates of PCV are also serologically distinct from each other. SBWMV and PMTV also have a distant relationship with tobacco mosaic virus (TMV). BBNV is unrelated to TMV, but is related to two other tobamoviruses, cucumber green mottle mosaic virus and odontoglossum ringspot virus. Otherwise, all other viruses in the four genera appear to be serologically distinct. Mouse monoclonal antibodies (MAbs) against SBWMV failed to react with 13 other viruses including TMV, and SCSV. For quantitative enzyme-linked immunosorbent assay (ELISA) and long-term storage, SBWMV-infected leaf samples were best after $CaCl_2$ desiccation. MAbs against

Table 3 Genome structure and expression of four fungus-transmitted, rod-shaped plant viral genera: comparison with *Tobamo-* and *Alphavirus* genera

Genus	No. of RNA segments	Terminal structures 5′/3′	RNA polymerase phylogeny/ expression	Putative fungus transmission factor	MP
Furovirus	2	Cap / tRNA-like	*Tobamo* / 59K RT (UGA)	84K CP-RT (UGA)	37K
Pecluvirus	2	Cap / tRNA-like	*Tobamo* / 60K RT (UGA)	39K Leaky scanning	TGB
Pomovirus	3	Cap / tRNA-like	*Tobamo* / 59K RT (UAA)	104K CP-RT (UAG)	TGB
Benevirus	2	Cap / Poly(A)	*Alpha* / Pro?	75K CP-RT (UAG)	TGB
Tobamovirus	1	Cap / tRNA-like	*Tobamo* / RT (UAG)	n.a.	30K
Alphavirus	1	Cap / Poly(A)	*Alpha* / 69K RT (UGA) and Pro	n.a.	n.a.

MP, cell-to-cell movement protein; RT, readthrough (leaky termination codon); CP-RT, capsid readthrough protein (leaky termination codon); TGB, triple gene block proteins; Pro, proteolytic processing by papain-like proteinase; n.a., not applicable.

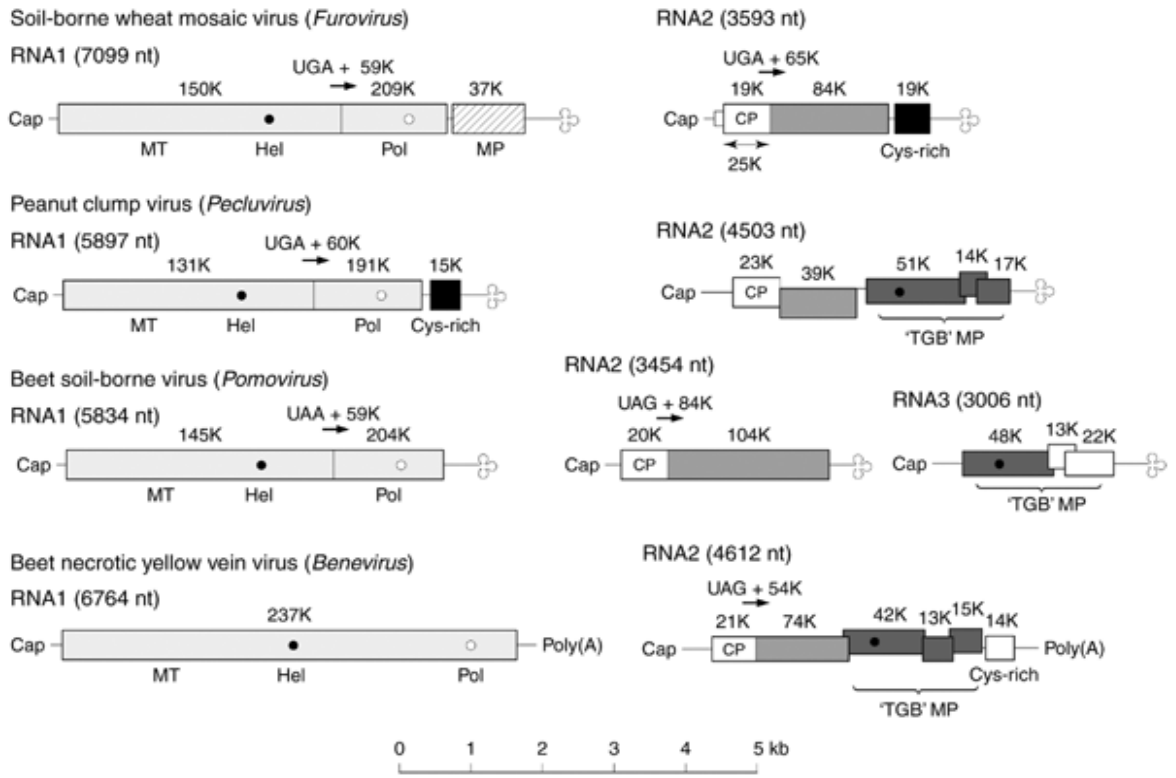


Figure 1 Genome structures of the four genera of fungus-transmitted, rod-shaped plant RNA viruses. Wild-type RNA species encoding all the essential functions required for replication, encapsidation, systemic infection and fungus transmission are drawn. Field isolates of BNYVV contain two or three additional smaller RNAs, which facilitate efficient replication in specific host tissues and transmission by the fungal vector. MT, methyltransferase domain; Hel, helicase domain; Pol, RNA polymerase domain; MP, movement protein; Cys-rich, cysteine-rich protein; 'TGB' MP, triple gene block movement proteins; filled circle, NTP-binding helicase motif (GXXGXGKS/T); open circle, RNA polymerase motif (GDD); cloverleaf, tRNA-like structure; rightward arrow, readthrough at a leaky termination codon.

SBWMV, PMTV or BNYVV have been used for coat protein epitope mapping in virions.

Genome Structure and Expression

Fig. 1 shows the genome organizations of SBWMV, PCV, BSBV and BNYVV, representing the four genera, *Furo-*, *Peclu-*, *Pomo-* and *Benevirus*, respectively. Characteristics of the genomes of the four genera are shown in **Table 3** and described briefly as follows:

- Fundamental viral functions of replication, systemic infection and vector transmission are coded on two RNA segments in furo-, peclu- and beneviruses, whereas those of the pomoviruses are coded on three RNA segments.
- The 5′ end of the RNA is shown to be capped in furo-, peclu- and beneviruses and is probably also m^7GpppX in pomoviruses.
- The 3′ end of the RNA can be folded into a tRNA-like structure in furo-, peclu- and pomoviruses which is shown to be valylated *in vitro*, similar to those of tymoviruses and sunn hemp mosaic tobamovirus, whereas that of the beneviruses is polyadenylated.
- RNA replicase proteins are coded in the 5′-terminal region of RNA1 in all four genera with the capping enzyme, helicase and polymerase domains positioned sequentially from the 5′ end.
- The RNA polymerase domain is located downstream of a leaky termination codon in furo-, peclu- and pomoviruses, whereas in the beneviruses there is no termination codon between the helicase and polymerase domains.
- Capsid protein is encoded in the 5′-terminal region of RNA2 in all four genera.
- The termination codon for the capsid protein can be readthrough to express the downstream region as a capsid fusion protein in furo-, pomo- and beneviruses, whereas in the pecluviruses the down-

stream region is expressed separately by leaky ribosome scanning.

- The cell-to-cell movement proteins of peclu-, pomo- and beneviruses are composed of triple gene block (TGB) proteins, whereas that of furoviruses is a single 37 kDa protein.
- A small cysteine-rich protein of 8–19 kDa is encoded in the 3′-proximal region of one of the RNA segments, except for the BSBV genome.

The molecular details of each genus are as follows.

Furovirus (type virus, SBWMV)

The complete nucleotide sequences of RNA1 and RNA2 of a USA-Nebraska isolate of SBWMV were determined and the details are described below. Partial nucleotide sequences of RNA2 of a French and a Japanese isolate of SBWMV, and a UK isolate of OGSV, have been determined. Amino acid sequences of the capsid proteins of the Nebraskan and French SBWMV, and OGSV, are more than 97% identical to each other, whereas the coat protein of the Japanese SBWMV is about 80% identical to those of the Nebraskan and French SBWMV, and OGSV.

SBWMV RNA1 (7099 nt) codes for a 150 kDa protein at its 5′ terminus (nt 102–4061), the N-terminal and C-terminal regions of which contain the capping enzyme domain and an NTP-binding, helicase domain, respectively. The UGA termination codon of the 150 kDa protein can be readthrough with approximately 10% frequency to produce a 209 kDa protein (nt 102–5585). The readthrough region contains the canonical Gly-Asp-Asp polymerase motif. After a short intergenic region, the 37 kDa, putative cell-to-cell movement protein (nt 5653–6633) is encoded by the 3′-proximal region, which is likely to be expressed via a subgenomic mRNA. The 3′-proximal region, is 466 nt long and consists of four pseudoknot motifs and a tRNA-like structure, which is shown to be valylated *in vitro*.

SBWMV RNA2 (3593 nt) codes for the 19 kDa capsid protein at its 5′ terminus (nt 334–861), the UGA termination codon of which is frequently suppressed to produce an 84 kDa readthrough protein (nt 334–2595). In the 3′-proximal region, a 19 kDa cysteine-rich protein (nt 2665–3186) is encoded, which should be expressed from a subgenomic mRNA. The 3′ terminal untranslated region is 407 nt long and again is composed of four pseudoknot motifs and a tRNA-like structure similar to RNA1. In addition, there is a CUG 'initiation codon' at positions 214–216 for a 25 kDa protein, which represents a 40 amino acid, in-frame extension to the N terminus of the capsid protein. In wheatgerm extracts the CUG initiation site for the 25 kDa protein is as efficient at initiation as the AUG for the capsid protein.

Pecluvirus (type virus, PCV)

The complete nucleotide sequences of RNA1 and RNA2 of an Ivory Coast isolate of PCV were determined and the details are described below. The complete nucleotide sequence of RNA1 and a partial sequence of RNA2 of an Indian H isolate were also determined. Amino acid sequence identities of the capsid proteins and RNA polymerase domains between the two geographical isolates are 59% and 95%, respectively.

PCV RNA1 (5897 nt) codes for a 131 kDa protein (nt 132–3566), containing the capping enzyme domain in the N-terminal region and the NTP-binding helicase domain in the C-terminal region. The UGA termination codon can be readthrough occasionally to express the downstream polymerase domain in a 191 kDa protein (nt 132–5147). The 3′-proximal region encodes a cysteine-rich 15 kDa protein (nt 5225–5596) which should be translated from a subgenomic mRNA. The 3′-terminal untranslated region is 301 nt long, and the last 80 nt can be folded into a tRNA-like structure, which could be valylated *in vitro*.

PCV RNA2 (4503 nt) contains six open reading frames (ORFs). After the 390 nt 5′ untranslated region lies the 23 kDa capsid protein ORF (nt 391–1011). The second ORF, for a 39 kDa protein, starts at the third nucleotide of the C-terminal proline codon of the capsid protein and terminates at nt 2111. ORF 2 was shown to be translated from genomic RNA2 by leaky ribosomal scanning. The third (nt 2250–3590), fourth (nt 3556–3924) and fifth (nt 3767–4225) ORFs code for 51 kDa, 14 kDa and 17 kDa proteins, respectively, which comprise the TGB proteins for cell-to-cell movement. The 51 kDa protein contains the NTP-binding, helicase motif. The TGB proteins should be translated from a tricistronic subgenomic mRNA. The 3′-terminal untranslated region is 279 nt long and shows extensive nucleotide sequence similarity with PCV RNA1.

Pomovirus (type virus, PMTV)

The complete nucleotide sequences of RNAs 1, 2 and 3 of an Ahlum isolate of BSBV were determined and the details are described below. RNA2 and RNA3 of a UK isolate of PMTV were also determined. Amino acid sequence identity between BSBV and PMTV capsid proteins is 52%.

BSBV RNA1 (5834 nt) codes for the 145 kDa (nt 130–3939) and 204 kDa (nt 130–5463) RNA replicase proteins, which are equivalent to the SBWMV

150 kDa and 209 kDa proteins, and the PCV 131 kDa and 191 kDa proteins. The termination codon for the 145 kDa protein is UAA, which is unique as a leaky readthrough codon. The 3′-terminal untranslated region is 371 nt long.

BSBV RNA2 (3454 nt) codes for the 20 kDa capsid protein (nt 383–877) in its 5′-proximal region, the UAG termination codon of which can be readthrough to produce a 104 kDa capsid fusion protein (nt 383–3178). The 3′-terminal untranslated region is 279 nt long.

BSBV RNA3 (3006 nt) has three ORFs which code for the 48 kDa (nt 405–1685), 13 kDa (nt 1675–2022) and 22 kDa (nt 1889–2458) TGB proteins for cell-to-cell movement. The TGB proteins should be translated from a tricistronic subgenomic mRNA. The 3′-terminal untranslated region is 548 nt long.

In all three BSBV RNA segments, the 3′-terminal 80 nt could be folded into a tRNA-like structure.

In the case of PMTV RNA2, which codes for the TGB proteins, there is an additional short ORF for an 8 kDa cysteine-rich protein in the 3′-proximal region.

Benevirus (type virus, BNYVV)

The complete nucleotide sequences of RNAs 1, 2, 3 and 4 of a French isolate and a Japanese isolate (including RNA5) of BNYVV were determined. Details of the French isolate are given below. Amino acid sequence identities of the RNA polymerase protein and the capsid protein between the French and Japanese isolates are 99% and 98%, respectively. RNA 3, 4 and 5 sequences were also determined for a German isolate of BNYVV. Besides BNYVV, the complete nucleotide sequence of BSBMV RNA2 was determined. The capsid proteins of BNYVV (French isolate) and BSBMV are 58% identical at the amino acid level.

BNYVV RNA1 (6746 nt) codes for a single protein of 237 kDa (nt 154–6480) without interruption by a leaky termination codon as in the RNA1 of furo-, peclu- and pomoviruses. There is also a putative papain-like proteinase domain between the helicase and polymerase domains. The 3′-terminal untranslated region is 266 nt long, followed by a poly(A) tail.

BNYVV RNA2 (4612 nt) codes for the 21 kDa capsid protein (nt 145–708), the UAG termination codon of which could be readthrough occasionally to express the downstream region as a 75 kDa, capsid fusion protein (nt 145–2217). In the central region of RNA2, there are three ORFs for the TGB, 42 kDa (nt 2133–3284), 13 kDa (nt 3287–3640) and 15 kDa (nt 3627–4022) proteins. The TGB proteins should be translated from a tricistronic subgenomic mRNA. In the 3′-proximal region, there is an ORF for a 14 kDa protein (nt 4043–4423) which is rich in cysteine residues and probably translated from a subgenomic mRNA. The 3′-terminal untranslated region is 189 nt long, followed by a poly(A) tail.

The two RNAs mentioned above are sufficient for BNYVV replication, systemic infection and vector transmission. However, all field isolates contain two or three additional smaller RNA species, which are designated RNAs 3, 4 (and 5).

BNYVV RNA3 (1775 nt) codes for a single protein of 25 kDa (nt 447–1103). The 5′- and 3′-terminal untranslated regions are quite long, 446 nt and 672 nt, respectively. The 3′ end is polyadenylated.

BNYVV RNA4 (1431 nt) also codes for a single protein of 31 kDa (nt 344–1189). The 5′- and 3′-terminal untranslated regions are 343 nt and 242 nt, respectively. The 3′ end is polyadenylated.

Some Japanese and German BNYVV isolates contain an RNA5 (1347 nt), which codes for a 26 kDa protein (nt 449–1132). The 5′- and 3′-terminal untranslated regions are 448 nt and 215 nt, respectively. The 3′ end is polyadenylated.

Genetics and Replication

Furovirus (type virus, SBWMV)

Both RNA1 and RNA2 are required for systemic infection. RNA1 codes for the 150 kDa and 209 kDa RNA replicase proteins and the 37 kDa, putative cell-to-cell movement protein. RNA2 codes for the capsid protein, the putative fungus-transmission factor in the capsid readthrough region, and a putative replicational/transcriptional regulator protein. The function of the 25 kDa protein derived from a 40 amino acid N-terminal extension to the capsid protein is not known. Neither the 25 kDa nor the 84 kDa proteins are required for virus particle formation, or systemic spread in wheat plants.

Replication requires temperatures lower than 20°C. Above this temperature infection either does not occur, or systemically infected plants recover from the virus.

When systemically infected wheat plants are grown for prolonged periods, or the virus is repeatedly passaged by mechanical inoculation at about 17°C, deletion mutants of RNA2 occur spontaneously. Deletions occur exclusively within the capsid readthrough region, are of various lengths and are mostly single (or occasionally double) deletion events. There is a tendency for larger deletions to make the mutants more virulent on wheat plants. Since deletions occur when virus is maintained or passaged without its fungal vector, the capsid readthrough region has been implicated in fungal transmission.

OGSV requires low temperatures for replication, whereas the optimum temperature for replication of SCSV is around 25°C.

Pecluvirus (type virus, PCV)

Both RNA1 and RNA2 are required for infection. RNA1 codes for two N-coterminal replicase proteins and a small cysteine-rich protein which may function in the regulation of RNA replication and transcription. RNA2 codes for the capsid protein, followed by a 39 kDa protein translated separately from the capsid protein, from genomic RNA, by leaky ribosome scanning; and finally, the TGB, cell-to-cell movement proteins. Spontaneous deletion mutations in RNA2 have been found in virus isolates propagated in *Nicotiana benthamiana*. The deletions occurred within the 39 kDa protein, but the mutants could form virions and infect host plants systemically, suggesting that the 39 kDa protein is involved in virus–fungus interactions.

Pomovirus (type virus, PMTV)

All three RNA segments are required for replication, systemic spread and fungus transmission, as shown for the genome structure and organization of BSBV. RNA1 codes for two N-coterminal, replicase proteins. RNA2 codes for the capsid protein and the capsid readthrough protein, which is believed to be required for fungal transmission. RNA3 codes for the TGB proteins for cell-to-cell movement.

Contrary to BSBV, PMTV RNA2 codes for the TGB proteins and RNA3 codes for the capsid and readthrough proteins. The sizes of RNAs 2 and 3 are reversed in BSBV and PMTV due to the shorter readthrough region (47 kDa) in PMTV compared with 84 kDa in BSBV, and the 3′-terminal untranslated regions (218 nt in PMTV; 548 nt in BSBV). The former may have resulted from spontaneous deletion in wild-type RNA2, while the latter could be due to a cDNA cloning artifact.

Using an antiserum prepared against the PMTV capsid readthrough domain, the readthrough region was found to be present at one end of rod-shaped virus particles.

Benevirus (type virus, BNYVV)

Among the four genera, the molecular genetics of BNYVV have been studied most extensively, using infectious *in vitro* transcripts produced from full-length cDNA clones.

BNYVV RNA1 and RNA2 code for all essential virus functions required for replication, systemic infection and vector transmission. The 237 kDa protein coded by RNA1 is the viral RNA replicase. The TGB 42 kDa, 13 kDa and 15 kDa proteins, and the capsid protein coded on RNA2, are required for cell-to-cell movement and long distance movement of the virus, respectively. The cysteine-rich 14 kDa protein may be required for regulation of RNA transcription and replication.

The RNA1-coded 237 kDa replicase protein contains a putative papain-like proteinase domain between the helicase and polymerase domains. The 237 kDa protein is processed into 150 kDa and 66 kDa polypeptides, and the latter has been detected in infected leaf tissues, indicating that processing occurs *in vivo*. A requirement for this proteolytic processing in viral RNA replication is uncertain.

The capsid readthrough region is required for fungus transmission. Deletion mutants in the capsid protein readthrough region, isolated in the greenhouse or generated artificially by site-directed mutagenesis, were not fungus-transmissible. By alanine scanning mutagenesis, a KTER motif at positions 553–556 in the 75 kDa capsid readthrough protein was shown to be important for efficient transmission by *Polymyxa betae*. The N-terminal domain of the readthrough region also appears to be important for virus particle assembly.

Using an antiserum prepared against the BNYVV capsid readthrough domain, the readthrough region was found to be present at the ends of rod-shaped virus particles.

The RNA3-encoded 25 kDa protein is soluble and responsible for the yellow lesion phenotype on *Tetragonia expansa* leaves. It also facilitates the proliferation of fine rootlets in sugarbeet, thus increasing BNYVV virulence in infected plants. A short ORF overlaps the C-terminus of the 25 kDa protein and induces a severe necrotic response when activated by deleting upstream sequence.

The RNA4-encoded 31 kDa protein increases the efficiency of transmission of the virus by the vector fungus.

An RNA5 is also detected in field isolates from Japan and Germany. The 26 kDa protein contains an FRGPGN stretch in the C-terminal region, as found in the RNA3-coded 25 kDa protein. RNA5 may be related to the virulence of BNYVV, as is RNA3.

Virus–Host Interactions

In ultrathin tissue sections of infected plants, virus particles are observed both scattered and in aggregates, usually as parallel arrays. Virions appear in both the cytoplasm and vacuole. SBWMV, BBNV, PMTV and OGSV also create inclusion bodies consisting of masses of tangled tubules which can be seen easily in the light microscope. For SBWMV, the

shape and size of the inclusion body depends on the nature of RNA2, implying that the extended coat protein readthrough portion forms or determines inclusion body morphology.

In general, the concentration of many of these viruses in infected plants is fairly low, which presents problems for virus purification. Several (e.g. BNYVV and PMTV) are poorly systemic. Yields are typically 20–30 mg kg^{-1} fresh leaf (or taproot) weight, at best. In part, this reflects particle fragility, as well as problems of releasing virions from aggregates sedimented with the cell debris during low-speed centrifugation. In the case of PMTV and NVMV, the first low-speed pellet is taken as the best source for subsequent virus extraction.

In addition to their effects on the grana of chloroplasts, resulting in leaf mosaics and yellowing, PMTV and BNYVV primarily infect root and tuber tissues. Movement of BNYVV from roots to leaves is rare, but causes vein yellowing and necrosis, hence the name of the virus. It has been speculated that the viruses may also affect phytohormone levels and thereby cause growth abnormalities such as severe stunting, 'mop-tops' or 'rhizomanias'. BNYVV infects the vascular tissues of young taproots, causing browning, taproot stunting and loss of sugar content. If serious, the young taproot dies and, through loss of apical dominance or hormone effects, other fine rootlets or lateral roots then develop into new taproots. They too become infected in turn, resulting in the 'hairy root' phenotype. PMTV causes 'spraing' disease (brown necrotic rings) in a few potato cultivars, e.g. Arran Pilot, Saturna.

Best studied is BNYVV, where the RNA3 25 kDa product has been shown to perturb chloroplast structure and cause yellow lesions, despite the fact that the 25 kDa protein is found predominantly in the 30 000 *g* supernatant fraction of infected cells. The availability of full-length cDNA clones and infectious transcripts of BNYVV and SBWMV will add considerably to our understanding of the molecular pathology of these viruses through reverse genetics, production of transgenic plants and complementation studies.

Transmission and Vectors

The natural vector for SBWMV, PCV, OGSV and RSNV is *Polymyxa graminis*. BNYVV, BSBV and BSBMV are transmitted by zoospores of the closely-related species, *P. betae*, whereas PMTV is spread by *Spongospora subterranea*.

All three vectors for fungus-transmitted, rod-shaped viruses belong to the Class Plasmodiophoromycetes and are obligate parasites inhabiting roots and tubers. *P. graminis* and *P. betae* have little effect on their host plants and are morphologically indistinguishable, but have different host ranges. *S. subterranea* is the causal agent of powdery scab on potato tubers. The life cycle of each consists of biflagellate zoospores, multinucleate plasmodia, zoosporangia and thick-walled resting spores in clusters or cystosori, but all lack a mycelial stage. The optimum temperature for growth of *P. graminis* is 18°C and zoospores become immobile at 28°C. Thus most fungus-transmitted, rod-shaped viruses become a problem when soil temperatures are around 15–18°C. In contrast, PCV spreads efficiently in soil at 28°C (or above) in West Africa and India. At higher temperatures, resting spores of the fungal vector produce zoospores more rapidly. The major source of field infections is viruliferous resting spores in cystosori in virus-infected root debris. Cystosori can even survive passage through the alimentary tract of herbivores.

Since zoospores require moisture for release and motility, virus infections are most evident after extended damp weather or irrigation, and then mostly in low-lying areas of fields. The viruses can persist in the vector for many months and, for PMTV for over 12 years, in resting spores in moist field soil growing crops other than potatoes. Only a small fraction (1–10%) of the zoospore population appears to contain virions, although it is not formally proved that virus particles are the form of infection transmitted by the fungal vector. It is still unclear whether or not these viruses replicate inside their fungal vectors. Electron microscopy has shown increasing numbers of virus particles in successively older spore preparations; however, continuous propagation of initially viruliferous fungi on plants which do not support virus replication eventually leads to virus-free fungus. Viruses are acquired by fungi only *in vivo*, from virus-infected root cells by an unknown mechanism (possibly phagocytosis or pinocytosis). Mixing suspensions of fungal spores and virions does not create viruliferous vectors. Virus particles are internal, hence transmission cannot be reduced by treating motile viruliferous zoospores with virus-specific antisera or by washing resting spores with acid or alkali.

Once contact is made with a healthy plant root, the zoospore becomes encysted and the contents, including virions, enter the epidermal cell, which is penetrated by a rigid, subcellular dart-like structure (the 'stachel'). During growth of the plasmodium, virus is probably released into the host cell cytoplasm. The plasmodium becomes either a zoosporangium or a cystosorus with clusters of resting spores, from which viruliferous zoospores are eventually released for infection of further host plants. Clearly a

component of virus–host plant specificity refects some specific fungus–plant interactions.

At the molecular level, it has been shown that RNA4 of BNYVV confers efficient transmission by *P. betae*, and that RNA3 is necessary for efficient virus spread in root tissue. Repeated mechanical transmission through single lesions in leaves of *Tetragonia expansa* or *Chenopodium quinoa* results in greatly decreased levels (or loss) of RNA3 and RNA4. In addition, a natural deletion mutant of BNYVV RNA2 showed that the 75 kDa readthrough protein (the C-terminally extended 21 kDa coat protein) was essential for virus transmission by *P. betae*. By *in vitro*, site-directed mutagenesis using full-length cDNA clones, a KTER motif at positions 553–556 in the 75 kDa coat readthrough protein was shown to be important for efficient transmission of BNYVV by *P. betae*.

No vectors are known for BBNV and SCSV, but *Polymyxa* spp. are most likely. NVMV also has no known vector, but a high rate of seed transmission to several *Nicotiana* spp. may aid its survival in the semiarid region of South Australia where it was isolated. In addition to their fungal vectors, peclu-viruses are also seed-transmitted in groundnuts (peanuts), and PMTV is spread vegetatively through infected 'seed' potatoes.

Zoospores of the unrelated chytrid fungus *Olpidium brassicae* transmit tobacco stunt virus and lettuce big vein virus nonpersistently; however, these labile rod-shaped particles have capsid proteins of 48–52 kDa and genomes consisting of two double-stranded RNAs and, as such, are now considered members of the genus *Varicosavirus*. *P. graminis* and *P. betae* also transmit bipartite, flexuous filamentous viruses belonging to the genus *Bymovirus*, e.g. oat mosaic virus, wheat spindle streak virus, barley mild mosaic virus and barley yellow mosaic virus.

Epidemiology and Control

Because fungus-transmitted, rod-shaped viruses are retained in resting spores in soil for years it is difficult to control the disease once a field becomes infested with viruliferous fungi. Winter wheat is second only to rice as a major crop in global agriculture (584 million metric tonnes in 1996; Food and Agriculture Organization) and, for example, in the USA 34.5×10^6 hectares of wheat have an annual value of $\$7.7 \times 10^9$. SBWMV can cause up to 80% crop loss, although values of 10–50% are more typical and the US national average (1976–1983) was about 3% loss. Losses due to BNYVV in European sugarbeet can amount to 30%.

Conventional control measures, including rotation of nonsusceptible with susceptible crops, reduce, but do not eliminate, the effective 'titer' of field inoculum (e.g. for SBWMV this means growing maize, oats and soybeans for 3 years between wheat sowings). Delayed crop sowing in the autumn reduces the number of SBWMV-infected winter wheat plants, presumably because a less prolonged damp period, and colder conditions, prevent zoospore invasion. Using only PCV-free groundnut seed, or PMTV-free seed potatoes, and 'roguing' (manually uprooting) obviously infected plants can reduce the incidence of disease. Removing potential weed hosts in which the fungal vectors might overwinter is also beneficial. Virus-resistant cultivars are used extensively to reduce the effects of infection. For SBWMV, a single dominant gene in hard red winter wheat has been used successfully to slow virus production and accumulation until later in the season, when symptoms are less pronounced anyway. Thus the wheat plants are not completely resistant to either the fungus or the virus, nor are they tolerant to infection. In fact, roots are more damaged by SBWMV in resistant than in susceptible cultivars. It is the movement of virus from the roots to the foliage that is alleged to be inhibited by the resistance gene. Chemical sterilants and fungicides (methyl bromide, formaldehyde, captan and benomyl) can reduce the incidence of PMTV and SBWMV in small test plots, but are financially and environmentally impractical for large-scale field control. Similarly, reducing the soil pH to 5.0 with sulfur, or adding mercuric or zinc compounds, can kill zoospores, but not resting spores, of the fungal vector (*S. subterranea*) of PMTV. In Japan, sugarbeet seedlings are germinated in sterile soil and later transplanted in paper pots in infested fields. Although BNYVV infection of fine rootlets is severe, the taproots often escape infection. Nevertheless they are still frequently stunted and have a lower sugar content.

Genetic engineering of crop plants to express portions of the virus genome constitutively has provided effective dominant monogenic protection by interfering with the natural life cycle of the pathogen. Some candidate sequences for such experiments include: the viral coat protein gene, the 3′ untranslated viral RNA sequence – to compete with the viral replicase, the viral nonstructural (replicase) polypeptides, catalytic RNA-cleaving ribozyme sequences targeted to essential viral genes, defective RNA or protein species to block cell-to-cell spread, fungus acquisition or transmission. In addition to pathogen-derived resistance, transgenic expression of a single-chain antibody fragment specific to the BNYVV capsid protein in *Nicotiana benthamiana*

has provided some degree of resistance to BNYVV infection.

Geographic Distribution

Table 1 shows that, in common with their widespread fungal vectors, fungus-transmitted, rod-shaped viruses are found throughout the world in temperate as well as tropical regions. Records of their geographic location probably only reflect sites where particular agronomic problems have arisen and where research as been focused. Airborne viruliferous resting spores offer a global opportunity to spread these viruses.

See also: **Tobamoviruses; Tobraviruses; Hordeiviruses (*Hordeivirus*); Plant virus disease – economic aspects.**

Further Reading

Brunt AA and Richards KE (1989) Biology and molecular biology of furoviruses. *Adv. Virus Res.* 36: 1.

Cooper JI and Asher MJC (eds) (1988) *Developments in Applied Biology.* Vol. 2: *Viruses with Fungal Vectors.* Suffolk, UK: Association of Applied Biologists, Lavenham Press.

Jupin I, Tamada T and Richards KE (1991) Pathogenesis of beet necrotic yellow vein virus. *Semin. Virol.* 2.2: 112.

Koenig, R and Loss, S (1997) Beet soil-borne virus RNA 1: genetic analysis enabled by a starting sequence generated with primers to highly conserved helicase-encoding domains. *J. Gen. Virol.* 78: 3161.

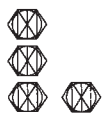
Shirako Y and Wilson TMA (1993) Complete nucleotide sequence and organization of the bipartite RNA genome of soil-borne wheat mosaic virus. *Virology* 195: 16.

Torrance L and Mayo MA (1997) Proposed re-classification of furoviruses. *Arch. Virol.* 142: 435.

G

G4 Bacteriophage *see* **Coliphage ϕX174 and related phages (*Microviridae*)**

GEMINIVIRUSES (*GEMINIVIRIDAE*)



K. W. Buck, Department of Biology, Imperial College of Science, Technology and Medicine, London, UK

History

The description in an eighth century Japanese anthology of yellow vein of *Eupatorium chinense*, now known to be caused by a geminivirus, may be the earliest record of a plant virus disease. Some of the world's most serious diseases of crop plants, such as beet curley top, African cassava mosaic and maize streak, have been known for over 100 years, but it was not until the 1970s that the causative agents of these and some other diseases were characterized as viruses with an unusual twinned-isometric or geminate morphology and a genome of circular single-stranded (ss) DNA. The *Geminivirus* group was established by the International Committee on the Taxonomy of Viruses in 1978 and upgraded to a family, the *Geminiviridae*, in 1995. Currently there are over 90 recognized virus species, and several tentative species, in the family.

Taxonomy and Classification

Viruses in the *Geminiviridae* family are characterized by geminate particles (about 20 nm × 30 nm) containing circular ssDNA molecules (2.5–3.0 kb). They have been classified into three genera, *Mastrevirus* (type member, maize streak virus, MSV), *Curtovirus* (type member, beet curly top virus, BCTV) and *Begamovirus* (type member, bean golden mosaic virus, BGMV), which are distinguished largely on the basis of differences in genome organization, modes of gene expression, functions of virus-encoded proteins and sequence similarity.

Virion Structure

Chloris striate mosaic virus (CSMV) capsids, and probably those of other geminiviruses, are composed of 110 polypeptide subunits. The geminate structure arises by fusion of two incomplete $T = 1$ icosahedra with 22 pentameric capsomers in total. Each geminate particle contains only one ssDNA molecule, whether the genome consists of one DNA component (e.g. CSMV) or two components (e.g. BGMV).

Genome Organization and Expression

Mastrevirus Genus

The genome of mastreviruses is composed of one circular ssDNA molecule of 2.6–2.8 kb. The complete nucleotide sequences of infectious clones of the DNA of bean yellow dwarf virus (BeYDV), CSMV, digitaria streak virus (DSV), MSV, miscanthus streak virus (MiSV), panicum streak virus (PanSV), sugarcane streak virus (SSV), tobacco yellow dwarf virus (TYDV), and wheat dwarf virus (WDV) have been determined. The DNA has four open reading frames (ORFs), two on the virion DNA strand (V1 and V2) and two on the complementary DNA strand (C1 and C2) (**Fig. 1**; **Table 1**).

Transcription is bidirectional from within a large intergenic region (LIR) that separates the V and C ORFs and contains sequences essential for promoter activity and DNA replication. The other ends of the V and C ORFs are separated by a second smaller intergenic region (SIR) which contains transcriptional polyadenylation signals.

ORF V1 encodes the virus movement protein (MP). MP is not required for DNA replication or accumula-

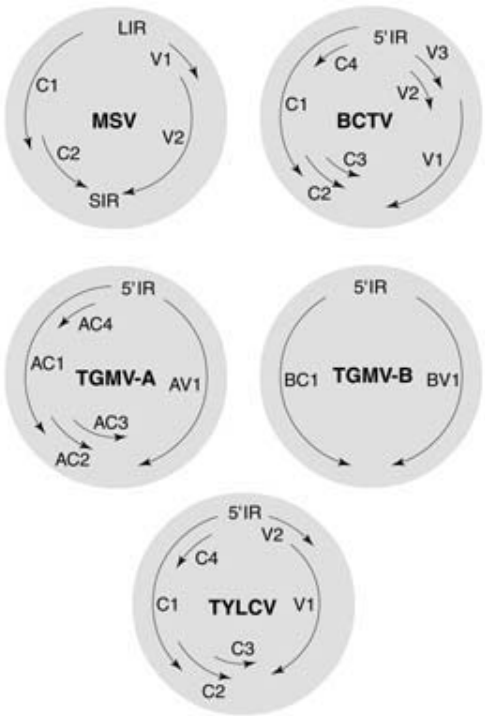


Figure 1 Genome organization and ORFs in the DNA of MSV (*Mastrevirus* genus), BCTV (*Curtovirus* genus), TGMV (*Begamovirus* genus; New World; Bipartite), TYLCV-Sar (*Begamovirus*; Old World; Monopartite). New World begamoviruses lack ORF AV2. Monopartite begamoviruses lack DNA B.

tion of ssDNA in protoplasts. The protein is essential for virus cell-to-cell movement. It is associated with secondary plasmodesmata and a transmembrane domain has been predicted in its amino acid sequence.

ORF V2 encodes the virus capsid protein (CP). In single cells, CP is not required for virus DNA replication, but is needed for accumulation of ssDNA. CP is needed for systemic infection of plants. It binds to double-stranded (ds) DNA as well as ssDNA in a sequence nonspecific manner with a DNA binding domain and/or nuclear localization sequence near the N-terminus. It may be involved in viral DNA nuclear transport as well as in encapsidation. CP is also needed for insect transmission and may be the sole determinant of insect vector specificity.

For MSV, there are two classes of V-sense transcripts, starting 1 and 142 nucleotides upstream of the MP translational initiation codon, respectively. CP appears to be expressed from highly abundant short spliced transcripts of the first class, from which a 76 nucleotide intron in ORF V1 has been removed. MP appears to be expressed from low abundance unspliced long transcripts with a 142 nucleotide leader sequence. Spliced V-sense transcripts have also been identified in DSV-infected plants. Only one (unspliced) transcript spanning ORFs V1 and V2 has been detected in WDV-infected plants and it has been suggested that the CP may be expressed from this dicistronic mRNA by ribosomal frameshifting or by a 'relay-race' mechanism. Spliced V-sense transcripts have also not been detected from TYDV-infected plants.

The product of ORF C1 (Rep A protein) activates V-sense gene expression, possibly in conjunction with the Rep protein. It is probably expressed from an unspliced transcript which spans ORFs C1 and C2. The RepA protein is not required for virus DNA replication in protoplasts, but is required for systemic infection of plants probably due to its activation of V-sense and host gene transcription (see section on DNA replication). For all sequenced mastreviruses except MSV, ORF C2 lacks an ATG translational initiation codon. ORF C2 is expressed as a C1:C2 fusion protein (Rep protein) from a spliced transcript from which an intron, spanning sequences near the 3′-end of the C1 ORF and at the 5′-end of the C2 ORF, has been removed. The Rep protein is essential for virus DNA replication.

Single-stranded DNA from virions of CSMV, MSV, TYDV and WDV has been shown to contain a tightly bound oligodeoxyribonucleotide (about 80 nucleotides) with a few ribonucleotides covalently linked at the 5′-terminus. The sequence of this oligonucleotide is complementary to that of the virion DNA and maps to the SIR. The function of the bound oligonucleotide is probably to act as a primer for complementary-strand DNA synthesis.

DNA from WDV-infected plants contains, in addition to the genomic DNA, a number of deletion mutants (subgenomic DNAs). The deletions in most of these map to the V1/V2 region, but mutants with deletions in the C1/C2 region have also been described. All these subgenomic DNAs retain the LIR and most of the primer binding site. These sequences are required in *cis* for DNA replication. Other replication functions are supplied in *trans* by the wild-type virus genomic DNA which acts as a helper.

Curtovirus Genus

The genome of curtoviruses consists of one circular ssDNA molecule of 2.9–3.0 kb. The complete nucleotide sequences of infectious clones of the DNA of BCTV (three strains), horseradish curly top virus (HrCTV), and tomato pseudo-curly top virus (TPCTV) have been determined. The DNA of BCTV and TPCTV has four ORFs, three on the virion DNA strand (V1, V2 and V3, also called R1, R2 and R3,

Table 1 Geminivirus genes and functions

Genus	*ORF*[a,b]	*Protein encoded*	*Function*
Mastrevirus	C1	RepA	Transactivation of V-sense transcription; binds Rb protein
	C1:C2	Rep	Initiation of DNA replication; sequence-specific endonuclease/ligase
	V1	MP	Cell-to-cell movement
	V2	CP	Encapsidation of virus ssDNA; systemic movement; vector transmission
Curtovirus	C1	Rep	Initiation of DNA replication; sequence-specific endonuclease/ligase
	C2	C2	Suppression of host defense response?
	C3	REn	Enhancement of DNA replication
	C4	C4	Initiation of cell division
	V1	CP	Encapsidation of virus ssDNA; systemic movement; vector transmission
	V2	V2	Regulation of viral ssDNA synthesis
	V3	V3	Movement
Begamovirus			
Monopartite	C1	Rep	Initiation of DNA replication; sequence-specific endonuclease/ligase
	C2	TrAP	Transactivation of V-sense transcription
	C3	REn	Enhancement of DNA replication
	C4	C4	Initiation of cell division? Movement?
	V1	CP	Encapsidation of virus ssDNA; systemic movement; vector transmission
	V2	V2	Virus movement in some Old World begamoviruses
Bipartite	AC1	Rep	Initiation of DNA replication; sequence-specific endonuclease/ligase; transcriptional repression of Rep gene; binds Rb protein; activates transcription of PCNA gene
	AC2	TrAP	Transactivation of AV1 and BV1 transcription; suppression of host defense response?
	AC3	REn	Enhancement of DNA replication
	AC4	AC4	Contributes to transcriptional repression of Rep gene
	AV1	CP	Encapsidation of virus ssDNA; vector transmission; systemic movement in some hosts
	AV2	AV2	Virus movement in some Old World begamoviruses
	BC1	MP	Cell-to-cell movement
	BV1	NSP	Shuttles viral DNA in and out of the nucleus

[a] In an alternative ORF nomenclature, C is replaced by L and V is replaced by R.
[b] ORF V2 (AV2) is found in Old World, but not New World, begamoviruses.

respectively) and four on the complementary DNA strand (C1, C2, C3 and C4, also called L1, L2, L3 and L4, respectively) (**Fig. 1**; **Table 1**). The HrCTV genome lacks ORF C3. As with the mastreviruses, there is an intergenic region (5′-IR) separating the start of the V and C ORFs, which contains sequences essential for promoter activity and DNA replication.

ORF V1 encodes the CP, which is not needed for virus DNA replication in protoplasts, but which is required for systemic infection of plants. It is also required for insect transmission specificity. ORF V2 appears to be involved in the regulation of virus DNA synthesis. In transient replication assays, V2 mutants overaccumulate dsDNA, but produce about 10-fold less ssDNA than the wild-type virus. The V2 gene product is needed for systemic infection of the natural host *Beta vulgaris*, but is not needed for systemic infection of *Nicotiana benthamiana*. ORF V3 is not needed for virus DNA replication. It encodes a protein involved in systemic movement.

ORF C1 encodes the Rep protein, essential for DNA replication, and is the counterpart of the mastrevirus Rep protein. ORF C2 encodes a protein of unknown function. It may possibly suppress a host defense response. BCTV C2 mutants show only a small reduction in virus DNA replication in protoplasts and do not abolish CP synthesis. The C2 protein does not transactivate transcription of the V1 (CP) gene, but may suppress a host defense response. ORF C3 encodes a replication enhancer (REn). REn is

not an absolute requirement for virus DNA replication, but BCTV C3 mutants accumulated three- to five-fold less DNA than the wild-type virus in protoplasts. ORF C4 encodes a protein which can initiate cell division.

BCTV subgenomic DNAs have been described which retain *cis*-essential sequences for DNA replication, but which require a helper virus to provide replication proteins in *trans*.

Begamovirus Genus

The genome of most begamoviruses consists of two ssDNA components, DNA A and DNA B, each 2.5–2.8 kb in size, although some begamoviruses, such as Acalypha yellow mosaic virus (AYVV), tomato yellow leaf curl virus-Israel (TYLCV-Is), TYLCV-Sardinia (TYLCV-Sar), tomato leaf curl virus-Australia (ToLCV-Au), and probably cotton leaf curl virus-Pakistan (CLCuV-Pk), lack a DNA B component. The complete nucleotide sequences of infectious clones of the DNA of abutilon mosaic virus (AbMV), African cassava mosaic virus (ACMV), bean dwarf mosaic virus (BDMV), bean golden mosaic virus-Puerto Rico (BGMV-PR), pepper huasteco virus (PHV), potato yellow mosaic virus (PYMV), squash leaf curl virus (SLCV), tomato golden mosaic virus (TGMV), tomato mottle virus (ToMoV), TYLCV-India (TYLCV-In), TYLCV-Is, TYLCV-Thailand (TYLCV-Th) and ToLCV-Au have been determined, and sequence information for over 40 begamoviruses is available.

Both the DNA A and DNA B components of the bipartite begamoviruses, AbMV, ACMV, PYMV, TGMV and TYLCV-In, are required for efficient infection of plants. However, DNA A can replicate alone and produce virions in single cells. DNA B cannot replicate in the absence of DNA A and is required for systemic virus movement in plants. TYLCV-Th has DNA A and DNA B components, but DNA B is not required to infect plants.

DNA A of all begamoviruses has five ORFs, of which one (AV1, also called AR1) is on the virion DNA strand and the other four (AC1, AC2, AC3 and AC4, also called AL1, AL2, AL3 and AL4, respectively) are on the complementary strand (**Fig. 1**). Begamoviruses from the Old World (Europe, Asia, Africa, Australia) possess an additional ORF (AV2, **Fig. 1**), not found in New World (North America, South America) begamoviruses.

ORF AV1 (V1 for monopartite begamoviruses) encodes the CP. CP is not an absolute requirement for DNA replication, but protoplasts infected with AV1 mutants accumulate decreased amounts of ssDNA and increased amounts of dsDNA, suggesting a regulatory role for CP in ssDNA synthesis. CP is not absolutely required for systemic infection of some plants by bipartite begamoviruses, such as ACMV, TGMV and TYLCV-Th, although mutations in CP can delay symptom development and evidence suggests that CP contributes to systemic movement in a virus- and host-dependent fashion. CP is required for systemic movement of the monopartite begamovirus ToLCV-Aus. As for the mastreviruses and curtoviruses, CP is important for vector transmission of begamoviruses.

Mutational analysis suggests that ORF AV2 (V2 for monopartite begamoviruses) may not be functionally equivalent in different Old World begamoviruses. The AV2 protein is not required for replication of TYLCV-In DNA, although AV2 mutants can reduce the amount of ssDNA produced. TYLCV-In AV2 is required for efficient virus movement in plants. Disruption of ORF V2 in ToLCV-Aus also greatly impaired systemic infection of plants. In contrast, disruption of ORF AV2 in ACMV had no effect on infection of plants.

ORF AC1 (C1 for monopartite begamoviruses) encodes the Rep protein, which is essential for DNA replication, is the counterpart of the mastrevirus and curtovirus Rep proteins. The begamovirus Rep protein negatively regulates its own transcription by binding to a sequence between the TATA box of the Rep promoter and the Rep transcriptional initiation site. The N-terminal region of the TGMV Rep protein mediates this binding. Transgenic plants expressing antisense RNA to the Rep gene of TGMV or TYLCV, or truncated forms of the Rep gene of ACMV or TYLCV in the sense orientation, show resistance to virus infection and reduced viral DNA replication in protoplasts or leaf discs.

ORF AC2 (C2 for monopartite begamoviruses) encodes a transcriptional activator protein (TrAP) which transactivates transcription of the CP gene on DNA A and the BV1 gene on DNA B. Evidence suggests that transcription of the CP gene is activated in mesophyll cells and derepressed in phloem cells. The TrAPs of PYMV and TYLCV-Is have been shown to bind ssDNA with high affinity and dsDNA with low affinity in a sequence-nonspecific manner. The TrAP DNA-binding domain maps to a region rich in cysteine and histidine residues which might form a zinc finger to mediate DNA binding. The TrAP protein may also suppress a host defense response.

ORF AC3 (C3 for monopartite begamoviruses) encodes a replication enhancer (REn), which is the counterpart of the REn proteins of the curtoviruses. It is not an absolute requirement for begamovirus DNA replication, but AC3 mutations reduce DNA replica-

tion by 10- to 50-fold. REn has been shown to interact with Rep via an N-terminal region of the latter.

ORF AC4 (C4) is conserved in most begamoviruses and is contained completely within the AC1 coding sequence, but in a different reading frame. It is apparently not functionally equivalent in different viruses. The TGMV AC4 protein is much more highly expressed than the AC1 (Rep) protein *in vitro* and evidence suggests that it may contribute to transcriptional repression of the AC1 gene. However, TGMV AC4 mutants show no phenotype in a variety of hosts. In contrast ToLCV-Aus C4 mutants show reduced symptoms, while retaining wild-type levels of all viral DNA species. It is possible that the C4 proteins of the monopartite begamoviruses, and possibly those of some Old World bipartite begamoviruses, are functionally equivalent to the C4 proteins of the curtoviruses, and may induce cell division. It has also been suggested that the TYLCV-Is C4 protein might play a role in virus movement.

Like the mastreviruses and curtoviruses, an intergenic region (5′-IR) separates the start of the begamovirus AV1 (or AV2 for Old World begamoviruses) and AC1 genes. This contains transcriptional promoters for these genes, as well as sequences essential for DNA replication. It is likely that the Rep and AC4 proteins are translated from one mRNA, possibly by a leaky scanning or internal initiation mechanism. The promoter for the Rep/AC4 mRNA is located in a region 60 bp immediately upstream of the mRNA start site and contains TATA and G boxes, both of which are important for promoter activity. The TrAP and REn proteins may also be translated from one mRNA; the mRNA for these two proteins is probably synthesized from a promoter which lies within the AC1 ORF.

Begamovirus DNA B has two ORFs, one on the virion DNA strand (BV1, also called BR1) and one on the complementary DNA strand (BC1, also called BL1). The start of these ORFs is separated by an intergenic region, much of which is almost identical to the 5′-IR of the DNA A component of the same virus and this near identical region in both DNAs has been called the 'common region'. The DNA B intergenic region contains promoters for the BV1 and BC1 genes and *cis*-essential sequences for DNA replication. The 3′ ends of the V and C transcripts from both DNA A and DNA B are close together and are adjacent to inverted repeat sequences in the DNA, which act as bidirectional polyadenylation signals.

The BV1 and BV2 proteins are both required for virus movement through the plant. The SLCV BV1 protein binds strongly to ssDNA and less strongly to dsDNA *in vitro*. It localizes to the cell nucleus and has a bipartite nuclear localization signal within the N-terminal 113 amino acid residues. The SLCV BC1 protein binds only weakly to ssDNA and not at all to dsDNA *in vitro*. It is associated with endoplasmic reticulum-derived microtubules in developing phloem cells and with the plasma membrane later in infection. The BC1 protein interacts with a C-terminal domain of the BV1 protein and can relocalize BV1 to the cell surface. A model has been proposed in which the BV1 protein acts as a nuclear shuttle for transport of the virus DNA in and out of the nucleus as a complex that is recognized by the BC1 protein for transport to adjacent cells, possibly through the plasmodesmata via the endoplasmic reticulum-derived tubules. The BV1 protein has been named the nuclear shuttle protein (NSP) and the BC1 protein, the movement protein (MP).

Some isolates of ACMV, PYMV and TGMV contain, in addition to the genomic DNAs, subgenomic DNA B species of about half the size of DNA B. The deletions in these subgenomic DNAs have been mapped to BR1, and in some cases, additionally to the C-terminal part of BC1. All the subgenomic species retain the 5′-IR. Subgenomic DNAs of ACMV act as defective-interfering DNAs and transgenic plants containing an integrated dimer of a subgenomic DNA B, from which a monomer can be released by replication, show partial resistance to infection by ACMV. Defective forms of monopartite begamoviruses (AYVV, CLCuV-Pk and TYLCV-Is) have also been described. The CLCuV-Pk defective DNAs have various combinations of sequence deletion, duplication, inversion, rearrangement, and in some cases, insertion of sequences of unknown origin.

A 682 nucleotide satellite DNA has been found associated with ToLCV-Aus. The satellite DNA (ToLCV-Aus satDNA) is encapsidated by the CP of ToLCV, has no significant ORFs and is completely dependent on the Rep protein of its helper virus for its replication. ToLCV-Aus satDNA has no sequence similarity to ToLCV-Aus DNA, except for *cis*-acting sequences required for virus DNA replication.

Geminivirus DNA Replication

Geminiviruses replicate in the nucleus of infected cells. Curtoviruses and begamoviruses induce morphological changes in the nucleus which include the formation of characteristic fibrillar rings, composed of deoxyribonucleoprotein. Viral and host DNA appear to become compartmentalized with viral DNA forming inclusions in the interior of the nucleus and host DNA localized mainly at the periphery. Viral proteins involved in replication (Rep, REn) and transcriptional regulation (RepA, TrAP) are localized to the nucleus.

Replication of all geminiviruses probably takes place by basically the same mechanism, although there are some differences between the replication of geminiviruses in different genera, partly determined by the virus and partly by the host. Replication is envisaged to occur in two stages in a similar way to that of the ssDNA bacteriophage φX174: (1) synthesis of a minus-strand on the virion plus-strand template to produce dsDNA; and (2) synthesis of plus-strands from the minus-strand template of the dsDNA. Early in the replication cycle, plus-strands synthesized in stage (2) are probably converted into dsDNA to amplify the templates required for virus transcription. Open circular and covalently closed circular (supercoiled) dsDNA, as well as chromatin-like structures, have been isolated from infected plant tissue. Later in the cycle, plus-strand synthesis may be coupled to encapsidation.

As ssDNA extracted from virions is infectious for plants, at least the initial synthesis of minus strands must be carried out by plant proteins and enzymes, before any geminivirus replication proteins have been synthesized. Differentiated plant cells lack detectable levels of DNA replication enzymes and in mature plants, DNA replication and cell division are limited to apical meristems, developing leaves and the cambium. The initial minus-strand synthesis may therefore be confined to existing meristematic cells or cells in which DNA replication and cell division have been induced by wounding during the inoculation process. Subsequent synthesis of plus strands and minus strands will also require host proteins and enzymes. It has been suggested that replication of geminiviruses which are phloem-limited, such as AbMV, BCTV and SLCV, may occur in procambial cells. Other geminiviruses are able to replicate in a variety of differentiated cells, For example, TGMV has been found in nuclei of differentiated cells of leaves, stem and roots of infected *N. benthamiana* plants and MSV DNA is located in mesophyll cells, as well as vascular cells, of mature maize plants; neither virus was found in meristematic cells. Several geminiviruses have been shown to be able to induce the synthesis of proteins and enzymes needed for DNA replication and switch differentiated cells into S phase. For example, the TGMV Rep protein can induce the transcription of the gene for proliferating cell nuclear antigen (PCNA) the processivity factor for host DNA polymerases δ and ε in differentiated cells. Geminivirus Rep proteins may induce synthesis of host replication proteins by binding to plant retinoblastoma (Rb) homologues. In mammalian cells, the Rb protein controls entry into the cell cycle by binding to and inactivating members of the E2F family of transcription factors. When Rb becomes phosphorylated by cyclin-dependent kinases, it can no longer bind E2F transcription factors, which are then able to induce the transcription of DNA replication genes. The genes of several animal viruses which require cellular replication enzymes for virus DNA replication, such as the SV40 large T antigen and adenovirus E1A protein, bind Rb and prevent it from interacting with E2F transcription factors, which are free to induce progression into the S phase. These proteins bind Rb via a conserved motif LXCXE which is also found in the Rep proteins of mastreviruses. The RepA proteins of WDV and other mastreviruses contain the LXCXE motif and can bind to mammalian Rb proteins and plant homologues. The TGMV Rep protein has also been found to bind to maize Rb-related proteins, although it lacks the LXCXE motif. The C4 protein of BCTV is able to induce cell division, possibly by binding to cellular protein kinases.

Little is known about the synthesis of the minus strand, but based on current knowledge of plant DNA replication, it is likely that the origin of minus-strand synthesis is recognized by a DNA polymerase α-primase complex which catalyzes the synthesis of an oligoribonucleotide which acts as a primer for subsequent DNA synthesis. The presence of oligodeoxyribonucleotides with ribonucleotides at their 5′ends bound to virion DNA of several mastreviruses is consistent with this hypothesis. The position of these oligonucleotides suggests a mastrevirus origin of minus-strand replication within or close to the SIR, or somewhere upstream of this region. Such oligonucleotides have not been found bound to virion DNA of curtoviruses or begamoviruses, but analysis of intermediates of replication of ACMV DNA indicates that the minus-strand origin lies within the common region.

Synthesis of geminivirus plus-strand DNA occurs by a rolling circle mechanism, probably similar to that of ssDNA bacteriophages and some bacterial plasmids. All geminiviruses that have been sequenced contain a sequence in the LIR or 5′-IR that can potentially be folded into a stable stem–loop structure, the loop of which contains the invariant sequence TAATATTAC. This sequence is also found in ToLCV-Aus satDNA. The initiation site for plus-strand synthesis *in vivo* lies between the seventh and eigth nucleotide of this sequence. Rep proteins of several geminiviruses have been shown to be site-specific endonucleases which cleave plus-strand viral DNA at the same position *in vitro*. After cleavage, the Rep protein remains covalently bound to the 5′end of the cleaved strand via a tyrosine residue. Rep proteins also have ligase activity. The endonuclease/ligase activity could catalyze the release and circularization

of genome-sized ssDNA after each round of rolling circle replication. The Rep protein probably acts as a dimer with each subunit cleaving the DNA at alternate rounds of replication. The endonuclease/ligase activities map to an N-terminal region of the Rep protein.

Rep proteins can cleave ssDNA, but not dsDNA *in vitro*. Mutational analysis has also indicated the importance of maintaining the stem–loop structure for geminivirus DNA replication, indicating that the dsDNA must be unwound *in vivo* before cleavage can occur. Rep proteins contain ATP-binding motifs in their C-terminal regions, shown to be essential for virus DNA replication, and ATPase activity has been demonstrated for TGMV and TYLCV-Sar Rep proteins. However the ATPase activity is not DNA-dependent, only weak sequence similarity to consensus helicase motifs has been found and helicase activity has not been demonstrated for any geminivirus Rep protein. It is possible therefore that the dsDNA is unwound by one or more cellular helicases, as found for phage φX174.

A conserved stem–loop containing the invariant TAATATTAC sequence is the only structural element in common between plus-strand origins of geminiviruses in different genera. Indeed, replication origins of viruses within the curtovirus and begamovirus genera tend to be virus specific. For example, the BGMV and TGMV origins are not interchangeable between the two viruses. An exception is ToLCV-Aus satDNA which can replicate with either curtovirus or begamovirus helpers. The TGMV origin of replication overlaps the promoter for transcription of the Rep mRNA and includes the stem–loop structure and upstream sequence elements, a G box, AG-motif, TATA box, Rep-binding site and CA-motif. The G box and TATA box, which are transcription factor binding sites, and the CA-motif, are not absolute requirements for TGMV DNA replication, but mutations reduce the efficiency of replication. The Rep-binding site and AG-motif are essential for replication. The TGMV Rep-protein-binding site **GGTAG**TAA**GGTAG** contains two pentanucleotide repeats (shown in bold) and similar repeats were found in Rep-protein-binding sites of other begamoviruses and curtoviruses. The Rep-protein-binding sites are necessary, but not sufficient, to confer virus-specific origin recognition on begamoviruses and curtoviruses. Mastrevirus LIRs contain repeated sequences (iterons) located in the stem of the conserved stem–loop structure and between the TATA box and the transcriptional start site of the Rep mRNA. Iterons may be recognition sites for proteins involved in DNA replication. The WDV origin region consists of a minimal *ca.* 200 bp *cis*-acting element within the LIR, which contains 28 bp and *ca.* 70 bp, downstream and upstream, respectively, from the replication initation site. This is flanked by auxillary elements of *ca.* 70 bp (5′-aux) and 30 bp (3′-aux) which are needed for efficient DNA replication. There is a Rep-protein-binding site upstream of the TATA box.

Transmission, Host Range and Geographical Distribution

In the field, most geminiviruses are transmitted by either whiteflies (begamoviruses) or leafhoppers (mastreviruses and curtoviruses). TPCTV, a curtovirus, is transmitted by a leafhopper. There is no evidence for transmission through seed or by contact between infected and uninfected plants. Once acquired by the vector, the viruses are circulative and persist in it for days or for life, but apparently do not replicate. Transovarial transmission of TYLCV-Is has been reported for at least two generations of whiteflies, suggesting that the vector could act as a virus reservoir between growing seasons of crop plants.

The whitefly-transmitted geminiviruses are transmitted exclusively by *Bemisia tabaci*, although the 'B' biotype is sometimes referred to as *B. argentifolii*. In contrast, each leafhopper-transmitted geminivirus has a different principal vector, e.g. the principal vectors for MSV, WDV and BCTV are *Cicadulina mbila*, *Psammotettix alienus* and *Circulifer tenellus*, respectively. The CP is a major determinant of vector specificity. The CP sequences of the whitefly-transmitted geminiviruses are more closely related to each other than are those of the leafhopper-transmitted geminiviruses. Transmission of ACMV by *B. tabaci* requires sequences from DNA A and DNA B. Some begamoviruses found in ornamental plants, e.g. AbMV and honeysuckle yellow vein mosaic virus, appear to be nontransmissible by *B. tabaci*.

The field host range and geographical distribution of a geminivirus will depend partly on the host range and distribution of the insect vector, and partly on the ability of a virus to infect a particular plant. The host range of *B. tabaci* is very wide, although there may be biotypes with different feeding preferences. Both whitefly-transmitted and leafhopper-transmitted geminiviruses are found predominantly in tropical and subtropical regions, although some geminiviruses, such as WDV, have been found in countries with cooler climates, such as Sweden. Mastreviruses have narrow host ranges and with the exception of BeYDV and TYDV, which infect dicotyledonous plants (dicots), are limited to monocotyledonous plants (monocots) (*Gramineae*). Curtoviruses have

only been found in dicots. BCTV has a very wide host range, infecting over 300 plant species in 44 families. Begamoviruses generally have narrow host ranges and have only been found in dicots. Geminiviruses have been found in a number of weed plants, e.g. AYVV in *Ageratum conyzoides*, which may act as reservoirs for infections of crop plants. TYLCV has been isolated from *Solanum nigrum* in Spain and an Almerian isolate of TYLCV has been found to be transmissible by *B. tabaci* from tomato to *S. solanum* and *vice versa*.

The host range of geminiviruses can be investigated, and potentially extended, by use of inoculation methods that do not involve the vector. Many bipartite begamoviruses can be transmitted mechanically. In contrast, geminiviruses with one DNA component (monopartite begamoviruses, mastreviruses, curtoviruses) either have not been transmitted mechanically or have been transmitted with difficulty. It is possible that mechanical inoculation is aided by DNA B which enables the virus to be transported from cells initially infected to the phloem parenchyma. Agroinoculation and biolistic delivery have been developed as general procedures for infecting plants with geminiviruses. An *Agrobacterium*/Ti plasmid vector system or a high-velocity microprojectile bombardment system is used to introduce a dimer or partial dimer of the cloned viral dsDNA into plant cells. Monomeric viral DNA molecules are then produced either by replication between origins (functionally equivalent to rolling circle replication) or, less efficiently, by intramolecular recombination between homologous sequences.

The ability of a geminivirus to infect a plant will depend on the ability of the virus to replicate in single cells and its ability to move from cell to cell and systemically through the plant via the phloem. Both of these processes will involve interactions between host and viral proteins and nucleic acids and such interactions must be compatible, i.e. able to carry out a particular function, if a productive infection is to occur. For example, DSV and WDV can replicate in monocots, but not in dicots. Synthesis of the DSV or WDV Rep protein in monocots requires the production of a spliced mRNA. In transgenic tobacco plants containing an integrated dimer of DSV DNA, transcription of the C1 region occurs, but no splicing of the pre-mRNA and therefore no production of the Rep protein takes place. Hence failure of DSV and WDV to replicate in dicots is probably due, at least partly, to differences in splicing mechanisms between monocots and dicots. Analysis of host range variants of SLCV suggest that sequences of the common region could be important determinants of host range, perhaps as a result of productive or nonproductive interactions of host DNA primase with the minus-strand origin. Compatibility of virus cell-to-cell movement proteins with plant plasmodesmatal proteins could also be important in determining host range.

Gene Amplification Vectors based on Geminivirus Replicons

The finding that the CP of begamoviruses is not required for DNA replication or systemic infection of some plants led to the development of CP replacement vectors for ACMV and TGMV. Agroinoculation of plants with DNA A vectors containing a reporter gene of similar size to the CP gene, such as *neo*, together with DNA B, leads to systemic infection of plants, stable replication of the vector to average copy numbers up to 500 and concomitant increases in enzyme activity, e.g. neomycin phosphotransferase. Vectors containing reporter genes larger than the CP gene, such as the *gus* gene, show instability in infected plants, undergoing deletions and rearrangements.

Apparently cell-to-cell and systemic movement by begamoviruses in plants requires that the recombinant DNA A construct is of a similar size to the wild-type. However, in transgenic plants containing a master copy (partial dimer) of the vector integrated into the plant chromosomal DNA, vectors up to at least 1.1 kb larger than DNA A replicated stably; in such plants replication occurs in single cells in the absence of DNA B and cell-to-cell movement of the vector is not required. Furthermore, since the plant chromosomal DNA contains a master copy of the vector, gene amplification is heritable. Begamovirus vectors have also been used to study plant splicing signals, to amplify ribozymes in plant cells and to silence endogenous host genes.

Coat protein replacement vectors have also been constructed from mastreviruses. Replication of WDV vectors up to 4 kb larger than the wild-type virus DNA and increased expression of reporter genes occurs in plant protoplasts or cultured plant cells. Replication of WDV replicons smaller than the wild-type virus DNA also occurs. The efficiency of replication of vectors is inversely proportional to their size. WDV vectors have been used to introduce transposable elements into plant cells, to show that transposition requires DNA replication and to determine *cis* requirements for transformation. Since mastreviruses require the CP for systemic infection of plants, infection of plants by mastrevirus CP replacement vectors is precluded. However, a chromosomally integrated TYDV binary vector replicated and maintained a *gus* reporter gene in tobacco plants. MSV mutants with small insertions in the SIR are

viable, but recombinants containing a herbicide resistance gene (*bar* gene), flanked by cauliflower mosaic virus 35S promoter and terminator sequences, inserted into the SIR did not move systemically through plants, probably because even the smallest recombinant with the CP gene deleted was larger than the wild-type virus DNA.

Pathogenesis

The symptoms induced by geminiviruses include striate mosaics and streaks, stunting, enations, leaf curling, vein clearing and vein chlorosis, and yellow and green leaf mosaics. The severity and type of symptoms will depend on the number and type of cells infected, the amount of virus replication per cell and any interactions between virus-encoded proteins and cellular constituents which affect cell function. Since geminiviruses replicate and induce morphological changes in the nucleus, interference with normal nuclear function probably contributes to pathogenic effects at the cellular level. Inappropriate induction of cellular DNA replication and cell division could also result in pathogenic effects Alterations to the plasmodesmata, needed for cell-to-cell virus movement, may contribute to symptoms. For some viruses, such as AbMV, direct effects on the chloroplasts are suggested by the detection of virus ssDNA and dsDNA in these organelles.

Symptoms such as stunting, leaf curling, streaking, vein clearing and veinal chlorosis can be explained by replication of the viruses in phloem cells with consequent disruption of the plant's vascular system. More extensive chlorosis on leaves of dicots indicates wider tissue invasion. Similarly the width of streaks in monocots may be determined by the ability of the virus to spread out of the phloem into other cell types.

Mutational analysis and expression of virus proteins in transgenic plants indicate that several different virus genes contribute to pathogenicity. TGMV AC3 deletion mutants that have reduced DNA replication in single cells produce attenuated symptoms in plants. A mutation in the LIR of MSV which disrupted a TATA box in the promoter for the Rep gene caused a change in symptoms from severe to mild, probably as a result of synthesis of limited amounts of Rep and RepA proteins. Transgenic tobacco plants expressing the TGMV MP (BC1 protein) exhibit symptoms of viral disease, possibly due to an effect of this protein on plasmodesmata. Similarly a mutation within the V1 (MP) gene of MSV, which reduced streak width, reduces the cell-to-cell and/or systemic movement of the virus. This mutation does not result in an amino acid change, but increases the proportion of spliced V-sense transcripts with a concomitant reduction in the amount of MP and an increase in the amount of CP. The C4 proteins of BCTV and ToLCV-Aus are major symptom determinants. Transgenic *Arabidopsis* and *N. benthamiana* plants expressing the BCTV C4 protein show a variety of abnormalities, including the production of tumorigenic growths, probably related to the capacity of this protein to induce cell division.

Evolution

The overall similarity of the genome organization of viruses in the three geminivirus genera suggests their evolution from a common ancestor. All geminiviruses have genes on both the virion DNA strand and its complement with bidirectional transcription and a 5′ intergenic region containing sequences important for transcription and replication, including a potential stem–loop containing the conserved TAATATTAC sequence. The CP genes are located in similar positions on the virion DNA strand and the replication genes are all on the complementary DNA strand.

Despite these similarities, significant differences have emerged, probably as a result of selective pressures dictated by the host and the insect vector. An attractive hypothesis is that the ancestral geminivirus had a single DNA component, resembling that of mastreviruses. Old World monopartite begamoviruses may have evolved from the ancestral mastrevirus. It is noteworthy that the Old World monopartite begamoviruses and the mastreviruses both have V-sense ORFs (V2 and V1, respectively) which encode proteins needed for virus movement and which may be functionally equivalent. Evolution of Old World monopartite begamoviruses from ancestral mastreviruses would have involved several changes. (1) The CP sequence would be altered to allow transmission by whiteflies instead of leafhoppers. (2) An intron would be lost from the Rep gene. A consequence of this would be that the RepA protein, postulated to be a transactivator of the CP gene, would no longer be produced. The TrAP gene, which serves the same purpose in the begamoviruses, may have coevolved with the loss of the Rep gene intron. (3) The REn gene would be acquired. Although the mechanism of replication enhancement by the REn protein, other than its interaction with the Rep protein, is not known, differences in the structure of the LIR and 5′ IR between mastreviruses and begamoviruses, including the presence of iterons in the former, could have led to the evolution of this gene. Old World begamoviruses could then have acquired a DNA-B component to enhance movement of the virus to different tissues and cell types, producing more striking symptoms and increasing

whitefly transmissibility. The formation of bipartite begamoviruses in which the DNA B component is not essential for systemic infection, e.g. TYLCV-Th, could represent the first stage in this evolutionary pathway. The DNA B component probably evolved from the DNA A component, as suggested by their almost identical common regions, the low, but significant sequence similarities between the AV1 (CP) and BV1 (NSP) proteins, the fact that both these proteins bind strongly to ssDNA, and the transactivation of transcription of both genes by TrAP. Acquisition of cell-to-cell movement genes on DNA B could have led to the loss of cell-to-cell movement function of the AV2 protein in some Old World bipartite begamoviruses, e.g. ACMV, and loss of this gene completely in the New World bipartite begamoviruses. The greater sequence diversity within the *Mastrevirus* genus than within the *Begamovirus* genus is consistent with the evolution of begamoviruses from ancestral mastreviruses. Sequence comparisons suggest that curtoviruses, such as BCTV and HrCTV, may have arisen by recombination acquiring the CP gene, and hence leafhopper transmissibility, from a mastrevirus and the Rep gene from a begamovirus.

Geminiviruses have some similarities to another group of multipartite ssDNA viruses (proposed *Nanovirus* genus), comprising banana bunchy top virus, subterranean clover stunt virus, faba bean necrotic yellows virus and milk vetch virus, which all have Rep proteins with the Rb-binding LXCXE motif, have intergenic regions containing a putative stem–loop structure with a conserved consensus TAG/TTATTAC (similar to the geminivirus TAATATTAC sequence) sequence and which probably replicate by a rolling circle mechanism. Geminiviruses and nanoviruses also have some affinities to viruses in the *Circoviridae* family of animal ssDNA viruses. Recently it has been found that ToLCV-Aus DNA can replicate in *Agrobacterium tumefaciens* suggesting that plant ssDNA viruses may have arisen from ssDNA bacteriophages or plasmids which employ rolling circle replication. *Agrobacterium* species are widely distributed plant pathogens known to be able, via their Ti plasmids, to insert DNA into plant genomes. Consistent with this theory is the finding that remnants of geminivirus DNA have been found in the genome of some (uninfected) plants which are hosts for geminiviruses.

See also: **Pathogenesis: Plant viruses.**

Further Reading

Hanley-Bowdoin L, Settlage SB, Orozco BM, Nagar S and Robertson D (1999) Geminiviruses – models for plant DNA replication, transcription and cell cycle regulation. *Crit. Rev. Plant Sci.*, (in press).

Padidam M, Beachy RN and Fauquet CM (1995) Classification and identification of geminiviruses using sequence comparisons. *J. Gen. Virol.* 76: 249.

Palmer KE and Rybicki EP (1997) The use of geminiviruses in biotechnology and plant molecular biology, with particular focus on mastreviruses. *Plant Sci.* 129: 115.

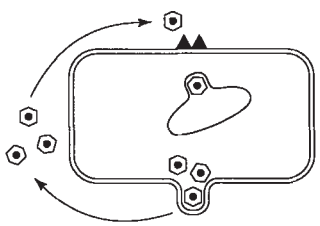
Palmer KE and Rybicki EP (1998) The molecular biology of mastreviruses. *Adv. Virus Res.* 50: 183.

Rybicki EP, Briddon RW, Fauquet CM *et al* (1999) Family *Geminiviridae*. In: Van Regenmortel MHV, Fauquet C, Bishop D *et al* (eds) *Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses*. New York: Academic Press. (in press)

Sanz-Burgos AP and Gutiérrez C (1998) Organization of the *cis*-acting element required for wheat dwarf geminivirus DNA replication and visualization of a rep-protein–DNA complex. *Virology* 243: 119.

GENETICS OF ANIMAL VIRUSES

Frank Fenner, John Curtin School of Medical Research, Canberra, Australia



History

Although genetic variation was the basis of some of the earliest work with animal viruses, such as Pasteur's attenuation of rabies virus, the first formal demonstration of the occurrence of mutation in an animal virus did not occur until over 60 years later, when Burnet used limiting dilution methods to clone influenza virus. A few years later, in the early 1950s, Burnet demonstrated 'high frequency recombination' (later called genetic reassortment) between different strains of influenza A virus. Intramolecular recombination was demonstrated with herpes simplex virus in 1955 and with vaccinia virus in 1958 and the first

HEPADNAVIRUSES (*HEPADNAVIRIDAE*): HEPATITIS B VIRUSES

Contents

General features

William S Robinson, Stanford University School of Medicine, Stanford, California, USA

History

Infectious icterus or jaundice has been recognized for more than 2000 years and has occurred frequently as epidemic disease in military and civilian populations during wars. Much of this disease was probably hepatitis A now known to occur in epidemics when transmitted by sewage-contaminated water and by fecal-contaminated food. Hepatitis transmitted by serum and blood was only recognized in the mid-nineteenth century when venipuncture, use of the same needle without sterilization for many individuals and vaccines containing human serum came into use. Only in the twentieth century was hepatitis shown to be transmitted by 'filterable' agents (i.e. that could pass through filters that retain bacteria) and those agents fit the emerging concept of viruses as being infectious agents smaller than bacteria and not visible by light microscopy. Epidemiologic and human transmission studies in the 1940s and 1950s led to the clear recognition that 'infectious hepatitis' (hepatitis A) and 'serum hepatitis' (hepatitis B and probably C and D) were caused by distinct viral agents. Hepatitis B virus (HBV) was discovered as a physical entity only in the 1960s.

Hepatitis B surface antigen (HBsAg) was discovered serendipitously in human serum in the mid-1960s and initially was named Australia antigen because it was first found in serum of an Australian aborigine. It was later associated with acute serum hepatitis and eventually identified as a viral antigen (specifically the envelope antigen of hepatitis B virus). The most common form of this antigen in the blood was found to be small lipid-containing envelope particles (i.e. incomplete virus particles without viral nucleocapsids). This led to rapid advances in understanding the epidemiology of the virus, the course of infection and recognition of associated diseases, and the physical nature of the virus. Early during this period, serum HBsAg was found in populations all over the world, and HBV infection was recognized as commonly becoming chronic and as being associated with chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC).

The complete virus or 'Dane particle' was found to be a 42 mm diameter spherical particle with a lipid-containing envelope with HBsAg on its surface and surrounding a 20 nm core particle or nucleocapsid containing a small circular partly single-stranded viral DNA and a DNA polymerase activity that repaired the single-stranded region of the virion DNA. Within a decade, new viruses which were very similar to HBV were discovered in several species and it was clear that these viruses represented a previously unrecognized virus family.

Taxonomy and Classification

HBV and related viruses make up the virus family designated *Hepadnaviridae* for hepatotropic DNA viruses. This virus family includes hepatitis B virus of man, woodchuck hepatitis virus (WHV) of *Marmata monax*, ground squirrel hepatitis virus (GSHV) of *Spermophilus beecheyi*, duck hepatitis B virus (DHBV) found in several varieties of domestic ducks, and similar viruses in tree squirrels, Richardson ground squirrels, snow leopards and German herons. Less well documented findings in other rodents, marsupials and cats suggest that other hepadnaviruses may have been detected. There may be many undiscovered members of this virus family since naturally occurring infections can be relatively silent for many years.

Hepadnaviruses share many epidemiologic and biological features. All hepadnaviruses exhibit moderately narrow host ranges, strong relative tropism for hepatocytes, *in vivo* production by infected hepatocytes of large amounts of noninfectious viral envelope particles (as well as infectious virus) that can be detected in high concentration (up to 500 $\mu g\ ml^{-1}$) in the blood, and the common occurrence of persistent infections with viral forms in the liver

and blood continuously for years and often for the lifetime of the host. Infections with hepadnaviruses may be associated with acute and chronic hepatitis, immune complex (viral surface antigen-antibody)-mediated disease and HCC.

Hepadnaviruses share unique features of virion size and ultrastructure with an envelope surrounding an electron dense spherical nucleocapsid or core; characteristic polypeptide and antigenic composition; common virion DNA size and structure (3200 bp circular DNA being among the smallest of all known animal viruses) and genetic organization; the presence of DNA polymerase activity in the virion; and an unusual mechanism of viral DNA replication which includes reverse transcription of a viral RNA that is greater than the genome length. These biological and molecular features define this virus family.

Mammalian viruses have four recognized genes and avian viruses only three (the mammalian virus X gene is not present). Viral core and envelope polypeptide sizes are quite similar among mammalian hepadnaviruses and different in DHBV. Different mammalian hepadnaviruses contain cross-reacting antigens which do not cross-react with the respective antigens of avian hepadnaviruses. These and other differences suggest that mammalian and avian hepadnavirus should be considered to represent different genera within the family *Hepadnaviridae*.

Geographic and Seasonal Distribution

Studies of geographic distribution have revealed HBV in all regions of the world, but unevenly distributed. The highest prevalences of serum HBsAg are in eastern Asia and sub-Saharan Africa (for example approximately 10% in China compared with 0.1% in the United States). There are no known seasonal preferences for primary HBV infections. WHV has been found in wild woodchuck populations over a broad region of the eastern United States. GSHV has been found only in wild Beechey ground squirrels in a small area near Palo Alto, California, but not in other areas of California or elsewhere. DHBV has been found in domestic ducks of several varieties and in many eastern, mid-western and western areas of the United States as well as in domestic ducks in parts of China. There is no documented seasonal preference for primary infections. However, wild rodents such as woodchucks and ground squirrels breed and deliver offspring during a narrow time period in the spring in North America and thus perinatal transmission of hepadnaviruses would be expected to occur only during that time period.

Host Range and Virus Propagation

Hepadnaviruses have narrow host ranges. HBV infect humans, chimpanzees, some other great apes but not monkeys or lower species. WHV can infect woodchucks but not ground squirrels, rodents or other species tested. GSHV infects both Beechey ground squirrels and woodchucks but not other rodents or species tested. DHBV infects several varieties of domestic ducks and geese but not chickens, other birds or mammals. Only DHBV readily infects cells in culture (only primary duck hepatocyte cultures). Mammalian hepadnaviruses have not been shown readily or reproducibly to infect any cell type in culture.

Genetics

Genetic studies have been limited by the failure of mammalian hepadnaviruses to propagate in cell culture.

Evolution

Nucleotide sequence homology and genome organization show that mammalian hepadnaviruses are more closely related to each other than to avian hepadnaviruses.

Hepadnaviridae appear to be phylogenetically related to members of two other virus families: cauliflower mosaic virus and *Retroviridae* and related transposable elements. Similarity in gene number, function and order; shared genome nucleotide sequence homology in genomic regions of similar function; and utilization by all of a reverse transcriptase step in genome replication, indicate these virus families are related. Reverse transcription is an unusual mechanism of genome replication for DNA viruses (known at this time only for hepadnaviruses and cauliflower mosaic virus). Undoubtedly it is a mechanism used by these viruses because their evolution from a common ancestor is shared with retroviruses, and differs from the evolutionary pathway of other DNA viruses. Notable differences in the viruses of these families include: (1) the form of the genome packaged in virions is different (linear RNA in the case of retroviruses and nicked or gapped double-stranded circular DNA in hepadnaviruses and cauliflower mosaic virus); (2) retroviruses package two copies of the viral genome in each virion resulting in high rates of homologous recombination and hepadnaviruses one genome copy; and (3) details of the replication mechanisms are different.

Another important difference is in the mechanism of viral integration and the requirement for DNA genome integration in cellular DNA for virus replica-

tion. The orderly integration of retroviral DNA 'provirus' is mediated by a virus-encoded integrase with preservation of the genome sequence and including a terminally repeated sequence (the long terminal repeat or LTR) containing *cis*-acting transcriptional and other regulatory elements. Expression of viral genes and synthesis of new RNA genomes from the integrated provirus is an integral feature of retrovirus replication occurring in all infected cells. This feature is not shared by hepadnaviruses whose replication appears to involve only episomal (unintegrated) viral nucleic acid forms. Hepadnaviral integration appears to be a less well controlled event by a mechanism such as illegitimate recombination without involvement of viral integrase and without preservation of the genome sequence, making it impossible for the viral integrant to function as a template for virus replication.

Serologic Relationships and Variability

There are three major antigens of hepadnaviruses and these are: (1) the surface or envelope antigen (e.g. HBsAg in the case of HBV) contained in the small (s), medium (preS2+S) and large (preS1+preS2+S) surface antigen polypeptides of the viral envelope and this antigen is found in blood exclusively on the surface of small spherical and long filamentous envelope particles and on enveloped virions; (2) the core or nucleocapsid antigen (e.g. HBcAg) contained in the structural polypeptide of assembled core particles; and (3) the e antigen (e.g. HBeAg) contained in a truncated, soluble form of the core gene-encoded polypeptide. The small (s) HBsAg polypeptide contains a constant or group-specific epitope *a* and at least two varying or subtype-specific epitopes, each one always found in one of two distinct antigenic specificities designated *d* or *y* and *w* or *r*. Thus HBsAg subtypes adw, adr, ayw and ayr (less common) have been found. Other minor subtype antigenic differences have also been described. Antigenic subtypes have been used as markers in epidemiologic studies. Different HBsAg subtypes have unique world-wide geographic distributions indicating that each arose in a different geographic region, and the current distribution must reflect that region of origin and the migrations of human populations since that time. No such antigenic variation is known for HBcAg or HBeAg. Recently, S gene and C gene mutants have been found which abolish or alter antigenicity and may be associated with altered virulence.

The respective c and e antigens of mammalian hepadnaviruses cross-react (s more weakly than c) but no cross-reactions between mammalian and avian hepadnavirus antigens have been detected. S antigen subtypes have not been reported for animal hepadnaviruses.

Epidemiology

Most primary infections in highly endemic populations occur at very early ages (between 10 and 50% of HBV infections that become persistent in different high-prevalence geographic regions are acquired perinatally from infected mothers), are silent or subclinical, and many become persistent. In Western countries most primary HBV infections are in adults (acquired by sexual contacts or percutaneous exposure). They are more often associated with clinically apparent acute hepatitis B and only 5–10% become persistent. The prevalence of HBsAg carriers is greater among males than among females in most populations. There are estimated to be more than 200 million HBsAg carriers in the world; these are the only reservoir of HBV for new human infections. The associated chronic liver disease and HCC are among the most important human health problems in high-prevalence regions. On a world-wide basis, HBV is probably the most common single cause of chronic liver disease and HCC in man. The incubation period of hepatitis B (time from infection to onset of hepatitis) is typically 6–24 weeks and accounts for an early name for this disease being 'long incubation hepatitis'.

Transmission and Tissue Tropism

As with other viruses that persist in the blood of infected individuals, HBV is transmitted by heterosexual and homosexual contacts; percutaneously (for example by skin puncture with contaminated shared needles for illicit drug use) and by inoculation with contaminated blood and blood products; and perinatally from infected mothers to their newborn infants. Very few infants of HBV-infected mothers are infected *in utero*. Different HBV carriers may have greatly different degrees of contagiousness for contacts. This is due to different levels of virus replication in the liver and quantity of infectious virus in the blood and body fluids of different carriers. Serum HBeAg and levels of viral DNA-containing virions in the blood are reliable markers indicating the presence of infectious virus. Some HBsAg carriers with no detectable serum HBeAg or viral DNA and reactive for anti-HBe may have no infectious virus in the blood and do not transmit infection.

Hepadnaviruses are hepatotropic (i.e. infect primarily liver cells) although less frequent and less permissive infection sometimes occurs in bone marrow, circulating leukocytes, B cells, T cells and

pancreas. Virtually all active infections are accompanied by high concentrations of HBsAg and often infectious virus in the blood.

Pathogenicity

Hepadnaviruses infect hepatocytes and the most common disease manifestations are in the liver. Acute infection may result in little disease or in clinically apparent acute hepatitis B of varying severity from mild to fulminant hepatitis with extensive liver necrosis. Persistent infection can be associated with very mild changes in liver, chronic persistent hepatitis, or chronic active hepatitis with chronic liver cell necrosis, an inflammatory response, lymphocytic infiltration and liver regeneration. Chronic hepatitis B can progress to macronodular cirrhosis characterized by liver cell necrosis, fibrosis and regenerative nodules.

The mechanism of liver injury in acute and chronic hepatitis B is not completely defined but a cellular immune mechanism is considered to be likely. Some evidence indicates that cytotoxic T cells directed at the viral core antigen (HBcAg) displayed on the surface of hepatocytes can lead to hepatocyte killing. Other studies have suggested that HBcAg expression in infected cells may have a direct cytopathic effect. Another nonimmune mechanism involves accumulation of viral envelope polypeptides in liver cells and resulting liver cell necrosis, a mechanism demonstrated in transgenic mice. Hepatocytes that accumulate HBsAg *in vivo* during natural HBV infection can be recognized by light microscopy, have been termed 'ground glass cells' and are characteristic of HBV infection.

Hepatocellular carcinoma (HCC) may develop after many years of HBV infection and most HBV-infected patients that develop HCC have cirrhosis. A carcinogenic mechanism for HCC has not been established, although genetic changes that could contribute to carcinogenesis have been found in HCC. In WHV-infected woodchucks more than 50% of HCC contain activated c-*myc* or N-*myc* genes related to viral promoter/enhancer insertion near the respective cellular gene. In GSHV-infected ground squirrels, the c-*myc* gene is amplified and overexpressed in more than 50% of HCC but a role for the virus in causing this change has not been shown. Neither of these changes has been found in HCC of HBV-infected humans. Mutations in the p53 gene, however, have been found in more than 50% of human HCC but no role for HBV in causing these mutations has been shown.

It is possible that in man HBV is not carcinogenic by a direct viral mechanism like those shown for numerous other tumor viruses in animals. Instead, HBV may play a role by causing chronic liver cell necrosis with associated host responses of inflammation and liver regeneration that continues for many years. This pathologic process, particularly when it leads to cirrhosis, may be carcinogenic without involving a direct oncogenic action of the virus (e.g. no viral oncogene, insertional mutagenesis, or *cis*- or *trans*-activation of oncogenic cellular genes has been consistently found). Chronic liver injury caused by numerous other agents, such as hepatitis C virus, alcohol and iron overload, can result in a similar chronic pathologic process leading to cirrhosis and ultimately HCC.

The final pathogenetic mechanism known for HBV is in initiating immune complex disease apparently related to HBsAg–anti-HBs complexes. A serum sickness-like illness with fever, rash and arthritis precedes the onset of acute hepatitis B in 10–15% of cases, and syndromes of glomerulonephritis and necrotizing vasculitis (polyarteritis) with respectively glomerular membrane and vascular endothelial deposits of HBsAg-anti-HBs and complement are unusual but not rare.

Clinical Features of Infection

Most HBV infections are asymptomatic with mild serum alanine aminotransferase (ALT) changes that resolve, although clinically apparent acute hepatitis and occasionally fulminant hepatitis with hepatic failure and death can occur. The severity of acute viral hepatitis B appears to be related to age of infection (young children generally have milder disease than adults), the infecting virus dose (higher doses appear to result in shorter incubation period and generally more severe hepatitis), and immunosuppression which may be associated with mild disease and frequent persistent infection.

Chronic HBV infection is most often asymptomatic ('healthy carriers') and symptomatic chronic active hepatitis with periods of fever, malaise and hepatic pain is much less common. In highly endemic regions of the world such as eastern Asia, most HBV infections occur in newborns or young children and are relatively asymptomatic; many of these infections become persistent and remain silent for many years until cirrhosis or HCC develop in middle or old age and become symptomatic with pain, ascites, abdominal mass, etc. In the United States, more primary HBV infections occur in adults and more often result in symptomatic acute hepatitis B, and fewer become persistent.

Pathology and Histology

Acute hepatitis B is not clearly distinguishable from hepatitis A or hepatitis from many other causes in most individual cases. It is characterized by liver cell necrosis, an inflammatory response, lymphocytic infiltration and liver cell regeneration.

Immune Response

Primary infection leads to an antibody (IgM and IgG) response to HBcAg within a few days of the appearance of HBsAg in serum and near the time of onset of hepatitis. Antibody to HBsAg can usually be detected in serum only several weeks later when serum HBsAg can no longer be detected. Similarly serum anti-HBe can usually be detected when HBeAg is no longer detected in serum.

During chronic HBV infection almost all patients have HBsAg in the blood and some have HBeAg. All have a high titer of anti-HBc. A high titer of anti-HBc suggests ongoing or recent HBV infection. Anti-HBs is usually not detected in serum of HBsAg-positive patients, probably because of excess HBsAg. In many HBsAg-positive patients, HBsAg–anti-HBs complexes can be found in serum, indicating an anti-HBs responsive in many HBsAg carriers. Anti-HBe usually appears when HBeAg can no longer be detected in serum of HBsAg carriers.

T cells which proliferate in response to HBcAg can be detected in the blood of many patients with hepatitis B. T cell proliferative responses to HBsAg appear to be less common.

The presence of anti-HBs and/or anti-HBC from previous HBV infection indicates immunity to HBV and a high level of resistance to re-infection.

Prevention and Control

HBV infections are prevented by measures that interrupt routes of transmission. Recognizing and identifying HBV-infected individuals permits avoidance of contacts which may lead to transmission. Changes in behavior with respect to sexual contacts and illicit intravenous drug use can reduce spread of HBV. Elimination of infected blood and plasma donors by HBsAg screening has largely eliminated HBV infections by blood transfusion and by other blood products. Screening all pregnant women for HBsAg permits protection of newborns of infected mothers by use of hepatitis B immune globulin and HBV vaccination (such passive-active immunization protects up to 95% of newborns of HBV-infected mothers).

Clearly the most important advance in controlling HBV in populations is vaccination. Studies with synthetic peptides indicate the group-specific *a* epitope of the small HBsAg polypeptide can elicit protective immunity and there also appear to be protective epitopes in the preS1 and preS2 sequences. However, HBV vaccines containing only the small HBsAg polypeptide in a particulate form are highly immunogenic and protective without preS1 or preS2 sequences. HBsAg particles containing the small HBsAg polypeptide produced in yeast by recombinant DNA methods is currently used in the United States and provides a highly effective vaccine. HBV vaccine use in high-risk populations in the United States over the past decade (e.g. medical and dental personnel, newborns of HBV-infected mothers, homosexual men, etc.) has protected many individuals but has had little impact on overall numbers of HBV infections. This has led to a recent change in the recommendation for HBV vaccine use in the United States to make it a universal childhood vaccine to be given with DPT (diphtheria, pertussis, tetanus). In addition, vaccination of newborns of HBV-infected mothers and nonimmune uninfected adults in high-risk groups is still indicated. Vaccination of all newborn babies is a goal in almost all countries of the world with high HBsAg carrier rates.

Future Perspectives

Among the important and interesting unanswered questions about hepadnavirus are their mechanistic role in HCC and the immunologic and other factors that lead to persistent infection. At the clinical level, achieving full immunization in countries with high HBsAg carrier rates is a goal unmet due to high cost of the vaccine and logistical problems in delivery in some populations. A big challenge is to develop effective strategies for antiviral therapy of chronic HBV infections. Investigations of therapeutic agents directed at blocking virus replication as well as strategies that may act on host antiviral mechanisms such as the immune response are important to pursue.

See also: **Hepatitis C virus (*Flaviviridae*); Hepatitis Delta virus; Hepatitis E virus; Retroviruses – type D (*Retroviridae*).**

Further Reading

Colacino JM, Staschke KA (1998) The identification and development of antiviral agents for the treatment of chronic hepatitis B virus infection. *Progress in Dry Research* 50: 259–322.

Lee WN (1997) Hepatitis B virus infection. *New England Journal of Medicine* 337: 1733–1745.

Marion PL, Oshiro L, Regnery DC, Scullard GH and

whitefly transmissibility. The formation of bipartite begamoviruses in which the DNA B component is not essential for systemic infection, e.g. TYLCV-Th, could represent the first stage in this evolutionary pathway. The DNA B component probably evolved from the DNA A component, as suggested by their almost identical common regions, the low, but significant sequence similarities between the AV1 (CP) and BV1 (NSP) proteins, the fact that both these proteins bind strongly to ssDNA, and the transactivation of transcription of both genes by TrAP. Acquisition of cell-to-cell movement genes on DNA B could have led to the loss of cell-to-cell movement function of the AV2 protein in some Old World bipartite begamoviruses, e.g. ACMV, and loss of this gene completely in the New World bipartite begamoviruses. The greater sequence diversity within the *Mastrevirus* genus than within the *Begamovirus* genus is consistent with the evolution of begamoviruses from ancestral mastreviruses. Sequence comparisons suggest that curtoviruses, such as BCTV and HrCTV, may have arisen by recombination acquiring the CP gene, and hence leafhopper transmissibility, from a mastrevirus and the Rep gene from a begamovirus.

Geminiviruses have some similarities to another group of multipartite ssDNA viruses (proposed *Nanovirus* genus), comprising banana bunchy top virus, subterranean clover stunt virus, faba bean necrotic yellows virus and milk vetch virus, which all have Rep proteins with the Rb-binding LXCXE motif, have intergenic regions containing a putative stem–loop structure with a conserved consensus TAG/TTATTAC (similar to the geminivirus TAATATTAC sequence) sequence and which probably replicate by a rolling circle mechanism. Geminiviruses and nanoviruses also have some affinities to viruses in the *Circoviridae* family of animal ssDNA viruses. Recently it has been found that ToLCV-Aus DNA can replicate in *Agrobacterium tumefaciens* suggesting that plant ssDNA viruses may have arisen from ssDNA bacteriophages or plasmids which employ rolling circle replication. *Agrobacterium* species are widely distributed plant pathogens known to be able, via their Ti plasmids, to insert DNA into plant genomes. Consistent with this theory is the finding that remnants of geminivirus DNA have been found in the genome of some (uninfected) plants which are hosts for geminiviruses.

See also: **Pathogenesis: Plant viruses.**

Further Reading

Hanley-Bowdoin L, Settlage SB, Orozco BM, Nagar S and Robertson D (1999) Geminiviruses – models for plant DNA replication, transcription and cell cycle regulation. *Crit. Rev. Plant Sci.*, (in press).

Padidam M, Beachy RN and Fauquet CM (1995) Classification and identification of geminiviruses using sequence comparisons. *J. Gen. Virol.* 76: 249.

Palmer KE and Rybicki EP (1997) The use of geminiviruses in biotechnology and plant molecular biology, with particular focus on mastreviruses. *Plant Sci.* 129: 115.

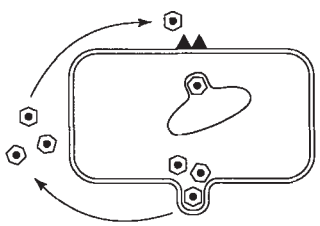
Palmer KE and Rybicki EP (1998) The molecular biology of mastreviruses. *Adv. Virus Res.* 50: 183.

Rybicki EP, Briddon RW, Fauquet CM *et al* (1999) Family *Geminiviridae*. In: Van Regenmortel MHV, Fauquet C, Bishop D *et al* (eds) *Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses*. New York: Academic Press. (in press)

Sanz-Burgos AP and Gutiérrez C (1998) Organization of the *cis*-acting element required for wheat dwarf geminivirus DNA replication and visualization of a rep-protein–DNA complex. *Virology* 243: 119.

GENETICS OF ANIMAL VIRUSES

Frank Fenner, John Curtin School of Medical Research, Canberra, Australia



History

Although genetic variation was the basis of some of the earliest work with animal viruses, such as Pasteur's attenuation of rabies virus, the first formal demonstration of the occurrence of mutation in an animal virus did not occur until over 60 years later, when Burnet used limiting dilution methods to clone influenza virus. A few years later, in the early 1950s, Burnet demonstrated 'high frequency recombination' (later called genetic reassortment) between different strains of influenza A virus. Intramolecular recombination was demonstrated with herpes simplex virus in 1955 and with vaccinia virus in 1958 and the first

crude maps (of vaccinia virus) were described soon after. Hirst demonstrated that intramolecular recombination could occur among RNA viruses in experiments with poliovirus in 1962 and Cooper published a genetic map of poliovirus in 1968.

Apart from the demonstration of the remarkable variety to be found in the genomes of animal viruses of different groups, animal virus genetics advanced slowly until the discovery of recombinant DNA by Berg, Boyer and Cohen in 1972. This led to the explosive development of molecular genetics. Since animal viruses multiply only in eucaryotic animal cells, viruses were soon found to be a powerful way of investigating the molecular biology of animal cells, especially after the discovery that the DNA of tumor viruses was integrated into the genome of the host cell.

The Genomes of Animal Viruses

Historically, animal viruses were first classified on the basis of the morphology of the virion, but this criterion failed to distinguish between many small isometric viruses or between several different families of enveloped viruses. Morphology was powerfully supplemented as the basis for classification as the genomes of viruses were analyzed and their extraordinary variety demonstrated. Finally, as work proceeded on the replication mechanisms, a third criterion for classification was developed, the strategy of viral replication. Currently eight families of DNA and 16 families of RNA animal viruses are recognized. The genomes of most DNA viruses are double stranded (ds) and range in size from 3.2 to 280 kbp; others have genomes of ssDNA ranging from 1.8 to 5 kb. Some genomes are linear, others circular; among viruses with ssDNA some are of positive sense, some of negative sense.

Viruses are the only living things that use RNA as the repository of their genetic information. The genomes of the RNA viruses are much smaller than those of most DNA viruses, and different families of RNA viruses show a remarkable diversity in genome structure. All except one family of viruses have haploid genomes; the retroviruses, however, have diploid RNA genomes and they also have an obligatory DNA stage in their replication cycle. Among other families the RNA genome may be a single linear strand varying in size from 3.5 to 24 kb, a circular molecule of 1.7 kb, or it may consist, in different genera, of 2, 3, 7 or 8 pieces of ssRNA, or it may consist of segmented dsRNA in 2, 10, 11 or 12 pieces.

Mutations

The most common and important changes in the nucleic acid sequences of viral genomes are due to mutations. In every viral infection of an animal or a cell culture, one or a small number of virus particles replicate to produce millions of progeny. In such large populations, errors in copying the nucleic acid, i.e. mutations, inevitably occur. Many such mutations are lethal. Whether a particular nonlethal mutation survives in the genotype depends upon whether the resultant change in the gene product affords the mutant virus some selective advantage, or whether it is neutral or disadvantageous.

In the laboratory, reasonable genetic constancy of viral stocks (e.g. those used for making viral vaccines or retained as reference strains) is achieved by: (1) isolating a clone, i.e. a population of viral particles originating from a single particle, usually by growth from a single plaque in cell culture, followed by replaquing; then, (2) growing 'seed' stock from this clone; and (3) as far as practicable avoiding or strictly limiting further passage of the virus.

Mutation Rates

Rates of mutation involving single base substitutions (point mutations) are probably the same in DNA viruses as they are in the DNAs of procaryotic and eucaryotic, since viral replication is subject to the same 'proof-reading' exonuclease error correction as operates in cells. Such errors are estimated to occur at a rate of 10^{-8} to 10^{-11} per incorporated nucleotide (i.e. per base pair per replication). Point mutations in the third nucleotide of a triplet often do not result in an altered amino acid, because of coding redundancy, and some point mutations are lethal, because they produce a stop codon or other aberrant regulatory sequences. Viable mutations that are neutral or deleterious in one host may provide a positive selective advantage in a different host.

The error rate in the replication of viral RNA is much higher than that of viral or cellular DNA, because there is no cellular 'proof-reading' mechanism for RNA. For example, the base substitution rate per incorporated nucleotide in the 11 kb genome of vesicular stomatitis virus is $10^{-3} - 10^{-4}$, which is about a million times higher than the average rate in eucaryotic DNA. Of course, most of these base substitutions would be deleterious and the genomes containing them would be lost. Even so, there is growing evidence that nonlethal mutational changes in the genome of RNA viruses occur very rapidly.

Types of Mutations

Mutations can be classified according to the kind of change in the nucleic acid. The most common are nucleotide substitutions (point mutations), deletions

and insertions. Each point mutation has a characteristic frequency of reversion which can be accurately measured. The physiological effects of mutations depend not only on the kind and location of the mutation but also on the activity of other genes. The phenotypic expression of a mutation in one gene may be reversed not only by a back mutation in the substituted nucleotide but, alternatively, by a suppressor mutation occurring elsewhere in the same, or even in a different gene.

Mutations are usually classified by their phenotype – hence temperature-sensitive, cold adapted, host range, plaque size, etc. mutants have been described. Each of these kinds of mutant has been used for the analysis of viral functions, temperature-sensitive mutants being particularly useful (see later). Cold-adapted and temperature-sensitive mutants have been used extensively in attempts to produce attenuated live virus vaccines. Mutations affecting antigenic determinants of virion surface proteins may be strongly favored when viruses replicate in the presence of antibody, and are of importance both in persistently infected animals (e.g. in equine infectious anemia virus) and epidemiologically, as with influenza virus.

Conditional lethal mutants

These are produced by mutations that so affect a virus that it cannot grow under certain conditions, determined by the experimenter, but can replicate under 'normal' or permissive conditions. Their importance is that a single selective test can be used to obtain and analyze mutants in which mutations may be present in any one of several different genes. The conditional lethal mutants most commonly studied are those whose replication is blocked in certain host cells, or at certain defined temperatures. With temperature-sensitive mutants; the selective condition used is a high temperature of incubation of infected cells. A point mutation in the genome, leading to an amino acid substitution in the translated polypeptide product, results in a structurally abnormal protein which, although functional at the permissive temperature, cannot maintain its structural integrity and functional conformation when the temperature is raised by a few degrees.

Defective-interfering (DI) mutants

Mutants of this class have been demonstrated in all families of RNA viruses and in some DNA viruses. They occur when viruses are passed at high multiplicity of infection, because more cells then receive helper virus to support their replication, and the ratio of defective-interfering to infectious particles increases dramatically on serial passage at high multiplicity. The properties that all DI virus particles have in common are: (1) they are defective, i.e. they cannot replicate alone, but can in the presence of a parental wild-type virus; and (2) they decrease the yield of wild-type virus (interference).

All RNA DI particles studied are deletion mutants. In the case of influenza viruses and reoviruses, which have segmented genomes, the defective virions lack one or more of the larger segments and contain instead smaller segments consisting of an incomplete portion of the encoded gene(s). In the case of viruses with a nonsegmented genome, DI particles contain RNA which is shortened – as little as one-third of the original genome may remain in the DI particles of vesicular stomatitis virus. Morphologically, DI particles resemble the parental virions, having a comparable envelope or capsid, but they are sometimes smaller. Sequencing studies of the RNA of DI particles reveal simple deletions and a great diversity of structural rearrangements.

Mutagenesis

Spontaneous mutations arise because of chance errors during replication, the occurrence of which is probably influenced by natural background ionizing radiation. Mutation frequency can be enhanced by treatment of virions or isolated viral nucleic acid with physical agents such as UV or X irradiation or with chemicals such as nitrous acid, hydroxylamine, ethylmethyl sulfonate or nitrosoguanidine. Base analogues, such as 5-fluorouracil or 5-bromodeoxyuridine, are mutagens only when virus is grown in their presence because they are incorporated into the viral nucleic acid.

Site-directed mutagenesis

This process enables the experimenter to introduce mutations at a selected site in a DNA molecule (a DNA genome or cDNA transcribed from an RNA genome). This technique has opened up new research areas; for example: (1) the function of individual genes and the proteins for which they code, or of particular regions of these genes and proteins, can be dissected; (2) mutations can be introduced into particular genes, e.g. those concerned with viral virulence, to produce mutants suitable for use as attenuated live virus vaccines.

Recombination

When two different viruses simultaneously infect the same cell, several kinds of genetic recombination may occur between the newly synthesized nucleic acid

molecules: intramolecular recombination, reassortment, reactivation (when one of the virions has been inactivated) and marker rescue.

Intramolecular recombination

Intramolecular recombination (**Fig. 1A**) involves the exchange of nucleic acid sequences between different but usually closely related viruses. It occurs with all dsDNA viruses, presumably because of strand switching by the viral DNA polymerase. Intramolecular recombination has been demonstrated among RNA viruses only with foot-and-mouth disease virus, poliovirus and coronavirus.

In rare cases, intramolecular recombination occurs between unrelated viruses; the best example is between SV40 (a papovavirus) and adenoviruses. Both SV40 and adenovirus DNAs become integrated into cellular DNA, so that it is perhaps not surprising to find that when rhesus monkey cells which harbor a persistent SV40 infection are super-infected with an adenovirus, not only does complementation occur (see later), the SV40 acting as a helper in an otherwise abortive adenovirus infection, but recombination occurs between SV40 and adenovirus DNAs to yield hybrid (recombinant) DNA which is packaged into adenovirus capsids.

In addition to recombining with each other, or sometimes, as with SV40, with an unrelated virus (adenovirus), many DNA tumor viruses recombine with the genome of cells and in the process the viral oncogenes that they carry may transform the infected cell to a neoplastic state. Integration of proviral DNA into the cellular DNA by a process of intramolecular recombination is an essential part of the replication cycle of retroviruses. Although the genome of these viruses is positive-sense ssRNA, replication does not occur until this is transcribed into DNA by the virion-associated reverse transcriptase and the resultant copy DNA is integrated into the cell's DNA.

Reassortment

A type of recombination called reassortment (**Fig. 1B**) occurs with viruses that have segmented genomes, whether these are ssRNA or dsRNA and consist of two (*Arenaviridae, Birnaviridae*), three (*Bunyaviridae*), seven or eight (*Orthomyxoviridae*), or 10, 11 or 12 segments (*Reoviridae*). In cells infected with two related viruses, there is an exchange of segments with the production of various stable reassortants. Reassortment occurs in nature, and is epidemiologically important in generating new subtypes of influenza virus for example.

Reactivation

The term multiplicity reactivation is applied to the production of infectious virus by a cell infected with two or more viruses of the same strain, each of which has suffered a lethal mutation in a different gene, e.g. after exposure to UV irradiation. Multiplicity reactivation could theoretically lead to the production of infectious virus if animals were to be inoculated with UV irradiated vaccines; accordingly this method of inactivation is not used for vaccine production. Cross-reactivation or marker rescue are terms used to describe genetic recombination between an infectious virus and an inactivated virus of a related but distinguishable genotype or a fragment of DNA from such a virus.

Interactions between Viral Gene Products

As well as interactions between viral genomes, interactions between viral gene products may occur in mixedly infected cells.

Complementation

The term complementation is used to describe situations where in mixedly infected cells one virus provides a gene product that the other cannot make, thus enabling the latter to replicate. Complementation can occur between different strains of the same species of virus and between unrelated viruses; indeed adeno-associated viruses can replicate only if complemented by genes of an adenovirus.

Phenotypic mixing

This term refers to a situation where, after mixed infections, the envelope or capsid of some of the progeny contains gene products of both parents, such as the spikes of enveloped viruses (**Fig. 1E**), the genomes being those of one or other parent, so that on passage the phenotypically mixed particles produce only virions resembling that parent. Phenotypic mixing is an essential part of the life cycle of defective retroviruses, progeny virions being called pseudotypes, with the genome of the defective parental virus but the envelope glycoproteins of the helper retrovirus.

With nonenveloped viruses, phenotypic mixing can take the form of transcapsidation, in which there is partial or usually complete exchange of capsids (**Fig. 1F, G**). For example, poliovirus nucleic acid may be enclosed within a coxsackievirus capsid, or adenovirus 7 genome may be enclosed within an adenovirus 2 capsid.

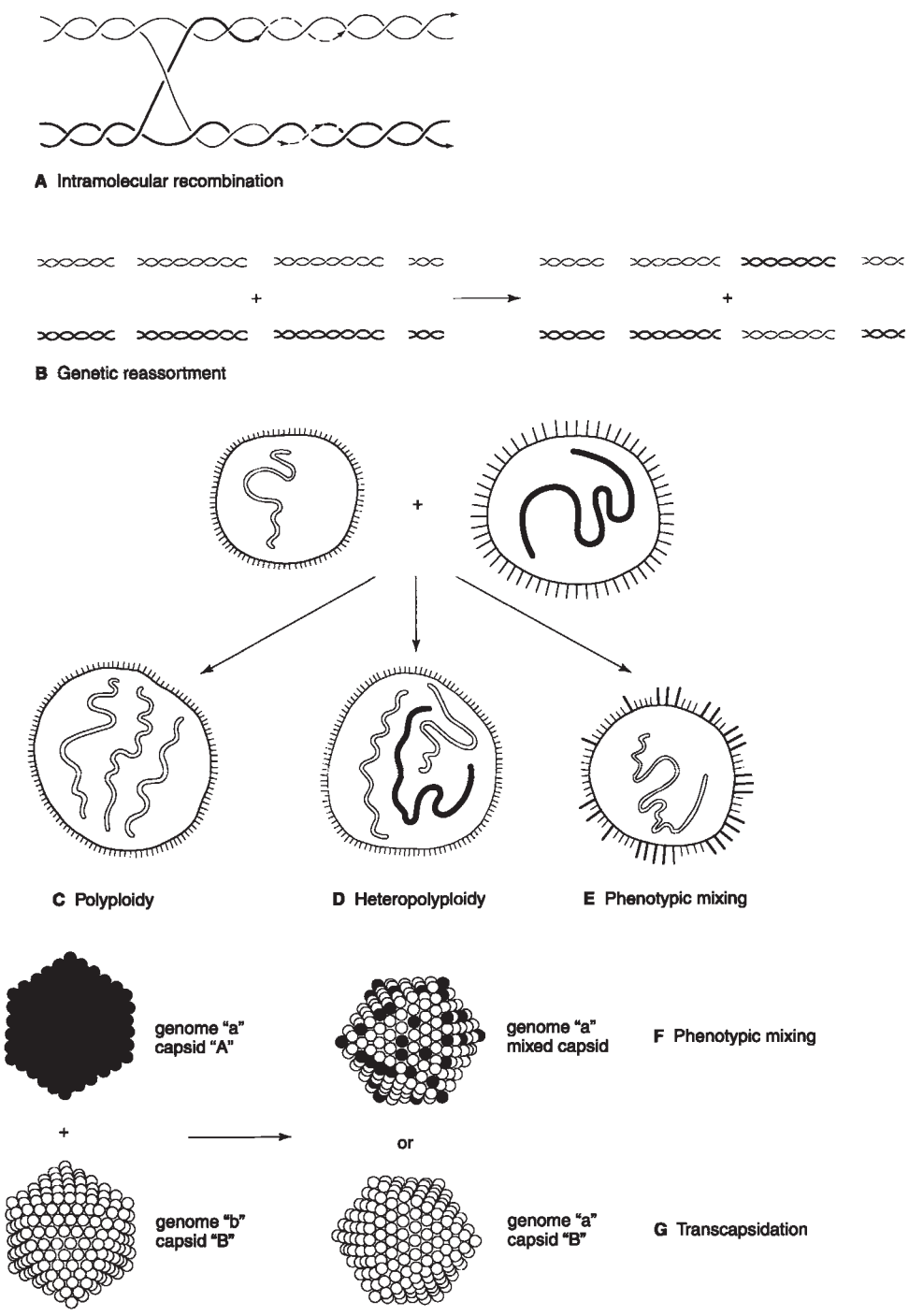


Figure 1 Genetic recombination, polyploidy, phenotypic mixing and transcapsidation. (**A**) Intramolecular recombination. (**B**) Reassortment of genome fragments, as in reoviruses and orthomyxoviruses. (**C**) Polyploidy, as seen in unmixed infections with paramyxoviruses. (**D**) Heteropolyploidy, as may occur in mixed infections with paramyxo-viruses and other enveloped RNA viruses. (**E–G**) Phenotypic mixing: (**E**) with enveloped viruses; (**F**) viruses with icosahedral capsids; (**G**) extreme case of transcapsidation or genomic masking.

Polyploidy

With the exception of the retroviruses, which are diploid, all viruses of vertebrates are haploid, i.e. they contain only a single copy of each gene. However, among viruses that mature by budding from the plasma membrane, e.g. paramyxoviruses, it is commonly found that several nucleocapsids (and thus genomes) are enclosed within a single envelope, i.e.

the virion is polyploid (**Fig. 1C**). If cells are doubly infected with recognizably different strains of such viruses, many of the multiple genome progeny particles are heteropolyploid (**Fig. 1D**) and they may also have phenotypically mixed envelope antigens.

Mapping Viral Genomes

Viral genomes can be mapped several ways. Genetic maps can be constructed on the basis of recombination or complementation tests, and physical maps may show the cut sites of various restriction enzymes or may be obtained by determining the sequence of nucleotides that constitute the viral genome.

Recombination maps

Among viruses that undergo intramolecular recombination, the probability of recombination occurring between two markers reflects the distance between them and recombination frequencies in adjacent intervals are approximately additive. Two-factor crosses are used to determine recombination frequencies between pairs of mutants; for very close or distant markers three-factor crosses are used to resolve ambiguities. Recombination maps have been made for several large DNA viruses, notably herpes simplex virus, and for poliovirus. With the determination of nucleotide sequences the genetic markers of a number of viruses have been located on the relevant physical maps.

For viruses that have segmented genomes, reassortant maps can be constructed by crossing mutants of different serotypes that have electrophoretic polymorphisms for each of the genome segments. They have confirmed that mutations able to recombine reside on different genome segments.

Restriction maps

During the 1960s it was shown that a phenomenon that had been called 'restriction' in bacteriophages, whereby certain bacteriophages failed to replicate in particular species of bacteria, was due to the rapid degradation of the bacteriophage DNA by specific bacterial endonucleases, which were therefore called restriction endonucleases. Subsequently several hundred of these enzymes have been identified and purified from various bacteria. Each recognizes a unique short, palindromic sequence of nucleotides (a sequence that reads the same backwards as forwards), generally four to eight nucleotide pairs long. Depending upon the location and frequency of the specific unique sequence in a particular DNA molecule, a particular restriction endonuclease cleaves the DNA into a precise number of fragments of precise sizes. Other endonucleases, recognizing different sequences, cleave the same DNA into different numbers and sizes of fragments. These DNA fragments, produced by a panel of endonucleases, may be separated by gel electrophoresis. Different viruses, often even very closely related strains of the same virus, yield characteristically different restriction endonuclease fragment patterns, sometimes called fingerprints or restriction fragment length polymorphisms (RFPL). These have been invaluable for distinguishing between different species of viruses with large genomes, such as the various poxviruses. Restriction enzymes can also be used to analyze the molecularly cloned cDNA copies of genes or genomes from RNA viruses. Once the restriction maps of a virus have been determined, the location of many genetic markers, such as temperature-sensitive mutations, at specific locations on the viral genome can be determined by rescue of the deleterious mutation by the corresponding wildtype restriction fragment.

Complementation groups

Complementation tests are used to divide collections of mutants into functional groups, and are particularly useful with virus groups in which recombination mapping is not possible because of the absence of recombination, such as togaviruses and paramyxoviruses.

Molecular Genetics

Since about 1970 there has been an explosive development of techniques that have revolutionized viral genetics, namely methods for molecular cloning, DNA and RNA sequencing, fine mapping of transcripts and expressing genes in procaryotic and eucaryotic cells. In conjunction with site-directed mutagenesis, these techniques have made it possible to introduce into viral genomes virtually any selected mutation and to remove genes and *cis*-acting elements from viral genomes and study their activities in isolation from the rest of the genome. The basic strategy is molecular cloning by the use of recombinant DNA techniques.

Recombinant DNA

The identification of the cleavage sites of the restriction endonucleases and the development of knowledge of the enzymes involved in DNA synthesis opened up the possibility of deliberately introducing specific foreign DNA sequences into DNA molecules. When these recombinant molecules replicate, there is a corresponding amplification of the foreign DNA.

The process is called molecular cloning. When the replicating recombinant molecules are placed in a situation where their genetic information can be expressed, the polypeptide specified by the foreign DNA is produced. These results are usually achieved by incorporating the foreign DNA into a bacteriophage or a plasmid, which serves as a cloning vector for introducing the foreign DNA into bacterial or other cells. Vectors are available that replicate in bacteria, yeasts, animal cells and intact animals. For animal cells, a variety of animal viruses can be used as vectors, SV40, retroviruses and vaccinia virus being popular choices. The development of recombinant DNA methodology has been facilitated by great improvements in the techniques of sequencing DNA and the perfection of methods for making complementary DNA (cDNA) from either viral RNA or mRNA. The cluster of techniques used is often called 'genetic engineering'.

Sequence analysis

A great deal of information can be gleaned from knowledge of the sequence of part or all of a viral genome. Open reading frames (ORF), which are long translational sequences uninterrupted by stop codons (UAA, UAG, UGA), suggest the presence of protein coding sequences, especially if the codons for given amino acids occur with the frequency found in known protein coding sequences of the virus. The function of the predicted protein can sometimes be surmised by the similarity of its imputed sequence to that of a protein of known function. Such comparisons are carried out by searching international computer-based databases of nucleotide and amino acid sequences. Thus the existence of vaccinia growth factor was predicted when sequence analysis of the vaccinia virus genome revealed an ORF similar to that of known mammalian growth factors. It is also possible from an examination of the sequence to predict that specific portions of a predicted protein will have particular functions, such as signal sequences for targeting proteins to the endoplasmic reticulum or the plasma membrane, transmembrane sequences, glycosylation sites and nucleotide binding sites. Short sequence motifs can be identified which serve as signals in gene expression. Methionine codons (AUG) used to initiate translation are found at the beginning of ORFs, embedded in a consensus sequence GCCGCC/GCCAUGG. Sites of mRNA cleavage and polyadenylation are usually signaled by AAUAAA followed by certain other signals. The start sites for transcription by RNA polymerase II are usually about 30 bp downstream from TATA boxes.

Uses of Genetic Engineering of Animal Viruses

Practical applications of genetic engineering of viruses include the development of nucleic acid probes for diagnosis and novel methods for the production of vaccines, such as the use of vaccinia virus as a vector. Combined with the availability of simple and fast methods of sequencing nucleic acids, genetic engineering has also led to studies of animal virus genomes that could not be contemplated before it became possible to produce large quantities of selected fragments of viral nucleic acid by the use of the polymerase chain reaction. Among the achievements so far are:

1. Complete sequencing of the genome of DNA viruses of several families, including the 230 kbp genome of cytomegalovirus (a herpesvirus) and the 192 kbp genome of vaccinia virus.
2. Complete sequencing of cDNA corresponding to the entire genome of several RNA viruses.
3. Recognition of the number and sequence of viral or proviral DNAs that are integrated into the DNA of transformed cells.
4. Development of diagnostic probes for use in *in vitro* assays, including *in situ* hybridization.
5. The polymerase chain reaction can be used to detect low copy number of a viral genome.
6. Marker rescue by transfection with gene fragments, as a method of genetic mapping.
7. Production of proteins coded by specific viral genes, using bacterial, yeast, baculovirus and animal cell expression systems or by cell-free translation.
8. Synthesis of peptides based on DNA sequence data.

Transgenic Mice

Transgenic mice provide a new tool for investigating many problems in virology, immunology and developmental biology. They are produced by injecting selected fragments of DNA into the nuclei of fertilized eggs washed out of the mouse oviduct. After replacement, some ova develop normally to form the base of a colony of transgenic mice. The technique provides insights into the potential role in viral pathogenesis of individual viral gene products in the context of the intact animal. For example, transgenic mice have been produced in which every cell contained greater than genome length hepatitis B DNA sequences. All mice had HBs antigen in plasma, but the viral sequences were selectively expressed only in cells of the liver, kidney and pancreas. Likewise, transgenic mice

containing the DNA for the early region of bovine papilloma virus developed skin tumors at 8–9 months of age. Extrachromosomal viral DNA was detected in tumor cells and integrated viral DNA in normal tissues.

See also: **Defective interfering viruses; Herpesviruses 6 and 7 – human (*Herpesviridae*); Interference; Reoviruses (*Reoviridae*): General features; Vaccinia virus (*Poxviridae*); Vectors: Plant viruses.**

Further Reading

Becker Y (1995) A short introduction to the origin and molecular evolution of viruses. *Virus Genes* 11(2–3): 73.

Coen DM and Ramig RF (1996) Viral genetics. In: Fields BN, Knipe DM, Howley PM *et al.* (eds.) *Fields Virology*, 3rd edn, p. 113. Philadelphia, Lippincott-Raven.

Domingo E and Holland JJ (1997) RNA virus mutations and fitness for survival. *Annu. Rev. Microbiol.* 51: 151.

Fitch WM (1996) The variety of human virus evolution. *Mol. Phylogenet. Evol.* 5(1): 247.

García-Sastre A and Palese P (1993) Genetic manipulation of negative-strand RNA virus genomes. *Annu. Rev. Microbiol.* 47: 765.

Gibbs AJ, Calisher CH and García-Arenal F (eds) (1995) *Molecular Basis of Virus Evolution.* Cambridge, Cambridge University Press.

Nathanson N and Murphy FA (1997) Evolution of viral diseases. In: *Viral Pathogenesis*, Nathanson N *et al.* (eds.), p. 353. Philadelphia, Lippincott-Raven.

Prusiner SB and Scott MR (1997) Genetics of prions. *Annu. Rev. Genet.* 31: 139.

White BA and Totowa NJ (eds.) (1997) PCR cloning protocols: from molecular cloning to genetic engineering. *Methods in Molecular Biology*, Vol. 67. Clifton, New Jersey, Humana Press.

[This article is reproduced from the 1st edn (1994).]

GIARDIAVIRUSES (*TOTIVIRIDAE*)

Alice L Wang and **Ching C Wang**, University of California, San Francisco, California, USA

History

In 1986, a 6.3 kb linear double-stranded (ds) RNA molecule was observed in nucleic acid extracts of *Giardia lamblia* Portland I trophozoites. *G. lamblia* is an anaerobic parasitic flagellate that inhabits the upper gastrointestinal tracts of humans as well as many other mammals. When infecting humans, the parasitic protozoan causes giardiasis, an acute diarrhea that often progresses to chronic, carrier-stage for adults and severe malnutrition for children. Further examination of this 6.3 kb dsRNA isolated from the extract of *G. lamblia* revealed it to be the genome of a small isometric virus, hence named giardiavirus (GLV), that specifically infects this protozoan.

It is interesting to note that although GLV was first detected in an isolate of *G. lamblia* Portland I (P1) obtained from Dr D. G. Lindmark of Cleveland State University, the same P1 isolate from the American Type Culture Center is virus-free. It therefore remains a mystery as to how the Cleveland P1 became exposed to the virus. All the information included here has been derived from studies of the virus originally isolated from this Cleveland P1 strain.

Taxonomy and Classification

GLV belongs to the family *Totiviridae*, genus *Giardiavirus*, of RNA viruses. This family is characterized by the nonsegmented dsRNA genome and simple virion structure. Members of *Totiviridae* include the yeast dsRNA virus (ScV-L). Many of the viruses recently discovered from protozoa, such as the *Trichomonas vaginalis* virus (TVV), *Leishmania braziliensis* virus (LBV), and *Eimeria stiedae* virus (ESV), also belong to this family.

Host Range and Geographic Distribution

Purified GLV readily infects many virus-free isolates of *G. lamblia* trophozoites, but not any other parasitic protozoa tested, including *Tritrichomonas foetus, Trichomonas vaginalis, Trypanosoma brucei brucei, Entamoeba histolytica* and *Eimeria stiedae.* The virus has also been shown not to infect two transformed human intestinal cell lines. It is therefore believed that giardiavirus has a rather narrow host range. It probably infects only *G. lamblia* in nature.

containing the DNA for the early region of bovine papilloma virus developed skin tumors at 8–9 months of age. Extrachromosomal viral DNA was detected in tumor cells and integrated viral DNA in normal tissues.

See also: **Defective interfering viruses; Herpesviruses 6 and 7 – human (*Herpesviridae*); Interference; Reoviruses (*Reoviridae*): General features; Vaccinia virus (*Poxviridae*); Vectors: Plant viruses.**

Further Reading

Becker Y (1995) A short introduction to the origin and molecular evolution of viruses. *Virus Genes* 11(2–3): 73.

Coen DM and Ramig RF (1996) Viral genetics. In: Fields BN, Knipe DM, Howley PM *et al.* (eds.) *Fields Virology*, 3rd edn, p. 113. Philadelphia, Lippincott-Raven.

Domingo E and Holland JJ (1997) RNA virus mutations and fitness for survival. *Annu. Rev. Microbiol.* 51: 151.

Fitch WM (1996) The variety of human virus evolution. *Mol. Phylogenet. Evol.* 5(1): 247.

García-Sastre A and Palese P (1993) Genetic manipulation of negative-strand RNA virus genomes. *Annu. Rev. Microbiol.* 47: 765.

Gibbs AJ, Calisher CH and García-Arenal F (eds) (1995) *Molecular Basis of Virus Evolution.* Cambridge, Cambridge University Press.

Nathanson N and Murphy FA (1997) Evolution of viral diseases. In: *Viral Pathogenesis*, Nathanson N *et al.* (eds.), p. 353. Philadelphia, Lippincott-Raven.

Prusiner SB and Scott MR (1997) Genetics of prions. *Annu. Rev. Genet.* 31: 139.

White BA and Totowa NJ (eds.) (1997) PCR cloning protocols: from molecular cloning to genetic engineering. *Methods in Molecular Biology*, Vol. 67. Clifton, New Jersey, Humana Press.

[This article is reproduced from the 1st edn (1994).]

GIARDIAVIRUSES (*TOTIVIRIDAE*)

Alice L Wang and **Ching C Wang**, University of California, San Francisco, California, USA

History

In 1986, a 6.3 kb linear double-stranded (ds) RNA molecule was observed in nucleic acid extracts of *Giardia lamblia* Portland I trophozoites. *G. lamblia* is an anaerobic parasitic flagellate that inhabits the upper gastrointestinal tracts of humans as well as many other mammals. When infecting humans, the parasitic protozoan causes giardiasis, an acute diarrhea that often progresses to chronic, carrier-stage for adults and severe malnutrition for children. Further examination of this 6.3 kb dsRNA isolated from the extract of *G. lamblia* revealed it to be the genome of a small isometric virus, hence named giardiavirus (GLV), that specifically infects this protozoan.

It is interesting to note that although GLV was first detected in an isolate of *G. lamblia* Portland I (P1) obtained from Dr D. G. Lindmark of Cleveland State University, the same P1 isolate from the American Type Culture Center is virus-free. It therefore remains a mystery as to how the Cleveland P1 became exposed to the virus. All the information included here has been derived from studies of the virus originally isolated from this Cleveland P1 strain.

Taxonomy and Classification

GLV belongs to the family *Totiviridae*, genus *Giardiavirus*, of RNA viruses. This family is characterized by the nonsegmented dsRNA genome and simple virion structure. Members of *Totiviridae* include the yeast dsRNA virus (ScV-L). Many of the viruses recently discovered from protozoa, such as the *Trichomonas vaginalis* virus (TVV), *Leishmania braziliensis* virus (LBV), and *Eimeria stiedae* virus (ESV), also belong to this family.

Host Range and Geographic Distribution

Purified GLV readily infects many virus-free isolates of *G. lamblia* trophozoites, but not any other parasitic protozoa tested, including *Tritrichomonas foetus, Trichomonas vaginalis, Trypanosoma brucei brucei, Entamoeba histolytica* and *Eimeria stiedae.* The virus has also been shown not to infect two transformed human intestinal cell lines. It is therefore believed that giardiavirus has a rather narrow host range. It probably infects only *G. lamblia* in nature.

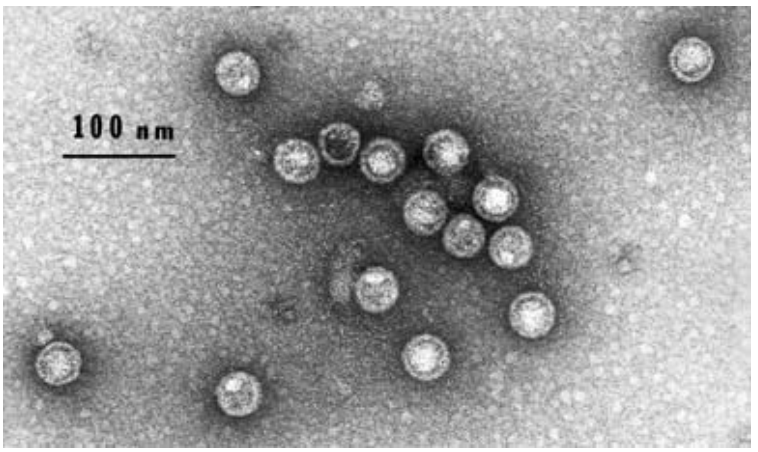


Figure 1 Giardiavirus.

On the other hand, the cellular host for this virus, *G. lamblia*, parasitizes many mammals other than humans. Viruses that are identical in size, shape and size of genomic dsRNA and share dsRNA sequence homology with one another have been detected from many *G. lamblia* strains and isolates obtained from humans, guinea pigs, cats, beavers, llamas and sheep. The human isolates were collected from Belgium, Poland, England, Israel, Ecuador, Puerto Rico and various states in the USA. Since *G. lamblia* is found in almost all parts of the world, affecting developing as well as developed countries, it is expected that GLV follows its host and is distributed worldwide.

Physical and Biochemical Characteristics

Under the electron microscope, GLV appears as an icosahedron of 36 nm diameter, consisting of an electron-dense core encapsidated in a shell 5–6 nm thick (**Fig. 1**). In addition to the 6.3 kb linear dsRNA genome, the virion contains a major polypeptide of 100 kDa (p100) which is most likely the viral capsid. Purified virions are good antigens in mice. Polyclonal antibodies raised against whole virions react with p100 in Western blots and can effectively block viral infection in the *in vitro* culture of *G. lamblia* trophozoites. The same antiserum also reacts positively with a minor component of the virion, a polypeptide of 190 kDa, which is believed to be the viral RNA-dependent RNA polymerase (RDRP). Indeed, GLV-encoded RDRP activity has been detected in the infected cell extract as well as the purified GLV fractions.

Antisera raised against synthetic dsRNA crossreact only with the viral dsRNA and are not protective against viral infection of *G. lamblia* trophozoites *in vitro*. Giardiavirus does not contain any lipids nor is the p100 polypeptide glycosylated.

The entire nucleotide sequence of the linear GLV dsRNA genome has been determined. It consists of 6277 bp and is flush at either end. As the dsRNA can be readily radiolabeled by [^{32}P]pCp and RNA ligase, this molecule must have a free 3′-OH group at one or both of its 3′ termini. The exact structure at the 5′ termini of the dsRNA has not yet been elucidated. It is known that the dsRNA molecule cannot be phosphorylated by T4 polynucleotide kinase, and that the denatured dsRNA molecule can be circularized with T4 RNA ligase. Therefore, the 5′ termini of the GLV genomic dsRNA are probably phosphorylated. Additionally, no covalently linked protein is found at these termini such as in the case of poliovirus.

Organization and Molecular Biology of the GLV dsRNA Genome

The 6277 nucleotide (nt) GLV dsRNA genome contains only two large open reading frames (ORFs), both on the same strand of RNA (**Fig. 2**). The first ORF (nts 368–3015) encodes the precursor polypeptide for the GLV capsid protein. Thirty-two amino acid residues from the N-terminus of this precursor protein are apparently removed by a cellular cysteine protease before the processed capsid protein is assembled into the virion.

The second ORF (nts 2806–5976) is −1 relative to ORF 1, and the two ORFs overlap by 220 nucleotides. Amino acid sequence motifs conserved for all RDRPs have all been found in ORF 2. It is now known that the 190 kDa GLV RNA polymerase is synthesized as a fusion protein of ORFs 1 and 2 at a level that is 2–5% of p100. Apparently, ribosomes carrying the growing nascent polypeptide chain are stalled when they encounter the pseudoknot structure residing within the 220 nt overlap of these two ORFs. At a 2–5% frequency, the ribosomes slip back one reading frame and proceed to translate ORF 2 as the C-terminus of a

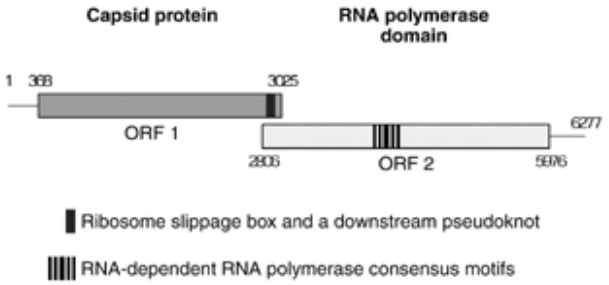


Figure 2 Organization of the giardiavirus dsRNA genome.

fusion protein. The ability of this GLV overlap fragment to induce −1 ribosomal frameshifting has been demonstrated in a reporter system in yeast.

Flanking the two ORFs, there are 367 and 301 nt untranslated regions respectively on the 5′ and 3′ sides. These two regions contain sequence elements that are critical for initiation of transcription and replication of GLV RNA. For example, deletion of a single nucleotide from the 5′ terminus of the (+) strand GLV RNA totally abolishes transcription of GLV mRNA. Similarly, deletion or alteration of sequences in these two regions drastically reduces the level of progeny viral RNA.

In recent years, GLV has been successfully used as a vector for the introduction of foreign genes into *G. lamblia*. When a portion of the GLV genome is replaced with the firefly luciferase gene in a cDNA construct downstream from a T7 promoter, chimeric RNA can be synthesized *in vitro* using T7 polymerase. *Giardia* cells that are infected with wild-type GLV and electroporated with the chimeric RNA show luciferase activity of a millionfold above background. The chimeric RNA is replicated as dsRNA and packaged into recombinant virions that are shed into the culture supernatant. These recombinant viruses, together with wild-type GLV, can in turn infect naive *Giardia* trophozoites to produce luciferase activity. The list of foreign genes tested in this system include genes encoding neomycin phosphotransferase, hygromycin phosphotransferase and green fluorescence protein. All can be delivered and expressed at high levels. Inclusion of a fragment, nts 368–631 in the coding region of p100, as the N-terminus of a fusion protein with the foreign gene product has been shown to enhance translation level by 5000-fold. This region therefore may contain elements that can promote interaction between GLV mRNA and ribosomes of *G. lamblia*.

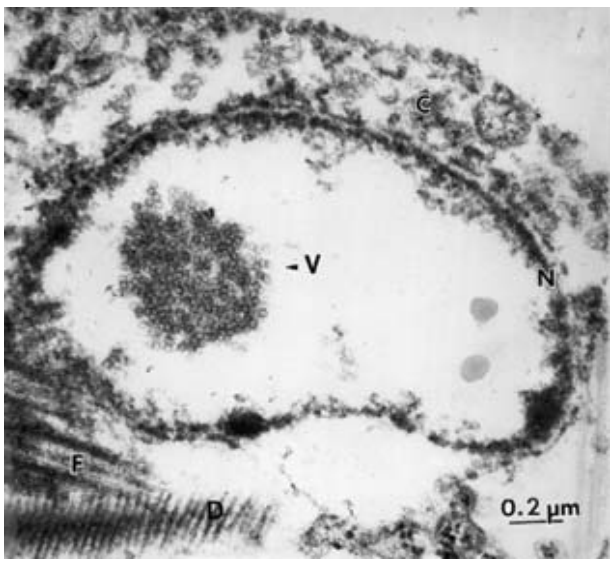


Figure 3 Thin section of giardiavirus-infected cell. C, cytoplasm; N, nuclear envelope; F, flagella; D, ventral disc; V, aggregates of virus particles.

Infection and Replication

GLV infects susceptible isolates of *G. lamblia* very efficiently. It has been estimated that demonstrable infection can be achieved at a multiplicity of infection as low as ten GLV particles per cell. The exact mode of viral entry into the cell has not been elucidated. However, a number of virus-free *G. lamblia* strains have been found resistant to GLV infection. When trophozoites of these strains are electoporated with the single-stranded (ss) GLV RNA (see below), they become infected by GLV and can support and complete the cycle of replication, producing progeny virions that are fully infective to sensitive strains. It is therefore probable that certain cellular component(s) such as viral receptors are involved in the initial interaction between GLV and susceptible strains.

Using purified GLV to infect virus-free *G. lamblia* WB strain, it was shown by *in situ* hybridization that dsRNA replication was first detected in the cytoplasm. Towards the late stage of infection, viral dsRNA was found to spread into the twin nuclei of the infected flagellate. Transmission electron microscopic examination of thin sections of infected cells at stationary phase also reveals paracrystals of virus-like particles in the nuclei (**Fig. 3**). It has been estimated that an infected cell may harbor as many as 10^5 GLV particles without lysis. Meanwhile, mature and infectious GLV particles begin to appear in the supernatant of *Giardia* culture medium 42 h after infection. It is not known whether a specific cellular process is involved in the release of giardiavirus into the culture supernatant, although cell lysis has not been observed as a consequence of viral infection.

The growth rate of newly infected cells decreases with increasing multiplicity of infection. As the ratio

Table 1 Physical properties of some small dsRNA viruses

Virus	Shape	Diameter (nm)	Density (g ml^{-1})	dsRNA (μm)	dsRNA (kb)	Capsid protein (kDa)
GLV	Isometric	33	1.368	1.50	6.277	100
TVV	Isometric	33	1.468	1.50	4.3–4.8	85
LRV	Isometric	32	—	—	5.3	—
ESV	Isometric	35	—	1.63	6.5	—
BBV	Isometric	38	1.358	—	5.5	—
ScV-L	Isometric	33–41	1.368	1.31	4.3–4.8	88
UmV-P1	Isometric	41–43	1.418	—	6.3	73

GLV, *Giardia lamblia* virus; TVV, *Trichomonas vaginalis* virus; LRV, *Leishmania* RNA virus; ESV, *Eimeria stiedae* virus; BBV, *Babesia bovis* virus; ScV-L, yeast double-stranded RNA virus; UmV-P1, *Ustilago maydis* virus.

of the infecting virus to cell increases, the percentage of the nonadhering (nondividing) trophozoites also increases. However, in established infected cell lines or at moderate multiplicity of infection (<1000), the infected cell assumes normal appearance and maintains the same growth rate as its uninfected counterpart. Furthermore, GLV infection persists indefinitely throughout repeated subculturings.

In addition to the 6.3 kb genomic dsRNA, an ssRNA (SS) of identical length that is homologous to only one of the two strands of GLV dsRNA is also found in the GLV-infected cellular extract. Studies of the infection time course showed that, in contrast to GLV dsRNA which increases steadily from 23 h to 141 h after infection, SS becomes detectable at about the same time, peaks at 42–50 h after infection, then gradually declines. Electroporation of gel-purified SS into the uninfected WB cells resulted in the recovery of dsRNA from the cell extract and infectious GLV particles in culture supernatant, demonstrating that SS is the viral messenger RNA as well as the full-length replicative form of the viral genome. Recent nucleotide sequence analysis of GLV cDNA clones also verifies that it is the SS strand that encodes the large open reading frames. There is no subgenomic viral RNA detected inside the infected cell.

The RNA products synthesized *in vitro* by the virion-associated RDRP are homologous to SS and complementary to the negative strand. Transcription of viral message therefore must proceed conservatively by utilizing the dsRNA as its template.

Evolution

The extent to which a person is affected by giardiasis varies widely. The outcome of the severity of parasitic infection depends on many variables derived not only from the parasite but also from the human host. Attempts at correlating the presence or absence of GLV with the severity of giardiasis have been inconclusive, and we still do not know the role GLV might play in the delicate balance of the host–parasite interaction, if any.

Some of the characteristics of GLV are listed with those of other dsRNA viruses of *Totiviridae* in **Table 1**. When compared with ScV-L, the best known virus of this group, GLV and ScV are similar in many ways: (1) single molecules of genomic dsRNAs of similar sizes; (2) single major capsid polypeptides of similar sizes; (3) virion-associated RDRP with similar amino acid sequence motifs deduced from the genomic sequence, (4) ability to synthesize viral messages from intact virions *in vitro;* and (5) use of ribosomal frameshifting for the synthesis of viral polymerase. However, the two viruses do not crossinfect and the two dsRNAs do not crosshybridize in Northern blots. GLV dsRNA has also been shown not to cross-hybridize with dsRNAs of TVV or LRV. Comparison of the nucleotide sequence from GLV cDNAs with that of ScV-L, LRV or TVV indicates that, despite the similar organization of the viral genomes, GLV share very little overall sequence identity with any of these viruses whose genomic sequences have been completely determined. GLV is therefore not closely related to any of these viruses evolutionarily.

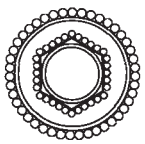
See also: **Totiviruses (*Totiviridae*): General features, *Ustilago maydis* viruses; Vectors: Animal viruses, Plant viruses.**

Further Reading

Wang AL and Wang CC (1991) Viruses of the protozoa. *Ann. Rev. Microbiol.* 45: 251.

Wang AL and Wang CC (1995) The double-stranded RNA genome of *Giardiavirus*. In: Boothroyd JC (ed.) *Molecular Approaches to Parasitology*, p. 179. New York: Wiley.

GIBBON APE LEUKEMIA VIRUS (*RETROVIRIDAE*)



Marvin S Reitz Jr, Institute of Human Virology, University of Maryland, Baltimore, USA

Introduction

Gibbon ape leukemia virus is the designation for a related group of retroviruses which have been seroepidemiologically linked to leukemias in captive gibbon apes (species *Hylobates lar*). The occurrence of leukemias and lymphomas in gibbons was first described in the late 1960s. Outbreaks of multiple cases were reported within a gibbon ape colony used for malaria research in Thailand by the Southeast Asia Treaty Organization (SEATO), in a colony at the animal care facility of the San Francisco Medical College, University of California, and a colony maintained for psychological testing on Hall's Island in the Caribbean.

Taxonomy and Classification

The gibbon ape leukemia viruses are typical mammalian type C retroviruses (genus) and are transmitted horizontally (either congenitally or from one individual to another) rather than vertically (via germ lines). Horizontal transmission is a property shared by all other leukemia viruses, such as those of cats, mice, chickens, cattle, and, more recently, humans.

The gibbon ape leukemia viruses (GaLV) and the related simian sarcoma virus and its helper virus, simian sarcoma-associated virus, form a closely related group and as a group are most like the murine leukemia viruses. The gibbon ape viral receptor on target cells is the same receptor used by feline leukemia virus subgroup B.

Clinical Features of Infection

The disease at the San Francisco facility was characterized as lymphoblastic lymphosarcoma, with lymph node, liver, spleen and bone marrow involvement. A more current description would probably be T-cell leukemia/lymphoma. A lymphoid cell line called UCD-144 (alternatively MLA-144) was established from one of the leukemic animals, and the first GaLV isolate was obtained from this cell line. UCD-144 cells have the phenotype of relatively mature T cells, including the production of IL-2. Indeed, IL-2 was first cloned from this cell line. The strain of GaLV isolated from this cell line is designated as the San Francisco strain of GaLV (GaLV-SF).

A second GaLV isolate was obtained from tissues of gibbons from the SEATO Medical Project colony in Bangkok, Thailand, with a disease resembling human chronic myelogenous leukemia. Other gibbons in the colony were found to be seropositive for the virus. Inoculation of this virus, which has been designated GaLV-SEATO, into young gibbons induces a myeloproliferative disease within a year.

The third isolate of GaLV was from the frozen brain tissues of three nonleukemic gibbons. Two of these had been inoculated with brain extracts of human patients with the progressive neurodegenerative disease kuru. These isolates, designated GaLV-Br, have not been associated with hematopoietic neoplasms.

A fourth strain of GaLV was isolated from a gibbon ape with T-cell leukemia who was a member of a gibbon colony kept in isolation on Hall's Island, near Bermuda, for psychological studies. A lymphoid cell line was established from this animal, and retrovirus was readily detected. The cell line had properties characteristic of immature T cells, including the expression of terminal transferase. The distribution of virus corresponded closely to the degree of infiltration of leukemic cells, which was extensive and evident in blood, heart, liver, spleen, lymph nodes and salivary glands. The tongue was coated with viral particles, as determined by electron microscopy. This virus was designated GaLV-H.

Serologic Relationships and Variability

All four subtypes of GaLV are closely related to each other by protein serology and by nucleic acid sequence homology, but are readily distinguishable by both criteria. Differences among the envelope genes are probably greater than in other regions of the viral genome.

As mentioned earlier, the GaLVs as a group appear to be most closely related to the murine leukemia viruses, but the relationship appears to be closest to an endogenous virus of the Asian mouse *Mus caroli*. This suggests that GaLV could have originated by a trans-species transmission, but it is not clear when this might have happened or what mouse might be the source. It is not yet known if GaLV is found in feral gibbons.

Table 1 Pathogenicity

Virus	*Isolated from*	*Pathogenic activity*
Herpesviruses		
H. saimirii	Normal squirrel monkey	Rapidly lymphomagenic in heterologous hosts
H. ateles	Normal spider monkey	Rapidly lymphomagenic in heterologous hosts
Retroviruses		
GaLV-SF	Lymphoid leukemia of gibbon ape	Highly associated with and probably causes lymphoid leukemia in host of origin
GaLV-H	Lymphoid leukemia of gibbon ape	Highly associated with and probably causes lymphoid leukemia in host of origin
GaLV-SEATO	Myeloid leukemia of gibbon ape	Causes myeloid leukemia in host of origin
SSV	Fibrosarcoma of woolly monkey	Causes fibrosarcoma in host of origin and other species
MPMV	Breast adenocarcinoma of rhesus monkey	Causes immunosuppression and thymic atrophy in host of origin
HTLV-I	Adult T-cell leukemias and lymphomas of man	Highly associated with and probably causes adult T-cell leukemia and lymphoma in man
HIV-1	T-cells from human AIDS patients	Causes AIDS in man

The simian sarcoma virus (SSV) and its associated helper virus, simian sarcoma associated virus (SSAV) were isolated from a spontaneous fibrosarcoma in a pet woolly monkey. These viruses were, in retrospect, actually the first isolate of the GaLV group. SSAV is genetically as similar to the other members of the GaLV group as the latter are to each other. The woolly monkey from which SSV was isolated lived in the same household as a pet gibbon ape, and it has become clear, from the otherwise restricted distribution of GaLV to gibbons and their absence in New World monkeys, that the woolly monkey was infected by a strain of GaLV from the gibbon.

Pathogenicity (Table 1)

SSV is able to transform fibroblasts *in vitro*, and it causes fibrosarcomas when inoculated into marmosets. SSV is a defective virus whose genome was formed by a deletion of all of the reverse transcriptase gene and parts of the genes for the envelope and core structural proteins and a recombinational insertion of a cell-derived gene, called *sis*. It is the last gene which gives SSV the ability to transform fibroblasts *in vitro*. SSAV and the GaLVs are not transformation competent for cultured fibroblasts, and SSV, due to its lack of any functional viral genes, is not replication competent. Consequently its replication requires the presence of its helper virus, namely SSAV. The *sis* gene was subsequently shown to be identical to β-platelet-derived growth factor, a cellular gene involved in the process of wound healing and which promotes the growth of fibroblasts *in vitro*. The normal human analogue of *sis* has been shown to directly transform fibroblasts when transfected *in vitro* and can confer tumorigenic potential *in vivo* when its expression is constitutively high. The *sis* gene appears to have been derived from the woolly monkey, indicating that the SSV genome was formed by recombination subsequent to transmission from the gibbon.

The reason for the disease specificity of these viruses is unclear. Presumably differences in the ability to replicate or be expressed in different cell types helps to determine the type of leukemia they cause, but the situation is not simple. All strains of GaLV apparently use the same cell surface receptor, a transmembrane sodium-dependent phosphate transporter, yet have somewhat different host cell specificities *in vitro*, suggesting that envelope–receptor binding is not the basis for disease specificity. The ability to grow in different cell types, however, is also influenced by events following binding of the viral envelope proteins to their cell surface receptor, including entry, uncoating of the viral core, reverse transcription of viral DNA, transport to the nucleus, integration into the host cell genome, and transcription of the viral genome. Presumably the ability of the different strains of GaLV to induce different kinds of leukemia is determined at one or more of these levels.

The basic mechanisms by which these viruses cause leukemia in their hosts is not known, although it presumably shares features with the other leukemia viruses. The infected host shows a persistent immune response to the virus, but the response is not always protective. Very little work on infected gibbons has been performed beyond the mid 1970s. This is primarily due to the appearance of gibbons on the endangered species list and to the unfortunate dissolu-

tion of the gibbon colonies in which these viruses were described, through lack of financial support.

Recent work involving GaLV has primarily involved the adaptation of viral genes, especially the envelope gene, for the construction of improved retroviral vectors for gene delivery and gene therapy. The PG13 gene delivery vector system uses a standard murine retroviral core and packagable genome, along with the GaLV-SEATO envelope, and has been reported to give significantly improved efficiencies with some human hematopoietic cells. Further developments of GaLV-related retroviral vectors include substitution of a GaLV core and/or packagable genome for the murine analogues and use of the envelope proteins from strains of GaLV other than SEATO. These may well provide superior retroviral gene delivery vehicles to those currently available.

See also: **Avian type C retroviruses (*Retroviridae*); Feline leukemia and sarcoma viruses (*Retroviridae*).**

Further Reading

Gallo RC, Gallagher RE, Wong-Staal E *et al* (1978) Isolation and tissue distribution of type-C virus and viral components from a gibbon ape (*Hylobates lar*) with lymphocytic leukemia. *Virology* 84: 358.

Kawakami TG and Buckley PM (1974) Antigenic studies in gibbon type-C viruses. *Transplant. Proc.* 6: 193.

Kawakami TG, Huff SD, Buckley PM, Dungworth DC and Snyder JP (1972) Isolation and characterization of a C-type virus associated with gibbon lymphosarcoma. *Nature New Biol.* 246: 170.

Kawakami TG, Kollias G and Holmberg C (1980) Oncogenicity of gibbon type-C myelogenous leukemia virus. *Int. J. Cancer* 25: 841.

Miller AD, Garcia JV, Suhr NV *et al* (1991) Construction and properties of retrovirus packaging cells based on gibbon ape leukemia virus. *J. Virol.* 65: 2220.

Goat Pox Virus *see* **Poxviruses**

Gonometa Virus *see* **Picornaviruses – Insect**

Goose Parvovirus *see* **Parvoviruses**

Granuloviruses *see* **Baculoviruses**

Guanarito Virus *see* **Lassa, Junin, Machupo and Guanarito Viruses**

H

HANTAVIRUSES (*BUNYAVIRIDAE*)

Connie S Schmaljohn, Virology Division, United States Army Medical Research Institute of Infectious Diseases, Ft Detrick, Frederick, Maryland, USA

History and Classification

Hantaviruses are rodent-borne viruses which may be transmitted to humans in aerosolized urine, feces or saliva, and occasionally by bite. Certain hantaviruses cause hemorrhagic fever with renal syndrome (HFRS) or hantavirus pulmonary syndrome (HPS). Other hantaviruses are not known to be human pathogens.

The term HFRS was adopted in 1983 by the World Health Organization to collectively describe clinically similar diseases such as Korean hemorrhagic fever, epidemic hemorrhagic fever, and nephropathia epidemica. Although HFRS was undoubtedly a disease problem historically, it first became a widely recognized health threat in 1951 when an intense epidemic occurred in military personnel stationed in Korea. A mortality rate of 10–15% was reported among the more than 3000 United Nations soldiers hospitalized with HFRS during the Korean war. Hantaan virus (HTNV) was first associated with HFRS in 1978 when Korean HFRS patient sera were found to react with antigens in the lungs of striped field mice (*Apodemus agrarius*). After the isolation of HTNV in cell culture in 1981 it was possible to characterize the virus for the first time and to determine that molecular properties were consistent with those of other viruses in the family *Bunyaviridae*. Based on those findings, the *Hantavirus* genus was added to the family in 1987. In addition to HTNV, at least three other hantaviruses, Seoul virus (SEOV), Puumala virus (PUUV) and Dobrava-Belgrade virus (DOBV), cause HFRS.

HPS was first described in 1993 when a cluster of cases of acute adult respiratory distress, with mortality rates of approximately 50%, were reported in the southwestern part of the United States. A previously unknown hantavirus, Sin Nombre virus (SNV), was detected in deer mice (*Peromyscus maniculatus*), and was subsequently shown to be responsible for most of the approximately 200 confirmed cases of HPS in North America. At least three other hantaviruses, New York virus (NYV), Bayou virus (BAYV), and Black Creek Canal virus (BCCV), also cause HPS in North America.

The discovery of HPS in North America prompted prospective and retrospective studies in South America to determine if hantaviruses were responsible for unexplained cases of respiratory distress. In Argentina, more than 140 cases of HPS were confirmed. Investigations involving a cluster of cases in a family from southern Argentina identified Andes virus (ANDV) in long-tailed pygmy rice rats (*Oligoryzomys longicaudatus*). ANDV is the only known hantavirus to be transmitted person-to-person. In 1995–1996, an outbreak of 17 cases of HPS in Paraguay led to the discovery of another HPS-associated virus, Laguna negra virus (LNV), carried by the vesper mouse (*Calomys laucha*).

Viruses in the Genus *Hantavirus*

Since the discovery of HPS, a number of novel hantaviruses have been detected in rodents throughout the western hemisphere. Many of these were not isolated in cell culture, but instead were identified by reverse transcription and polymerase chain reaction (RT-PCR) with hantavirus crossreactive primers. Therefore, although distinct species of hantaviruses are defined historically by fourfold or greater differences in serological assays, particularly in neutralization assays, genetic relationships are used to define some of the newly discovered viruses. In general, viruses that are antigenically distinct also display at least an 8% difference in predicted amino acid sequence of one or more of their proteins. Additionally, distinct viruses are usually associated with different rodent hosts. A single hantavirus species has been identified in an insectivore; however, it is possible that this virus, Thottapalayam virus, may

Table 1 Viruses of the genus *Hantavirus*, family *Bunyaviridae*

Virus	*Abbreviation*	*Original source*	*Location*	*Disease*
Murinae subfamily-associated viruses				
Hantaan	HTN	*Apodemus agrarius*	Korea	HFRS
Seoul	SEO	*Rattus norvegicus, R. rattus*	Korea	HFRS
Dobrava-Belgrade	DOB	*Apodemus flavicollis*	Slovenia	HFRS
Thai-749	THAI	*Bandicota indica*	Thailand	Unknown
Arvicolinae subfamily-associated viruses				
Puumala	PUU	*Clethrionomys glareolus*	Finland	HFRS
Prospect Hill	PH	*Microtus pennsylvanicus*	USA	Unknown
Tula	TUL	*Microtus arvalis*	Russia	Unknown
Khabarovsk	KBR	*Microtus fortis*	Russia	Unknown
Topografov	TOP	*Lemmus sibiricus*	Siberia	Unknown
Isla Vista	ISLA	*Microtus californicus*	USA	Unknown
Sigmodontinae subfamily-associated viruses				
Sin Nombre	SN	*Peromyscus maniculatus*	USA	HPS
New York	NY	*Peromyscus leucopus*	USA	HPS
Black Creek Canal	BCC	*Sigmodon hispidus*	USA	HPS
Bayou	BAY	*Oryzomys palustris*	USA	HPS
Caño Delgadito	CANO	*Sigmodon alstoni*	Venezuela	Unknown
Rio Mamore	RM	*Oligoryzomys microtis*	Bolivia	Unknown
Laguna Negra	CHP	*Calomys laucha*	Paraguay	HPS
Muleshoe	MULE	*Sigmodon hispidus*	USA	Unknown
El Moro Canyon	ELMC	*Reithrodontomys megalotis*	USA	Unknown
Rio Segundo	RIOS	*Reithrodontomys mexicanus*	Costa Rica	Unknown
Andes	AND	*Oligoryzomys longicaudatus*	Argentina	HPS
Insectivore-associated virus				
Thottapalayam	TPM	*Suncus murinus*	India	Unknown
Other possible species (limited sequence; no other information available)				
Monogahela		*Peromyscus maniculatus*	N. America	
Blue River		*Peromyscus leucopus*	N. America	
Maciel		*Bolomys obscurus*	S. America	
Juquitiba		Unknown	S. America	

HFRS, hemorrhagic fever with renal syndrome; HPS, hantavirus pulmonary syndrome.

also have a rodent reservoir. A current list of hantavirus species is shown in **Table 1.**

Virion Properties

Morphological features of hantaviruses provided the first clues relating them to viruses in the *Bunyaviridae* family. When examined by electron microscopy, hantaviruses are roughly spherical with a diameter of 80–120 nm (**Fig. 1**). Elongated, oval-shaped particles are sometimes evident with reported lengths of up to 210 nm. Negatively stained particles exhibit a grid-like pattern of morphological subunits on their surfaces. Sectioned virions display a distinct, bilayer envelope with a fringe of short projections. Immunoelectron microscopy with monoclonal antibodies identified the projections as the viral G1 and G2 glycoproteins. The envelope surrounds a granulofilamentous interior, which consists of ribonucleoprotein structures. RNA-dependent RNA polymerase activity is associated with virions (**Fig. 1**).

Physical Properties

Like other enveloped viruses, hantaviruses are sensitive to detergents and organic solvents. Other physical properties of virions include sedimentation densities in sucrose of 1.16–1.17 g ml^{-1} and in cesium chloride of 1.20–1.21 g ml^{-1}. Disruption of virion particles with nonionic detergent releases the nucleocapsids, which sediment at densities of approximately 1.18 g ml^{-1} in sucrose and 1.25 g ml^{-1} in cesium chloride. HTNV infectivity is generally stable for up to 12 h at 0–4°C. Nonphysiological pH extremes (<7.0 or

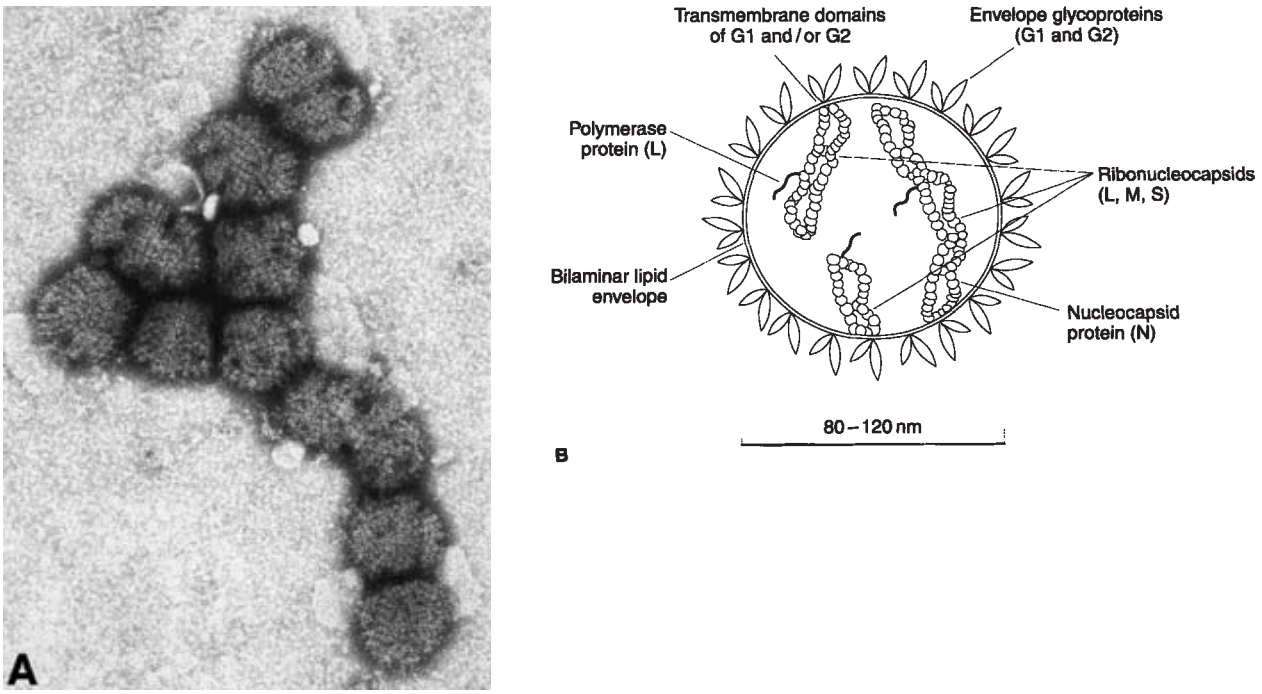


Figure 1 (**A**) Electron micrograph of negatively stained Hantaan virions. Surface morphology shows a grid-like structure. (**B**) Virions contain three noncovalently closed circular ribonucleocapsids designated L, M and S. The ribonucleocapsids are surrounded by a bilaminar lipid envelope of host origin into which are embedded two viral glycoproteins, G1 and G2. Transmembrane regions of G1 and/or G2 are believed to interact with the nucleocapsid protein, N, to trigger budding at Golgi membranes. The viral polymerase protein L is associated with virions and is needed to copy the negative sense vRNA to mRNA.

>8.0) and high salt conditions, such as 32% potassium tartrate, inactivate HTNV infectivity completely after 12 h at 4°C. After drying HTNV may remain viable for 1–3 days. The presence of 10% serum considerably extended the pH and temperature ranges as well as the length of time after drying during which infectious virus could be recovered.

Genome Properties

Hantaviruses have tripartite negative sense genomes with a total size of approximately 13 kb. The large (L), medium (M) and small (S) segments, respectively, encode the viral polymerase, envelope glycoproteins and nucleocapsid protein (**Fig. 2**). Each gene segment has conserved complementary nucleotide sequences which can base pair to form panhandle structures (**Fig. 3**).

Properties of Viral Proteins

A single nucleocapsid protein (N) of approximately 48 kDa is found associated with each of the three gene segments of hantaviruses. The nucleocapsid protein is always associated with virion and complementary sense RNAs but not with messenger RNAs.

The G1 and G2 proteins are integral membrane proteins. Like those of other viruses in the family, hantavirus envelope proteins have asparagine-linked

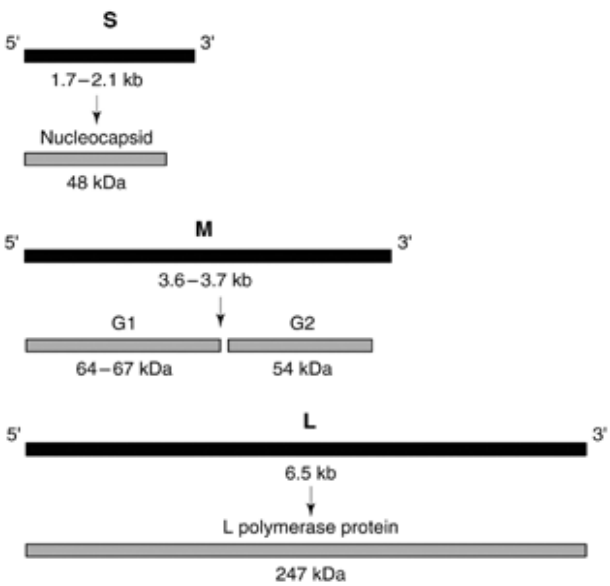


Figure 2 Coding strategy and gene organization of the L, M and S segment sequences of hantaviruses. Approximate nucleotide and predicted amino acid sizes are indicated.

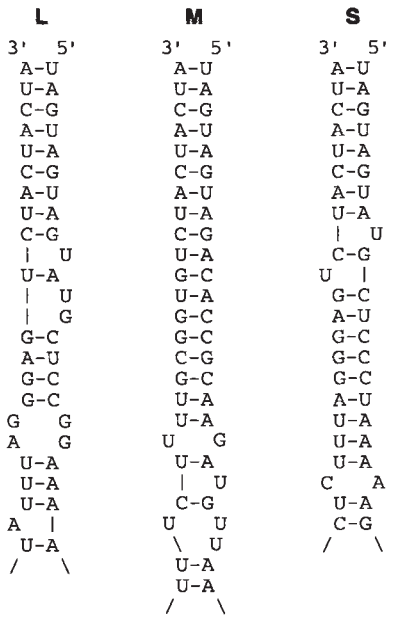


Figure 3 Predicted panhandle structures formed by base-pairing of complementary terminal nucleotides of the L, M and S segments of HTNV.

sugars, are rich in cysteine residues, and induce neutralizing antibody responses in animals. Amino acid sequences typical of signal sequences precede both G1 and G2 and stretches of hydrophobic amino acids are found at their carboxy termini.

The L protein serves several functions necessary for viral replication. It acts as an endonuclease to cleave host mRNAs approximately 10–18 nucleotides beyond the 5′ cap structure. The resultant capped oligonucleotides are used to prime mRNA transcription (**Fig. 4**). The L protein also serves as a transcriptase for generating exact copies of the genome (cRNA), which in turn are used as templates for synthesis of genomic RNA (vRNA). The L protein may also act as a helicase for unwinding the encapsidated RNAs during transcription.

Replication: Strategy of Replication of Nucleic Acid

Transcription

A prime and realign model for hantavirus transcription of vRNA and cRNA was proposed to explain experimental data indicating that the 5′ residue of

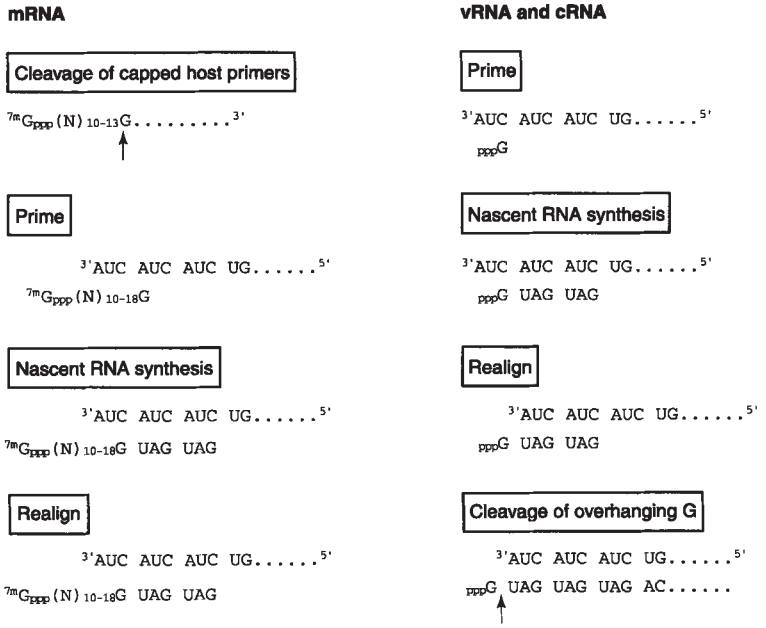


Figure 4 Transcription and replication model for hantaviruses. For synthesis of mRNA, the L protein cleaves capped oligonucleotides approximately 10–18 nucleotides long from host-cell messenger RNAs (preferably after G residues) and uses them to prime transcription. According to the proposed prime and realign model, the terminal G residue aligns at position 3 (C). After several nucleotides are added, the RNA slips backward and realigns with the G residue adjacent to the templated hantavirus nucleotides. Transcription is terminated at undefined signals shortly after the end of the ORF. For cRNA and vRNA synthesis, the L protein uses nonprimed synthesis and must ignore transcription termination signals to produce exact copies of the vRNA (or cRNA) template. According to the prime and realign model, the first nucleotide added is a G which aligns at position 3 (C). After several nucleotides are added, the nascent RNA slips backward and realigns. The overhanging G residue is then cleaved, leaving a terminal U monophosphate residue at the 5′ end of the newly synthesized RNA.

HTNV vRNA is a monophosphorylated U residue. According to the model, transcription is initiated when pppG aligns at the third nucleotide position (C). After synthesis of several nucleotides, the polymerase slips, allowing the nascent RNA to realign such that the initial priming G residue is overhanging (**Fig. 4**). The L protein then serves as an endonuclease to remove the G, leaving a monophosphorylated U residue at the nascent 5′ end.

Transcription of mRNAs may also follow a prime and realign strategy, but instead of a triphosphate residue, capped oligonucleotides cleaved from host mRNAs are used for priming transcription. There appears to be a preferential, but not absolute preference for cleavage of the host oligonucleotides after G residues, as indicated by the preponderance of Gs adjacent to the templated hantavirus residues. As for vRNA and mRNA, this G residue could align at the third C, and after synthesis of a few oligonucleotides, the nascent RNA could realign by slipping backward two nucleotides. The frequent deletion of one or two of the triplet repeats in mRNA also supports this sort of slippage mechanism and suggests that sometimes the initial priming might start at the C residue of the third triplet in the conserved sequence rather than at the C of the second triplet.

Slippage by the viral polymerase is likely to be a general feature of polymerases in the family as well as within the *Hantavirus* genus. Polymerase slippage has been suggested to account for the large amount of noncoding and repeated sequences present at the 3′ ends of the S segments of certain hantaviruses such as SNV.

Distinct transcription termination signals are not clearly defined for hantaviruses, but mRNAs appear to terminate shortly after the open reading frames (ORFs) present in each gene segment as indicated from sequence analysis of mRNAs. The mRNAs are therefore extended at their 5′ ends by 10–18 nucleotides derived from host messages and are truncated at their 3′ ends by approximately the length of the noncoding information. It is not known what factors are involved in the ability of the L protein to recognize or ignore transcription termination signals when switching from mRNA to vRNA and cRNA synthesis, but it is suspected that it involves accumulation of N.

Translation

The L, G1 and G2, and N proteins are the only known translation products of the L, M and S genome segments, respectively. Hantaviral L and N proteins are translated on free ribosomes and G1 and G2 on membrane-bound ribosomes.

Post-translational processing

The G1 and G2 envelope glycoproteins of hantaviruses are expressed from a single mRNA and are probably cotranslationally cleaved, as evidenced by the absence of a precursor polypeptide of a size corresponding to that encoded by the complete M segment ORF. It is believed that cleavage is mediated by host cell protease (i.e. signalase). Typical signal sequences precede both G1 and G2 and probably dictate insertion of the nascent proteins into membranes.

Both G1 and G2 are glycosylated with asparagine-linked carbohydrates, predominantly of the high mannose, endo-H sensitive type. Other post-translational modifications, such as phosphorylation or myristoylation, have not been described.

Assembly and transport

In general, hantaviruses assemble by budding into Golgi cisternae, as do other viruses in the family *Bunyaviridae*. Complete virions are then transported to the cell surface within Golgi vesicles. However, at least two hantaviruses, BCCV and SNV, also bud from cell membranes.

The specific steps in hantaviral assembly are poorly understood. Nascent vRNA and cRNA molecules probably associate with N immediately on transcription. It is presumed that a specific interaction of N and viral nucleic acid is required for their encapsidation. If hantaviruses are analogous to viruses in the *Bunyavirus* genus, this interaction probably involves some sort of signal at the 5′ ends of vRNA and cRNA. The cap structure and/or the deletion of the 3′ terminal complementary sequences of mRNA most likely prevents its association with N.

For all hantaviruses studied to date, the G1 and G2 proteins are synthesized in the endoplasmic reticulum (ER) then transported to the Golgi without the need for other viral proteins or nucleic acid. For HTNV, G1 and G2 must dimerize in the endoplasmic reticulum to achieve correct conformation and efficient transport competency. When individually expressed, both G1 and G2 remain predominantly in the ER, although some G1 migrated to the Golgi. In contrast, the G1 and G2 proteins of SNV are able to exit the ER and transport to the Golgi when expressed independently, although G1 transport depended on the formation of homotrimers. Coexpression of G1 and G2 resulted in quicker transport to the Golgi.

After G1 and G2 are transported to the Golgi, their carbohydrates are modified. It is assumed that either or both proteins have a Golgi retention signal, but if so, it has not been defined. The interactions of G1 and G2 proteins with N during morphogenesis have not

yet been studied in detail. Hantaviruses have no matrix proteins to facilitate interation between the envelope proteins and ribonucleocapsids. It is suspected that there is a direct interaction between transmembrane regions of G1 or G2 and N which triggers budding.

Cytopathology

Hantaviruses persistently infect cultured cells as well as their natural rodent hosts. In both cell cultures and rodents hantaviruses cause no obvious cytopathology.

Geographic and Seasonal Distribution

HTNV causes severe HFRS throughout Korea, The People's Republic of China, and the eastern part of the former Soviet Union. Before the discovery of DOBV in Slovenia in 1992, severe HFRS occurring in Balkan countries was attributed to HTNV infections. Today, however, it is not clear if both HTNV and DOBV circulate in the Balkans or if DOBV is solely responsible for severe HFRS in this region.

SEOV infections occur worldwide. The global distribution of SEOV coincides with the distribution of its rodent host, the urban rat (*Rattus norvegicus*). Although not native to many parts of the world, these rats are now ubiquitous because of their transport on cargo ships over the past several hundred years.

A mild form of HFRS caused by PUUV occurs throughout Sweden, Finland, Norway, the western part of Russia and western Europe.

The seasonal distribution of hantaviruses coincides with rodent population densities and activities. For example, in Korea there are two seasonal peaks of HFRS caused by HTNV in late spring and fall when the populations of infected *Apodemus* field mice are highest. PUUV infections exhibit similar bimodal peaks coinciding with infected *Clethrionomys* densities. Year-to-year variation is also observed, reflecting rodent population periodicity. HFRS caused by SEOV transmission from infected house rats occurs throughout the year but also tends to be more frequent in the fall and winter seasons in temperate climates.

The occurrence of HPS has also been linked to factors that influence rodent density such as rainfall and availability of food. The rodent hosts of HPS-causing viruses are primarily associated with rural environments, but may invade homes in more urban areas either when food supplies are short or in cold weather. The initial recognition of HPS in the spring of 1993 involved a cluster of approximately 27 cases; however, the majority of cases in North America that have occurred since then have appeared sporadically throughout most of the United States and in three Canadian provinces, suggesting an infrequent yet endemic presence. To date, five countries of South America have reported HPS. Although cases have occurred throughout the year in an endemic fashion similar to that seen in North America, clusters of cases in South America generally appear in the spring and summer months (September–January).

Host Range and Virus Propagation

Rodents in the family Muridae are the natural hosts and reservoirs of hantaviruses (**Table 1**). Each hantavirus species is generally (but not always) associated with a single rodent host species. Thus, the natural distribution of a hantavirus is limited to the range of its rodent host. Infection of rodents is generally asymptomatic and persistent despite the presence of neutralizing antibodies. All known HPS-causing hantaviruses are associated with the Sigmodontinae subfamily of rodents. HFRS-causing hantaviruses are associated with the subfamilies Murinae or Arvicolinae.

Although members of the genus replicate in a variety of different vertebrate cell lines and viral antigen can be detected by fluorescent antibody and other techniques, cytopathology is not a consistent characteristic of these infections. Virus isolation in cell culture requires multiple passages, frequently without evidence of viral multiplication; however, once infected, cultures remain persistently infected, with virtually all cells in a culture exhibiting viral antigen. Vero E6 cells are used most frequently for laboratory propagation and assay of hantaviruses.

Genetics

Hantaviruses display classic negative-strand, viral gene-coding properties. That is, all of the recognized viral proteins are encoded in the virus-complementary sense RNA. Comparative sequence analysis suggests that nucleotide sequence conservation parallels antigenic differentiation of viruses defined by plaque-reduction neutralization tests.

Evolution

The high concordance between rodent and hantavirus phylogenies supports a coevolutionary relationship of each hantavirus and its reservoir, probably over thousands of years. Because each hantavirus usually circulates only within a defined rodent population, there are probably restricted possibilities for genome reassortment and genetic mixing. Natural reassortment among strains of SNV has been described, and is probably also common for other hantaviruses. A single example of reassortment between two closely

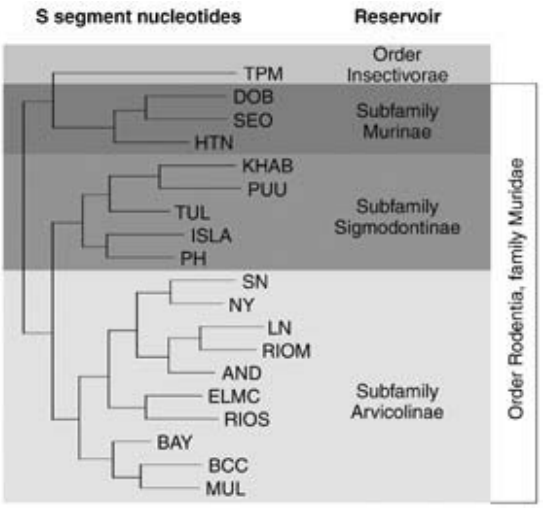


Figure 5 Genetic relationships of hantaviruses and association with natural reservoirs. The phylogenetic tree was constructed by pairwise comparison of S segment nucleotides encoding the nucleocapsid protein. Abbreviations for viruses are as in **Table 1.**

related species (BAYV and BCCV) is known. It is likely that reassortment between more distant species is not possible. Humans are generally dead-end hosts of hantaviruses, and thus probably play no role in their evolution.

Serologic Relationships and Variability

All hantaviruses are serologically related. Techniques such as the indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assays (ELISA) are commonly used to measure the degree of that relationship. Many viruses within the genus have been classified by reciprocal plaque-reduction neutralization assays with convalescent antisera from experimentally infected laboratory animals. However, some hantaviruses have not been isolated in cell culture and are characterized strictly by gene sequence comparisons. A total of 22 distinct hantaviruses have been described to date (**Table 1**), and others will undoubtedly be discovered as additional rodents are screened. Because the phylogenetic relationships among hantaviruses generally reflect their antigenic diversity, such genetic analyses are often used to study the variability among hantaviruses (**Fig. 5**).

Epidemiology

There are three basic epidemiological forms of hantaviral diseases: rural, urban and laboratory-acquired. Rural HFRS is usually associated with transmission of HTNV or DOBV from *Apodemus* or with transmission of PUUV from *Clethrionomys*. Urban HFRS is usually caused by *Rattus*-borne SEOV and is globally distributed with different epidemiological features that vary between countries and geographic regions. Laboratory-acquired HFRS is associated with the infection of both wild-caught and laboratory-bred rodents. Mortality rates are estimated as $< 1\%$ for PUUV infections, 1–5% for SEOV infections, and 5–15% for HTNV and DOBV infections.

HPS in both North and South America is primarily rural. Although case numbers are small, serosurveys suggest that there may be a higher seroprevalence rate among South American populations than those in North America. Whether this implies that there is less asymptomatic infection with HPS-causing hantaviruses in North America is currently unknown. The overall fatality rate for HPS in North America is approximately 45%. Lower case-fatality rates for more recent HPS infections probably reflect both better physician awareness and improved patient care. In South America, case fatality rates have been estimated at from 12% to more than 60% in various outbreaks. Although it is possible that these rates reflect infection by less and more virulent viruses, it is also possible that either or both estimates are incorrect. More consistent diagnosis and reporting of HPS should help to clarify the true fatality rate of HPS in South America.

Transmission and Tissue Tropism

Among rodents, hantaviruses are transmitted by aerosol and bite. HFRS and HPS are almost always traceable to contact with rodents or their excreta. HFRS is associated with activities such as farming, military exercises or other activities which disturb hantavirus-infected rodent nests or burrows. HPS is associated with activities such as inhabiting dwellings invaded by infected rodents, opening or cleaning seasonally vacant cabins or other buildings, or disturbing rodent-infested areas during recreational or occupational pursuits. There is no evidence for person-to-person transmission of HFRS. Similarly, HPS is not yet known to be transmitted among humans in North America. However, in South America there is good evidence for human-to-human transmission of ANDV in one Argentinian outbreak.

Hantaviruses can be isolated early in infection from human plasma, peripheral blood mononuclear cells, and urine sediment obtained from severely infected individuals. Samples taken later in disease are normally associated with high-titer neutralizing antibody which is presumed to reduce isolation efficiency.

Limited unsuccessful attempts to detect virus in clarified urine, saliva, feces, milk, and semen from infected humans have been made. Isolates have been obtained from autopsy material, although most deaths occur beyond the suspected viremic period and after the development of neutralizing antibody. Experimental infection of human endothelial cells and demonstration of endothelial cell infection in laboratory animal infections suggest one possible tropism for these viruses.

Pathogenicity

The pathogenesis of HFRS and HPS are incompletely understood, largely due to the absence of animal models of human disease. Rodent pathogenesis studies for HPS-causing hantaviruses have not been reported, most likely because Biosafety Level 4 containment is suggested for rodent work with these viruses. Pathogenesis studies for HFRS-causing viruses indicate that experimentally infected rodents display viral antigen in virtually all organ systems, but antigen is most apparent in lungs, nerve tissue and vascular endothelium. Infection of newborn rodents is often fatal but infection of adult animals results in a chronic or persistent infection without apparent histopathology, despite the presence of large amounts of viral antigen in numerous tissues including brain.

Clinical Features of Infection

The clinical features of HFRS are well defined, and although they may vary slightly for the different viruses in various geographic settings, most severe human infections can be divided into five characteristic phases. After a variable incubation period of 1 or 2 weeks with extremes from 4 to more than 40 days, a febrile phase is initiated with the sudden onset of high fever, chills, malaise and myalgia, which are often accompanied by significant headache, backache and orbital pain. Flushing is observed on the face, neck and anterior chest as well as conjunctival hemorrhage and petechiae on the soft palate, face, neck and axillary folds. Clinical features of this phase appear to correlate closely with disease prognosis. Albuminuria appears early with microscopic hematuria and tubular cells in the urine, which may progress to massive proteinuria in this phase. Within the first week of illness, the hypotensive phase develops abruptly and can last from hours to days. Massive edema and hemorrhage may lead to hypovolemia, hypotension and clinical shock. About one-third of all deaths are associated with irreversible shock in this phase. Capillary hemorrhages are prominent, electrolyte imbalance is common, hematocrit increases and numbers of platelets drop. Blood urea and creatinine begin to rise in this phase. The oliguric phase can last from 3 to 7 days and blood pressure returns to normal. Some patients become hypertensive because of their hypervolemic condition. Urine output decreases and blood urea and creatinine increase often associated with hyperkalemia, hyponatremia, hypocalcemia and occasional metabolic acidosis. Cardiac failure, pulmonary edema, and cerebral bleeding are complications of severe cases, and half of all fatalities occur during this phase. Clinical recovery is initiated with the onset of the diuretic or polyuric phase in which a daily urine output of 3–6 liters is not uncommon. Lassitude and anorexia are common in this phase, which normally lasts a few days but can continue up to a few weeks due to dehydration, electrolyte imbalance, or infection. The convalescent phase generally lasts for 2–3 months with a progressive improvement in glomerular filtration rate, renal blood flow and urine concentrating ability. Clinical diagnosis is very good in endemic regions with experienced clinicians but HFRS may not be easily recognized if infected individuals suddenly present with symptoms in nonendemic areas.

HPS presents in four phases which parallel the five phases of HFRS: febrile, shock or pulmonary edema, diuretic and convalescent phases. The incubation period for HPS has been estimated to be 4–30 days and the febrile phase 1–12 days. The prodromal symptoms of HPS are similar to those of HFRS and include fever, chills, dizziness, headache, muscle aches, nausea and abdominal pain. A rapid progression to shock and pulmonary edema may ensue (within 4–24 h). At the onset of pulmonary edema, hematological findings include thrombocytopenia, left-shifted myeloid series and immunoblasts. Symptoms of pulmonary edema include tachypnea, exertional dyspnea and non-productive cough. Shock is indicated by hypotension and oliguria. Elevated creatinine and blood urea nitrogen reflect the degree of shock and hypovolemia, rather than renal disease as seen in HFRS. Microscopic hematuria and elevated liver enzymes are found in most cases. Capillary leakage in HPS patients is found exclusively in the lungs and large amounts of serum may accumulate in the airways. Death from HPS is due to shock and cardiac complications. Convalescence may be protracted with lung capacity diminished for many months.

Pathology and Histopathology

The onset of clinical symptoms in both HFRS and HPS patients coincides with specific immune re-

sponses. The most significant pathological event of HPS or HFRS is increased vascular permeability which appears to arise from endothelial damage. An immune component is probable for both HFRS and HPS, although how the shock in these diseases relates to factors such as viral distribution in vascular endothelia and immunological and chemical mediators of capillary permeability remains to be explained.

Autopsy specimens from HFRS patients show tubular damage in the kidneys as well as hemorrhagic necrosis in the renal medullary junctional zone, cardiac right atrium and anterior pituitary. Histopathological changes in the kidney correlate with severity and clinical staging of disease, beginning with congested subcortical medullary vessels in late febrile and hypotensive phases. Tubular damage progresses and late stage autopsies often reveal swollen kidneys with a hemorrhagic, congested medulla under a pale cortex. Tubular lesions, which include patterning and differentiation of epithelium with loss of the brush border and epithelial cell vacuolization, have been reported for both severe and mild HFRS. In the majority of HFRS patients studied between days 5 and 30 of disease, it was possible to identify cytoplasmic foci of viral glycoproteins in tubular epithelial cells and in sloughed cells displaying tubular degenerative changes. It was suggested that direct viral invasion of renal tubules may partly explain the pathogenesis of acute renal failure in HFRS.

Hemorrhagic manifestations are rare in HPS patients and necrotic lesions, other than those due to shock, are not seen. Histology of autopsy specimens shows moderate interstitial pneumonitis characterized by variable degrees of edema and an interstitial mononuclear cell infiltrate composed of a mixture of small and enlarged mononuclear cells with the appearance of immunoblasts. Focal hyaline membranes composed of condensed proteinaceous intra-alveolar edema fluid, fibrin and variable numbers of inflammatory cells are observed. Neutrophils are reported to be typically sparse and the alveolar pneumocytes are unaltered. Other commonly reported characteristics of HPS include immunoblasts within the splenic red pulp and periarteriolar white pulp, lymph nodal paracortical zones, hepatic portal triads, and in the peripheral blood.

In both HPS and HFRS, viral antigens are found extensively in endothelial cells of various tissues. This accumulation, however, does not coincide with obvious cytopathology.

Immune Response

Rodents with their characteristic chronic or persistent infections exhibit high levels of circulating antibody for life. Infectious virus can be readily demonstrated in antibody-rich sera of infected animals, suggesting that immune complexes are either infectious or readily dissociable under the conditions of infectious virus isolation.

In HFRS and HPS patients, immunoglobulin (Ig)M antibody appears rapidly after onset of disease; this is the most commonly used diagnostic indicator of these diseases. In a retrospective analysis of patient sera collected during the Korean conflict of 1951–1954, specific IgM antibodies were present in the first sera available (frequently as early as the first day of disease), and all patients had measurable IgM antibodies by day 7. IgG can also be detected relatively early in disease by ELISA with hantaviral antigen attached directly to the plate and specific anti-IgG reagents. The IFAT has historically been used to diagnose this disease and remains a reliable test for IgG but not IgM antibody. IgM persists for at least 3–6 months after infection, and IgG can be detected for years and possibly the life of the recovered patient. Both IgM and IgG can be virus-specific in their reaction with infected cell antigens. This can cause some concern that low-level reactivities might be missed in geographic areas where multiple hantaviruses overlap if sera are tested only against a single viral antigen. Antibody has been detected in the urine of HFRS patients and immune complexes may be important in disease; however, immune complex excretion appears sporadic and does not appear to correlate with clinical severity or prognosis.

Humoral immunity is probably the most important factor in protection from HFRS and HPS, as indicated by the ability to passively protect animals from hantavirus infection with neutralizing monoclonal antibodies. Thus, for vaccine development purposes, the elicitation of neutralizing antibodies is often regarded as a correlate of protection. Cell-mediated immunity may also play a role in protection or recovery from infection. Consistent with this was the finding that baculovirus-expressed nucleocapsid protein of HTNV protected hamsters from challenge with HTNV. Also, crossreactive cell-mediated immune responses to a number of hantaviruses were observed in mice. The type of cell-mediated response and its overall importance to disease prevention or recovery need to be further examined.

There is mounting evidence for an immune component of both HFRS and HPS. It is likely that much of the pathogenesis of both diseases is related to damage of vascular endothelial cells caused by the release of as yet undefined cytokines that are triggered by immune cells in response to the presence of viral antigens.

Prevention and Control of Infection

The risk factors for HFRS and HPS are clearly associated with exposure to infected rodent excreta. Although avoiding any rodent excrement may be difficult for persons who must work outdoors in endemic regions, knowledge of these significant risk factors can be very helpful in reducing aerosol transmission.

Direct exposure to infected laboratory rodents has been responsible for numerous infections and some deaths. Complete eradication of all infected animals, disinfection of the facilities, and replacement with uninfected animals from a clean source is the only accepted solution to a laboratory hantavirus infection problem. Animals reared under strict sanitary conditions involving delivery by cesarean section and foster nursing on known negative mothers has resulted in a good source of hantavirus-negative animals.

Inactivated cell-culture derived and rodent brain-derived vaccines for HFRS have been developed, tested, and are licensed for use in Asia. Preliminary information suggests that these vaccines are well-tolerated and that they can elicit antibody responses detectable by ELISA. Some of the vaccines also elicit neutralizing antibody responses in humans. Controlled efficacy studies have not been reported.

A recombinant vaccinia virus vaccine for HFRS was developed in the US and tested in phase I and phase II clinical trials. The vaccine elicited neutralizing antibodies in the majority of vaccinia-naive individuals but not in vaccinia preimmunes. Other genetically engineered vaccines are under development but have not yet been tested in humans.

Future Perspectives

The successful application of PCR technology to detect hantaviruses has greatly expanded our knowledge of the ecology and epidemiology of these viruses. Undoubtedly more hantaviruses remain to be discovered in as yet unstudied rodents. Additional oligonucleotide and amino acid sequence homology comparisons may provide insight into the genetic variation within the genus, the basis for variable virulence among the isolates, and evidence for genetic interaction between viruses, if it exists.

Because of the plenitude of different hantaviruses that cause human disease, it is likely that multivalent vaccines will be needed. Genetically engineered vaccines containing multiple hantavirus genes may most readily meet that need.

Finally, a reverse genetics system based on an infectious clone of a hantavirus would be invaluable for helping to describe specific aspects of viral replication. To date, such a system has proven elusive.

See also: ***Bunyaviridae*****: General features, Replication.**

Further Reading

Butler JC and Peters CJ (1996) Lessons learned from the hantaviruses and other hemorrhagic fever viruses. *Am. J. Med. Sci.* 311: 55.

Clement J, Heyman P, McKenna P, Colson P and Avsic-Zupanc T (1997) The hantaviruses of Europe: from the bedside to the bench. *Emerg. Infect. Dis.* 3: 205.

Plyusnin A, Vapalahti O and Vaheri A (1996) Hantaviruses: genome structure, expression and evolution. *J. Gen. Virol.* 77: 2677.

Schmaljohn C and Hjelle B (1997) Hantaviruses: a global disease problem. *Emerg. Infect. Dis.* 3: 95.

Schmaljohn CS and LeDuc JW (1998) *Bunyaviridae*. In: Collier L, Balows A, Sussman M and Mahy BWJ (eds) *Topley and Wilson's Microbiology and Microbial Infections*, 9th edn, vol. 1, p. 601. London: Arnold Press.

Hare Fibroma Virus *see* **Poxviruses**

Nassal M (1996) Hepatitis B virus morphogenesis. *Curr. Top. Microbiol. Immunol.* 214: 297.

Seeger C and Mason WS (1996) Replication of the hepatitis virus genome. In: dePamphilis E (ed.) *DNA Replication in Eukaryotic Cells*, p. 815. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Avian Hepatitis B Virus

William Mason, Fox Chase Cancer Center, Philadelphia, Pennsylvania, USA

Patricia Marion, Division of Gastroenterology, Stanford University Medical Center, Stanford, California, USA

History

At present, only two species of avian hepadnaviruses have been identified and well characterized: duck hepatitis B virus and heron hepatitis B virus. Duck hepatitis B virus was first discovered in 1979 in duck (*Anas domesticus platyrhynchos*) serum samples collected in the People's Republic of China. The discovery that ducks might be carriers of a hepadnavirus followed the observation that ducks in certain areas of China had a high incidence of primary hepatocellular carcinoma. This cancer was already known to be of common occurrence in humans infected with hepatitis B virus, which led to the idea that a similar virus might be present in ducks. Subsequent research has neither sustained nor refuted the possibility that infection contributed to the liver cancer that had been observed in the ducks, which may, instead, have been caused by environmental carcinogens.

A second avian hepadnavirus species was discovered in 1988 in the sera of grey herons (*Ardea cinerea*) from Germany. The discovery of this second virus was facilitated by the fact that its DNA genome hybridized with a radioactive probe prepared from the cloned genome of the duck hepatitis B virus, indicating that the two viruses share considerable DNA sequence homology, as was subsequently shown by comparing the complete sequences of the two viruses.

A preliminary report suggests the existence of an avian hepadnavirus, possibly distinct from the duck or heron viruses, in the Australian woodduck (*Chenonetta jubata*). A novel strain of duck hepatitis B virus has also been isolated from the Ross goose (H. Shi, J. Cullen, and J. Newbold, personal communication; GenBank accession no. M95589).

Classification

The *Hepadnaviridae* family has been divided into two genera: *Orthohepadnavirus*, of which human hepatitis B virus is the prototype, and *Avihepadnavirus*, of which duck hepatitis B virus is the prototype.

Properties of the Genome

All members of the *Hepadnaviridae* family have a partially double-stranded DNA genome of about 3 kb that is held in a circular conformation by a short cohesive overlap between the 5′ ends of the two DNA strands (**Fig. 1**). One strand is always complete in virus particles, whereas the second strand is incomplete, with a 3′ end that is heterogeneous in location. The incomplete strand is of plus polarity and the complete strand of negative polarity. When these viruses infect a cell, the plus strand is completed and the fully double-stranded DNA is then converted to a covalently-closed circular (CCC) molecule, which serves as a template for viral RNA synthesis (**Fig. 2**). The two genera of hepadnaviruses are thought to differ in that orthohepadnaviruses encode four open reading frames (ORFs) whereas the prototypic avihepadnavirus, duck hepatitis B virus, encodes only three, lacking the X ORF. This ORF appears to encode a protein that modulates transcription, possibly by acting on signal transduction pathways. There is now evidence for a fourth ORF in heron hepatitis B virus and in Ross goose virus, capable of coding for a 7–8 kDa protein, in the same genomic location as the C-terminal half of the X ORF of the orthohepadnaviruses. Functional studies on this ORF have not been reported. With one exception, the known products of the different ORFs are each translated from distinct mRNAs, transcribed from one of the four (*Orthohepadnavirus*) or three (*Avihepadnavirus*) viral promoters. The major nucleocapsid protein and the product of the polymerase gene appear to be encoded by the same mRNA, the pregenome, which also serves as the template for viral DNA synthesis. It was originally thought that mRNA splicing did not have a role in virus replication; however, it was recently found that the larger (L) of the two envelope proteins of duck hepatitis B virus is translated both from a spliced mRNA as well as from an unspliced mRNA. The spliced mRNA is transcribed from the major nucleocapsid promoter, located about 1200 bp upstream of the start codon for the large envelope protein. The unspliced mRNA is synthesized from a promoter located immediately upstream of the ORF for the large envelope protein (**Fig. 1**).

All hepadnaviruses replicate their DNA by reverse transcription of a viral RNA, a process which takes

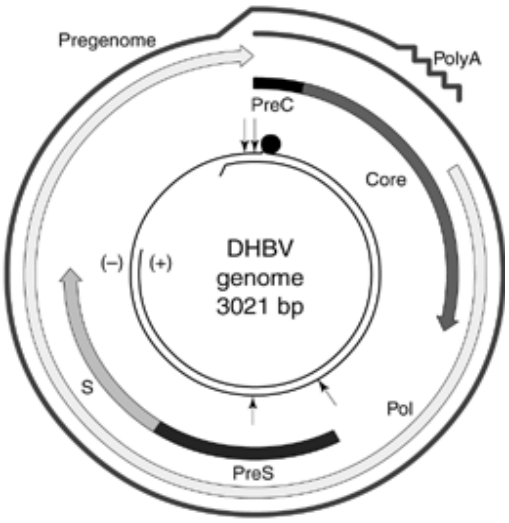


Figure 1 The DHBV genome. Duck hepatitis B virus (DHBV) the prototypic avihepadnavirus, has a 3021 bp genome which is held in a relaxed circular conformation by a short cohesive overlap between the 5′ ends of the two DNA strands. The minus strand is slightly larger than unit length, with a 9 base terminal redundancy. The plus strand is always less than unit length within virions. The viral pol gene product, a reverse transcriptase, is found covalently attached to the 5′ end of the minus-strand DNA, reflecting its dual role as a polymerase and as a protein primer of reverse transcription. A short, 17 base capped RNA, derived from the 5′ end of the pregenome (the template for reverse transcription) is covalently attached to the 5′ end of the plus strand. The three open reading frames are shown. The core reading frame encodes the 262 amino acid viral nucleocapsid subunit, while the PreC/core frame encodes a secretory protein (e-antigen). The pol reading frame encodes the 836 amino acid viral DNA polymerase, a reverse transcriptase. The PreS/S reading frame encodes the 364 amino acid L envelope protein, while the S region encodes the 167 amino acid S envelope protein. The start sites for transcription of the viral mRNAs are shown by arrows. The mRNA for the core protein is the pregenome mRNA, which also acts at a lower frequency as the mRNA for the reverse transcriptase. When reverse transcriptase is made, it binds to the 5′ end of its own message and directs it into the reverse transcription pathway. Reverse transcription takes place following packaging of the pregenome/polymerase complex into viral nucleocapsids. The pregenome is terminally redundant, as shown, by 270 bases. A second mRNA, initiating *c.* 30 bases upstream of the pregenomic mRNA, serves as the message for the PreC/core protein. The mRNA for L initiates just upstream of the open reading frames, while the S protein mRNA initiates within the PreS domain. All viral mRNAs end at the same polyadenylation site as the pregenome.

place within viral nucleocapsids found in the cytoplasm of the infected cell (**Fig. 2**). At the cellular level, infection can be chronic and productive, or it may be latent. Infections seen in the liver are generally productive, and evidence for true latent infection of hepatocytes has not so far been obtained. The reverse transcription process, observed in productively infected cells, differs mechanistically from that employed by members of the *Retroviridae* family. Following synthesis of viral DNA, the nucleocapsids are enveloped with a lipoprotein coat, possibly by budding into the endoplasmic reticulum.

Properties of the Proteins

For DHBV, the coat probably contains only two proteins, L and S, that appear to be both structurally and functionally important. L, the larger of the two, is transcribed from the same ORF as S, spans all of S, but initiates at an AUG further upstream in the ORF, so that it is almost twice as large as S. Some investigators believe that a third envelope protein, M, is also produced by this ORF, initiating at an AUG located between the start sites of L and S and extending to the end of S. The M protein is in fact produced by all orthohepadnaviruses. The protein domain unique to L would be referred to as PreS1, the domain common to L and M as PreS2, and the domain common to all three as S. Here, we keep to the convention that only two proteins, L and S, are produced; the PreS1 and PreS2 domains are combined to PreS. PreS sequences of L are often located on the external surface of the viral envelope, where they are believed to specify a critical step in viral host range by interacting with specific cell surface receptors. For a portion of the large envelope protein molecules the PreS sequences remain on the cytoplasmic side of the viral envelope, where they probably interact with the viral nucleocapsid to facilitate virion formation during budding.

Hepadnaviruses produce a large excess of the envelope proteins, above the amount needed for virion assembly, and the excess is secreted as surface antigen particles. The S protein appears to be sufficient for production of surface antigen by orthohepadnaviruses, though L can also be incorporated into these particles. Both L and S have been detected in surface antigen particles produced by duck hepatitis B virus, the prototype avihepadnavirus. The surface antigen particles produced by avihepadnaviruses are pleomorphic spheres, 40–60 nm in diameter, whereas orthohepadnaviruses produce regular, 22 nm diameter rods and spheres. The particles produced by the orthohepadnaviruses may have a much lower lipid content than those produced by the avihepadnaviruses, and in neither case has the mechanism of assembly been determined, although, as for the virions, the particles first appear in the cisternae of the endoplasmic reticulum.

In addition to the envelope proteins, DHBV produces 3 other proteins (**Fig. 1**). The 262 amino acid core protein forms the nucleocapsid shell. The

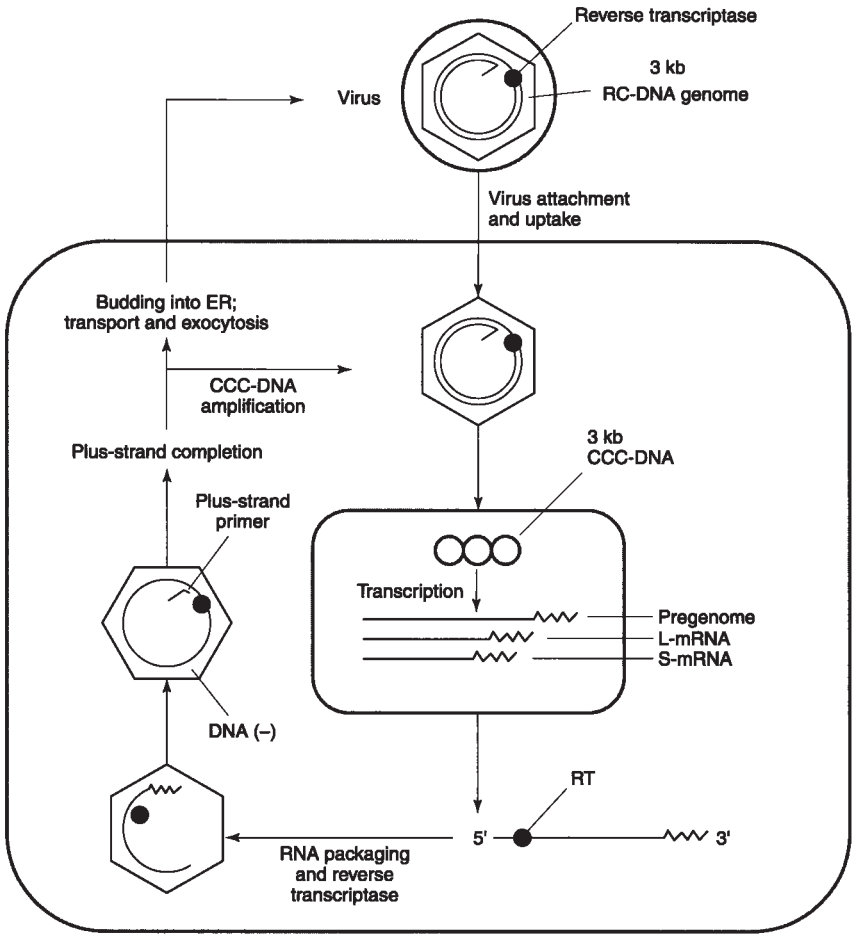


Figure 2 The replication of duck hepatitis B virus within hepatocytes. When virus, shown at the top, infects a cell, the DNA migrates to the nucleus and is processed to a unit length, covalently-closed circular species (CCC-DNA) which serves as the template for all viral mRNAs. If pol is translated from a pregenome mRNA, the translation product binds near to the 5′ end, to a stem loop structure called epsilon, and the protein–RNA complex is then packaged into nucleocapsids, composed of a core protein subunit, where reverse transcription actually takes place. Early in infection, newly made viral DNA is transported to the nucleus, resulting in an increase of the CCC-DNA copy number to *c.* 10–50. Accumulation of CCC-DNA is eventually shut down because the abundant synthesis of viral surface antigens leads to envelopment of mature nucleocapsids and subsequent secretion of the virus from the cell. For all hepadnaviruses, envelope proteins are made in vast excess over the amount used in virion assembly, with the excess being secreted in the form of noninfectious surface antigen particles. Overproduction of envelope proteins may be needed to effectively shut down CCC-DNA formation, as excess CCC-DNA accumulation can lead to cell death. ER, endoplasmic reticulum. RC, relaxed-circular.

polymerase, with protein priming, reverse transcriptase, and RNase H activity, carries out viral DNA synthesis. The 305 amino acid PreCore/core protein is processed and secreted from the infected cell, as the viral c-antigen. The function of the e-antigen is unknown. It has been conjectured that this core related protein protects infected cells from the antiviral immune response.

Geographic and Seasonal Distribution

Except for the Muscovy duck (*Cairina moschata*), most varieties of domestic ducks are thought to derive from the wild mallard (*Anas p. platyrhynchos*) and, despite wide differences in appearance due to domestication, belong to a single species. Duck hepatitis B virus has been found both in the wild mallard and in domestic flocks of this species throughout the world, but not in Muscovy ducks. The incidence of chronic infection in individual flocks can range from zero to 100%. Infections are primarily acquired congenitally, by virus passed into the egg laid by a viremic duck. Transmission by this route leads to a chronic viremia that is probably lifelong. Horizontal spread of infection may also occur. Since the virus is bloodborne, horizontal spread presumably occurs through

open wounds. Sexual modes of transmission have not been studied. Except during the first few days of life, horizontal spread would be expected to lead to a transient infection, with subsequent immunity, rather than to a chronic infection (see Pathogenicity and Clinical Features of Infection, below). In domestic flocks, the only seasonal effects that might be expected to arise would be attributable to breeding and the associated congenital transmission, together with the presence of hatchlings that would be more susceptible than older birds to chronic infection resulting from horizontal transmission.

The geographic and seasonal incidence of the heron hepatitis B virus has not been described.

Host Range and Virus Propagation

Duck hepatitis B virus infects wild mallards and related domestic ducks (*Anas platyrhynchos*). Transmission to the Muscovy duck and the chicken has not been successful, but the virus can be transmitted to domestic Embden and Toulouse geese and has also been detected as an apparently natural infection in domestic geese. The distribution of duck hepatitis B virus in geese and other waterfowl has not been studied in detail. The virus is not known to be infectious to mammals. The virus will infect and replicate in primary duck hepatocyte cultures, and can be transmitted to developing duck embryos. *In vivo*, hepatocytes produce infectious virus, which is released into the bloodstream, producing a persistent viremia in the chronic carrier.

The apparently limited host range of duck hepatitis B virus is probably due to a lack of cell surface receptors for the virus in nonsusceptible species. Indeed, the virus will replicate efficiently in a chicken hepatocellular carcinoma cell line transfected with the cloned viral genome, and even in transfected human hepatocellular carcinoma cell lines. Some of the viral promoters are relatively inactive in cell lines that are not of hepatocellular origin, suggesting that a lack of appropriate transcriptional factors may also limit viral host range or lead, following virus uptake, to a latent infection. Evidence for latent infection of lymphocytes by human and woodchuck hepatitis viruses in their respective hosts has been reported, but latent infections have not, so far, been demonstrated in the duck.

Transmission of heron hepatitis B virus has not yet been achieved in the laboratory. The virus does not infect ducks. As with the duck virus, replication of heron hepatitis B virus can be demonstrated following transfection of the cloned viral genome into hepatoma cells.

Genetics

The mutation rate of the avihepadnaviruses has not been measured, but would be expected to resemble that of the woodchuck hepadnavirus, which has been estimated to be $\leq 2 \times 10^{-4}$ base substitutions per site per year. This relatively low rate of genetic change is one or two orders of magnitude lower than that estimated for other viruses which lack polymerase-associated proofreading functions, including the retroviruses. The apparently low rate of mutation may reflect two factors: (1) constraints on the viabity of genetic changes that are imposed by the compact coding of genetic information by the hepadnaviruses, with multiple overlapping reading frames; (2) a low rate of turnover of infected hepatocytes and, accordingly, of the template for transcription of the RNA that is reverse transcribed into viral DNA.

Evolution

As there is amino acid sequence homology between hepadnaviruses and retroviruses in both the polymerase and core genes, it has been suggested that these viruses have a common origin, with hepadnaviruses arising from deletions of a retrovirus or retrovirus-like ancestor. These virus families also share a common genomic sequence: a conserved hepadnavirus sequence near the initiation site for minus-strand DNA synthesis is homologous over 67 nucleotides to the U5 region in retroviral long terminal repeats. On the basis of nucleotide and amino acid homologies, it has been proposed that the avihepadnaviruses and the orthohepadnaviruses diverged from a common ancestor around 30 000 years ago. The least conserved proteins among the hepadnaviruses are the preS regions of the surface protein, which are thought to be those involved in binding to the cellular receptors. As each of the hepadnaviruses is fairly species specific, variation in the preS region could be necessary to correspond to the host variation of cellular receptors.

Serologic Relationships and Variability

There are currently no defined subtypes or serotypes of duck hepatitis B virus, though there are monoclonal antibodies which distinguish between different amino acid sequences of the surface antigen. The amount of variation in nucleotide sequence in the published sequences of duck hepatitis B virus (1.2–11.6%) appears to resemble that of hepatitis B virus subtypes (8–10%). Sequences from three genomes cloned from ducks from the USA and Germany appear more closely related to each other (1.2–5.9% variation) than to five sequences cloned from ducks

from China (9.1–11.6% variation). The Pekin ducks in the USA and Germany are thought to have come from China about 140 years ago. Within the sequences cloned from ducks from China, there is variation ranging from 3.7 to 9.4%.

Five sequences of heron hepatitis B virus have been published. Up to 7% sequence diversity has been found in the heron hepatitis B virus isolates. The prototype heron hepatitis B virus sequence differs from duck hepatitis B virus sequences by 21.6%, somewhat more than the 16.4% variation between the woodchuck and ground squirrel hepatitis viruses.

Epidemiology

Congenital transmission to virtually all progeny of infected female ducks appears to be the major route for maintenance of duck hepatitis B virus infection in a natural setting. It is not known whether transmission to the embryo will occur when a viremic drake mates with a nonviremic duck. Preliminary observations suggest that transmission from infected males to progeny is, at best, a low frequency event. The virus accumulates in the yolk of eggs laid by viremic birds and infects the yolk sac, liver and pancreas of the developing embryo. This early exposure produces an immunological tolerance to the virus, and, in fact, there is no evidence of an immune-mediated liver disease, including hepatitis, in these ducks. A mild hepatitis has been observed when ducks are experimentally infected with duck hepatitis B virus. This does not seem to lead to the extensive lobular destruction, fibrosis, or cirrhosis associated with severe or long-term infections of humans by hepatitis B virus, and, although hepatocellular carcinomas with integrated viral DNA have been observed in infected ducks, a strong correlation between liver cancer and virus infection has not been found. The finding of integrated DNAs is perhaps not surprising if the cancers arise from infected hepatocytes, as spontaneous integration may be quite common during virus replication. It is presumed that the worldwide distribution of duck hepatitis B virus reflects its presence in the wild flocks from which the domestic were derived, as well as the intermingling and interbreeding that may occur between wild ducks and domestic ducks maintained in fields or pens that are not completely enclosed.

Compared to the situation with duck hepatitis B virus, virtually nothing is known about the epidemiology of heron hepatitis B virus. Approximately 30% of the grey herons examined in Germany were virus infected.

Transmission and Tissue Tropism

Duck hepatitis B virus, as already indicated, is transmitted congenitally and, in crowded conditions, horizontal spread has been observed, and presumably took place via a parenteral route. There is no evidence, however, that is either for or against other routes of transmission. Rather, it is generally assumed that transmission parallels that of the better studied human hepatitis B virus, that blood is the major source of virus and that introduction through the parenteral route is the only source of horizontal infection that is of any importance. In ducks, as in mammals, hepatocytes are the quantitatively most significant target, and in chronic carriers virtually every hepatocyte appears to be productively infected. Virus replication intermediates have also been detected in bile duct epithelium in the liver, in endocrine cells and scattered exocrine cells in the pancreas, and in tubular epithelium in the kidney. Except for the endocrine islets of the pancreas, which can be totally infected, infection appears to be limited in extrahepatic organs to only a few percent of the potential target cells, although the basis for this restriction in the presence of high titers of circulating virus is not known. There is also evidence for virus in germinal centers in the spleen, probably in dendritic cells (A. Jilbert, personal communication), although the results may be consistent with accumulation of viral components from the bloodstream rather than actual infections. In contrast to the mammalian hepadnaviruses, duck hepatitis B virus infection of lymphocytes has not been demonstrated. Cell-free preparations of virus derived from the blood of an infected duck are, indeed, highly infectious. The specific *in vivo* infectivity of fresh virus preparations is probably close to 1.

Heron hepatitis B virus also infects the liver and produces a viremia in its natural host, the grey heron. Modes of transmission remain to be characterized.

Pathogenicity and Clinical Features of Infection

Studies of pathogenicity in the infected duck have generally focused on the liver as the main target of infection. From this limited point of view, congenital infection is apathogenic. Therefore, infection with wild-type virus does not kill host cells in the absence of an immune response. Experimental infections of birds less than 1 week of age usually produces a chronic infection, with a mild to severe, transient, hepatitis. Infection of older ducks, in contrast, almost always produces a transient infection, followed by recovery and the development of a humoral immune

response to the virus envelope, with only a mild hepatitis. Infection has not been associated with a significant rise in liver enzymes in the bloodstream, or with any other clinical features, again suggesting that hepatocellular destruction is mild. It is not known if horizontal infection accelerates the development of secondary amyloidosis of the liver in ducks, a common condition in waterfowl which is seen in infected ducks, as well as in ducks that are not infected with duck hepatitis B virus.

Experiments with variants of duck hepatitis B virus have been performed to address the consequences of direct cytotoxicity of hepadnaviral infections. These variants were mutated so that the transcriptional template (**Fig. 2**) would be overproduced, a situation which leads to cell death. Infection of ducks with the variants led to severe but transient liver disease, followed by repopulation of the liver with spontaneously arising viral mutants that exhibited wild-type behavior. Thus, there is a strong selection against variants that are cytopathic in hepatocytes.

Pathogenicity due to extrahepatic virus infection would not, for the most part, be expected to be clinically important, as so few cells are infected. Only in the endocrine pancreas are the majority of parenchymal cells infected. An effect on β islets has, in fact, been observed in ducks chronically infected by the congenital route. In these ducks there is a marked deficiency in the rise in plasma insulin levels normally seen in response to arginine, a physiological secretagogue.

Pathology and Histobiology

Laboratory infection of ducklings or older birds can produce a variable degree of hepatic injury, usually mild, including portal hepatitis with penetration of the limiting plate by mononuclear lymphoid cells and, occasionally, lobular hepatitis. Portal hepatitis, by itself, is quite common in domestic ducks, irrespective of exposure to duck hepatitis B virus, whereas a periportal or lobular hepatitis can be correlated with experimental viral infection. Piecemeal and focal necrosis have also been observed, as have occasional apoptotic (eosinophilic) bodies. Chronic persistent and chronic active hepatitis have not so far been observed as a response to infection in a controlled setting. Histopathologic features have not been described that distinguish transient from chronic infection resulting from experimental inoculation of duck hepatitis B virus. The deficiency in insulin secretion reported in congenitally infected ducks is not due to an overt pancreatitis and is more probably a direct consequence of virus infection (i.e. cytotoxicity).

Immune Response

A humoral immune response to viral nucleocapsid antigens has not been detected in congenitally infected ducks. In contrast, antibodies to determinants of the viral nucleocapsid have been detected in ducks that have become chronically infected as a result of experimental inoculation, and an immune response to epitopes of viral coat proteins eliciting both neutralizing and nonneutralizing antibody has been detected in ducks recovering from a transient infection. Presently four epitopes on the DHBV preS protein region and one on the S protein are known to be involved in neutralization of virus infection in *in vitro* studies, and antibody to some of the PreS epitopes can significantly reduce the level of viremia and extend its onset in experimental infection of ducklings.

The presence of a mononuclear infiltrate (hepatitis) in the liver during transient infection suggests a T cell recognition of viral antigens, though the actual targets are unknown. Antibodies to viral coat, core and *pol* proteins might also function in antibody-dependent cellular toxicity. The observation that infections can rapidly resolve, even after infection of up to 70–80% of the hepatocytes, but without evidence of major liver damage, raises questions about the quantitative importance of killing of infected cells in the large-scale disappearance of virus from the host. Experiments with hepatitis B virus transgenic mice suggest that cytokines produced by the cellular immune response may induce degradation of viral DNAs and proteins that are present in the cytoplasm of infected cells. It is speculated that the transcriptionally active DNA in the nucleus is lost as infected hepatocytes proliferate to replace those cells killed by the antiviral response. It is not clear if neutralizing antibodies are needed for elimination of an infection.

Prevention and Control

Because duck hepatitis B virus has not been demonstrated to cause morbidity, mortality or even minor adverse effects on the health and viability of domestic ducks, a serious effort at primary prevention (i.e. vaccination) has not seemed warranted. However, because the *in vivo* replication of the duck hepatitis B virus is virtually identical to that of human hepatitis B virus, this animal model has been used to study antiviral agents and strategies that might eventually be tried in humans. Selected nucleoside analogues have been shown to have good specificity against virus DNA synthesis, mediated by a viral encoded reverse transcriptase, and to cause a reduction of viremia and a drop in hepatic levels of replicating viral DNA.

With one exception, prolonged (3–6 months) administration of inhibitors of viral DNA synthesis has not been found to significantly reduce the number of infected hepatocytes from the >95% characteristic of chronic infection. One nucleoside analogue, 2′-carbodeoxyguanosine, caused a 10–100-fold reduction in the number of infected hepatocytes in about a month, an effect that could be attributed to the hepatotoxicity of the analogue rather than to inhibition of virus replication *per se*. The spontaneous and complete recovery usually seen following virus inoculation of adult ducks implies that a more effective treatment than has been found, so far, is theoretically possible. The main issue is whether this can be achieved in the absence of a major contribution from the immune system of the host or if, in a duck or human carrier infected at an early age, and perhaps tolerant to some viral antigens, particularly those that are targets for neutralization, a life-time of multidrug therapy might be required to control the infection (i.e. keep the hepatocyte population substantially, but not 100%, virus free).

Future Perspectives

Though a vaccine effective against human hepatitis B virus has been produced for more than a decade, availability or delivery have not been universal, and perinatal and transfusion-mediated infections still remain common, as do infections among intravenous drug users. For infections acquired in adult life, interferon therapy has demonstrated some promise, and the hope remains that immune modulators and/or combined therapy with direct inhibitors of viral replication can be utilized to produce even greater efficacy. Unfortunately, infections acquired perinatally or during childhood do not respond well to interferon therapy, and new treatment strategies are needed for this population group which, worldwide, may number in excess of 1×10^8. The chronically infected duck remains as a particularly attractive model for chemotherapeutic approaches to antiviral therapy, especially because of the ready availability and simplicity of housing and handling of infected animals. Moreover, for the duck hepatitis B virus there is a simple and dependable cell culture system in which to evaluate antiviral strategies before initiating *in vivo* trials.

Some of the same considerations discussed above have made the duck hepatitis B virus a favorite of molecular biologists interested in modes of hepadnavirus replication. The role of reverse transcription in hepadnavirus DNA synthesis was first deduced with duck hepatitis B virus, and subsequent studies with this virus have revealed not only the fine details of genome DNA synthesis but also the role of cytoplasmic viral DNA synthesis in amplification to 10–50 copies per cell, of CCC-DNA, the nuclear template for viral RNA synthesis (**Fig. 2**). Recent studies have also shown how CCC-DNA copy number is controlled through a feedback mechanism employing the viral L and S proteins (**Fig. 2**). In the near future, we should expect ongoing research to produce a detailed picture of all the major intracellular processes required to maintain an infection that is both chronic and productive.

See also: **Hepadnaviruses (*Hepadnaviridae*): Hepatitis B Virus: General features, Molecular biology.**

Further Reading

Fields BN, Knipe DM, Howley PM *et al.* (eds) (1996) *Fields Virology*, 3rd edn. Philadelphia: Lippincott-Raven.

Gong SS, Jensen AD and Rogler CE (1996) Loss and acquisition of duck hepatitis B virus integrations in lineages of LMH-D2 chicken hepatoma cells. *J. Virol.* 70: 2000.

Lenhoff RJ and Summers J (1994) Construction of avian hepadnavirus variants with enhanced replication and cytopathicity in primary hepatocytes. *J. Virol.* 68: 5706.

Luscombe C, Pedersen J, Uren E and Locarnini S (1996) Long-term ganciclovir chemotherapy for congenital duck hepatitis B virus infection *in vivo*: effect on intrahepatic-viral DNA, RNA, and protein expression *Hepatology* 24: 766.

Marion PL (1988) Use of animal models to study hepatitis B virus. In: *Progress in Medical Virology*. Basel: Karger.

Obert S, Zachmann-Brand B, Deindl E *et al.* (1996) A spliced hepadnavirus RNA that is essential for virus replication. *EMBO J.* 15: 2565.

Robinson WS (1980) A virus in Beechey ground squirrels that is related to hepatitis B virus of man. *Proc. Natl Acad. Sci. USA* 77: 2941.

Mason WS, Seal G and Summers J (1980) Virus of Pekin ducks with structural and biological relatedness to human hepatitis B virus. *J. Virol.* 36: 829.

Miller RH and Robinson WS (1986) Common evolutionary origin of hepatitis B virus and retroviruses. *Proc. Natl Acad. Sci. USA* 83: 2531.

Robinson WS (1990) Biology of human hepatitis viruses. In: Zakim D and Boyer T (eds) *Hepatology: A Textbook of Liver Disease*. Philadelphia: WB Saunders.

Robinson WS (1990) *Hepadnaviridae* and their replication. In: Fields BN *et al.* (eds) *Virology*, 2nd edn. New York: Raven Press.

Summers J, Smolec JM and Snyder R (1978) A virus similar to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks. *Proc. Natl Acad. Sci. USA* 75: 4533.

Szmuness W (1978) Hepatocellular carcinoma and the hepatitis B virus: evidence for a causal association. *Prog. Med. Virol.* 24: 40.

[This article is reproduced from the 1st edn (1994).]

Molecular Biology

Christoph Seeger, Fox Chase Cancer Center, Philadelphia, Pennsylvania, USA

Properties of the Virion

The virion or 'Dane particle' of human hepatitis B virus (HBV) appears as a spherical structure with a diameter of approximately 42–47 nm. Within the sphere is an electron-dense core with an estimated diameter of 22–25 nm. The viral envelope consists of a lipid bilayer containing three polypeptides, termed large (L), middle (M) and small (S) surface protein (also known as pre-S1, pre-S2 and HBsAg). Incubation of the virion with nonionic detergent releases the nucleocapsid (core particle), which contains the 3.2 kb-long, partially double-stranded viral DNA genome and the viral DNA polymerase, a reverse transcriptase. The nucleocapsid is composed of 240 monomers of core protein (core antigen, HBcAg) arranged to form an icosahedral structure with a triangulation number $T = 4$. Dane particles and subviral core particles present in infected hepatocytes exhibit DNA polymerase (endogenous polymerase) and protein kinase activities. Although the former can add nucleotides to the 3′ ends of the incomplete positive-strand DNA, the latter phosphorylates predominantly serine residues at C-termini of core polypeptides.

In HBV-infected patients the titer of virions can vary from 10^3 to 10^9 per milliliter of blood. In addition to Dane-particles, sera of infected patients also contain nucleic acid-free, lipid-containing sphere- and rod-like (filamentous) subviral structures composed largely of S and some M protein. The spheres have a diameter of about 22 nm and the rod-like structures are approximately 20 nm wide and vary in length from approximately 20 nm to 200 nm. The concentration of these subviral particles can exceed that of virions by a factor of 10^3–10^5.

The rodent hepadnaviruses, woodchuck hepatitis virus (WHV) and ground squirrel hepatitis virus (GSHV) share their principal ultrastructural features with those described for HBV and, with HBV, are within the genus *Orthohepadnavirus*. The DNA-containing virus particles are somewhat larger than the Dane particles, reaching 47–55 nm in diameter. Subviral filamentous forms in sera of GSHV infected Beechey ground squirrels are more abundant and about three times longer than their HBV counterparts.

Properties of the Genome

The mammalian hepadnaviruses have a partially double-stranded, 3.2–3.3 kb-long DNA genome held in a circular conformation by an approximately 220 nucleotide-long cohesive overlap demarcated by the 5′ ends of the two DNA strands (**Fig. 1**). Whereas negative-strand DNA contains a complete copy of the viral genome, and hence, is referred to as 'the complete DNA strand', the positive-strand DNA is incomplete, only extending on average to approximately 60% of the length of negative-strand DNA. The 5′ end of negative-strand DNA is covalently linked to a tyrosine residue located near the N terminus of the viral polymerase polypeptide. The 5′ end of the positive-strand DNA is linked to an approximately 18 nucleotide-long RNA sequence, which is derived from the 5′ end of pregenomic RNA. Negative-strand DNA is the template for the synthesis of the viral mRNA transcripts and bears four known open reading frames (ORFs) encoding the core, polymerase, envelope and X genes.

The genomes of WHV and GSHV have the same general structural properties as those described for HBV. The length of their genomes exceeds that of HBV by approximately 140 nucleotides due to additional nucleotide sequences in the region encoding the N-terminal domain of the L envelope protein. Whereas WHV and GSHV share 82% nucleotide sequence homology, HBV and the rodent viruses exhibit approximately 55% sequence identity. The

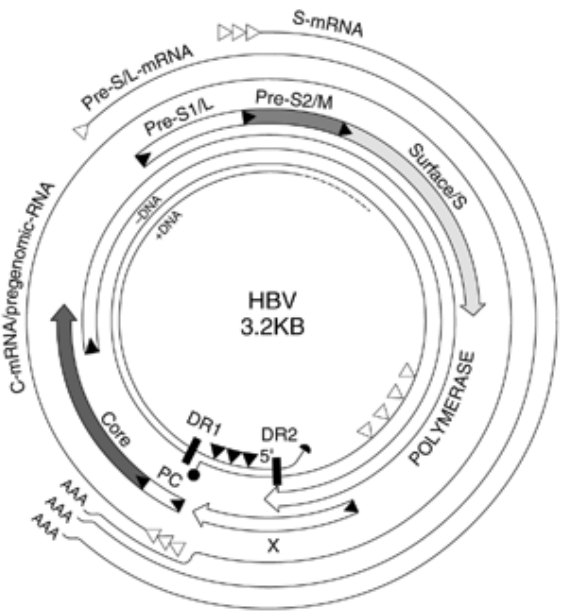


Figure 1 Physical map of the hepatitis B virus (HBV) genome. The inner circle depicts the viral genome with the complete, 3.2 kb-long, negative-strand DNA and the incomplete positive-strand DNA. The viral polymerase covalently linked to the 5′ end of the negative-strand is indicated with a filled circle and the cap-structure at the 5′ end of the RNA oligomer attached to the 5′ end of positive-strand DNA with a solid half circle. Interrupted lines show the heterogeneous 3′ ends of positive-strand DNA. The sites for initiation of viral DNA synthesis (DR1 and DR2) are shown as filled rectangles. Open and solid triangles illustrate the positions of the two transcriptional enhancer elements, enhancer I and II, respectively. The viral genome is surrounded by the viral coding regions, which are depicted by four circular arrows (for details see text). The initiation sites for the translation of the seven known viral gene products are indicated with the filled triangles. (PC, precore). The three major viral RNA species are shown in the outer circle. The 5′ and 3′ termini of individual transcripts are delineated with open rectangles and the letters AAA, respectively. RNAs with multiple initiation sites are marked with three symbols at their 5′ ends.

GC content of the mammalian hepadnavirus genomes is about 48%.

Physical Properties

Infectious virions (Dane particles) have a buoyant density of 1.24–1.26 g cm^{-3} in CsCl and a $s_{20,w}$ of 280S. The density of intracellular core particles is 1.36 g cm^{-3}. Subviral HBsAg particles, in which lipid accounts for approximately 25% of their weight, have a density of 1.18 g cm^{-3} in CsCl. The viral nucleic acid is a circular partially double-stranded DNA molecule with a mol. wt of about 1.6×10^6 kDa and a $s_{20,w}$ of 15S. Virions are inactivated following incubation in formaldehyde (1:2000) at 37°C for 72 h, heat treatment at 98°C for 2 min or incubation in 2% glutaraldehyde at room temperature for 5 min.

Properties of the Viral Proteins

The four protein-coding regions of the mammalian hepadnaviruses are translated into seven known proteins (**Fig. 1**). The core ORF codes for the 21 kDa viral capsid protein, also called hepatitis B core antigen (HBcAg) and the 16–18 kDa precore polypeptide, termed hepatitis B e-antigen (HBeAg). The HBcAg is the most highly conserved polypeptide among the mammalian hepadnaviruses showing 68% amino acid homology between HBV and GSHV (GSHcAg) and 92% between GSHV and WHV (WHcAg). It is a cytoplasmic and nuclear phosphoprotein with a basic C-terminus that is essential for the packaging of viral RNA. Core polypeptide monomers form dimers that assemble into spherical shells with icosahedral symmetry consisting of 120 dimer subunits. The formation of these dimers is mediated by two long alpha helices that form spikes on the surface of nucleocapsids. Dimers are further stabilized through the formation of two homologous intermolecular disulfide bridges.

The HBeAg is a secreted protein of unknown function that is antigenically distinct from HBcAg. This polypeptide is translated from an initiation codon preceding the core AUG in the same phase. The primary translation product is proteolytically processed resulting in the removal of 10 and 34 amino acids from its N- and C-terminus, respectively.

The surface or envelope ORF encodes the co-C-terminal proteins of 39 kDa (L, pre-S1), 31 kDa (M, pre-S2) and 24 kDa (S, HBsAg). S is an integral transmembrane protein that is present in both unglycosylated and glycosylated (gp27) forms and bears the signals necessary for assembly into 42 nm viral and 20 nm subviral particles, a process leading to the formation of disulfide bridges between the S components. The S proteins of the mammalian hepadnaviruses show strong antigenic crossreactivity and reveal amino acid homologies of 61% between HBV and GSHV and 90% between WHV and GSHV. The S polypeptides of WHV and GSHV have a molecular weight of about 22 kDa and are, as in HBV, present in both unglycosylated and glycosylated forms.

The M and L proteins are translated from AUG initiation codons located upstream of the initiation site for the translation of S. Hence, these polypeptides share their C-termini with S. The L and M proteins are expressed at a level of approximately 5–15% and 1–2% compared to S. Whereas M protein is present in both viral and subviral structures the L protein is

primarily found in Dane particles. The ratio of L:M:S in 42 nm Dane particles is about 1:1:4. In contrast, 20 nm subviral structures lack L and consist mainly of S and some M polypeptides. Like S, both L and M proteins contain N-linked oligosaccharides (gp33 and gp42). Glycosylation sites have been localized to the S region and to the N-terminal domain of the M polypeptide. The L polypeptide is further modified with myristic acid at its N terminus. The function of the M protein is obscure, but L protein is believed to carry the binding site for the viral receptor on susceptible cells.

All three envelope components are integral transmembrane proteins with at least two, but possibly four, membrane-spanning domains present in the S domain. Both N- and C-termini of L, M and S are exposed on the outside of virions. In the absence of S, L is retained in membranes of the endoplasmic reticulum with its N-terminus on the cytoplasmic site of the membranes. Since the N-terminal domain of L is on the outside of mature virions, at least a fraction of L proteins must undergo a conformational change during viral morphogenesis. The L and M specific domains of HBV lack 43 amino acids compared to their GSHV and WHV counterparts and represent the least conserved regions between the mammalian hepadnaviruses.

The polymerase ORF encodes the viral reverse transcriptase. Based on the length of its coding region, the primary polymerase translation product has a predicted molecular weight of about 90 kDa. The polypeptide consists of two domains that are separated by a tether region. Whereas the longer C-terminal domain contains sequence motifs characteristic of other known reverse transcriptases, the shorter N-terminal domain is, so far, unique to hepadnaviruses. It plays a critical role in the priming of viral DNA synthesis. The polymerase exhibits at least four enzymatic activities, which are required for the synthesis of the viral DNA genome from an RNA intermediate, called the pregenome. They include RNA- and DNA-directed DNA polymerase and RNAseH activities. Furthermore, the polymerase acts as a primer for reverse transcription of negative (first)-strand DNA. Enzymatic activity of the DNA polymerase has been demonstrated in permeabilized Dane particles (endogenous reaction), core particles isolated from infected liver tissue and with polymerase polypeptides expressed in insect cells and frog oocytes.

The X ORF encodes a 17 kDa protein, termed X, whose function for viral replication is not known. X is expressed in livers of WHV-infected woodchucks and is essential for viral replication *in vivo,* but dispensable for replication in tissue culture cells. Experiments in tissue culture cells revealed a plethora of functions for X. It can activate many viral and cellular promoters as well as several signal transduction pathways. Other activities include interactions with the tumor suppressor p53, the UV-damaged DNA binding protein and a subunit of the proteasome. In addition, overexpression of X in certain lineages of transgenic mice can lead to the formation of liver neoplasms. Next to the L region of the envelope products, the X protein is the least conserved protein among the hepadnaviruses with 33% amino acid homology between GSHV and HBV and 71% between the two rodent isolates.

Replication, Assembly and Release

HBV replication occurs primarily in hepatocytes. Circumstantial evidence suggested that tissue tropism is determined by the viral receptor. However, the nature of the receptor is still not known. Since uptake of virus in cultured cells is restricted to primary hepatocyte cultures it is likely that the expression of the putative receptor, or a component thereof, is suppressed in immortalized cell lines. In addition, tissue tropism is determined by liver specific transcription factors that orchestrate viral gene expression. A few differentiated hepatoma cell lines can support viral gene expression and produce infectious virus following their transfection with plasmids containing intact viral genomes.

Following infection and uncoating of the viral envelope, the relaxed circular (RC) DNA genome is transported by an unknown mechanism into the cell nucleus. During this step, RC DNA is converted into a covalently closed circular (CCC) molecule (**Fig. 2**). The 3.2 kb-long CCC DNA is the template for the transcription of a 3.4 kb-long RNA species, termed pregenomic (pg) RNA. pgRNA has redundant 5′ and 3′ ends and short sequence repetitions (DR1, DR2), one of which maps to the redundant termini (DR1). The RNA is packaged together with polymerase into core particles where it serves as a template for reverse transcription of negative-strand DNA. RNA packaging depends on the binding of the polymerase to a signal, termed epsilon, located near the 5′ end of pgRNA. This interaction also requires cellular proteins that include the molecular chaperone heat shock protein 90. The formation of this RNA–protein complex is believed to trigger assembly of nucleocapsids.

Negative-strand DNA synthesis is primed by a tyrosine residue located near the N-terminus of the reverse transcriptase. The protein-priming reaction leads to the formation of a phosphodiester bond between the tyrosine residue and the first nucleotide

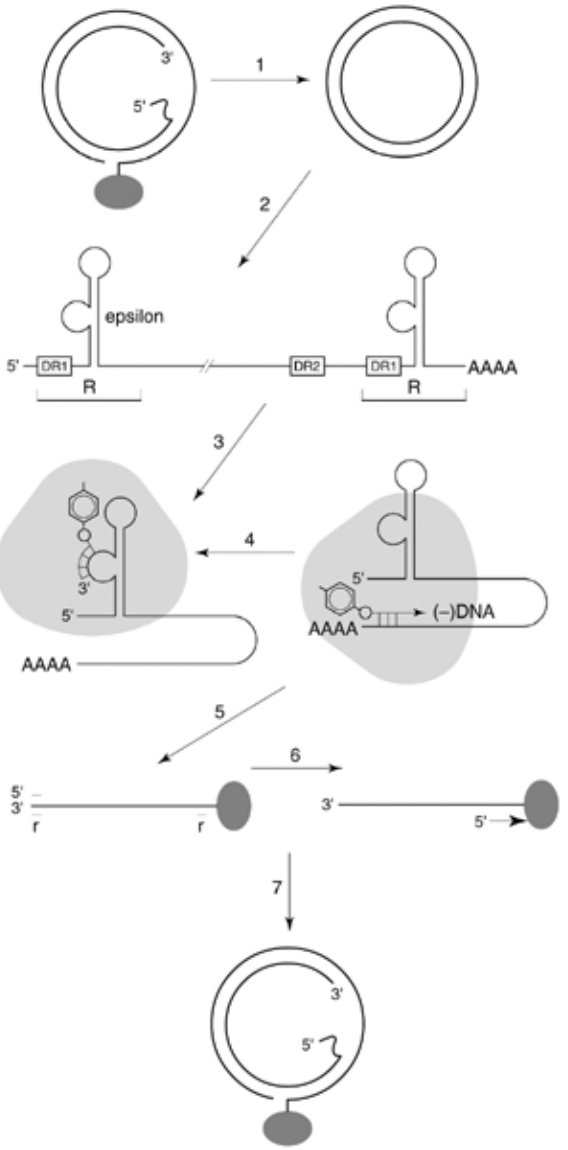


Figure 2 Model for the replication of the hepatitis B virus (HBV) genome. Relaxed circular (RC) virion DNA is converted into covalently closed circular (CCC) DNA (1), the template for transcription of pgRNA (2). The terminal redundancy (R), the packaging signal, termed epsilon, and the position of the direct repeats DR1 and DR2 on pgRNA are indicated. The polymerase (shaded oval) binds to epsilon at the 5′ end of the pregenome to prime negative-strand DNA synthesis from a tyrosine residue on the reverse transcriptase (3). Following a template switch to the 3′ end of the RNA template, DNA synthesis continues toward the 5′ end of pgRNA (4). The result is a negative-strand DNA with a short terminal redundancy (r) that remains covalently attached to the polymerase (5). An RNA oligomer spanning the 5′ end of pgRNA including DR1 is transferred to an internal acceptor site (DR2) on negative-strand DNA to prime positive-strand DNA synthesis (6). Following the synthesis of positive strands to the 5′ end of negative strand DNA a template switch, facilitated by the r region, is required to continue DNA synthesis and leads to the formation of RC DNA (7).

of negative-strand DNA and the synthesis of a 3–4 nucleotide-long DNA strand. The template for this reaction is the RNA packaging signal epsilon (**Fig. 2**). Following a template switch, the reverse transcriptase continues to elongate DNA from DR1 near the 3′ end of pgRNA. After the completion of negative-strand DNA synthesis, a capped RNA oligomer originating from the 5′ end of pgRNA containing a copy of DR1 is transferred to DR2 on negative-strand DNA, where it functions as a primer for positive-strand DNA synthesis. Once the nascent positive-strand reaches the 5′ end of negative-strand DNA, a template switch to the 3′ end of negative-strand DNA is necessary to continue DNA synthesis. Facilitated by a short redundancy at the ends of negative-strand DNA, this strand transfer accounts for the formation of the relaxed circular structure of virion DNA.

During viral DNA synthesis, core particles gain the ability to interact with L envelope polypeptides retained in membranes of the endoplasmic reticulum. This step leads to the translocation of cores into the lumen of the endoplasmic reticulum and to the assembly with the viral envelope components. Viral particles assemble into mature Dane particles as they pass through the secretory pathway. After they have traversed the Golgi, they exit the infected hepatocyte through a budding step. Aggregates of S antigen can enter the same pathway independently of nucleocapsids, which leads to the secretion of 20 nm subviral particles, the major viral components present in sera of HBV-infected patients.

Inferring from studies with an avian hepadnavirus, intracellular core particles can also enter an alternate pathway leading to the amplification of CCC DNA in the nucleus of the infected hepatocyte. Presumably, cores are redirected into the same pathway used for the formation of CCC DNA during infection. This mechanism is believed to play a major role in the maintenance of viral DNA in infected hepatocytes and thus, for the observed persistence of viral infection.

Transcription

CCC DNA is the template for the transcription of the viral RNAs. In HBV-infected liver, three classes of unspliced viral transcripts have been identified with 5′ ends located near the beginning of the precore, L and M ORFs (**Fig. 1**). All three transcripts terminate at a common polyadenylation site present in the HBcAg coding region. Transport of the unspliced mRNAs from the nucleus into the cytoplasm appears to depend on sequences located between the surface and X genes. It has been suggested that this region bears a signal similar to the so-called rev response

element (RRE) on human immunodeficiency virus (HIV). However, a protein factor that binds to this signal on HBV RNA remains to be identified.

The 3.4 kb transcript spans the entire genome and has terminally redundant ends. In addition to its role in DNA replication, it serves as mRNA for the translation of the HBcAg and the reverse transcriptase and is referred to as pgRNA or C-mRNA. A subspecies of C-mRNA with a short extension at its 5′ end is the template for the translation of the precore polypeptide (HBeAg). The 2.4 kb-long transcript, termed L- or preS-mRNA, directs the translation of the L protein. This transcript appears to be unique to HBV since it has, so far, not been detected in WHV- or GSHV-infected liver tissue. The 2.1 kb-long S-mRNA has heterogeneous 5′ ends that bracket the initiation codon for the M-protein. S-mRNA transcripts that initiate upstream of the translational start signal lead to the synthesis of the M polypeptide, whereas the shorter transcripts serve as templates for the synthesis of the S-protein. A fourth mRNA that could serve as the template for the translation of the X protein has been identified in tissue culture cells transfected with subgenomic HBV fragments. So far, an X-mRNA has not been identified in RNA purified from HBV-, WHV- or GSHV-infected livers. Transcription of the three mRNAs is controlled by distinct promoters located adjacent to the 5′ ends of each mRNA species and two enhancers (enhancer I and II) overlapping with the 3′ end of the polymerase gene and the X coding region (**Fig. 1**). Enhancer I bears binding sites for the transcription factors C/EBP, NF-1, HNF-3, RARE and EF-C and enhancer II for HNF-3, Sp1 and C/EBP.

Translation

Translation of the precore, core, preS1 and S transcripts occurs at the 5′ proximal AUG initiation codon of their respective mRNA templates. In contrast, translation of the polymerase polypeptide begins at an internal AUG codon on C-mRNA (**Fig. 1**). Expression of the polymerase is cap-dependent, suggesting that translation occurs by ribosomal scanning and not by internal landing of ribosomes upstream of the polymerase ORF. Translation of the X polypeptide most likely occurs from the first AUG present in this coding region. However, information on the exact mechanism of X translation *in vivo* depends on the identification of the mRNA used for X expression. As mentioned under Transcription, an mRNA for the expression of the L protein has so far not been identified in WHV or GSHV infected livers. Therefore, it is possible that the L protein of the rodent viruses is translated from C-mRNA by a mechanism similar to that postulated for the expression of the polymerase gene. Whereas translation of the three envelope proteins occurs on membranes of the endoplasmic reticulum the synthesis of the core, polymerase and X proteins takes place on free ribosomes in the cytoplasm of infected cells.

Role of Hepatitis B Virus in Liver Cancer

A consequence of chronic HBV infection is the development of hepatocellular carcinoma (HCC). Although viral DNA integration is not required for genome replication in hepadnaviruses, the large majority of tumors contain viral DNA sequences inserted into chromosomal DNA. The mechanism for DNA integration is not known. The pattern of integrated viral DNA generally reflects a clonal origin of the tumor and indicates that DNA integration is an early event in the development of HCC. Therefore, it has been proposed that viral DNA integration could play an active role in the progression of liver cancer, i.e. through the activation of proto-oncogenes as it occurs with certain retroviruses. Contrary to expectations, extensive analyses of DNA samples obtained from human HCCs failed to reveal any common integration sites for HBV sequences. However, analyses of DNA samples obtained from woodchuck HCCs identified a pseudogene of N-*myc* 2 as a major target for viral DNA integrations in this animal model.

In addition to viral DNA integration, expression of viral genes has also been considered to play a role in HCC formation. Spurred by a report demonstrating the occurrence of liver neoplasms in transgenic mice expressing the X polypeptide, much attention has been directed to understanding the function of this protein in tissue culture cells. As described under Properties of Viral Proteins, X can exhibit many different functions under selected assay conditions and, at present, it is not clear which, if any, of these properties contribute to HCC formation during a natural infection.

See also: **Hepadnaviruses (*Hepadnaviridae*): Avian hepatitis B virus; General features; Hepatitis A virus (*Picornaviridae*); Hepatitis C virus (*Flaviviridae*); Hepatitis Delta virus; Hepatitis E virus; Pathogenesis: Animal viruses.**

Further Reading

Ganem D (1996) *Hepadnaviridae*: the viruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al Fields Virology*, 3rd edn. p. 2703. Philadelphia: Lippincott-Raven.

Nassal M (1996) Hepatitis B virus morphogenesis. *Curr. Top. Microbiol. Immunol.* 214: 297.

Seeger C and Mason WS (1996) Replication of the hepatitis virus genome. In: dePamphilis E (ed.) *DNA Replication in Eukaryotic Cells*, p. 815. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Avian Hepatitis B Virus

William Mason, Fox Chase Cancer Center, Philadelphia, Pennsylvania, USA

Patricia Marion, Division of Gastroenterology, Stanford University Medical Center, Stanford, California, USA

History

At present, only two species of avian hepadnaviruses have been identified and well characterized: duck hepatitis B virus and heron hepatitis B virus. Duck hepatitis B virus was first discovered in 1979 in duck (*Anas domesticus platyrhynchos*) serum samples collected in the People's Republic of China. The discovery that ducks might be carriers of a hepadnavirus followed the observation that ducks in certain areas of China had a high incidence of primary hepatocellular carcinoma. This cancer was already known to be of common occurrence in humans infected with hepatitis B virus, which led to the idea that a similar virus might be present in ducks. Subsequent research has neither sustained nor refuted the possibility that infection contributed to the liver cancer that had been observed in the ducks, which may, instead, have been caused by environmental carcinogens.

A second avian hepadnavirus species was discovered in 1988 in the sera of grey herons (*Ardea cinerea*) from Germany. The discovery of this second virus was facilitated by the fact that its DNA genome hybridized with a radioactive probe prepared from the cloned genome of the duck hepatitis B virus, indicating that the two viruses share considerable DNA sequence homology, as was subsequently shown by comparing the complete sequences of the two viruses.

A preliminary report suggests the existence of an avian hepadnavirus, possibly distinct from the duck or heron viruses, in the Australian woodduck (*Chenonetta jubata*). A novel strain of duck hepatitis B virus has also been isolated from the Ross goose (H. Shi, J. Cullen, and J. Newbold, personal communication; GenBank accession no. M95589).

Classification

The *Hepadnaviridae* family has been divided into two genera: *Orthohepadnavirus*, of which human hepatitis B virus is the prototype, and *Avihepadnavirus*, of which duck hepatitis B virus is the prototype.

Properties of the Genome

All members of the *Hepadnaviridae* family have a partially double-stranded DNA genome of about 3 kb that is held in a circular conformation by a short cohesive overlap between the 5′ ends of the two DNA strands (**Fig. 1**). One strand is always complete in virus particles, whereas the second strand is incomplete, with a 3′ end that is heterogeneous in location. The incomplete strand is of plus polarity and the complete strand of negative polarity. When these viruses infect a cell, the plus strand is completed and the fully double-stranded DNA is then converted to a covalently-closed circular (CCC) molecule, which serves as a template for viral RNA synthesis (**Fig. 2**). The two genera of hepadnaviruses are thought to differ in that orthohepadnaviruses encode four open reading frames (ORFs) whereas the prototypic avihepadnavirus, duck hepatitis B virus, encodes only three, lacking the X ORF. This ORF appears to encode a protein that modulates transcription, possibly by acting on signal transduction pathways. There is now evidence for a fourth ORF in heron hepatitis B virus and in Ross goose virus, capable of coding for a 7–8 kDa protein, in the same genomic location as the C-terminal half of the X ORF of the orthohepadnaviruses. Functional studies on this ORF have not been reported. With one exception, the known products of the different ORFs are each translated from distinct mRNAs, transcribed from one of the four (*Orthohepadnavirus*) or three (*Avihepadnavirus*) viral promoters. The major nucleocapsid protein and the product of the polymerase gene appear to be encoded by the same mRNA, the pregenome, which also serves as the template for viral DNA synthesis. It was originally thought that mRNA splicing did not have a role in virus replication; however, it was recently found that the larger (L) of the two envelope proteins of duck hepatitis B virus is translated both from a spliced mRNA as well as from an unspliced mRNA. The spliced mRNA is transcribed from the major nucleocapsid promoter, located about 1200 bp upstream of the start codon for the large envelope protein. The unspliced mRNA is synthesized from a promoter located immediately upstream of the ORF for the large envelope protein (**Fig. 1**).

All hepadnaviruses replicate their DNA by reverse transcription of a viral RNA, a process which takes

HEPATITIS A VIRUS (*PICORNAVIRIDAE*)

Michael R Beard and **Stanley M Lemon**, Department of Microbiology and Immunology, The University of Texas Medical Branch at Galveston, Medical Research Building, 301 University Boulevard, Galveston, Texas, USA

History

Descriptions of epidemic human disease resembling acute hepatitis A extend back into antiquity, although the specific etiologies of these outbreaks remain unknown. Acute infectious hepatitis was first recognized as a transmissible entity in the early twentieth century and was clearly separated from homologous serum hepatitis (hepatitis B infection) during World War II. The virus responsible for hepatitis A was identified by immune electron microscopy in 1973 by Feinstone *et al*. Replication of hepatitis A virus (HAV) in monkey kidney cell cultures was reported by Provost *et al* in 1979. Although virus replication was noncytopathic, Lemon *et al* described a radioimmunofocus assay for quantitation of infectious HAV in 1983. The RNA genome of HAV was molecularly cloned by Ticehurst *et al* in 1983, and the first full-length nucleotide sequences of HAVs were reported in 1987 by Najarian *et al* and Cohen *et al*. These studies revealed that the genomic organization of HAV was similar to that of other picornaviruses, with a single large open reading frame (ORF) encoding a polyprotein which is cotranslationally processed during replication of the virus. The first infectious cDNA clone of HAV was reported by Cohen *et al* in 1988.

Taxonomy and Classification

HAV is classified within the genus *Hepatovirus* of the family *Picornaviridae*. This classification is based on several unique features of HAV: liver cell tropism, small and possibly absent VP4 protein, a unique VP1-2A precursor protein (pX), striking thermostability, a relatively slow and usually noncytopathic replication cycle and a strong tendency to initiate persistent infections in cell culture. The nucleotide sequence of HAV, while relatively well conserved among different isolates, is also widely divergent from those of the other picornaviral genera.

Properties of the Virion

The virion is approximately 27 nm in diameter and appears roughly spherical by electron microscopy. Based on the known structure of other picornaviruses, the HAV capsid is thought to have icosahedral symmetry and to contain 60 copies of each of three major polypeptides: 1B (VP2), 1C (VP3) and 1D (VP1). It is not known whether HAV contains a fourth, smaller capsid protein (1A, or VP4) that is present in other picornaviruses, although the genome of HAV potentially encodes a 1A protein of 21–23 residues. This 1A sequence contains an internal consensus myristoylation site, indicating the possibility of a very short L protein. However, mutagenesis of this site revealed no apparent requirement for myristoylation in HAV replication. HAV preparations made from infected cell cultures typically contain large quantities of empty capsids. These capsids are composed of three polypeptides 1AB (VP0, the precursor of VP2 and VP4), 1C and 1D. 1AB of the empty capsid is larger than 1B present in the complete virion, indicating that maturation cleavage of 1AB occurs in HAV as in other picornaviruses. What remains unknown, however, is whether the 1A protein is incorporated into the virion. Another difference from other picornaviruses is that virion precursors appear to contain unprocessed 1D2A. The function of this precusor protein (termed 'pX') is unknown.

Virions display a conformationally defined immunodominant neutralization antigenic site, in which Asp70 of 1C plays a prominent role. Amino acid residues of 1D also contribute to this antigenic site, as well as to at least one other functionally independent, minor antigenic site. This dominant antigenic site of HAV is highly conserved among all human strains. Reversion of neutralization escape mutants isolated in culture to the wild-type antigenic phenotype has been noted in experimentally infected New World owl monkeys, providing additional evidence for the importance of this antigenic site. Extensive digestion with high concentrations of trypsin and chymotrypsin results in cleavage primarily of 1B, but does not alter the antigenic characteristics, infectivity or exceptional thermal stability of the virion.

Properties of the Genome

The HAV genome is a single-stranded, positive-sense RNA, approximately 7500 bases in length (**Fig. 1**). As in other members of the picornavirus family, a short genome-linked protein (3B or VPg) is covalently

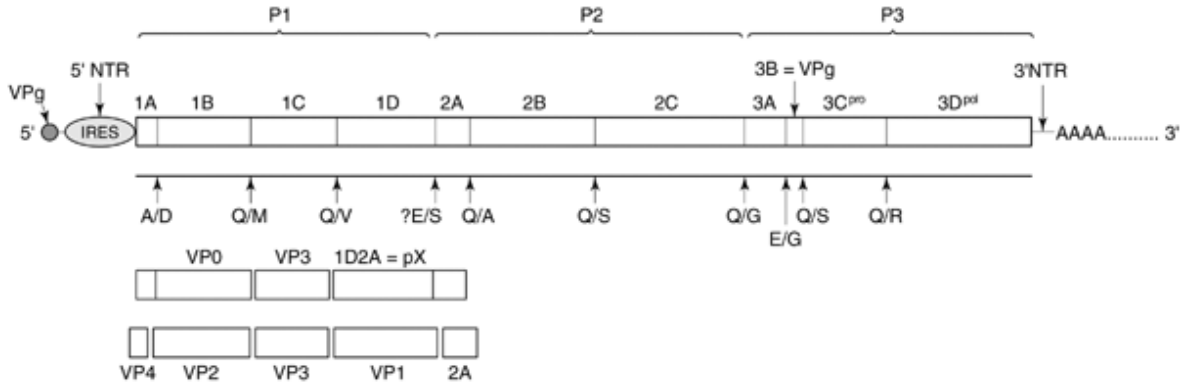


Figure 1 Organization of the positive-stranded RNA genome of hepatitis A virus. There is a small protein (VPg or 3B) attached to the 5′ terminus, followed by a nontranslated region of approximately 735 bases. A large open reading frame encodes the polyprotein that has three major domains: P1 (capsid proteins), P2 and P3 (nonstructural proteins). The primary cleavage event mediated by $3C^{pro}$ occurs at the 2A/2B junction and generates a P12A fusion, after which secondary cleavage by $3C^{pro}$ generates individual proteins and a 1D2A fusion protein termed pX. Arrows indicate authentic and predicted $3C^{pro}$ cleavage sites as determined experimentally. The processing of the P1 structural proteins that comprise the virus capsid is shown in detail. The final maturation cleavage of 1AB (VP0) to 1A (VP4) and 1B (VP2) occurs only after encapsidation of the RNA and is not mediated by $3C^{pro}$. The large open reading frame is followed by a short 3′ nontranslated region, and a 3′ terminal poly(A) tract. IRES, internal ribosome entry site.

linked to the 5′ end of the RNA, while at the 3′ end is a poly(A) tract. Flanking the single large ORF are 5′ and 3′ nontranslated regions (5′ NTR and 3′ NTR) of aproximately 735 and 64 bases, respectively. These regions form an extensive RNA secondary structure containing elements necessary for viral replication. In addition, the 5′ NTR plays an essential role in translation of the HAV polyprotein.

The 5′ NTR is relatively conserved among HAV strains (generally >92% nucleotide identity). There is no sequence similarity with other picornaviruses, but its structure and organization are similar to that of the 5′ NTRs of aphthoviruses and cardioviruses. There are two major functional domains. The larger, located between nucleotides 152 and 735, contains an internal ribosome entry site (IRES) which directs the 40S ribosomal subunit to bind to the RNA and initiate translation internally in a cap-independent manner. Several host cell proteins, including polypyrimidine tract-binding protein (PTB) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), bind to the *cis*-acting IRES and may influence translation. The smaller 5′ NTR domain, comprising the 5′ ~ 150 nt, contains three stem-loop structures (two of which likely form RNA pseudoknots), a pyrimidine-rich tract, 'pY1', and a short downstream single-stranded RNA segment. Recent evidence indicates that the pY1 segment forms extensive noncanonical base pair interactions involving U-U base pairs (**Fig. 2**). Removal of the pY1 domain does not impair HAV replication; however, extension of the deletion in a 3′ direction into the downstream single-stranded segment results in a temperature-sensitive replication phenotype due to defective RNA synthesis.

The ORF shares a general organization with other picornaviruses, and encodes the following proteins in a 5′ to 3′ order: 1A, 1B, 1C, 1D (the capsid proteins), 2A, 2B, 2C, 3A, 3B (or VPg), $3C^{pro}$ and $3D^{pol}$. These are expressed as a single large polyprotein. Considerably greater sequence variation is evident within the large ORF than in the 5′ and 3′ NTRs, and several distinct genotypes of HAV have been described. These have been defined as groups of strains with >15% nonidentity within the 1D/2A (pX) coding region.

The 3′ NTR is comprised of a short RNA segment which appears to be highly structured, and a 3′ terminal poly(A) segment of uncertain length.

Properties of the Viral Proteins

Compared with other picornaviruses, the proteolytic processing of the HAV polyprotein has been difficult to characterize due to the protracted replication cycle and lack of shut down of host-cell protein synthesis. None the less, most polyprotein cleavage sites have been identified. With the exception of the 1B/1A maturation cleavage, and possibly the 1D/2A cleavage, these cleavages are mediated by the nonstructural $3C^{pro}$ proteinase. The primary cleavage event occurs at the 2A/2B junction, as in the cardioviruses and aphthoviruses.

The structural proteins, 1A–D, are discussed above under Properties of the Virion. Less is known about

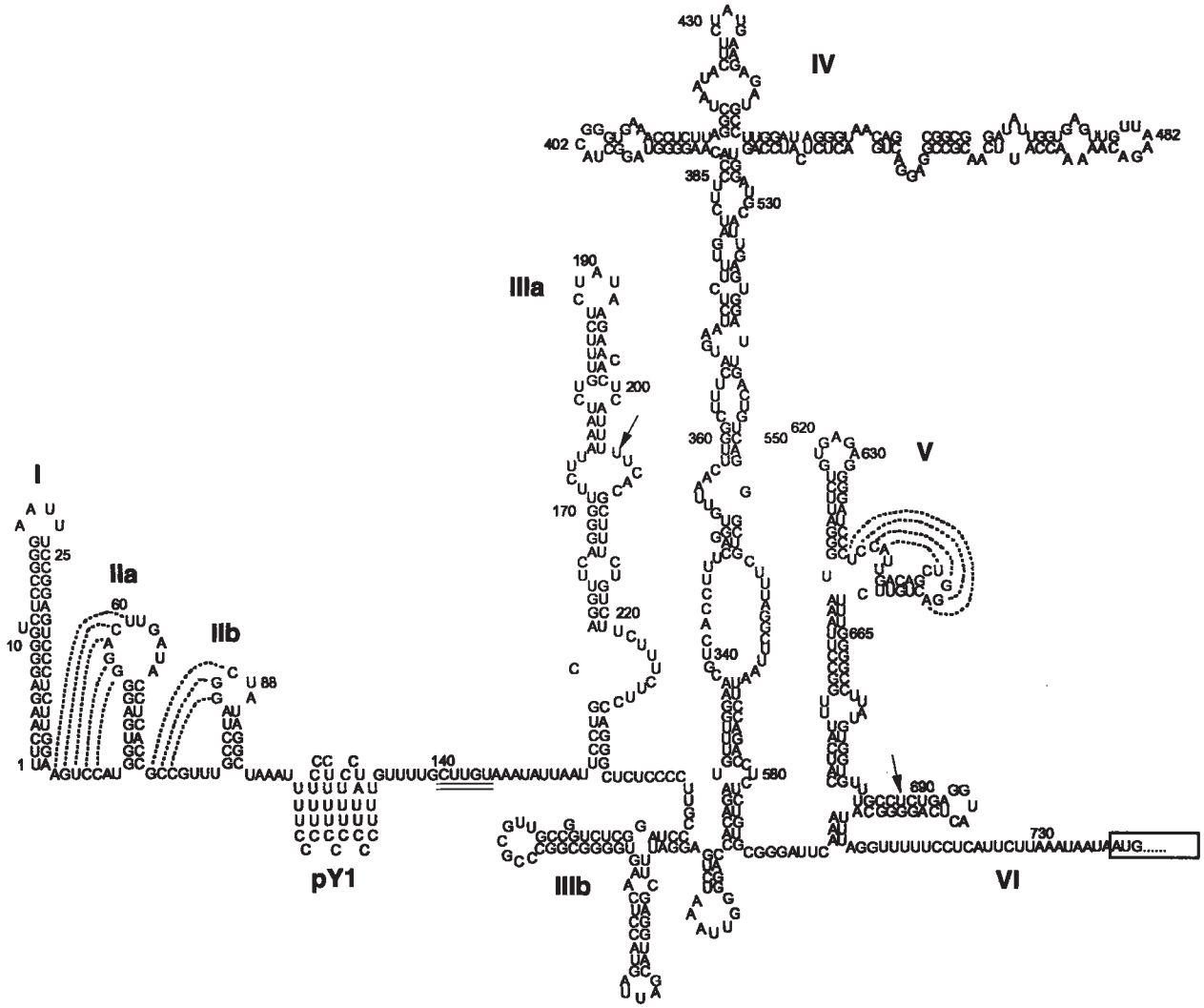


Figure 2 Putative secondary and tertiary structure of the 5′ nontranslated segment of HAV RNA from the wild-type HM175 strain as determined by a combination of comparative sequence analysis, thermodynamic modeling and nuclease probing of synthetic RNA. Major structural domains are labeled I through VI. Dotted lines indicate possible base pair interactions that result in RNA pseudoknots. The segment between nt 1 and nt 152 contains a pyrimidine-rich tract, pY1, within which there is prominent noncanonical U-U base pairing and an immediately downstream single-stranded segment, within which deletions confer a temperature-sensitive replication phenotype (indicated by double underline). The internal ribosome entry site extends from stem-loop IIIA to the first AUG within the large open reading frame (boxed). Arrows indicate sites of mutations that enhance translation and viral replication in cultured cells.

the specific functions of the nonstructural proteins of HAV. For example, the function of 2A remains unknown. It does not contain a proteinase sequence motif, such as found in the 2A protein of poliovirus, and it is unrelated to 2A sequences of other picornaviruses. Viruses with deletions of up to 15 amino acids within 2A have a reduced replication efficiency in cultured cells, but remain infectious when inoculated into chimpanzees. Proteins 2B and 2C play poorly characterized roles in replication. Expression of 2C and 2BC in cultured cells induces rearrangement of intracellular membranes. 2C has NTPase activity, and may be an RNA helicase. Mutations in these proteins are important with respect to a host range change that occurs during adaptation and passage of virus in cell culture (see below). These mutations also contribute to attenuation of the virus.

The function of the 3A protein is unknown, but preliminary data suggest it may associate with cellular membranes, possibly forming a membrane anchor for the replication complex. 3B is the 5′ genome-linked protein (VPg), and it is likely to serve as a primer for RNA synthesis. $3C^{pro}$ is the proteinase that directs the posttranslational processing of the HAV polyprotein.

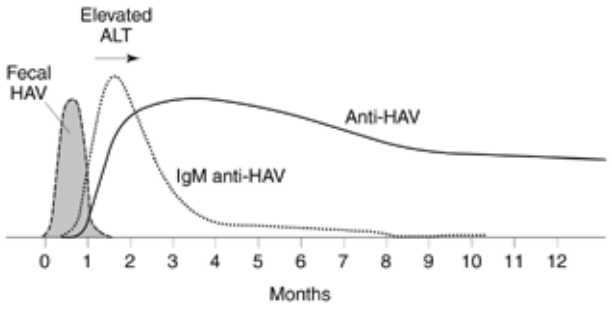


Figure 3 Clinical and virological course of events in acute hepatitis A. The time axis indicates months following exposure to the virus. ALT, serum alanine aminotransferase activity.

Among the nonstructural proteins, it has been characterized in greatest detail, with its structure defined by X-ray crystallography at a resolution of 0.2 nm (2.0 Å). The proteinase has a twofold domain structure characteristic of the chymotrypsin-like serine proteinases. In addition to its role in proteolysis, 3C contains an RNA-binding motif and is capable of binding to multiple sites within the 5′ terminal genomic RNA, suggesting a potential role in replication. Sequence analysis suggests that $3D^{pol}$ is an RNA-dependent RNA polymerase, but this activity has never been expressed *in vitro*.

Physical Properties

The HAV particle has a sedimentation constant of approximately 155 S, and a buoyant density of 1.325 g ml^{-1} in CsCl. 'Heavy particles', which have a buoyant density of about 1.44 g ml^{-1}, appear to have greater permeability to Cs^{2+} and thus resemble heavy poliovirus particles. Lighter particles which are also infectious and resistant to chloroform extraction have also been described, as well as very light particles which can be eliminated by chloroform extraction and most likely have residual associated lipids. A striking property of the HAV particle, which may well contribute to its potential for epidemic spread, is its resistance to thermal inactivation. Significant losses of infectivity (greater than a $\log_{10}$) over a 10 min exposure period are encountered only above 55°C. In the presence of 1 mol $MgCl^2$, this thermal stability is substantially enhanced, with the virus surviving incubation at 70°C for up to 10 min with only a 1–2 $\log_{10}$ loss of infectivity. The HAV particle is also remarkably resistant to low pH conditions, with little loss of infectivity reported at pH 1.0. The virus is relatively resistant to detergent inactivation, easily surviving 37°C for 30 min in 1% sodium dodecyl sulfate. There is no information concerning the radiation sensitivity.

The empty capsid has a sedimentation constant of about 70 S. Possible assembly intermediates (about 135 S) have been described which are enriched in their content of 1D2A (pX).

Replication

Although HAV demonstrates some unique features, such as a very protracted replication cycle, it is generally considered to replicate by a mechanism similar to that of other picornaviruses. Differences in various reports describing replication of HAV in cell culture may reflect significant differences in the extent to which the particular virus strains that have been studied have been adapted to growth in cell culture.

The virus binds to a wide range of cultured cells in a calcium-dependent manner. However, this binding does not necessarily correlate with internalization and establishment of a productive infection. A candidate HAV receptor protein has been recently identified. Expression cloning and nucleotide sequence analysis of a cDNA coding for this candidate receptor revealed it to be a novel, mucin-like class I integral membrane glycoprotein of 451 amino acids. Transfection of cDNA coding for this putative receptor resulted in HAV binding and increased susceptibility to infection in mouse LtK−cells. However, further studies are needed to confirm the functional significance of this putative receptor in HAV infection *in vivo*.

Replication of the genome proceeds via a negative-strand RNA intermediate, present in very small quantities in infected cells. Following infection of permissive cell cultures with cell culture-adapted virus, replication of the viral RNA occurs over several days. New RNA synthesis is then reduced in magnitude as persistent infection becomes established. The factors responsible for this downregulation in viral RNA synthesis remain unknown. Contrary to the situation with other picornaviruses, almost all positive-strand RNA present within infected cells is encapsidated. It has been suggested that a uniquely high affinity of this RNA for the capsid proteins results in its rapid encapsulation, thereby reducing the amount of RNA available to serve as template for further replication. While an interesting hypothesis, it is unlikely that this is the major cause of the relatively slow and restricted growth of HAV in cell culture. Viral antigen continues to accumulate after maximal infectivity has been reached in infections carried out at low multiplicity of infection. The reasons for this are not clear, but in some systems empty capsids represent the dominant form of antigenic material that is harvested from infected

cells. Early studies described defective particles containing RNA with large deletions putatively extending from the PI (capsid-encoding segment) into the nonstructural region of the genome. There have been no recent confirmations of these reports.

As in other picornaviruses, viral translation is initiated under control of an IRES located within the 5′ NTR. If any ribosome scanning is involved, it is confined to a short region just upstream of the two AUGs initiating the large ORF (codons 1 and 3). Compared with other picornaviral IRESs, the HAV IRES is extremely inefficient, a feature that may contribute to the generally slow and noncytolytic replication of the virus. The IRES interacts with *trans*-acting cellular factors that initiate cap-independent translation by positioning the 40S ribosomal subunit in a favorable position for translation. In contrast to other viral IRESs, the HAV IRES activity has a unique requirement for intact eukaryotic initiation factor eIF-4G, the p220 component of eIF-4F that is cleaved by proteinases expressed by several other picornaviruses. There is no cleavage of p220 in HAV-infected cells, explaining in part the absence of host cell shutdown and cytopathic effect. Several putative noncanonical *trans*-acting translational factors have been identified. Characterized in most detail is PTB, a 57 kDa protein that is normally involved in nuclear pre-mRNA splicing. PTB binds to picornavirus 5′NTRs, including that of HAV, and appears to be capable of significantly upregulating HAV translation *in vivo*. More recently a 5′ NTR-binding protein of 37 kDa was identified as GAPDH. GAPHD appears to destabilize important RNA base pair interactions within the IRES. Competition experiments have shown that PTB and GAPDH compete for binding to stem-loop IIIA, suggesting that the relative cytoplasmic levels of the two proteins may influence the translational activity of the HAV 5′ NTR.

There is very little information available concerning the specific mechanisms by which new viral RNA is primed and synthesized within infected cells. However, as in other picornaviruses, this occurs in an asymmetric fashion with much greater increases in positive-strand RNA than in the negative-strand RNA intermediate. It is likely that the 5′ and 3′ terminal structures participate in forming different and specific replicase complexes that are required for the initiation of synthesis of the two forms of viral RNA, and that each of the nonstructural proteins (2B-$3D^{pol}$) play roles in these processes. The site of virus assembly and the means by which HAV exits the cell are also unknown, although HAV has been found within cytoplasmic vesicles in infected cells in the liver. The virus is secreted from the apical surface of polarized, cultured gastrointestinal epithelial cells in the absence of cell death, and this may mimic the secretion of HAV across the apical hepatocyte membrane into biliary canaliculi *in vivo*.

Although HAV infection usually does not lead to demonstrable cytopathic effects, rapidly replicating, cytopathic (RR/CPE+) strains of HAV have been recovered from persistently infected cell cultures. These viruses induce a cytopathic effect characterized by vacuolation and cellular degeneration, but not associated with specific host cell metabolic shutdown.

Geographic and Seasonal Distribution

HAV is found in most human populations. However, the virus enjoys generally wider circulation in developing countries with relatively poor public sanitation standards. This is clearly related to the major mode of virus transmission, which is fecal–oral. Unlike enteroviruses, however, there is no clear-cut seasonal distribution for HAV infections. The incidence of hepatitis A infections may fluctuate widely in cycles extending over several decades.

Host Range and Virus Propagation

Only humans and several higher primate species are susceptible to human HAV. These primates include the chimpanzee (*Pan troglodytes*), New World owl monkey (*Aotus trivirgatus*) and various marmosets (*Saquinus mystax, S. labiatus*). Several macaque species have also been infected with HAV in various studies, but macaques are generally less susceptible than the other primates listed. Recently, several simian HAV strains that are genetically distinct but closely related to human HAV strains have been isolated from the African green monkey and from the cynomolgus monkey. It is not known whether humans are susceptible to infection with these simian 'biotypes', which appear to have a different host range than human HAV in other primates. These simian HAVs crossreact serologically with human HAV (see below) and are being evaluated for their ability to replicate and cause disease in chimpanzees.

The isolation of wild-type HAV in cell culture is a difficult, tedious and often lengthy procedure. Wild-type virus replicates slowly, is nearly always noncytopathic and tends to establish persistent infection. Generally speaking, only primate cells are permissive for HAV, although limited replication has been observed in mouse hepatocyte cultures and cells originating from dolphins. The most commonly used cell types are primary or continuous green monkey kidney cells. The initial isolation of virus in cell culture may be enhanced by prior passage in nonhuman primates. Certainly, with successive passage in

cell culture, the virus may become reasonably well adapted to propagation in a variety of primate cell types, including HeLa cells. Such cell culture-adapted strains often demonstrate a limited ability to replicate and cause disease in either humans or other susceptible primate species. This host-range change is the basis for the candidate attenuated HAV vaccines tested in humans thus far. Certain highly cell culture-adapted variants replicate more rapidly in cell culture and cause cytopathic effects (RR/CPE+ phenotype). However, even with these RR/CPE+ strains, up to 24 h is required to reach maximum virus yields in one-step growth curve experiments. This replication phenotype results from a number of mutations throughout the genome.

Genetics

Most HAV strains recovered from humans are closely related genetically. However, four distinct human HAV genotypes have been identified, each of which differs from the others in sequence at > 15% of base positions within the genome segment encoding the 1D/2A junction. In addition, there are three distinct simian genotypes of HAV.

Mutations responsible for several virus phenotypes have been identified by sequence comparisons and by mutagenesis of infectious cDNA. The altered host range associated with adaptation of HAV to growth in cell cultures and attenuation of the virus in primates has been associated with mutations located within the P2 region of the genome (proteins 2B and 2C) and in the 5′ NTR of the HAV genome. Although mutations in the P2 region are of primary importance in cell culture adaptation, mutations in the 5′ NTR also contribute to a cell culture-adapted phenotype and act cooperatively with P2 mutations in enhancing viral replication. Mutations within the 2B coding region (nt 3889, leading to an Ala to Val substitution) and in the 2C coding region at nt 4087 (Lys to Met) and nt 4222 (Phe to Ser) enhance the growth of wild-type HAV in cultured cells. In addition, selected mutations in the 5′ NTR (UU deletion at nt 203/204 and a U-G substitution at nt 687) both enhance translation and replication in African green monkey (BSC-1) cells but not in fetal rhesus monkey (FRhK-4) cells. The presence of mutations at nt 203/204 and nt 687 in three independently isolated cell culture-adapted HAV isolates and the appearance of the 687 mutation in HAV that has been adapted for growth in human diploid (MRC-5) cells suggests that these mutations play an essential role in overcoming a restriction for growth in cultured cells. The sequencing of multiple virus variants has suggested that RNA recombination occurs between HAV genomes in persistently infected cells.

Evolution

Little is known of the evolution of HAV. At the nucleotide sequence level, HAV shows less relatedness to other picornaviruses than that which exists between each of the other picornaviral genera. Within the region of the genome encoding the capsid proteins, there is only about 25–30% nucleotide identity with other picornaviruses. At the amino acid level, there is greater relatedness with the aphthoviruses and cardioviruses. However, this varies with individual proteins, as VP3 is more related to VP3 of poliovirus type 1, Mahoney strain (about 24%). Overall, however, HAV appears to be closest to the cardioviruses and aphthoviruses in terms of its genome organization, details of the polyprotein processing scheme and IRES structure. Limited studies suggest significantly less genetic drift among HAV strains isolated over several decades than that which has been observed with poliovirus. Strains isolated over a 30 year period have shown a <3% genetic divergence in the 1D/2A region.

Serologic Relationships and Antigenic Variability

Human HAV isolates show no evidence of antigenic variability, and share no antigenic determinants with any of the other picornaviruses or human hepatitis agents. In keeping with the lack of antigenic variability among human HAV strains, the amino acid sequences of the capsid proteins are highly conserved. Indeed, intense conservation of the immunodominant antigenic sites of human HAV appears to be an important biologic characteristic of this virus. One RR/CPE+ variant acquired a spontaneous neutralization escape mutation in residue 70 of 1C during persistent passage in cell culture, but this mutation was rapidly selected against on cell-free passage of the virus. However, simian HAV isolates differ significantly from human HAV at epitopes that are immunodominant in the human strains, even though the simian viruses are recognized by polyclonal human antibody to HAV. These simian viruses appear similar to neutralization escape mutants which have been recovered from human virus passaged in the presence of monoclonal neutralizing antibodies, in that the amino acid sequence of the capsid protein 1C differs at residue 70 (Asp in all human wild-type strains).

Epidemiology

Most commonly, hepatitis A occurs as a sporadic infection and the most commonly identified risk factor for acquisition of hepatitis A is to have lived in the same household with another infected person. Seroprevalence studies demonstrate that HAV infection is widespread in most human populations. The prevalence of HAV antibodies is generally higher in older age groups, with the shape of the age-related seroprevalence curve largely determined by present and past public sanitation standards. In less well developed countries, where hygiene is poor and fecal–oral transmission of infectious agents common, the age-related prevalence of HAV antibodies suggests acquisition of infection by almost all members of society within the first 5 years of life. In contrast, in well-developed countries of Western Europe even older adults have low antibody prevalence. Generally speaking, the incidence of new HAV infections appears to be declining in most populations, usually in keeping with improved public sanitation. This may lead to paradoxic increases in related disease activity in some rapidly developing countries, because a declining overall prevalence of the virus leads to acquisition of infection at older ages, when the risk of associated hepatitis A disease is significantly greater.

Hepatitis A rates increased significantly among illicit users of injected drugs in the United States during the late 1980s, but disease rates have since declined in this group. Several large outbreaks of hepatitis A have also occurred among adult homosexual males in several urban regions. Additional groups at special risk for acquisition of hepatitis A are children attending preschool daycare centers, the staff responsible for the management of such centers, and the older siblings and parents of these children. Increased risks for HAV transmission exist within closed institutions, such as prisons and institutions for the care of developmentally delayed individuals. Occasional hospital-associated outbreaks of hepatitis A have also been described, usually involving infected infants held in neonatal intensive care units.

The exceptional stability of the HAV particle and the fact that considerable amounts of virus are shed in the feces of infected individuals provide an unusual opportunity for common-source outbreaks of hepatitis A. Such epidemics are frequently reported in association with infection of a food handler, and are often linked to contaminated food or drink. Some outbreaks have been associated with ingestion of uncooked filter-feeding shellfish, as these shellfish are capable of concentrating HAV from polluted waters. Blood transmission is a very uncommon cause of infection, although several outbreaks of hepatitis A in hemophilic patients were related to administration of contaminated coagulation factor VIII preparations.

Transmission and Tissue Tropism

HAV is shed in the feces of acutely infected individuals, and transmission of the infection is almost always by the fecal–oral route. Virus shed in the feces is largely replicated within hepatocytes in the liver, and gains access to the intestinal contents by passage through the biliary tract. However, recent studies suggest that there is also a second, probably primary, enteric replication site within epithelial cells of the small intestinal crypts. The presence in infected primates of viral antigen in Kupffer cells, splenic macrophages and along the glomerular basement membrane may reflect antigen trapping rather than local virus replication. A significant viremia (perhaps as high as 10^5 infectious particles per ml serum) is present for several weeks during acute infection, and blood donations collected during the asymptomatic early stages of infection have caused rare, but well-documented, cases of transfusion-associated hepatitis A. It is also likely that needle-borne transmission has contributed to HAV spread among illicit users of injected drugs. Virus has been found in saliva late in the course of infection, but its source (possibly blood) is not known. Sexual activity may influence HAV transmission, especially among homosexual males. This is probably due to enhanced fecal–oral transmission related to oral–anal contact. Heterosexual activity may similarly influence hepatitis A transmission, but the data are less convincing.

Pathogenicity

There are no known differences in the pathogenicity of different HAV strains, although few studies have examined this in detail. The currently available evidence suggests that HAV is not directly cytopathic for the hepatocyte, but rather induces disease through an immunopathologic mechanism. The most important factor influencing disease severity associated with HAV infection is patient age at the time of infection. Very young children, under the age of 2 years, seldom develop symptoms of classic viral hepatitis. Their infections, although possibly not asymptomatic, are seldom icteric. The reverse may be said for adults. In individuals above 18 years of age, most infections are symptomatic and as many as two-thirds may be icteric.

Unlike hepatitis B and hepatitis C viruses, HAV causes only acute hepatitis and has never been unequivocally associated with chronic disease or persistent infection. Although it has been suggested

that premature infants may experience relatively protracted infections, even highly immunocompromised individuals appear capable of clearing HAV infection.

The disease manifestations of HAV infection are limited almost exclusively to the liver. Meningoencephalitis has been reported in association with acute hepatitis A infection in several cases, but this is clearly very exceptional. Acute renal failure has been more frequently reported, but its etiology in this setting is unclear.

Clinical Features of Infection

The incubation period of hepatitis A is approximately 4 weeks, with a range of perhaps 2–6 weeks. During the incubation period, virus replicates in the liver and is shed in the feces at high titers despite the absence of symptoms (**Fig. 3**). This is particularly true near the end of the incubation period. Disease is typically abrupt in onset and may be associated early in the course with high fever. More characteristically, the onset of hepatitis A is marked by malaise, nausea, right upper quadrant tenderness, and eventually scleral icterus and jaundice, dark urine (at times the color of Coca-Cola and quite frothy) and light, clay-colored stool. The latter manifestations represent disruption of the normal clearance of bilirubin by the infected liver. Diarrhea may be common in children (up to 60% of infected children in at least one study) but is usually not reported by infected adults. The disease manifestations taper off over a period of several weeks following their initial onset. However, convalescence may be prolonged and a number of months may elapse before the individual fully regains a sense of well-being. Chronic liver disease is not a recognized complication of HAV infection, but in some genetically predisposed individuals, HAV infection may trigger an autoimmune chronic hepatitis.

Although hepatitis may be recognized on clinical grounds alone, various chemical parameters are helpful in the diagnosis. The serum alanine aminotransferase (ALT) activity is typically elevated many-fold above normal levels. Aspartate aminotransferase (AST) activity is also elevated, but usually to a lesser degree. These enzyme abnormalities are soon followed by an elevated serum bilirubin, up to 20 or 30 times normal levels, and increases in the serum alkaline phosphatase activity. The aminotransferase elevations reflect hepatocellular damage with release of these hepatocellular enzymes into the circulation, while elevations in the serum bilirubin and alkaline phosphatase activity relate to intrahepatic cholestasis. The erythocyte sedimentation rate may be elevated. Serum immunoglobulin levels are generally increased nonspecifically, and rheumatoid factors are found in a large proportion of patients. Clinically and biochemically, acute hepatitis due to HAV cannot be distinguished from that due to the other hepatitis viruses. Thus, serologic tests are necessary for a virus-specific diagnosis.

Pathology and Histopathology

The pathology of the liver in acute hepatitis A has been studied in both infected humans and susceptible primates, including chimpanzees, owl monkeys and several species of marmosets. Characteristic changes include vacuolation and degeneration of hepatocytes, and intense mononuclear cell infiltration, which is usually most prominent in the portal regions. At times, this periportal inflammation may include piecemeal necrosis and thus the histopathologic picture may be confused with more chronic forms of viral hepatitis. These histopathologic changes are relatively short-lived with HAV infection, however, and are largely resolved within several months of the acute infection. During acute hepatitis, viral antigen can be identified (by immunofluorescence) in a large proportion of hepatocytes as punctate, cytoplasmic fluorescence. Electron microscopy may reveal virus particles packed in cytoplasmic vesicles.

Immune Response

The humoral immune response to hepatitis A is well characterized. IgM class antibodies to the virion are present in almost all patients by the onset of symptoms, and persist for up to 6–12 months following infection (**Fig. 3**). IgA and IgG antibodies to HAV also appear within a few days of symptom onset. Both IgG and IgM antibodies have virus-neutralizing activity, and the efficacy of passively administered immunoglobulins in prevention of symptomatic hepatitis A suggests that IgG anti-HAV is protective against subsequent symptomatic reinfection. IgG antibody generally persists for the life of the individual, although that it may fall to less than detectable levels after several decades in individuals who were infected at a very early age. Secretory antibody to HAV has been detected by solid-phase immunoassays, but saliva and feces generally do not contain neutralizing antibodies. Current evidence suggests that mucosal immunity plays little, if any, role in protection against hepatitis A.

Limited studies have examined the cellular immune response to HAV infection. NK cell activity against HAV-infected fibroblasts has been described. In addition, human leukocyte antigen (HLA)-restricted T cell cytotoxicity has also been demonstrated in

individuals with acute hepatitis A. The cytotoxic effector cell is a CD8+ T lymphocyte. Immune T cells release interferon γ, in an HLA-dependent but not restricted fashion, upon exposure to HAV-infected fibroblasts. Interferon γ may promote HLA-restricted cytotoxicity by upregulating the normally low-level display of HLA antigens by hepatocytes. Specific T cell epitopes have not been extensively characterized.

Prevention and Control

As almost all hepatitis A infections are transmitted by the fecal–oral route, good sanitation practices, including high standards of quality for public water supplies, have resulted in a low prevalence of HAV infections in many well-developed societies. Individual protection may be afforded by the passive administration of immune serum globulin, which consists of pooled IgG from a large number of individuals and normally contains a substantial titer of neutralizing antibodies to HAV. Such passive immunoprophylaxis provides transient protection of >80% efficacy. Passively administered IgG both prevents infection and modifies symptoms in those already infected, provided it is given within 2 weeks of exposure.

Active immunization against hepatitis A can be achieved with inactivated HAV vaccines produced by several commercial manufacturers. These vaccines consist of HAV that has been propagated in cell culture, purified and inactivated with formalin. The virus strains used in vaccine production have been adapted to growth in cell culture and are attenuated in their ability to cause disease in susceptible primates. A single dose of purified viral antigen, consisting of both full and empty viral particles, results in seroconversion in most individuals. Antibody levels are greater than those achieved by passive administration of immune serum globulin, but lower than those present following natural infection. The clinical efficacy of these vaccines has been demonstrated in controlled clinical trials to be in excess of 95%. Candidate attenuated HAV vaccines, containing virus adapted to growth in cell culture and extensively passaged in cell culture, have also been tested in humans. These candidate vaccine viruses have a markedly reduced ability to replicate in the liver following parenteral inoculation and have generally demonstrated poor immunogenicity.

See also: **Cardioviruses (*Picornaviridae*); Foot and mouth disease viruses (*Picornaviridae*); Hepadnaviruses (*Hepadnaviridae*): Avian hepatitis B virus, Hepadnaviruses (*Hepadnaviridae*): Hepatitis B Virus: General features, Molecular biology; Hepatitis C virus (*Flaviviridae*); Hepatitis Delta virus; Hepatitis E virus; Interferons: General features, Therapy of aids and cancer; Polioviruses (*Picornaviridae*): General features, Molecular biology.**

Further Reading

Gust ID and Feinstone SM (1988) *Hepatitis A*. Boca Raton: CRC Press.

Lemon SM (1985) Type A viral hepatitis: new developments in an old disease. *N. Engl. J. Med.* 313: 1059.

Lemon SM (1992) Hepatitis A virus: current concepts of the molecular virology, immunobiology and approaches to vaccine development. *Rev. Med. Virol.* 2: 73.

Lemon SM and Thomas DL (1997) Vaccines to prevent viral hepatitis. *N. Engl. J. Med.* 336: 196.

Lemon SM *et al* (1997) The molecular basis of the attenuation of hepatitis A virus. In: Harrison TJ and Zuckerman AJ (eds) *The Molecular Medicine of Viral Hepatitis*. Chichester: Wiley.

Ticehurst J *et al* (1989) Replication of hepatitis A virus: new ideas from studies with cloned cDNA. In: Semler BL and Ehrenfeld E (eds) *Molecular Aspects of Picornavirus Infection and Disease*, p. 27. Washington: American Society for Microbiology.

HEPATITIS C VIRUS (*FLAVIVIRIDAE*)

Robert H Purcell, Laboratory of Infectious Diseases, National Institute of Allergy & Infectious Disease, Bethesda, Maryland, USA

History

The recognition of the existence of hepatitis C virus (HCV) is a relatively recent event. In 1975, Feinstone and colleagues demonstrated that most cases of transfusion-associated hepatitis were caused by neither hepatitis A virus (HAV) nor hepatitis B virus (HBV), the only two recognized human hepatitis viruses at the time. In retrospect, there were clues to the existence of additional hepatitis viruses. These included the demonstration of multiple cases of hepatitis in drug addicts and hemophiliacs, the high rate of chronicity in non-B transfusion-associated hepatitis (HAV does not progress to chronicity) and the unimodal distribution of incubation periods of transfusion-associated hepatitis centering on 7–8 weeks, intermediate between the modal incubation periods of HAV infection (3–4 weeks) and HBV infection (12–14 weeks).

The disease associated with this newly recognized hepatitis was called 'non-A, non-B' hepatitis and the presumed etiologic agent 'non-A, non-B' hepatitis virus because it was not known whether the disease was caused by one or more viruses. It was not until 1989 that a test specific for the virus responsible for most transfusion-associated hepatitis was developed and the virus was named hepatitis C virus (HCV).

Taxonomy and Classification

Hepatitis C virus is the only member of the genus *Hepacivirus* within the virus family *Flaviviridae*. The genome of HCV is organized in a manner similar to that of the flaviviruses and pestiviruses, which constitute the other two genera, *Flavivirus* and *Pestivirus*, respectively, within the family *Flaviviridae*. Although only distantly related to these two groups of viruses, HCV does share some sequence identity and is slightly more closely related to the pestiviruses than to the flaviviruses. Interestingly, parts of the HCV genome appear to be distantly related to potyviruses and carmoviruses of plants. The former are members of the picornavirus-like superfamily and the latter are members of the alphavirus-like superfamily. These distant relationships suggest a common evolutionary origin of plant and animal viruses.

Properties of the Virion and Genome

Properties of the virion

Virions are approximately 50 nm in diameter, as determined by filtration and electron microscopy (**Fig. 1**). They have a spherical shape and are bounded by a lipid-containing envelope consisting of at least two structural glycoproteins. The nucleocapsid is spherical and approximately 30 nm in diameter.

Properties of the genome

The genome of HCV is single-stranded linear RNA of positive sense. It is unsegmented and consists of a 5′ untranslated region of approximately 340 nucleotides (nt) that contains an internal ribosomal entry site (IRES) and several small, presumably untranslated, open reading frames (ORFs); a protein-encoding region (ORF) of approximately 9400 nucleotides, which encodes a large polyprotein precursor of approximately 3000 amino acids; and a 3′ untranslated region that is surprisingly complex and contains a variable region (approximately 50 nt), a polypyrimidine region of approximately 100 nt but variable in length and a highly conserved terminal region of approximately 100 nt that is predicted to have a complex secondary structure.

The ORF encodes three (or four) structural proteins at its 5′ end and six (or seven) nonstructural proteins at its 3′ end (**Fig. 2**). The gene order is: 5′-core (*C*)–envelope 1(*E1*)–envelope 2(*E2*)–P7–nonstructural protein 2 (*NS2*)–nonstructural protein 3 (*NS3*)–nonstructural protein 4A (*NS4A*)–nonstructural protein 4B (*NS4B*)–nonstructural protein 5A (*NS5A*)–nonstructural protein 5B (*NS5B*)–3′. The small protein (p7) encoded between the three structural proteins and the six nonstructural proteins is of unknown function; it is not clear whether it is a structural or nonstructural protein but an analogous protein encoded by pestiviruses is not a part of the virion.

Properties of the Viral Proteins

The polyprotein of approximately 3000 amino acids that is encoded by the single ORF of HCV is cotranslationally and post-translationally cleaved into approximately 10 proteins. The proteins at the amino-terminus of the polyprotein are: a nonglycosyl-

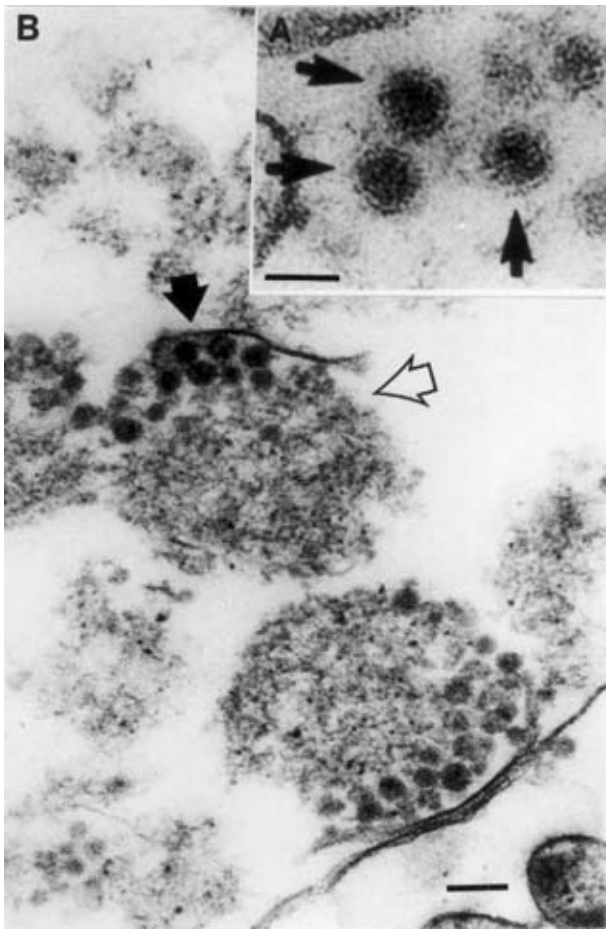


Figure 1 Thin section electronmicrograph of virus-like particles believed to be hepatitis C virus in a continuous line of human T-cells (HPBALL). **(A)** High magnification of the particles (arrows). **(B)** Cytoplasmic vesicles containing the virus-like particles (arrow) in close approximation to amorphous material (open arrow). Bar, 100 nm. (Reproduced, with permission from Shimizu YK *et al.* (1996) *Hepatology* 23: 205.)

ated nucleocapsid (core) protein of approximately 19 kDa and two glycosylated envelope proteins of approximately 31 and 70 kDa, respectively. The P7 protein is inefficiently cleaved from the precursor of E2 and exists in two forms, E2-P7 and P7; their ultimate disposition is unknown. The two envelope

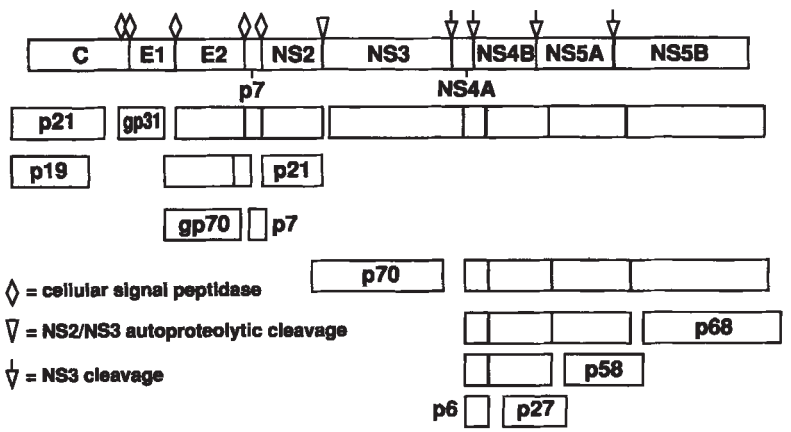


Figure 2 Predicted structure and processing of the HCV polyprotein by host and viral proteases. The viral coding region is displayed at the top. Boxes indicate precursors and mature proteins generated by processing. Approximate sizes of the mature proteins (p) and glycoproteins (gp) are indicated. (Adapted, with permission, from Major ME and Feinstone SM (1997) The molecular virology of hepatitis. *Hepatology* 25: 1527.)

glycoproteins form intermolecular noncovalently linked heterodimers. The nonstructural proteins include NS2 (a 21 kDa protein that, before cleavage, is part of a Zn-dependent protease that bridges NS2 and NS3 and mediates autocatalytic cleavage of the NS2/NS3 junction), NS3 (a 70 kDa protein encompassing three enzyme activities: a serine protease, a helicase and an NTPase; the protease cleaves remaining junctions between nonstructural proteins), NS4A (a 6 kDa protein that is a cofactor for NS3 serine protease function), NS4B (a 27 kDa protein of unknown function), NS5A (a 58 kDa serine phosphoprotein that exists in 56 and 58 kDa forms, depending on the degree of phosphorylation) and NS5B (a 68 kDa protein that is the viral RNA-dependent RNA polymerase). Each of the proteins with enzymatic function has been expressed as a recombinant protein and the enzymatic functions predicted from sequence motifs have been confirmed *in vitro*. Genetic heterogeneity has been identified in all the genes encoding viral proteins, but more variable and hypervariable regions occur in the envelope proteins than in the nonstructural proteins. With the exception of the serine protease, which has been crystallized, the three-dimensional structure of most of the HCV proteins is poorly understood.

Physical Properties

Hepatitis C virus has been recovered from cesium chloride fractions with a mean density of 1.24 g cm^{-3}. The isopycnic density of infectious HCV in sucrose has been measured at 1.06 g cm^{-3} in serum of acutely infected patients and at 1.12 g cm^{-3} for virus recovered from cell culture. The lower density of HCV recovered from serum results from the physical association of very low density lipoprotein with virions. Virus recovered from the serum of chronically infected patients generally has a density in sucrose of 1.15–1.18 g cm^{-3}, probably resulting from the formation of antigen–antibody complexes. The nucleocapsid of HCV was found to have a buoyant density in sucrose of 1.25 g cm^{-3}. The isopycnic density of HCV in potassium bromide is 1.12 g cm^{-3}. The s_{20w} is $\geq$ 150. The virus is stable in buffer at pH 8.0–8.7 but inactivated by exposure to lipid solvents or detergents, evidence that the virus envelope contains essential lipids. It is inactivated in aqueous solution by heat at 60°C for 10 h or 100°C for 2 min and is relatively unstable to storage at room temperature or repeated freezing and thawing. The virus is also inactivated by formaldehyde at a concentration of 1:2000 at 37°C for 72 h. Ultraviolet irradiation and β-propriolactone can also inactivate HCV.

Replication

Little is known about the replication HCV. Transcription and translation are thought to follow the strategies of flaviviruses and pestiviruses. Processing is thought to proceed in a manner similar to that of flaviviruses and pestiviruses. As with these viruses, post-translational cleavage of the structural proteins of HCV by microsomal membrane-bound cellular enzymes can be demonstrated *in vitro*. Translocation of the structural glycoproteins to the endoplasmic reticulum probably occurs via an internal signal sequence. Virus assembly is believed to occur by budding into vesicles.

Viral antigens can be detected in the cytoplasm of infected hepatocytes by immunofluorescent staining and virus-related RNA sequences can be similarly localized to the cytoplasm by *in situ* hybridization. Peculiar cytoplasmic tubular structures observed by electron microscopy in hepatocytes of chimpanzees infected with HCV are thought to result from local expression or action of interferon-related proteins and not directly from replication of the virus. Little is known about the methods of uptake, assembly or release of viral particles in infected cells and it is not known whether the virus is directly cytopathogenic or whether cytopathology is mediated by host immune responses.

Geographic and Seasonal Distribution

Serologic evidence for HCV infection has been found in all regions of the world in which it has been sought. In developed countries, 0.1–2% of the general population have antibody to the virus, as measured by currently available tests. In developing countries, the prevalence is somewhat higher but generally less than 5–10% of the population. An exception is Egypt, where prevalences as great as 20% have been recorded. Interestingly, similar high prevalences of anti-HCV have been found in certain high-risk populations in developed countries. Overall, it has been estimated that 3% of the world's population have been infected with HCV, resulting in more that 170 million chronically infected individuals.

From a clinical standpoint, HCV is thought to cause approximately 10–20% of acute hepatitis in many countries, but this may be as high as 50% in other countries, such as Japan. Infection with HCV has no recognized seasonal distribution.

Host Range and Virus Propagation

The recognized host range of HCV is quite limited. In addition to humans, the virus can be transmitted to chimpanzees and possibly to other greater and lesser

apes. Viral antigens and RNA can be detected in hepatocytes of naturally infected patients and experimentally infected chimpanzees and viral RNA has been detected in peripheral blood mononuclear cells (PBMC) and bone marrow cells of infected patients. Similar results have been obtained with hepatocytes and PBMC of experimentally infected chimpanzees.

Evidence has been obtained for replication of HCV in chimpanzee hepatocytes that were infected *in vivo* and then removed and maintained in culture. In addition, HCV has been reported to replicate in continuous lines of human T cells, B cells and monocytes. Such *in vitro* propagation systems have been used to study the neutralization of HCV by specific antibody and the inhibition of viral replication by antiviral agents such as interferon. However, all *in vitro* replication systems are difficult to reproduce and are limited in their application.

Genetics

The single-stranded RNA genome of HCV is replicated by a viral polymerase lacking proof-reading functions. As with other such viruses, HCV has a relatively high apparent evolutionary rate (approximately 2×10^{-3} base substitutions per genome site per year). This has resulted in genetic diversity among different strains of HCV and within the same strain over time. Strains of HCV can be grouped into six major genotypes, based on their genetic heterogeneity. Genotypes differ from each other by approximately 25–35% at the nucleotide level. The six genotypes have been further subdivided into over 100 subtypes. These differ from each other by about 15–25% at the nucleotide level. Although the genotypes are more or less distinct, discrimination of subtypes is less clear, owing to overlap in the degree of heterogeneity. This is demonstrated by the reported identification in Asia of major genotypes 7–11. More extensive analysis of the sequences of these genetic variants has demonstrated that genotype 10 is actually a subtype of genotype 3 and genotypes 7, 8, 9 and 11 are subtypes of genotype 6. Subtypes can be further subdivided into individual isolates that differ from each other by approximately 10% and, within individual isolates, into individual genomes that differ from each other by approximately 2%. The collection of variants within an individual isolate is called a 'quasispecies'.

Most strains recovered in the United States and Europe belong to genotype 1a or 1b (**Fig. 3**). Approximately 80% of strains recovered from Japanese patients represent genotype 1b. Subtypes a and b of genotype 1, together, are the two most common types of HCV and have a worldwide distribution. Genotypes 2 and 3 and their subtypes also have worldwide distributions. Genotype 4 and its variants are pan-African types; genotype 5 is found principally in South Africa. Genotype 6 and its many variants are mostly Asian strains.

Mutations in the genome of HCV are not uniformly distributed. Variable and hypervariable regions have been identified in portions of the HCV genome encoding the two envelope proteins. In particular, the amino-terminal end of envelope E2 has been designated the 'hypervariable region 1' (HVR1). In strains of genotype 1b, a second hypervariable region (HVR2) is found a short distance 3′ of HVR1. There is evidence that HVR1 is a neutralization epitope for the humoral immune response of infected patients. Thus, the sequence of HVR1 from HCV strains infecting agammaglobulinemic patients changes very little over time but changes rapidly in individuals subjected to immunostimulation. The rate of change of HVR1 in patients with a normal immune system is intermediate. Genetic variation has also been detected throughout the HCV genome. It is least in the 5′ UTR and, as noted, greatest in the genes encoding the envelope proteins.

Evolution

The origins of HCV are unknown. Its history cannot be traced back beyond several decades. Its limited host range and epidemiologic characteristics of transmission, principally by intimate exposure to blood or secretions, suggest that it is predominantly a human or primate virus. Recently, viruses related to HCV were recovered from various species of nonhuman primates. The virus most closely related to HCV, called 'GBV-B', was recovered from New World monkeys (tamarins) that had been inoculated with a virus previously believed to have been recovered from a human case of viral hepatitis. The origin of the virus remains obscure but it is probably a New World monkey virus. Other viruses, designated 'GBV-A' and 'GBV-A-like viruses', and a virus designated 'GBV-C' (also called 'hepatitis G virus'), are all somewhat more distantly related to HCV but are more closely related to this virus than to other members of the *Flaviviridae*. The GBV-A and GBV-A-like viruses have been recovered from a number of New World monkey species; GBV-C has been recovered from humans and has a worldwide distribution. Hepatitis C virus, the GBV agents, the flaviviruses and the pestiviruses form a group of transmissible agents with a probable common ancestry. Only HCV and GBV-B cause hepatitis; the GBV-A and GBV-C viruses appear not to be hepatitis viruses and, in fact, probably do not replicate in the

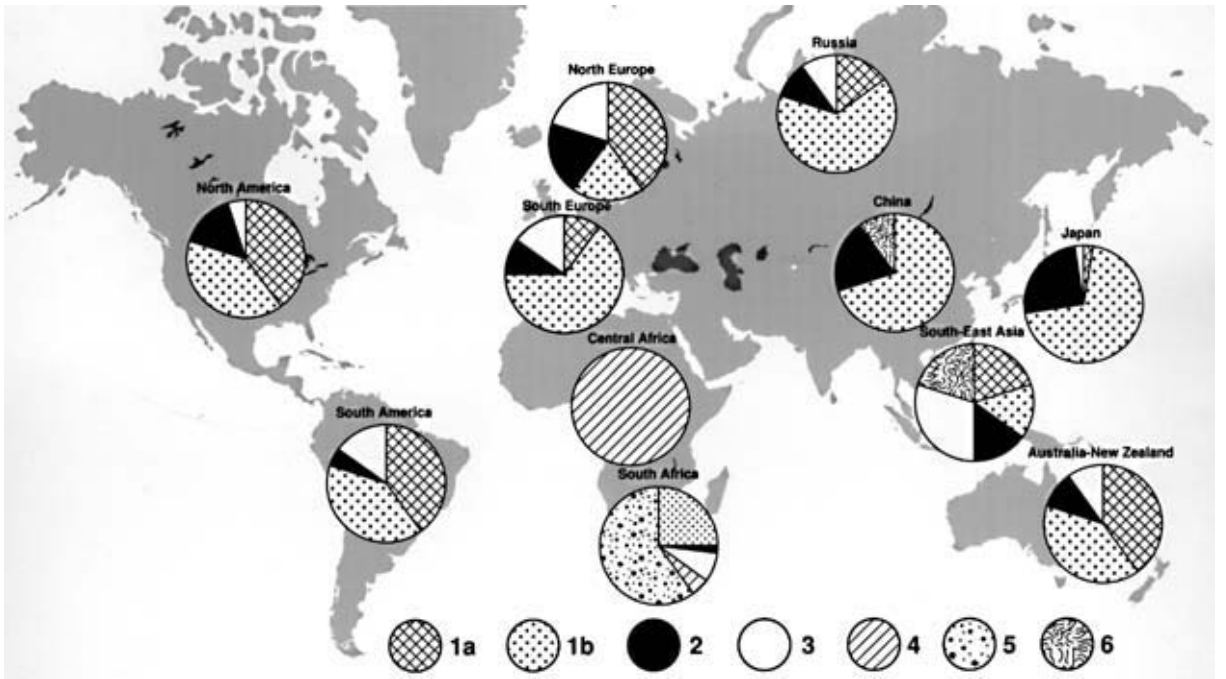


Figure 3 Worldwide distribution of HCV genotypes. (Reprinted with permission from Forns X and Bukh J (1998) *Viral Hepatitis Reviews* 4: 1.)

liver. Hepatitis C virus has no characteristics in common with any other recognized human hepatitis virus, except its ability to replicate in the liver and cause hepatitis.

Serologic Relationships and Variability

The inability to propagate HCV in standard cell cultures has made the serologic analysis of HCV strains very difficult. The search for different serotypes of HCV has been limited to crosschallenge experiments in chimpanzees. These have yielded results that have been difficult to interpret to date. In most studies, exposure to one strain of HCV has rendered chimpanzees resistant to hepatitis following rechallenge with a second strain. However, there have been exceptions. It is difficult to interpret these studies because of the propensity of HCV infection to progress to chronicity and the tendency of these chronic infections to cause recurrent hepatitis at unpredictable intervals. At least some of the strains involved in unsuccessful crosschallenge experiments have been shown to belong to the same subtype of HCV; strains involved in successful crosschallenges have not yet been examined to determine if they represent different subtypes or genotypes. However, even when rechallenge has not resulted in a second case of hepatitis, the chimpanzees have been reinfected with the second strain of HCV, as measured by detection of HCV RNA specific for that strain in the serum of challenged chimpanzees. Rechallenge with the same strain of HCV has also resulted in reappearance of HCV RNA, suggesting that solid immunity even to the homologous strain does not routinely follow infection with HCV. Thus, serologic differences among different HCV strains remain poorly understood.

Antigenic differences in the protein encoded by the *NS4* gene of HCV have been used for measuring antibody to the major genotypes of HCV. However, these have not been useful for typing of antibody to subtypes within a major genotype.

Epidemiology

Hepatitis C virus is predominantly spread by parenteral exposure to blood and blood products. Before the development of screening tests for carriers of hepatitis viruses, up to a third of recipients of large quantities of blood developed transfusion-associated hepatitis. The application of sensitive tests and screening measures to blood and plasma donation has virtually eliminated the transmission of viral hepatitis by transfusion and administration of blood products. This has been one of the great, and incompletely appreciated, achievements of research

on the hepatitis viruses. Another population at risk of acquiring infection with HCV and other blood-borne viruses consists of health care workers, especially those who come in contact with blood or tissues of patients. However, the largest proportion of community-acquired HCV cases among identifiable risk groups are the users of illicit parenteral drugs.

It has been proposed that HCV is a sexually transmitted disease but the evidence for this is controversial. The Centers for Disease Control have reported that up to 11% of community-acquired hepatitis C is caused by sexual transmission, mostly heterosexual, but others put the figure much lower. Perinatal transmission of HCV has been reported, principally to the offspring of mothers who are also infected with human immunodeficiency virus (HIV). The true incidence of such infections is not known, but sexual and perinatal transmissions probably play a real but relatively small role.

However, the largest proportion of individuals with community-acquired hepatitis C (approximately 40%) have no identifiable risk factor other than low socioeconomic status. Similarly, over 30% of patients with community-acquired hepatitis B are not identifiable members of a high-risk group. It is unclear whether these two viruses are spread through the community by still unrecognized means or whether the identification of individuals as members of high-risk groups is still imperfect. Overall, HCV is thought to account for approximately 33 000 cases of acute hepatitis C per year in the United States, approximately 12% of all cases of viral hepatitis. However, since HCV infections are much more likely to progress to chronicity than infections by the other recognized hepatitis viruses, HCV is thought to be responsible for approximately 42% of the estimated 1.5 billion dollar annual economic burden of viral hepatitis in the United States.

Transmission and Tissue Tropism

As noted earlier, transmission of HCV is predominantly through intimate and parenteral exposure to contaminated blood and body fluids. Hepatitis C virus replicates in hepatocytes and, possibly, mononuclear cells (B and T lymphocytes and monocytes).

Pathogenicity

The mechanism by which HCV causes hepatitis is not well understood. It is not known whether the virus is cytopathogenic or whether the clinical manifestations of disease are the result of the host's immune response.

Clinical Features of Infection

Hepatitis C cannot be distinguished clinically from other forms of acute hepatitis. The incubation period ranges from 4 to 13 weeks (mode: 4–8 weeks). The acute disease is often subclinical but approximately 80% of cases become chronic. Chronic HCV infection may be associated with relatively normal liver enzyme values, recurring liver enzyme elevations or, rarely, a rapidly progressive form of chronic hepatitis. Whether or not chronic hepatitis C is clinically apparent, up to 20–30% of cases progress to liver cirrhosis. The clinical progression of hepatitis C is generally more indolent than that of hepatitis B and its clinical manifestations are often not apparent until after the second or third decade of infection. Patients with chronic hepatitis C, especially those with cirrhosis, are at risk of developing hepatocellular carcinoma. Hepatitis C tends to be less clinically apparent in the immunologically immature or compromised, suggesting that, as with hepatitis B, immunity plays an important but poorly defined role in the pathogenesis of disease.

Pathology and Histopathology

The histologic changes in acute and chronic hepatitis C cannot generally be differentiated from those occurring in other types of viral hepatitis. The histologic changes in the liver during acute viral hepatitis are hepatic inflammation and hepatocellular necrosis. Inflammatory cells, principally lymphocytes and macrophages, are present in both the parenchyma and portal areas. Swelling and eosinophilic necrosis of hepatocytes are common. Chronic hepatitis C is characterized by inflammation (lymphocytes, macrophages and histiocytes) that is largely portal in distribution and hepatocellular necrosis that is typically periportal.

Immune Response

The immune response to HCV is complex and not well understood. Virus-specific antibodies against the structural proteins, principally the nucleocapsid and E2, and against nonstructural proteins, principally NS3, NS4 and NS5, have been detected with recombinant expressed antigens in individuals infected with HCV. Both linear and conformational epitopes are believed to be involved in the humoral immune response of the host to infection with HCV. Significant genetic heterogeneity throughout the genome is reflected in serologic heterogeneity of the humoral immune response, principally to the protein encoded by the *NS4* gene. The most extensive heterogeneity of HCV is found in HVR1, a probable

neutralization epitope of HCV. It is likely that neutralization-escape variants of HVR1 are positively selected by the host's humoral immune response. Other neutralization epitopes may exist but they have not been defined.

Cell-mediated immune responses against all of the HCV proteins, but most frequently against the nucleocapsid protein, have been detected but their significance is unknown. Because there is no widely available cell culture system for the propagation of HCV, it has not been possible to carry out extensive *in vitro* neutralization assays. However, data from experimental HCV infections in chimpanzees and from epidemiologic studies of natural infections in humans suggest that standard definitions of serotypes may not be applicable to HCV.

Prevention and Control

At present, control of HCV infection is achieved only by interdicting transmission via transfused blood or blood products by eliminating contaminated blood with tests capable of identifying most chronic carriers of the virus. Despite a number of controlled trials, normal immune globulin has not been shown to have a clear protective efficacy in prevention of HCV transmission associated with transfusion or accidental needle sticks. However, evidence for some degree of protection has been reported, especially when immune globulin has been administered before exposure. Currently, all units of plasma destined for plasma fractionation and production of immune globulin are prescreened to remove anti-HCV positive units. Therefore, current lots of normal immune globulin would not be expected to contain protective antibodies. There are no vaccines for HCV.

Future Perspectives

Knowledge about HCV is in a rapid state of flux. The licensed antibody tests for detecting HCV infection can detect antibody to a broad range of viral antigens. These tests have virtually eliminated transfusion-associated hepatitis and hepatitis among recipients of blood products. Vaccines against HCV are being developed but progress will be slow until more information is available about the serologic heterogeneity of HCV, the identity of viral epitopes involved in neutralization and the nature of the protective immune response.

Hepatitis C virus was the first virus to be characterized almost exclusively by molecular means. Progress was slow because of the low titers ($\leq 10^6$ infectious doses/ml) achieved by the virus during infection and the relatively weak and delayed immune response in many patients. It is unlikely that any progress could have been made without the development of an animal model (chimpanzees) in which the virus could be amplified biologically and specific reagents produced. The future development of vaccines will also depend heavily on the availability of an animal model.

See also: **Hepadnaviruses (*Hepadnaviridae*): Hepatitis B Virus: General features, Molecular biology; Hepatitis A virus (*Picornaviridae*); Hepatitis Delta virus; Hepatitis E virus; Potyviruses (*Potyviridae*); Poxviruses (*Poxviridae*): Capripoxviruses; Yellow fever virus (*Flaviviridae*).**

Further Reading

Bukh J, Miller RH and Purcell RH (1995) Genetic heterogeneity of hepatitis C virus: quasispecies and genotypes. *Semin. Liver Dis.* 15: 41.

Hadlock KG and Foung SKH (1998) GBV-C/HGV: A new virus within the *Flaviviridae* and its clinical implications. *Transfusion Med. Rev.* 12: 94.

Houghton M (1996) Hepatitis C viruses In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology* 3rd edn, p. 1035. Philadelphia: Lippincott–Raven.

Major ME and Feinstone SM (1997) The molecular virology of hepatitis C. *Hepatology* 25: 1527.

HEPATITIS DELTA VIRUS

Michael MC Lai, Howard Hughes Medical Institute, Department of Molecular Microbiology and Immunology, University of Southern California School of Medicine, Los Angeles, California, USA

History

In 1977, gastroenterologist Mario Rizzetto of Turin, Italy, reported the detection of a new hepatitis B-specific antigen in some patients with chronic B hepatitis. The antigen, termed 'delta antigen', was localized in the nuclei of hepatocytes, closely resembling the hepatitis B core antigen (HBcAg) in its subcellular localization. Its presence was always associated with hepatitis B virus (HBV) infection, and patients with delta antigen also developed antidelta antibodies. Initially, delta antigen was thought to be a previously undescribed form of HBV antigen. In 1980, Rizzetto, in collaboration with Gerin and Purcell, successfully transmitted this new antigen and its associated hepatitis to chimpanzees. A new virus particle distinct from HBV virions or HBV surface antigen (HBsAg) particles was identified in plasma of the infected chimpanzees. Thus, delta antigen was recognized as a component of a novel virus.

This new virus, hepatitis delta virus (HDV), was shown to rely on HBV for transmission because it utilized the HBsAg as its own virion coat. Subsequently, a radioimmunoassay for antidelta antibodies was developed, which revealed that HDV infections were prevalent throughout the world, particularly in Mediterranean countries. In 1986, the RNA genome of HDV was molecularly cloned and sequenced, revealing its unique, circular RNA structure, which closely resembled that of viroids, a class of plant pathogens. Thus, HDV was recognized as a new class of animal pathogen.

Taxonomy and Classification

HDV is a single-stranded RNA satellite virus, which depends on HBV as a helper virus for a complete viral life cycle. To date, HDV is the only member virus of the floating genus *Deltavirus* (no Family designation). No sequence or structural relationship exists between HDV and HBV. Structural and biochemical features of HDV RNA suggest some similarity to plant viroids or virusoids. However, *Deltavirus* is taxonomically distinct from viroids. HDV has been isolated only from humans. HDV isolates can be classified into three genotypes based on the extent of nucleotide sequence divergence, each genotype differing by over 35%; however, all of the isolates are antigenically related.

Geographic Distribution

Hepatitis delta virus infection is found worldwide and is endemic in certain areas, particularly southern Italy and the Mediterranean countries. Its distribution closely parallels that of HBV infections. However, its prevalence rate does not always match that of HBV infection in different geographical areas; for example, in some HBV-prevalent areas such as China, HDV infection is disproportionately low. In developed countries such as the USA, the rate of HDV infections is low in the general population and highest among intravenous drug abusers and hemophiliacs who receive blood products. Genotype I HDV has been isolated in every geographical region in the world. Genotype II so far has been found largely within Asia, primarily Taiwan and Japan. Genotype III, which is associated mostly with fulminant hepatitis, has been detected only in South America.

Host Range and Virus Propagation

In natural infections, HDV has only been isolated from humans. It has been experimentally transmitted to both chimpanzees preinfected with HBV and woodchucks preinfected with woodchuck hepatitis virus (WHV), causing clinical hepatitis similar to that in humans and producing HDV particles with HBV and WHV surface antigens, respectively. The presence of hepadnavirus in these animals is a prerequisite for successful HDV transmission. Viral infection is limited exclusively to hepatocytes. In tissue culture, woodchuck-passaged HDV can infect woodchuck primary hepatocytes, but infection is limited to one cycle in a small number of cells; it does not infect established tissue culture cell lines. An alternative method of virus propagation is the transfection of tissue culture cells with HDV cDNA or RNA. Such studies have shown that both hepatic and nonhepatic human and other mammalian cells lines are capable of supporting HDV replication even in the absence of a helper hepadnavirus. Thus, host range and tissue specificity are restricted primarily by the availability of a viral receptor in hepatocytes. However, these transfected cells do not produce virion particles unless

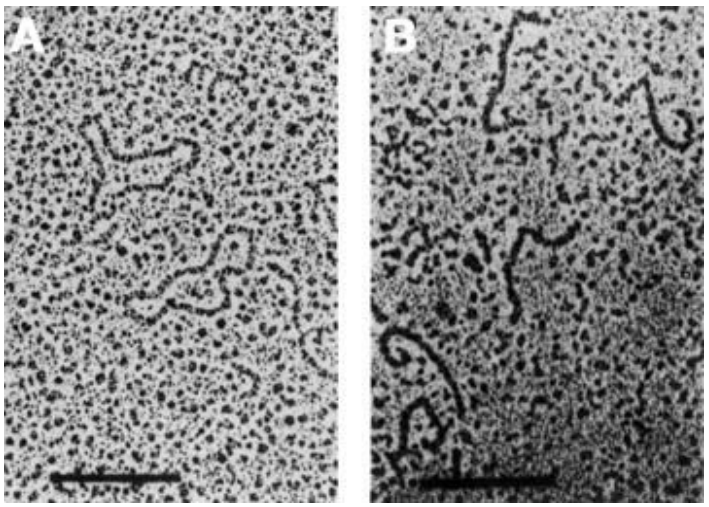


Figure 1 Electron micrograph of HDV RNA. (**A**) Denaturing condition showing the circular shape of HDV RNA. (**B**) Nondenaturing condition, showing the collapse of HDV RNA into a double-stranded RNA form as a result of the presence of intramolecular complementary sequence. Bar = 0.2 mm. (Reproduced with permission from *Nature* 323: 558–560, 1986).

a helper hepadnavirus is present. HDV RNA expressed in transgenic mice can replicate in every tissue.

Properties of the Virion

HDV virions are 36 nm, enveloped particles, which do not have surface projections but contain an internal, spherical nucleocapsid of 18 nm. Virus particles have a buoyant density in CsCl of 1.25 g^{-mL}. The envelope consists of HBsAg, while the nucleocapsid consists of hepatitis delta antigen (HDAg) and RNA. The intact virus particle is reactive to the anti-HBs antibody, but not the anti-HDAg antibody. The virus can survive extended periods of dry heat, up to 30 h at 60°C.

Properties of the Genome

The HDV genome is a single-stranded, circular RNA of 1.7 kb. It has a high degree of intramolecular complementarity, with about 70% of its nucleotides being base-paired to each other. Under nondenaturing conditions, HDV RNA forms a double-stranded, unbranched, rod-shaped structure (**Fig. 1**). The complete nucleotide sequence of more than 15 HDV isolates has been determined. The RNAs range from 1670 to 1683 nucleotides and contain about 60% of G + C.

The HDV RNA genome does not have a functional open reading frame (ORF); however, the antigenomic-sense HDV RNA detected in the infected cells has an ORF, which encodes the HDAg. This is the only protein made by the virus.

Both the genomic- and antigenomic-sense HDV RNA possess autocatalytic cleavage and ligation ('ribozyme') activities. Cleavage occurs at a specific site on both the genomic- and antigenomic-sense RNA and can take place in the absence of any protein. The ribozyme activity is contained within the region of HDV RNA that is considered to be a remnant of viroid RNA. However, the sequence and structural requirements of HDV ribozyme differ from those of known viroid or virusoid RNA ribozymes.

Properties of the Viral Proteins

HDV encodes a single protein, HDAg, which was the nuclear antigen originally detected by Mario Rizzetto. HDAg is not exposed on the virion surface; rather, it can be detected only after the virus envelope is disrupted. The protein consists of two species, small HDAg (24 kDa, 195 amino acids) and large HDAg (27 kDa, 214 amino acids). The two protein species are identical, except for the 19 additional amino acids at the C-terminus of the large HDAg. In most patients' sera, these two species are present in roughly equal amounts, but in infected liver the ratio often varies. They are complexed with HDV RNA genome in the virion and in the cells. In infected cells, they are primarily in the nuclei. HDAg is phosphorylated.

The large and small HDAg are translated from two distinct species of HDV RNA, which differ in the presence or absence of a termination codon near the C-terminus of the protein. During HDV RNA replication, small HDAg is synthesized first; large HDAg becomes detectable late in the viral replication cycle. Small HDAg is required for HDV RNA

replication, while large HDAg inhibits HDV RNA replication but is required for virion assembly. Thus, they play distinct roles in the viral life cycle.

HDAg contains several functional domains: The first contains a coiled-coil sequence, which enables the HDAg to form oligomers. The second is the nuclear localization signal, which transports HDAg and HDV RNA into the nucleus. The third contains the RNA-binding motif, which confers an RNA-binding property specific for HDV RNA. The final domain is unique to the large HDAg. It comprises the C-terminal 19 amino acids of the large HDAg and enables it to interact with the HBsAg during the assembly of HDV particles. It also causes the inhibition of HDV RNA replication. This domain is prenylated; prenylation is important for the various functions of the large HDAg.

The second protein present in the HDV particle is HBsAg, which constitutes the viral envelope. The protein is provided by the co-infecting HBV. It is essential for HDV particle assembly and virus transmission, but not necessary for HDV RNA replication. The envelope of HDV contains three HBsAg protein species, i.e. major, middle and large. The major HBsAg is the predominant form in the HDV virion.

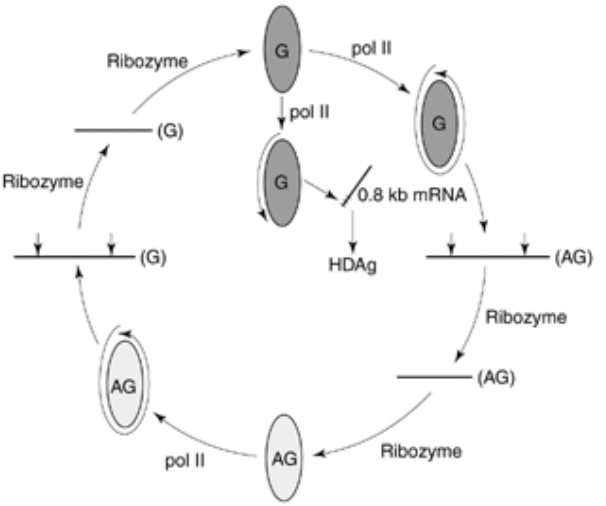


Figure 2 Proposed model of HDV RNA replication. The HDV RNA genome (G) is transcribed into a 0.8 kb mRNA (antigenomic sense) by cellular RNA polymerase II (pol II). The mRNA stops at a polyadenylation signal and is used for HDAg synthesis. The replication of genome length RNA goes through the synthesis of multiple-unit length antigenomic (AG) RNA intermediates, followed by cleavage and ligation by HDV 'ribozyme' to generate a circular antigenomic RNA. The synthesis of genomic RNA from the antigenomic RNA goes through a similar cycle.

Replication

The mode of virus entry into cells is not known, but may involve an interaction between HBsAg and a cellular receptor. Once inside the cell, HDV RNA can replicate in the absence of the helper HBV. The incoming viral RNA is transported into the nucleus by HDAg. RNA replication takes place in the nucleus via RNA-dependent RNA synthesis without a DNA intermediate. This process is most likely carried out by a cellular polymerase, as HDV does not encode its own polymerase. As HDV RNA synthesis can be inhibited by α-amanitin, it most likely utilizes cellular RNA polymerase II. It is not clear how this enzyme is converted from a DNA-dependent enzyme to an RNA-templated enzyme. Replication proceeds according to the double rolling circle model, yielding an antigenomic RNA product of multiple genome-unit length (**Fig. 2**). This RNA intermediate is subsequently cleaved into a single-unit length molecule by an autocatalytic process. The monomeric antigenomic RNAs are ligated into circular RNAs, also by an autocatalytic process. These circular RNAs then serve as templates for another round of rolling circle replication, yielding a genomic-sense RNA that repeats the cleavage and ligation process to form circular genomic-sense RNAs, thereby completing the cycle. The origins of replication for both genomic and antigenomic RNA have not been determined.

A subgenomic-size antigenomic-sense mRNA species of 0.8 kb which encodes the HDAg is also synthesized. The 5′ end of the mRNA begins at a point slightly upstream of the initiation codon and terminates immediately after a poly(A) addition signal in the HDV RNA. How mRNA transcription and genomic RNA replication are differentially regulated is not yet clear. This 0.8 kb RNA is associated with polysomes and used for translation of HDAg. The HDAg then is transported into the nucleus and participates in replication of HDV RNA; the precise mechanism by which the HDAg participates has not been elucidated. Only the small HDAg can promote HDV RNA replication.

During HDV RNA replication, a specific base mutation occurs on the termination codon of the small HDAg, resulting in a longer ORF encoding the large HDAg. This process is called RNA editing, which is probably mediated by cellular double-stranded RNA–adenosine deaminase. Thus, the large HDAg appears only later in the viral replication cycle. The large HDAg inhibits HDV RNA replication and triggers the formation of virus particles by interacting with HBsAg. Thus, the appearance of the large HDAg signals the switch from RNA replication to virion

assembly. How RNA editing is regulated is not yet clear.

The HDV genomic RNA is copackaged with HDAg into the virion. Both the large and small HDAg are packaged, although the small HDAg alone cannot trigger virus assembly. Only the genomic, but not the antigenomic, RNA is packaged into the virion. Virion assembly probably occurs in the cytoplasm, but the precise subcellular site has not been determined.

Genetics

HDV RNA shows very high genetic heterogeneity. Within the same HDV genotypes, different isolates could vary by as much as 21% sequence. Each virus isolate also consists of quasispecies with sequence microheterogeneity of nearly 0.1–0.5% of total nucleotides. Furthermore, mutations accumulate during chronic HDV infections in patients at an estimated frequency of approximately 2×10^{-3} to 1×10^{-2} mutations per nucleotide per year. An unusual characteristic of these mutations is that most are C → U or A → G transitions. In some cases, deletions also can be detected. However, the sequences around the catalytic cleavage sites of both the genomic and antigenomic RNA and the RNA editing site are highly conserved. No recombination has been reported.

Evolution

HDV RNA contains structural features characteristic of plant viroid or virusoid RNAs, but is significantly larger (1.7 kb versus 0.3–0.4 kb for viroids). It has been speculated that HDV RNA evolved from two different origins: a 'ribozyme' domain derived from viroids, which shares some sequence similarity with viroids, and another domain, which contains the ORF encoding HDAg and has been shown to share sequence similarity with a cellular protein termed 'Dip' (Delta antigen-interacting protein). Thus, HDV RNA could have evolved by recombination between a viroid RNA and a cellular mRNA. A portion of the HDV RNA is similar in sequence to the mammalian 7S RNA. HDV is the only animal virus with a circular RNA structure, although such an RNA structure is common among plant pathogens.

Serologic Relationships and Variability

HDV contains only one unique viral protein, HDAg. (The other protein, HBsAg, is shared with HBV.) Serological diagnosis of HDV infection is based on the antigenicity of this protein. Although there are three HDV genotypes, which differ in the amino acid sequence of HDAg, they cannot yet be distinguished serologically.

Epidemiology

HDV infection has been reported in every geographical region of the world. HDV transmission requires co-infection with HBV or occurs in individuals who are HBV carriers. The mode of transmission is parenteral. Approximately 5% of the HBsAg carriers in the world have been infected by HDV. Areas with a high prevalence rate of HDV infections include the Mediterranean Basin, particularly southern Europe, the Middle East, West Africa, the Amazon Basin of South America and certain South Pacific islands. Most of the HDV isolates from these regions (except South America) belong to genotype I. Severe fulminant-type delta hepatitis has been reported among Indians in South America, where genotype III HDV is prevalent. In Asia, the prevalence rate of HDV infections is relatively low, in contrast to the high prevalence rate of HBV infections. Genotype II is prevalent in east Asia (Taiwan and Japan). In nonendemic areas, HDV infection is sporadic and often associated with intravenous drug abusers. Sexual transmission has also been documented. In recent years, the prevalence rate of HDV has seen significant decreases across the world, probably as a result of HBV vaccination and routine blood screening for HBV, hepatitis C virus and human immunodeficiency virus.

Pathogenicity

Two possible mechanisms of HDV pathogenesis have been proposed: the first is the direct cytocidal effects of HDV; the second is an immune-mediated pathology. Consistent with the direct cytopathogenicity of HDV are the histopathology of delta hepatitis, which shows areas of hepatocellular necrosis with foamy degeneration, and the observation that immunosuppressive agents do not have significant effects on delta hepatitis. Furthermore, it has been shown that high levels of HDAg expression in mammalian cells can lead to cell death and that HDV infection of SCID (severe combined immunodeficiency) mice causes hepatic cell death, suggesting the direct cytotoxicity of HDV. However, permanent cell lines harboring integrated HDV cDNA have been isolated, which continuously express HDV RNA and HDAg without apparent cytopathology, and transgenic mice expressing HDV RNA or HDAg did not show any liver pathology; thus, HDV RNA replication alone may not be sufficient to cause cytopathology. Evidence supporting immune-mediated cytopathogenicity is derived from the finding that replication of HDV has often subsided and

immune responses have begun before the appearance of histopathology in delta hepatitis. Furthermore, experimental vaccination of chimpanzees with HDAg exacerbated the severity of subsequent HDV infections. The role of cellular immunity in the pathogenesis of HDV is not clear.

Clinical Features of Infection

The clinical features of HDV infection vary with the mode of transmission. In general, HDV infections have an incubation period of 3–7 weeks. The disease usually begins with a nonspecific prodrome of fatigue, anorexia, nausea and other nonspecific symptoms, followed by the typical signs of hepatitis, e.g. jaundice, increased serum alanine and aspartate aminotransferase activity. In cases resulting from simultaneous infection of HBV and HDV, acute symptoms are often followed by complete recovery within 12–16 weeks. In contrast, disease resulting from the superinfection of HDV in an HBV carrier often leads to chronic hepatitis. Furthermore, about 60–70% of patients with chronic hepatitis D develop cirrhosis. Fulminant hepatitis frequently results from HDV infection and is characterized by a high mortality rate. The probability of developing fulminant hepatitis is much higher for HDV than other types of viral hepatitis. There is no direct association between HDV infection and hepatocellular carcinoma; however, cirrhosis resulting from chronic delta hepatitis increases the risk of hepatocellular carcinoma.

Superinfection of HBV carriers by HDV often results in the inhibition of HBV replication. During the acute phase of HDV infection, synthesis of both HBsAg and HBV DNA are inhibited until the HDV infection is cleared. In chronic delta hepatitis, the HBV markers are suppressed throughout the entire clinical course of disease. The mechanism of suppression has not been elucidated.

During the acute phase of delta hepatitis, both HDV RNA and HDAg can be detected. This is soon followed by the appearance of IgM anti-HD antibody. In chronic delta hepatitis, HDV RNA, HDAg, and IgM and IgG anti-HD antibodies persist.

Pathology and Histopathology

The histopathology of acute delta hepatitis is not different from that of acute hepatitis caused by other viral agents. In delta hepatitis, hepatocytes often appear to be necrotic and surrounded by inflammatory cells, including lymphocytes and macrophages. The hepatocytes have deeply acidophilic cytoplasm and an irregular, rhomboid shape with pyknotic nuclei. In chronic delta hepatitis, there is a prominent necroinflammatory response in the parenchyma, with marked portal and periportal inflammatory changes. The HDAg can be detected in the nuclei of affected hepatocytes in both acute and chronic delta hepatitis. HDV RNA can also be detected in the nuclei by *in situ* hybridization. Furthermore, examination by electron microscopy reveals a 'microtubular structure' in the hepatocytes that is characteristic of, but not unique to, delta hepatitis.

Immunity

HDV infections induce both humoral and cellular immunity in patients. Antibodies are directed against most regions of the HDAg, but different regions are immunodominant in different individuals. Helper, suppressor and cytotoxic T cells are detectable in different areas of the infected liver. Their importance in HDV infection is not clear. Experimental immunization with HDAg in woodchucks did not provide protection for the animal. In fact, this immunization may exacerbate the clinical symptoms of subsequent HDV challenges. Anti-HD antibodies usually do not persist after acute infection is cleared. Thus, the serological evidence of past HDV infection is not easy to demonstrate.

Prevention and Control of Infection

Because HDV infection is dependent on a concomitant HBV infection, control of HDV infection is best achieved by HBV vaccination. However, there is no effective measure to prevent HDV infection of chronic HBV carriers. No routine blood screening for HDV is currently performed; however, HDV infection by contaminated blood sources can be prevented by screening for HBV. Currently, there is no specific treatment for delta hepatitis. Interferon α has been tried therapeutically with only marginal and transient effects. Ribavirin has been shown to inhibit HDV RNA replication in tissue culture.

Future Perspectives

Because of the severe hepatitis associated with HDV infection, its prevention and control is important. Although the molecular cloning of HDV RNA has opened up research on the replication of HDV, many questions remain unanswered, e.g. the enzymology of HDV RNA replication, the mechanisms of action of HDAg, the regulation of RNA editing, the regulation of the synthesis of HDAg, etc. HDV occupies a unique place in the taxonomy of viruses, possibly as a bridge between plant and animal viruses. Understanding these and other issues will be critical for the

future development of prevention measures and therapy for HDV infections.

See also: **Hepadnaviruses (*Hepadnaviridae*): Avian hepatitis B virus, General features, Molecular biology; Pathogenesis: Plant viruses; Persistent viral infection; Ribozymes; Taxonomy and classification – general; Viroids; Virus–host cell interactions.**

Further Reading

Lai MMC (1995) The molecular biology of hepatitis delta virus. *Annu. Rev. Biochem.* 64: p. 259.

Purcell RH and Gerin JL (1996) Hepatitis delta virus. In: Fields BN, Knipe DM, Howley PM *et al* (eds.) *Fields Virology*, 3rd edn, p. 2819. Philadelphia: Lippincott-Raven.

HEPATITIS E VIRUS

Albert W Tam and **Patrice O Yarbough**, Genelabs Technologies Inc, Redwood City, California, USA

Daniel W Bradley, Virology Laboratory Section, Hepatitis Branch, Centers for Disease Control, Atlanta, Georgia, USA

History

A significant proportion of acute viral hepatitis occurring in young to middle-age adults in Asia, Africa and the Indian subcontinent is caused by an enterically transmitted viral agent that is serologically unrelated to hepatitis A virus (HAV). The disease has been shown to occur in both epidemic and sporadic endemic forms and is primarily associated with the ingestion of fecally contaminated drinking water. The term enterically transmitted non-A, non-B hepatitis (ET-NANBH) was originally used to distinguish this disease from the parenterally transmitted form of NANBH. The latter disease is primarily associated with infection by hepatitis C virus (HCV), a small, enveloped flavi- or pesti-like virus. The viral agent of ET-NANBH was isolated in the mid-1980s, partially characterized, molecularly cloned and has been officially named hepatitis E virus (HEV) by the International Committee on Taxonomy of Viruses (ICTV).

Hepatitis E was first documented in New Delhi, India, in 1955, when 29 000 cases of icteric hepatitis were identified following widespread fecal contamination of the city's drinking water. A similar epidemic of viral hepatitis occurred between December 1975 and January 1976 in Ahmedabad City, India, again due to contaminated water supplies. Both outbreaks were originally thought to be caused by HAV; however, retrospective serologic analysis of paired serum specimens from documented cases revealed that neither HAV nor hepatitis B virus (HBV) was the etiologic agent.

Large epidemics of hepatitis E have also been observed in the Kirgiz Republic of the former USSR. Although these outbreaks were never described in the Western scientific literature, careful review of records and other related documents and manuscripts by D.W. Bradley during a visit to the former USSR in 1986 clearly revealed that HEV was the most likely etiologic agent. Between 1955 and 1956 more than 10 800 cases of acute viral hepatitis were documented in young to middle-age adults; approximately 18% of infected pregnant women died as a direct result of presumed infection with HEV. The epidemiological and clinical features of the Kirgiz outbreaks were remarkably similar to those associated with the 1955–1956 New Delhi outbreak.

Taxonomy and Classification

HEV is a small nonenveloped virus with morphologic features similar to those of Norwalk virus and other caliciviruses (see below). Based on the calici-like physiochemical properties, it was tentatively grouped into the *Caliciviridae* prior to its molecular identification. Subsequent to its cloning, sequence comparison reveals little, if any, homology between HEV and known viruses of *Caliciviridae*. Genomic organization analysis further supports a lack of genetic relationship. As a result, HEV has been declassified from the *Caliciviridae* by the ICTV, and currently remains unclassified in a floating genus, 'Hepatitis E-like viruses'.

future development of prevention measures and therapy for HDV infections.

See also: **Hepadnaviruses (*Hepadnaviridae*): Avian hepatitis B virus, General features, Molecular biology; Pathogenesis: Plant viruses; Persistent viral infection; Ribozymes; Taxonomy and classification – general; Viroids; Virus–host cell interactions.**

Further Reading

Lai MMC (1995) The molecular biology of hepatitis delta virus. *Annu. Rev. Biochem.* 64: p. 259.

Purcell RH and Gerin JL (1996) Hepatitis delta virus. In: Fields BN, Knipe DM, Howley PM *et al* (eds.) *Fields Virology*, 3rd edn, p. 2819. Philadelphia: Lippincott-Raven.

HEPATITIS E VIRUS

Albert W Tam and **Patrice O Yarbough**, Genelabs Technologies Inc, Redwood City, California, USA

Daniel W Bradley, Virology Laboratory Section, Hepatitis Branch, Centers for Disease Control, Atlanta, Georgia, USA

History

A significant proportion of acute viral hepatitis occurring in young to middle-age adults in Asia, Africa and the Indian subcontinent is caused by an enterically transmitted viral agent that is serologically unrelated to hepatitis A virus (HAV). The disease has been shown to occur in both epidemic and sporadic endemic forms and is primarily associated with the ingestion of fecally contaminated drinking water. The term enterically transmitted non-A, non-B hepatitis (ET-NANBH) was originally used to distinguish this disease from the parenterally transmitted form of NANBH. The latter disease is primarily associated with infection by hepatitis C virus (HCV), a small, enveloped flavi- or pesti-like virus. The viral agent of ET-NANBH was isolated in the mid-1980s, partially characterized, molecularly cloned and has been officially named hepatitis E virus (HEV) by the International Committee on Taxonomy of Viruses (ICTV).

Hepatitis E was first documented in New Delhi, India, in 1955, when 29 000 cases of icteric hepatitis were identified following widespread fecal contamination of the city's drinking water. A similar epidemic of viral hepatitis occurred between December 1975 and January 1976 in Ahmedabad City, India, again due to contaminated water supplies. Both outbreaks were originally thought to be caused by HAV; however, retrospective serologic analysis of paired serum specimens from documented cases revealed that neither HAV nor hepatitis B virus (HBV) was the etiologic agent.

Large epidemics of hepatitis E have also been observed in the Kirgiz Republic of the former USSR. Although these outbreaks were never described in the Western scientific literature, careful review of records and other related documents and manuscripts by D.W. Bradley during a visit to the former USSR in 1986 clearly revealed that HEV was the most likely etiologic agent. Between 1955 and 1956 more than 10 800 cases of acute viral hepatitis were documented in young to middle-age adults; approximately 18% of infected pregnant women died as a direct result of presumed infection with HEV. The epidemiological and clinical features of the Kirgiz outbreaks were remarkably similar to those associated with the 1955–1956 New Delhi outbreak.

Taxonomy and Classification

HEV is a small nonenveloped virus with morphologic features similar to those of Norwalk virus and other caliciviruses (see below). Based on the calici-like physiochemical properties, it was tentatively grouped into the *Caliciviridae* prior to its molecular identification. Subsequent to its cloning, sequence comparison reveals little, if any, homology between HEV and known viruses of *Caliciviridae*. Genomic organization analysis further supports a lack of genetic relationship. As a result, HEV has been declassified from the *Caliciviridae* by the ICTV, and currently remains unclassified in a floating genus, 'Hepatitis E-like viruses'.

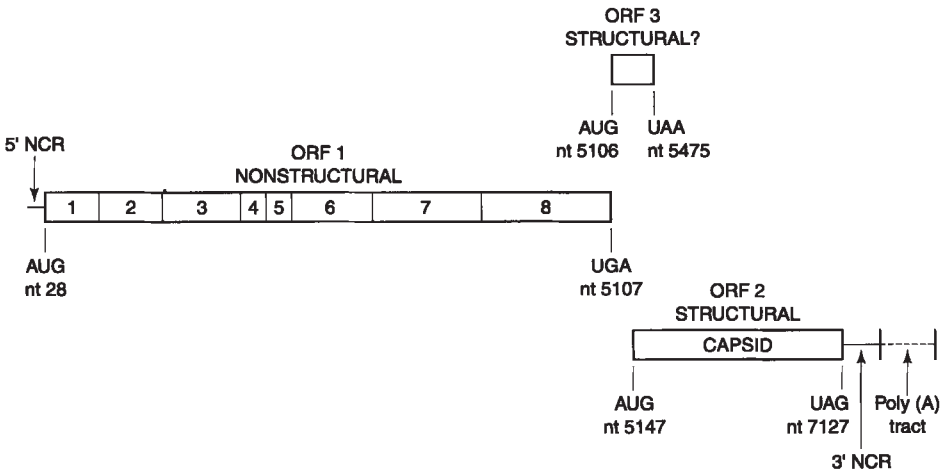


Figure 1 Genomic organization of hepatitis E virus (HEV) with three overlapping open reading frames (ORFs). Order of nonstructural genes and functional domains in ORF 1 is: (1) methyltransferase, (2) domain Y, (3) papain-like protease, (4) hypervariable region, (5) proline-rich hinge, (6) domain X, (7) helicase, and (8) RNA-dependent RNA polymerase. AUG, start codon; UAA/UGA/UAG, stop codons; NCR, noncoding region; nt, genomic nucleotide sequence number of HEB-Burma strain.

Physiochemical Properties of the Virion

Table 1 summarizes what is known to date about the major etiologic agent of hepatitis E. The virus appears to be extremely labile and will not tolerate exposure to high concentrations of salt, including cesium chloride. Pelleting of the virus from stool suspensions or gradient fractions frequently results in loss of virus. Rate-zonal banding of 32–34 nm virus-like particles (VLPs) in linear, preformed sucrose gradients, however, was found to yield partially purified virus suitable for further immune electron microscopy (IEM) studies. The computed sedimentation coefficient for HEV was approximately 183 S, in contrast to 157 S for that of HAV, a member of the *Picornaviridae*. Virus particles were sometimes found to sediment at 165 S; these particles are presumed to be defective and probably lack a complete viral genome. Isopycnic banding of an aliquot of the Telixtac no. 14 stool (HEV isolate from a 1986 outbreak in southern Mexico) suspension in a potassium tartrate/glycerol gradient revealed 32–34 nm VLPs at a buoyant density of 1.29 g ml^{-1}. These biophysical properties were consistent with the earlier notion that the major agent of hepatitis E was a calicivirus-like virus. Like many caliciviruses, the 32–34 nm VLPs described here are also sensitive to freeze-thawing, storage in liquid suspensions at temperatures between −70°C and +8°C, and pelleting from solutions of sucrose or buffer. HAS-15 HAV (partially purified from tissue

Table 1 Properties of hepatitis E virus (HEV)

Size:	32–34 nm mean diameter in 80% of virus particles visualized by immune electron microscopy, with a range of 27–38 nm
Morphology:	Spherical, nonenveloped particle with spikes and indentations visible on surface of virus
Biophysical properties:	Sedimentation coefficient = 183 S (defective particles, 165 S)
	Buoyant density = 1.29 g ml^{-1}
	Sensitive to cesium chloride, freeze-thawing, and pelleting
Genome:	Single-stranded, polyadenylated RNA of approximately 7.5 kb in size with three open reading frames 5′-nonstructural, 3′-structural genomic organization
Host range:	In addition to humans, natural infection reported in nonhuman primates, cattle, pigs, chickens and rodents
	Most reliable experimental infection models in cynomolgus and rhesus monkeys
	No effect on suckling mice inoculated intracerebrally with numerous case study stool suspensions (10% w/v)
In vitro *propagation:*	Reported virus replication mostly in primary cell cultures

culture lysates) and HEV contained in case stools from Burma, Mexico, Somalia and the Soviet Union were independently measured by three individuals at the Centers for Disease Control and Prevention in Atlanta, GA. HAV was found to have a mean diameter of 27–28 nm. HEV was found to have a mean diameter of 32–34 nm, with 78% of the VLPs within a range of 31–35 nm. There were no statistically significant differences in mean particle diameters in any of the four geographically different HEV isolates.

Molecular Biology of Genome Structure

HEV is a positive-sense, single-stranded RNA virus with a polyadenylated genome of 7.5 kb in length. cDNA cloning of HEV was initially attained with a virus isolate recovered from an epidemic outbreak in Burma. Additional strains identified and fully sequenced to date include those from China, India, Mexico, Pakistan and the former Soviet Union. Partial genomic sequences have also been determined from cases in Algeria, Chad, Morocco and the USA. Sequence analysis of all known HEV strains indicates the presence of three open reading frames (ORFs) in the virus genome. Two major ORFs of approximately 5 and 2 kb long are found in the first and second coding frames. A small ORF of 369 bp in length is present in the third forward frame that overlaps both of the two larger ORFs. Relatively short noncoding segments of 27 and 65 nucleotides are found at the 5′ and 3′ ends, respectively, of the viral genomic sequence.

HEV possesses a 5′-nonstructural, 3′-structural genomic organization. Conserved motifs corresponding to such viral proteins as the methyltransferase, papain-like protease, helicase and RNA-dependent RNA polymerase are found in the first open reading frame (ORF 1) that extends from the 5′ end of the virus genome. The nonstructural HEV proteins encoded by ORF 1 have yet to be extensively studied. The identities of these proteins, as depicted in **Fig. 1**, were revealed only by detailed computer alignment analysis of deduced amino acid sequence. Not much has been learned of their biological functionality. Just as the identification of a methyltransferase gene would suggest that HEV genomic RNA is capped, it remains to be empirically demonstrated. Domains X and Y, identified by their conserved motifs with other positive-strand RNA viruses, have no known assigned biological functions. The manner in which proteolytic processing of the ORF 1 polyprotein into individual protein species is also not known at present. The papain-like protease is presumed to play a role, but host cell proteases may also be involved in the process. Epitope mapping using a strategy of overlapping peptides revealed the presence of several potential immunogenic peptides, especially in the polymerase region. However, none has been shown to be of use in diagnostic assays. ORF 2 occupies the 3′-most 2 kb of viral genomic sequence and codes for the single capsid protein of HEV. A hydrophilic electropositive region rich in arginine residues found near the N-terminus of the capsid protein serves to neutralize and encapsulate the electronegatively-charged genomic RNA. A major immunogenic epitope is located at the C-terminus of the capsid protein, and has been found to be most useful in the diagnosis of hepatitis E (see below). The small ORF 3 also codes for an immunogenic protein of 123 amino acids; however, its biological role in the life cycle of the virus remains unclear.

In addition to the full-length genomic RNA, two subgenomic RNA transcripts of 3.7 and 2.0 kb have been detected in Northern blot analysis of infected liver RNA. All three RNA species coterminate at the 3′ poly(A) tail of the virus genome. The two subgenomic transcripts are believed to be involved in expression of the ORF 2- and ORF 3-encoded proteins. Hence, the HEV genome employs all three coding frames for its three ORFs as well as the use of subgenomic transcript in its expression strategy.

Genetics and Evolution

Genetic heterogeneity has been noted among the HEV strains fully sequenced to date. Of these, the Mexican isolate cloned from the Telixtac outbreak is the most divergent genetically. The Asian strains (Burma, China, India and Pakistan) are well-conserved among themselves, with nucleotide sequence identity between 93 and 100% in the coding regions and amino acid sequence identity between 98 and 100%. HEV-Mexico, on the other hand, differs from the Asian strains by about 25% at the nucleotide level and 17% at the amino acid level in ORF 1, 20% and 7% in ORF 2, and 10% and 13% in ORF 3. Unrooted phylogenetic analysis of the ORF 2/3 structural gene coding regions of the Burma, China, India, Mexico and Pakistan strains clearly shows the genetic distance between the Mexico isolate and the Asian strains (**Fig. 2**). These two genetic groups appear to represent the diverse ends of the heterogeneity based on geographical distribution. Limited sequence comparison of the more recently identified African strains (Algeria, Chad and Morocco) indicated that they are more closely related to the Asian strains, but within the phylogenetic continuum between the two ends.

The geographic heterogeneity found among the HEV strains suggests an 'Old World–New World'

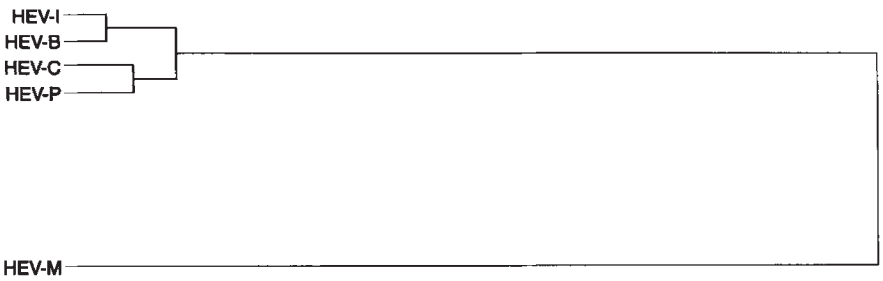


Figure 2 Phylogeny analysis of hepatitis E virus isolates with known structural gene coding sequences. The dendrogram depicts phylogenetic distances between the different strains of HEV and is derived from algorithms for phylogenetic tree generation using the CLUSTAL-V program.

evolution of the virus. Imported cases of hepatitis E in the Middle East region by religious pilgrims and labor workers from Central Asia have been reported. Interestingly, though, partial sequence information from the two most recently cloned HEV strains (USA and Taiwan) has provided conflicting evidence. The US isolate, identified from an acute hepatitis patient with no record of travel, yielded sequences as different from the Burma strain as they were from the Mexico strain. In another study, consensus ORF 1 sequence isolated from four patients in Taiwan was found to be divergent not only from the Mexico strain, but also more removed genetically than expected from strains described for Burma, China, India and Pakistan. Moreover, finding of atypical strains of HEV that did not serologically crossreact with the known strains has recently been reported by several laboratories, suggesting perhaps the existence of different serotypes of HEV. Full phylogenetic analysis of HEV must await the complete cloning and characterization of these recently discovered isolates as well as additional strains from other regions in which occurrence of hepatitis E has been documented.

Host Range and Virus Propagation

Humans are the natural hosts for hepatitis E. HEV is the major cause of acute viral hepatitis in young to middle-age adults living in underdeveloped regions of the world where water sanitation measures are lacking. Anti-HEV has been detected in wild-caught monkeys and rodents, suggestive of prior exposure to HEV or a related agent. Natural infection of various domestic species such as chicken, cattle and pigs has recently been described, especially in hepatitis E endemic regions. The zoonotic nature of HEV is further illustrated by the recent identification of a swine hepatitis E virus in Midwestern USA, where hepatitis E has rarely been reported. HEV researchers have long sought an explanation for the relatively high anti-HEV prevalence surveyed even in non-endemic countries. While the existence of a virus reservoir in animals as exemplified by the swine HEV and its potential transmission to the human population could serve to account for the observed seroprevalence rate, no record of animal strains of HEV infecting humans has been documented.

Nonhuman primates shown to be susceptible to experimental HEV infection include chimpanzees, cynomolgus macaques, rhesus monkeys, African green monkeys, tamarins, owl and squirrel monkeys. The most reliable animal transmission model to date remains the cynomolgus monkey (cyno). Multiple passages of the same virus in cynos has resulted in the shortening of the incubation period from more than 50 days to as little as 5 days, suggesting adaptation of the virus to the host or heightened response to an increased virus load.

As with other known hepatotropic viruses, development of a tissue culture system to replicate HEV has been problematic. Growing of three different strains of HEV in cultivated cells have been reported. Virus recovered in China was reported to be propagated in human embryo lung diploid cells. Cell-derived viruses were found to be reactive with anti-HEV obtained from HEV-infected human patients and nonhuman primates. Virus recovered from the Soviet Central Asia region was cocultivated in fresh fetal rhesus monkey kidney (FRhK-4) cells with primary cynomolgus monkey kidney cells isolated from experimentally infected animals. Nucleic acid hybridization and immunofluorescent detection of HEV antigen provided evidence for tissue culture-derived virus. More recently, the Burma strain HEV was propagated and produced in cell culture from *in vivo*- and *in vitro*-infected primary cynomolgus hepatocytes isolated and maintained in long-term culture using a serum-free medium formulation. The negative strand replicative RNA intermediates were directly detected by a sensitive strand-specific reverse transcription–polymerase chain reaction (RT-PCR)

assay. Presence of intracellular- as well as culture medium-derived viruses was demonstrated throughout the duration of the hepatocyte culture. Continued development of these *in vitro* culture systems may provide high titer virus samples suitable for further studies of HEV biology.

Transmission and Tissue Tropism

HEV can be experimentally transmitted to susceptible primates by either intragastric or intravenous inoculation. For humans, the normal route of infection is through consumption of contaminated drinking water. The incubation period in both nonhuman primates and humans is about 6 weeks, although adaptation or subpassaging of HEV in primates can significantly shorten the incubation period. Studies to date indicate that virus replicates primarily, if not exclusively, in liver cells of experimentally infected primates. There is no evidence for the existence of extrahepatic sites of virus replication, although sensitive *in situ* hybridization procedures may reveal the presence of nascent HEV RNA (negative strand) in cells other than hepatocytes.

The earlier development of an immunofluorescent antibody-staining procedure (FA) for HEVAg in liver tissue of experimentally infected primates permitted the visualization of disease-associated antigen (protein(s)) in liver cells. Fluorescent granules and deposits with a powdery appearance were found by FA in the cytoplasm of hepatocytes. Subsequent 'blocking' studies using a recombinant DNA-expressed, virus-specific protein to absorb the FITC-labeled IgG anti-HEV probe showed that these deposits were virus-specific.

HEV is excreted from the liver via the common bile duct into the duodenum of the small intestine. Although most stools obtained from acutely ill patients contain little or no virus detectable by IEM, numerous investigators have shown that HEV can be readily recovered from gall bladder bile, indicating that the lack of virus in stool samples is probably a function of the labile nature of the virus particle. Host proteases such as trypsin and chymotrypsin may contribute to the degradation in its passage through the gut. It is worth noting that the lower numbers of intact HEV particles present in patient stools may account for the generally lower rate of person-to-person transmission of hepatitis E when compared with that of hepatitis A. Viremia (HEV in blood/serum) is also associated with the course of disease, as shown by RT-PCR studies of HEV RNA in serum. HEV in serum (maximum level) usually precedes the major peak of alanine aminotransferase (ALT) activity.

Epidemiology

In developing countries, infection with HEV is a leading cause of morbidity and mortality. Since its initial documented outbreaks in India, hepatitis E epidemics have been reported in parts of Asia, Africa and North America, namely Nepal, Pakistan, Burma, the former Soviet Union, India, Borneo, Somalia, Sudan, China and Mexico. During epidemics the clinical attack rate has averaged 5% of the population, with the most severe disease observed in young adults. Two of the largest and most recent epidemics occurred in northwestern China between 1986 and 1988 with about 120 000 clinical cases reported and in 1991 in Kanpur, India affecting 79 000 persons. Virus-like particles recovered from stool specimens taken during the epidemics support the theory that one virus type was responsible for all the cases; in all of these outbreaks, hepatitis E was associated with the consumption of fecally contaminated water.

Sporadic outbreaks of hepatitis E have also been documented in Asia and Africa between the epidemic cycles. The virus is implicated in causing more than 50% of the sporadic acute viral hepatitis cases worldwide. The cause of sporadic hepatitis E in endemic regions is currently unknown, although recent demonstration of a swine hepatitis E virus may have implication for zoonosis.

Hepatitis E is not endemic in the USA and parts of Western Europe. With few exceptions, the confirmed cases of hepatitis E have been traced to immigrants and tourists from countries where HEV is endemic. These 'imported cases' establish that hepatitis E is a worldwide public health problem.

Clinical Features of Infection

Hepatitis E infection can be subclinical, acute or fulminant stage hepatitis. Some persons infected with HEV are anicteric without the classical symptoms of hepatitis. Asymptomatic hepatitis E is more likely to occur in infants and young children.

In acute hepatitis E, the diseased state usually becomes apparent within 2–9 weeks of exposure, the average incubation period being 6 weeks. Early symptoms of infection, such as chills, fever, nausea, vomiting, diarrhea, joint pain, headache and abdominal enlargement, can occur a week to 10 days before the onset of jaundice. The icteric phase usually persists for 15–40 days. Jaundice is accompanied by output of dark urine and clay-colored stools. Peak liver enzyme elevations occur usually in early icteric phase. Necroinflammatory changes observed in the liver are consistent with acute virus hepatitis. The period of recovery can take up to 14 weeks, after

which time liver biopsy returns to normal and liver enzyme levels return to baseline. Generally, hepatitis E is self-limiting, with full recovery and no progression to chronic liver disease.

Fulminant hepatitis E occurs in both men and women, but is mostly associated with high mortality rates in the third trimester of pregnancy in woman. Although the overall mortality for hepatitis E in the general population is estimated at 0.5–1.0%, mortality in pregnant women has been reported to be as high as 20%. The mechanism for fulminant hepatic failure during pregnancy is unknown. Endotoxin-mediated cytotoxicity, proposed as a cause for fulminating disease years ago, still cannot be ruled out as a cause. The absence of an animal model to study fulminant hepatitis E has hampered progress in this area.

Limited data from two human self-inoculation studies support the clinical course of disease already well-documented in experimentally infected non-human primates. HEV RNA was detected in serum and feces 22 days and 34 days respectively after self-inoculation. Anicteric symptoms of hepatitis began 30 days after inoculation. Icterus began 8 days later and lasted for more than 80 days. Antibodies to HEV were detectable at 41 days after infection and ALT elevations peaked 5 days later and persisted for 10 weeks. Jaundice was prolonged and hepatitis was severe; however, there was complete resolution of disease without any chronicity.

The clinical course of disease following HEV infection entails virus excretion in the feces, viremia, liver enzyme elevations (ALTs) and seroconversion, followed by the clearing of the virus and resolution of disease. As this clinical profile for HEV infection is similar to that of other forms of acute viral hepatitis, serologic markers for infection have proven most valuable for specific diagnosis.

Pathology and Histopathology

In the majority of hepatitis E outbreaks, liver specimens have rarely been available for histopathologic analysis. The only extensive studies of histopathological changes in the liver were carried out on biopsy specimens obtained from 78 patients during the large outbreak in New Delhi described earlier, and from 128 cases of endemic hepatitis E occurring in Ghana between 1962 and 1963. Two types of histopathologic changes were observed in the Indian outbreak: an obstructive or cholestatic type found in 58% of those examined, and a 'standard' type identified in 42% of cases. The cholestatic type was characterized by bile stasis in canaliculi and parenchymal cells which were arranged in a gland-like fashion. Cholestasis and glandular transformation of liver cell plates were much more significant in the cases from Ghana and West Africa (63% and 93%, respectively). Degenerative changes of liver cells (including acidophilic bodies) and focal necrosis were less frequently seen in the cholestatic types of hepatitis E in the New Delhi patients. Polymorphonuclear leukocytes were observed both in intralobular infiltrations and in portal tracts in larger amounts than seen in the 'classic' (standard) form of acute viral hepatitis, although in portal tracts monocytes (lymphocytes) remained the predominant cell type. In the standard type of viral hepatitis, ballooned hepatocytes, acidophilic degeneration of hepatocytes and acidophilic body formation were the most frequently encountered. Similarly, in experimentally infected primates, the acidophilic type of hepatocyte degeneration was a characteristic morphologic feature, particularly in cynos. Focal necrosis was observed in both patients and infected primates, with prominent accumulations of mononuclear macrophages and activated Kupffer cells and a much less striking presence of lymphocytes. In both human cases and infected primates, lymphocytes prevailed in portal infiltrations. In serial liver biopsy specimens from infected cynos, portal infiltrations seemed to occur later than intralobular changes. In summary, hepatitis E is associated with certain features of necrotic and degenerative changes in both humans and primates and is reminiscent of a toxic type of parenchymal lesion.

Serologic Markers and Immunodiagnosis

The traditional gold-standard diagnosis of HEV infection has been detection by IEM of virus-like particles in fecal specimens. Although highly specific, IEM is difficult to perform and most clinical specimens do not contain sufficient VLPs to be detected. An immunofluorescence-blocking assay using virus-specific antigen measures antibody to HEV in sera, but this assay does not distinguish between recent and past infection. Following the cloning and sequencing of the HEV genome, the identification of type-common viral epitopes within the structural genes led to the development of serologic assays for diagnosis of acute and past infection (**Fig. 3**).

An immunodominant epitope designated 3–2 and a broadly reactive peptide designated SG3 both map to the ORF 2 encoding a protein with properties consistent with being the capsid protein of the virus. A second epitope designated 4–2, that maps to ORF 3, is encoded by a cytoskeleton-associated phosphoprotein of unknown function. The immune response to ORF 3 has been shown to be more transient in experimental infections, waning within a few months

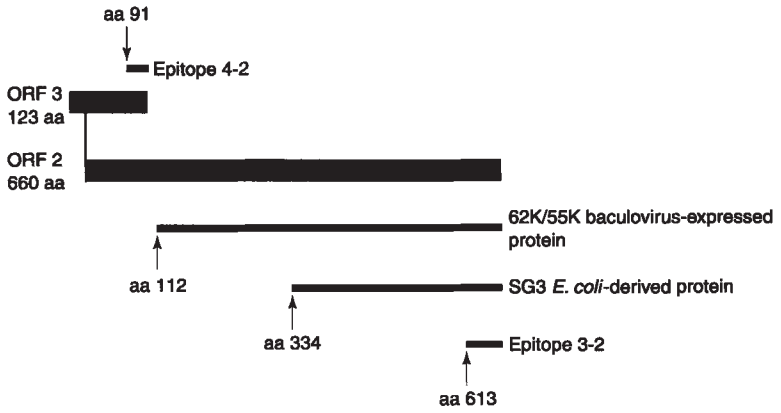


Figure 3 Localization of immunoreactive epitopes and immunogenic proteins encoded in the ORF 2 and ORF 3 structural regions of HEV. Arrows indicate the N-terminal amino acid residue numbers of expressed recombinant proteins. aa, Amino acids.

after ALT levels return to normal. Antibodies to ORF 2 have been shown to persist for years. An ELISA was developed using these virus-specific proteins expressed as recombinant proteins in *Escherichia coli*. The test is convenient, sensitive and specific, allowing the detection of IgG- and IgM-specific anti-HEV and therefore useful in diagnosing acute and past HEV infection. The specificity of the IgG and IgM ELISA is 99% and 97%, respectively. A sensitivity of 93.5% corresponds to IgM antibody measured in sera collected within 2 weeks of illness from acutely infected persons still shedding virus in the feces. The IgM antibodies to HEV decline quickly after acute infection resolves, although IgG antibodies persist at high titer for more than 1 year.

In an effort to preserve the immunologic structures that might be present only in the native structural protein and to improve the detection of acute phase antibodies to HEV, the structural capsid protein was expressed in insect cells by recombinant baculoviruses. The resulting soluble ORF 2 proteins, designated 62K and 55K deriving from the Burma and Pakistan strains, respectively, contain additional epitopes, presumably conformational, not present in the recombinant proteins expressed in bacteria (**Fig. 3**). These proteins are appropriate for large-scale epidemiologic studies and have been shown to be effective in experimental vaccination trials.

Nucleic Acid Diagnosis

HEV can be detected in acute phase stools and acute phase sera by RT-PCR. After recovery of the HEV viral RNA from stool or sera, cDNA is synthesized from RNA, followed by amplification by PCR using sense and antisense primers. PCR primers for the RNA-dependent RNA polymerase (RDRP) region within ORF 1 or the capsid protein within ORF 2 are mostly used to amplify target cDNA from divergent strains. HEV PCR products are analyzed by agarose gel electrophoresis and confirmed by Southern blot analysis with sequence-specific radiolabeled or biotinylated sequence-specific probes. It is important to note that viremia is usually short and often peaks before clinical signs of disease; therefore, depending on the timing of specimen acquisition, some acute phase sera may not contain enough viruses to be amplified. The assay, although useful for supplemental confirmation of infection, is not appropriate for screening.

Prevention and Control

Recent research on the identification of an effective subunit vaccine for hepatitis E has yielded encouraging results. Experimental vaccination of cynomolgus monkeys and rhesus monkeys with two different recombinant HEV capsid proteins, 62K and 55K, provides evidence for the potential to develop an effective vaccine for hepatitis E capable of providing crossprotection for the most divergent HEV strains. Unique antigenic and immunogenic properties of the ORF 2-encoded capsid protein expressed in baculovirus make them leading candidates as vaccine immunogens. Moreover, the amino acid sequence identity of these capsid proteins is 93% between the most divergent strains of HEV and 99% between the closely related Asian isolates of the virus, suggesting the presence of a well-conserved crossreactive immunologic determinant in the particle structure.

In two independent studies, nine cyno monkeys challenged with 1000–10 000 cynomolgus 50% infec-

tious doses (CID_{50}) of HEV following vaccination with 55K or 62K ORF 2 capsid protein were all protected from hepatitis. Postchallenge, there were no indicators of biochemical or histopathologic evidence for hepatocellular damage in any of the immunized animals. Vaccination clearly conferred protection against hepatitis in all animals. In one study there was a delayed and transient presence of viral RNA in the feces of only one of the three vaccinated animals, indicating limited 'breakthrough' viral replication. Although HEV infection was not prevented in this one animal, the presence of HEV-specific immunity at the time of challenge resulted in a limited infection and the absence of the characteristic features of HEV-induced disease.

A titration study with rhesus monkeys demonstrated that a dose of 0.4 μg of protein was effective in protecting the animals against hepatitis E following a challenge dose of virulent HEV. The capsid protein of HEV shows promise as a subunit vaccine candidate to: (1) prevent hepatitis E epidemics; (2) prevent sporadic cases of hepatitis E in developing countries; (3) protect pregnant women at high risk; and (4) provide protection to travelers.

Future Perspectives

Development of serologic tests for both IgM and IgG anti-HEV has allowed a comprehensive epidemiological survey of the distribution of HEV in the last few years. Cumulative data thus far suggest hepatitis E is not limited to endemic areas of the developing world. Clinical cases of HEV infection with no travel history have begun to be reported in Western developed countries. While hepatitis E disease burden in nonendemic areas remains to be fully evaluated, HEV will remain an important cause of morbidity and mortality in regions where fecal contamination of the environment and drinking water are still common. The need to further understand the biology of HEV and its replicative strategy within the host is made evident by recent discovery describing the virus as potentially a zoonotic agent. Development of a tissue culture system capable of supporting propagation of high titer HEV is essential. The mechanism leading to fulminant hepatic failure, especially in pregnant women, is still not understood and will require further studies. With the progressive realization of the medical significance of hepatitis E, recent development of an anti-HEV vaccine will be an important preventive measure for disease abatement in the future.

See also: **Diagnostic techniques: Detection of viral antigens, nucleic acids and specific antibodies, Isolation and identification by culture and microscopy; Epidemiology of viral diseases; Hepatitis A virus (*Picornaviridae*); Hepatitis C virus (*Flaviviridae*), Hepatitis Delta virus, Hepadnaviruses (*Hepadnaviridae*): Avian hepatitis B virus, General features, Molecular biology; Norwalk and related viruses (*Caliciviridae*); Immune response: Cell mediated immune response, General features; Pathogenesis: Animal viruses.**

Further Reading

Bradley DW *et al.* (1988) Aetiologic agent of enterically transmitted non-A, non-B hepatitis. *J. Gen. Virol.* 69: 731.

Fuerst TR *et al.* (1996) Prevention of hepatitis E using a novel ORF2 subunit vaccine. In: Buisson Y, Coursaget P and Kane M (eds) *Enterically Transmitted Hepatitis Viruses*, p. 384. Tours: La Simarre.

Tam AW *et al.* (1989) Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. *Virology* 185: 120.

Tam AW *et al.* (1997) *In vitro* infection and replication of hepatitis E virus in primary cynomolgus macaque hepatocytes. *Virology* 238: 94.

Tsarev SA *et al.* (1996) Prospects for prevention of hepatitis E. In: Buisson Y, Coursaget P and Kane M (eds) *Enterically Transmitted Hepatitis Viruses*, p. 373. Tours: La Simarre.

Yarbough PO *et al.* (1989) Hepatitis E virus: identification of type-common epitopes. *J. Virol.* 65: 5790.

HERPES SIMPLEX VIRUSES (*HERPESVIRIDAE*)

Contents

General Features

Laure Aurelian, Virology/Immunology Laboratories, Departments of Pharmacology and Microbiology, The University of Maryland School of Medicine, Baltimore, Maryland, USA,

History

The origin of the word 'herpes' as applied to spreading, usually ulcerative skin manifestations can be traced to Hippocrates. In 1736 Jean Astruc classified the condition as a sexually transmitted disease and noted its frequency among homosexuals. Its infectious nature was demonstrated in 1921, when material derived from herpetic lesions was shown to cause a serially transmissible keratoconjunctivitis in rabbits. In 1929 Goodpasture concluded that the virus travels along the axonal route from peripheral sites of infection and resides in a latent state within the nerve cells of the ganglion. In the 1930s it became evident that the manifestation is gingivostomatitis in seronegative subjects and recurrent 'fever blisters' in those who are seropositive. During the last 30 years distinct viruses were shown to cause facial and genital lesions. Molecular biology progress was applied towards herpes simplex virus (HSV) engineering for vaccine development and gene therapy. A critical advance has been the development of successful antiviral chemotherapy.

Classification

The herpes simplex virus serotypes (HSV-1 and HSV-2) are members of the family *Herpesviridae*. Classification is based on the virion architecture. The capsid is a 100–110 nm protein shell with icosadeltahedral symmetry composed of 162 capsomeres with a hole along the long axis. It is surrounded by the tegument, an electron-dense amorphous material which is asymmetrically distributed and may appear to be fibrous on negative staining. Tegument proteins involved in initiating virus replication include the regulatory immediate early (IE) proteins ICP4 and ICP0, the major *trans*-activating protein VP16, the large subunit of the viral ribonucleotide reductase (RR1), and vhs which inhibits host cell translation. The outer covering of the virus is the envelope, acquired from cellular membranes. It is decorated with spikes, approximately 8 nm long, containing HSV-specified glycoproteins (**Fig. 1**). Virions are 150–200 nm and contain 30–35 proteins.

The core is an electron-opaque structure approximately 77.5 nm in diameter, containing the 152 kbp linear, double stranded viral DNA. HSV DNA has two covalently linked components consisting of unique sequences (U_L and U_S) bracketed by inverted repeats. The U_L and U_S components can invert relative to each other, such that DNA extracted from virions or from infected cells consists of four equimolar populations differing in their relative orientation. Genes are contained within both the unique and repeat sequences. HSV genes RR1 and $\gamma_1$34.5 contain regions with cellular homology, presumably acquired from infected cells.

HSV belongs to the subfamily *Alphaherpesvirinae* and the genus *Simplexvirus*. Criteria for *Alphaherpesvirinae* classification are a variable host range, relatively short reproductive cycle, rapid spread in culture, efficient destruction of infected cells and the

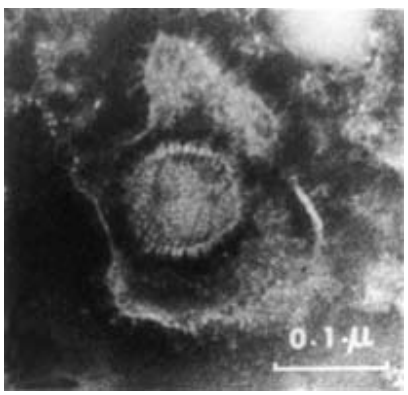


Figure 1 Negatively stained virion reveals icosadeltahedron capsid with 9.5 × 12.5 nm capsomeres (longitudinal section) and evidence of a 4 nm in diameter channel that runs from the surface along the long axis. The capsid is surrounded by an asymmetrical fibrous-like tegument and an envelope with spikes projecting from its surface. ×250 000.

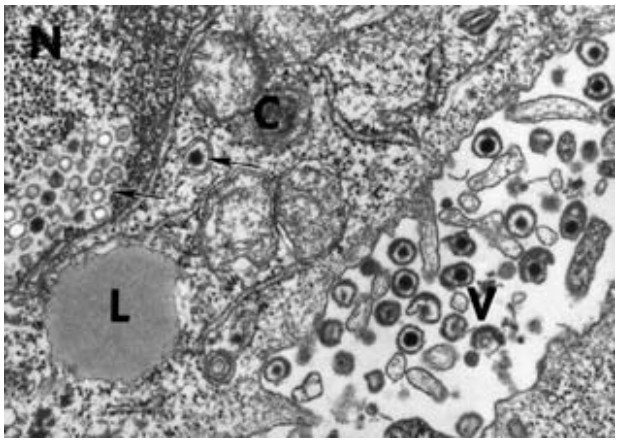


Figure 2 Thin section of HSV-2 infected cell (12 h after infection) stained with uranyl acetate and lead citrate. Intranuclear (N) capsids (arrow) are in different stages of assembly. Virus budding into the cytoplasm (C) through the nuclear membrane is identified (arrow). Extracellular mature virions (V) contain DNA and are enveloped. Lysosome-like cellular compartment (L). ×55 700.

capacity to establish latent infections in sensory ganglia. HSV shares four biological properties with all other *Herpesviridae:*

1. DNA codes for enzymes involved in nucleic acid metabolism;
2. DNA synthesis and capsid assembly occur in the nucleus; capsids are enveloped as they transit through the nuclear membrane;
3. Production of infectious progeny is accompanied by cell killing;
4. Viruses remain latent in their natural hosts.

Virus Replication and Host Range

After adsorption to cell surface receptors, the HSV envelope fuses with the plasma membrane. The capsid is transported to the nucleus, where its DNA is released. Three classes of genes are coordinately transcribed and temporally regulated. IE genes are transcribed first, in the absence of viral protein synthesis. ICP4, ICP27, ICP22 and ICP0 regulate the expression of the other viral genes. Early gene products include enzymes, such as thymidine kinase (TK) and DNA polymerase, that are required for virus DNA replication. Their expression requires functional IE genes. RR1 is the only viral gene regulated by cellular AP-1 transcription factors. The late genes require viral DNA synthesis for maximal expression. Onset of early gene expression coincides with irreversible shut-off of host cell macromolecular syntheses. Virus assembly begins in the nucleus. The envelope is acquired by budding through patches of altered nuclear membrane (**Fig. 2**). HSV replication is relatively inefficient with a low ratio of infectious/incomplete virus particles.

The biologic properties of some HSV proteins has been elucidated. Glycoproteins D (gD) and B (gB) are related to infectivity. gD is also a potent inducer of neutralizing antibodies. Glycoprotein G (gG) provides serotype specificity. The 5′-end of RR1 codes for a protein kinase (PK) activity related to the neoplastic potential of HSV-2 (HSV-2 oncogene). IE protein ICP47 downregulates HSV-specific immunity by interfering with antigen presentation to cytotoxic T cells (CTL).

Naturally, HSV causes disease only in humans. However, it infects experimental animals such as hamsters, mice, rats, guinea pigs, rabbits and embryonated chicken eggs. The animal species, virus type, route of inoculation, and state of immune competence affect the outcome of infection. In the mouse, intracerebral, footpad, intranasal and intraperitoneal inoculations are used as models of human fatal neurological or visceral disease. Eye infection is used as a model of encephalitic and latent disease. Certain mouse strains (e.g. C57BL/6) exhibit natural resistance to HSV infection whereas newborn mice are particularly sensitive. HSV-1 infection of the skin was described in mice, rabbits and guinea pigs following skin abrasion or intradermal inoculation. HSV-2 mucosal infections have been studied in the mouse and the guinea pig. The best approximation of human recurrent cutaneous disease was achieved in the guinea pig. A wide variety of cell lines support HSV growth. Primary and secondary cultures of human origin (e.g. MRC-5) are generally more sensitive than established nonhuman lines and they are used for virus isolation. Established lines (e.g. Vero) are used for the production of large quantities of virus for experimental studies and to determine virus titers by plaque assays.

Serologic Relationships

The HSV-1 genome has been entirely sequenced. It expresses at least 84 proteins. Of these, five open reading frames (ORFs) which map in the inverted repeats, are present in two copies/viral genome. A total of 38 HSV-1 ORFs cannot be deleted without ablating the capacity of the virus to replicate in cell culture. The HSV-2 genome has a similar organization, but the overall homology to HSV-1 is only 47–50%. Antigenic differences are evident in regions of low genome homology, such as those coding for gG and the PK domain of RR1. Differentiating biologic markers include virus titers in rabbit kidney cells,

plaque size and morphology in tissue culture, pock size on the chorioallantoic membrane of embryonated eggs, thermal stability, sensitivity to 5-iododeoxyuridine and interferon, properties of RR1 PK activity and neoplastic potential. Relatively few genes (e.g. homologues of HSV-1 gB) are conserved among members of different subfamilies.

Genetics

Epidemiologically unrelated isolates of the same HSV serotype are not identical (intratypical polymorphism). Differences result from base substitutions that add or eliminate a restriction endonuclease cleavage site or substitute an amino acid, occasional deletions, or due to variability in the number of repeated sequences at various genome sites. Intratypic polymorphism was also noted with monoclonal antibodies and isolates may differ with respect to neurovirulence. The restriction endonuclease cleavage patterns of a given strain are relatively stable even after many years of serial *in vitro* passage.

Epidemiology

HSV infections are worldwide. There are no differences in sex or seasonal variation. Animal vectors have not been described. Clustered outbreaks were reported in hospitals and orphanages, but there is no clinical or molecular epidemiologic evidence that HSV causes epidemic disease.

The mouth and lips are the most common sites of HSV-1 infection. Primary infection usually occurs by 5 years of age and is generally asymptomatic. With illness, gingivostomatitis is usually the manifestation. In young adults, primary infection was associated with pharyngitis and a mononucleosis-like syndrome. Geographic location, socioeconomic status and age influence the frequency of HSV infection. In developing countries 95% seroconversion was seen before the age of 15. This likely reflects the increased frequency of direct person to person contact in overcrowded conditions. In presumed middle-class individuals of industrialized societies, seroconversion occurred in 20% before the age of 5 years. Antibody prevalence was 60% in the third decade of life. Only five of 18 countries surveyed had an HSV-1 antibody prevalence of less than 70% at age 20–40 years.

Over a decade ago, conservative estimates were 300 000–500 000 new HSV-2 cases per year, calculated based on a ratio of one case of genital HSV for 5–10 gonorrhea cases. The prevalence of infection determined by cytopathological screening and virus isolation from cervicovaginal swabs was 0.09–0.24% in normal women and 0.002–7.0% in STD clinics. Recent estimates are one case of genital HSV-2 for 2.2 cases of gonorrhea. The current age-adjusted prevalence of HSV-2 in the US is 20.8%, a 30% increase over the last 13 years. Individuals with genital HSV range between 40 and 60 million.

Factors that influence acquisition of HSV-2 include: sex (women greater than men), race (blacks more than whites), marital status (divorced versus single or married), place of residence (city greater than suburb) and number of sexual partners (higher number increased risk). The highest prevalence of HSV-2 antibodies (75–98%) was seen in female prostitutes and homosexual men. Women are about 45% more likely than men to be infected with HSV-2. The risk of susceptible females for contracting HSV from infected males is 80% after a single contact. The probability of infection with HSV-2 is less than 10% for US women having one partner. It increases to 40%, 62% and greater than 80% as the number of partners increases to 2–10, 11–50 and greater than 50, respectively. For heterosexual men, these risks are 0, 20%, 35% and 70% for each of the risk groups respectively. For homosexual men, the risks are greater than 60% and 90% for those with 11–50 and greater than 50 partners, respectively. The frequency of HSV-1 isolation from genital lesions has increased significantly during the last 10 years. It is now estimated that 25 million US adults have genital HSV-1 infections.

HSV-2 infection is associated with acquisition of both human immunodeficiency virus type 1 (HIV-1) and human T cell lymphotropic virus type 1 (HTLV-1) infections. HSV-2 was also shown to activate HIV-1 expression in experimental systems.

The major risk to the fetus is maternal primary or initial genital infection with HSV, the rate of which is 0.5–10% per year. Infection acquired near the time of labor is associated with neonatal herpes and perinatal morbidity. Recurrent infection is the most common form of infection during gestation. The incidence of HSV-2 shedding at delivery is 0.01–0.6%, irrespective of past history and time of gestation. The incidence of cervical shedding in pregnant women with asymptomatic infection averages 3% (range 0.2–7.4%). The frequency of shedding does not vary by trimester during gestation. Most women whose children are infected (60–80%), are asymptomatic at the time of delivery and have neither a past history of genital HSV nor a sexual partner reporting a genital infection.

The estimated incidence of HSV encephalitis is 2.3 cases per million population per year. The age distribution is biphasic with one peak at 5–30 years of age and a second peak above 50 years of age. HSV-1

accounts for 95% of cases. HSV-2 was isolated from the cerebrospinal fluid in 0.5–3% of patients with aseptic meningitis.

Transmission and Tissue Tropism

HSV transmission occurs during close personal contact with active ulcerative skin lesions or virus-positive secretions from asymptomatic subjects. HSV-1 can be isolated from the mouth for 7–10 days after primary HSV-1 infection. Virus shedding in the saliva is uncommon in children less than 6 months of age. It was reported in 20% of children 7 months to 2 years old, 18% of those 3–14 years old, 2.7% in those over 15 years old and 2–9% of asymptomatic adults. This is consistent with a 2–5% shedding frequency reported by contemporary cross-sectional studies. HSV-1 DNA was detected by polymerase chain reaction (PCR) assay in oral secretions from 36–45% of patients at prodrome (24–48 h before clinical recurrence). However, since virus was not isolated, it is unclear whether patients are infectious during prodrome. Genital infection with HSV-1 results from self-inoculation or from oral sexual practices. It may be more prevalent in females than in males and less severe and prone to recur than HSV-2 infections.

Sexual contact is the primary route of HSV-2 transmission. HSV-2 was isolated from the genital tract of 0.3–5.4% of asymptomatic males and 1.6–8% of asymptomatic females attending sexually transmitted disease (STD) clinics. Approximately 18–23% of women evaluated after their first episode of genital HSV, shed virus in the absence of perineovulvar lesions. Protein and PCR assays suggest that asymptomatic vaginal shedding occurs at a rate of 1 day in 5. However, cervical lesions are generally unnoticed and they may be confused with asymptomatic shedding. Also, since virus titers in active lesions are 100–1000-fold higher than in salivary and genital secretions from asymptomatic subjects, the efficiency of transmission during acute episodes is significantly higher. Approximately one-third of the world population represents such a reservoir.

HSV-2 can be transmitted from mother to fetus, primarily during passage through an infected birth canal. Intrapartum transmission accounts for 75–80% of all cases. Approximately 30 babies were identified in the world literature with symptomatic congenital HSV disease. This frequently resulted from ascending infections in women who have had prolonged rupture of membranes before delivery.

The central nervous system (CNS) is a target of HSV infection. The trigeminal, vagal and sacral dorsal root ganglia are the major sites of latent HSV infection. HSV can also infect the liver, the lungs, adrenal glands, pancreas, small and large intestine and bone marrow.

Pathogenicity

For infection to occur virus must come in contact with mucosal surfaces or abraded skin. After replication at the site of infection, virus is transported by neurons to dorsal root ganglia where latency is established. Replication in the ganglia infrequently results in life-threatening CNS infection. However, latency generally predominates. Widespread organ involvement is the consequence of viremia in a host that is not capable of limiting virus replication to mucosal surfaces. The HSV-1 portal of entry is generally the oropharyngeal mucosa and the trigeminal ganglia are invariably colonized. Initial HSV-2 replication is at genital sites with colonization of the sacral ganglia. Wild-type isolates may differ in their ability to grow or invade the CNS. Virulence loci comprising both the ability to grow in CNS (after intracerebral inoculation of mice) and to invade the CNS (after peripheral inoculation) were ascribed to several genes, notably *TK* and $\gamma_1 34.5$.

Clinical Features of Infection

HSV infects neonates, children and adults. It produces a wide spectrum of diseases ranging from mild illness, undiscernible in the majority of patients, to severe and life-threatening disease in relatively few patients. The incubation period is 1–26 days (median 6–8 days). Factors that influence severity are age, gender, host genetic factors, immune competence, associated illnesses, and virulence of the infecting virus strain.

Most (70–90%) childhood HSV-1 infections are asymptomatic, but they may be followed by subsequent recurrent episodes of symptomatic disease. In children 1–3 years of age, the major manifestion of HSV-1 infection is gingivostomatitis, a serious infection of the gums, tongue, mouth, lip, facial area and pharynx, often accompanied by high fever, malaise, myalgias, swollen gums, irritability, inability to eat and cervical lymphadenopathy. Later in life the major clinical manifestation of HSV-1 infection is an upper respiratory tract infection, generally pharyngitis. Reactivated HSV-1 is associated with mucosal ulcerations or lesions at the mucocutaneous junction of the lip, presenting as small vesicles that last 4–7 days and are known as herpes *labialis*, cold sores or fever blisters. Other HSV-1 skin diseases include primary herpes dermatitis (a generalized vesicular eruption), eczema herpeticum (usually a manifestation of a primary infection in which the skin is the portal of entry), and traumatic herpes (resulting from trau-

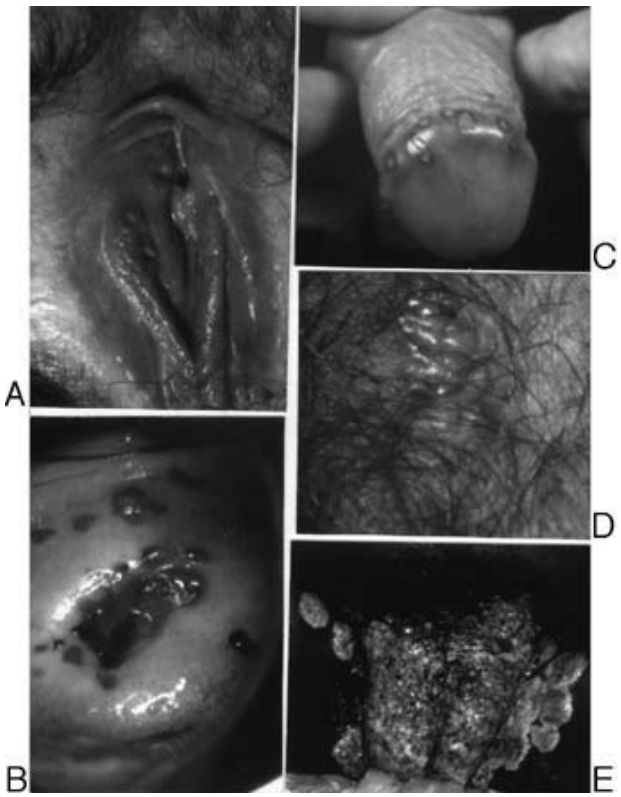


Figure 3 Genital HSV infection. Lesions are on the vulva (**A**), cervix (**B**), penis (**C**) and perineum (**D**). Hyperproliferative lesion caused by acyclovir-resistant HSV-2 evidences ulceration and yellow exudate and covers the entire perineum (**E**). **E**, reproduced with permission from Beasley KL *et al.* (1997) *J. Am. Acad. Dermatol.* 37: 860.

matic breaks due to burns or abrasions in the normal skin of a susceptible child). Herpetic whitlow is an occupational hazard (dentists, hospital personnel, wrestlers) resulting from infection of broken skin (often on fingers) in contact with virus on another individual. Erythema multiforme is a severe recurrent skin disease that follows HSV episodes. It is initiated by expression of the HSV DNA polymerase gene in the epidermis and has a T cell immunopathology involving, primarily, CD4+ Vβ2 T cells.

Acute HSV-1 rhinitis is a primary infection of the nose recognized by the appearance of tiny vesicles in the nostrils usually associated with fever and enlarged cervical lymph nodes. HSV-1 infections of the eye can lead to stromal keratitis, one of the leading causes of blindness in the Western world. This disease also has a T cell immunopathology involving HSV-specific CD4+ T cells. Chorioretinitis is a manifestation of disseminated HSV infection that may occur in neonates or in patients with acquired immune deficiency syndrome (AIDS). By using PCR HSV-1 DNA was recently found in gastrointestinal sensory neurons and in the geniculate ganglion and adjacent areas, implicating HSV in recurrent gastrointestinal disorders and Bell's palsy, respectively.

In females, HSV-2 infection is manifested by vesicles on the mucous membranes of the labia and the vagina. Severe forms result in ulcers that cover the entire area surrounding the vulva (**Fig. 3A**). Symptoms of primary infection include itching, pain and lymphadenopathy.

Cervical involvement is common, although it generally passes unnoticed (**Fig. 3B**). Common sites of primary HSV-2 infection in males are the shaft of the penis, the prepuce and the glans penis (**Fig. 3C**). Infection at adjacent body sites is also seen (**Fig. 3D**). Urethritis is the main local expression accompanied by a watery discharge often resulting in dysuria. Symptomatic urethritis is rare in recurrent disease, but virus often can be cultured from the urethra.

The infection is more severe in females than in males and systemic symptoms are more common. They often accompany the appearance of primary lesions and include fever, headache, photophobia, malaise and generalized myalgias. Dysuria, urinary retention, urgency and frequency, pain and discharge are also seen. Systemic symptoms are generally not seen in recurrent disease. A prodrome often signals a recurrence. It is characterized by a tingling sensation that may precede the lesions by a few hours to 2 days. This sensation may be accompanied by radiating radicular pain. Anal and rectal infections are seen primarily in homosexuals, as a result of anal intercourse. A newly described HSV-2 presentation takes the form of hyperproliferative lesions often associated with acyclovir resistant HSV-2 strains, co-infection with other agents or mild or severe immunodeficiency (**Fig. 3E**). HSV encephalitis is the most commonly reported viral CNS infection accounting for 10–20% of all cases. In children and young adults encephalitis usually results from a generalized primary infection due to virus that presumably enters the CNS through neurotropic spread by way of the olfactory bulb. In adults, CNS infections are rare and encephalitis is often preceded by recurrent lesions. Meningitis due to HSV-2 infection of the CNS was described in association with genital HSV-2 infections.

Immunocompromised adults can develop a severe generalized disease that is occasionally responsible for herpetic hepatitis. HSV pneumonitis accounts for 6–8% of cases of interstitial pneumonia in recipients of bone marrow transplants. Mortality due to HSV pneumonia in immunosuppressed patients is above 80%. Generalized HSV with involvement of adrenal glands, pancreas, small and large intestine and bone marrow was reported in the immunocompromised patient. HSV has also been isolated from 40% of patients with acute respiratory distress syndrome.

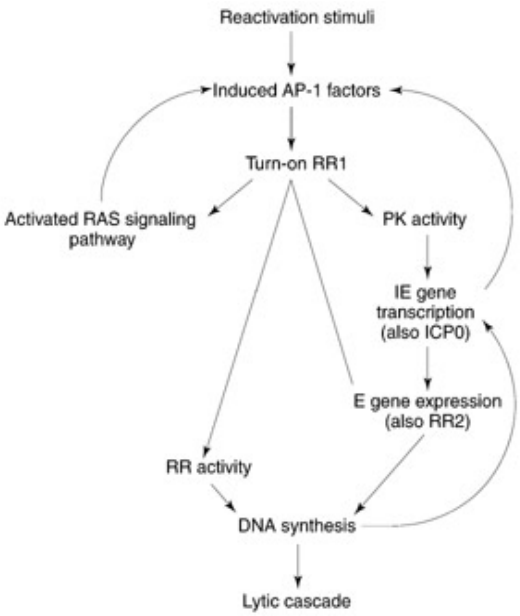


Figure 4 HSV-2 RR1 PK plays a crucial role in latency reactivation. Because RR1 is the only viral gene the basal expression of which is AP-1 dependent, we propose that it is the earliest viral response to latency reactivation stimuli which are known to induce AP-1 factors. The turned-on RR1 supplies the PK activity which is required for IE gene transcription, thereby initiating expression of other viral genes and DNA synthesis. The latter is amplified by the RR enzymatic activity resulting from complexation of RR1 and RR2. IE gene ICP0 interacts with AP-1 to further amplify RR1 expression. RR1 activates the RAS signaling pathway further amplifying AP-1 levels. The outcome is increased DNA synthesis and initiation of the lytic cascade. Reproduced by permission from Aurelian L, Herpes simplex virus type 2: unique biological properties include neoplastic potential mediated by the PK domain of the large subunit of ribonucleotide reductase. MS #97-190 published in *Frontiers in Bioscience* 3, D237–D249, 1998.

Other rather uncommon complications of HSV include monoarticular arthritis, adrenal necrosis, idiopathic thrombocytopenia and glomerulonephritis.

Latency

Latency is the ability of HSV to persist in the infected host in a nonreplicating state compatible with cell survival and followed by subsequent episodes of virus reactivation and clinical symptoms. Following replication in the skin, sensory or autonomic nerve endings are infected. Virions, or more likely capsids, are thought to be transported intra-axonally to the nerve-cell bodies in the ganglia. At this stage, in some animal models there is a short (1–2 days) period of virus replication in the ganglia, which can be detected by virus isolation from cell-free homogenates of the ganglia. It is unknown whether this also occurs in humans and it may be an artifact due to the animal species, route of inoculation or the large virus inoculum used in experimental models. At approximately 2–4 weeks post infection, a latent infection is established in which viral DNA is maintained in the neurons as an episome and there is limited HSV gene expression. Infectious virus is no longer detected in cell-free homogenates, but it can be detected by explantation and organ culture of the ganglia. Human trigeminal ganglia contain approximately 20 copies of the HSV genome per latently infected neuron. Persistence at peripheral sites (e.g. eye) was demonstrated by PCR, but its role in disease pathogenesis is unclear. Virus isolates obtained at various times from the same patients have different restriction endonuclease patterns, indicating that ganglia can be colonized by multiple HSV strains.

Certain stimuli cause reactivation of virus replication in latently infected ganglia (detected within 3–5 days of exposure) with concomitant reverse axonal transport of virus progeny to a peripheral site, at or near the portal of entry. Reactivating stimuli include fever, axonal injury, exposure to ultraviolet irradiation (sunlight), stress and possibly hormonal irregularities. The development of clinically apparent recurrent lesions is probably influenced by the amount of virus, virus–cell interactions, and the rapidity of the host's immune response in clearing virus. In the guinea-pig model latent virus reactivates spontaneously. In other animal models it is reactivated by neurectomy, ganglionic trauma, electrical stimulation, epinephrine iontophoresis or cadmium treatment. Depending on the host immune response, the outcome of virus reactivation may vary from severe debilitating lesions (in immunosuppressed individuals) to no lesions, or barely visible ones.

Viral gene products that govern the establishment and maintenance of latency are unknown. A region of the HSV-1 genome, termed latency associated transcript(LAT) and two ORFs (ORF-O and ORF-P) which are read antisense to LAT on the HSV genome, are the only transcripts in latently infected sensory neurons. LAT may be involved in site-specific (trigeminal and sacral neurons) reactivation of HSV-1 and HSV-2. A hypothetical virus reactivation pathway is shown in **Fig. 4**. It is based on the finding that reactivating stimuli function by inducing AP-1 transcription factors and argues that they induce expression of *RR1*, the only viral gene that can respond to AP-1. In turn, *RR1* amplifies AP-1 levels through activation of the RAS signaling pathway by its PK activity (**Fig. 5**). Our recent studies of a HSV-2 mutant deleted in RR1 PK indicate that the PK activity is required for early onset of *IE* gene

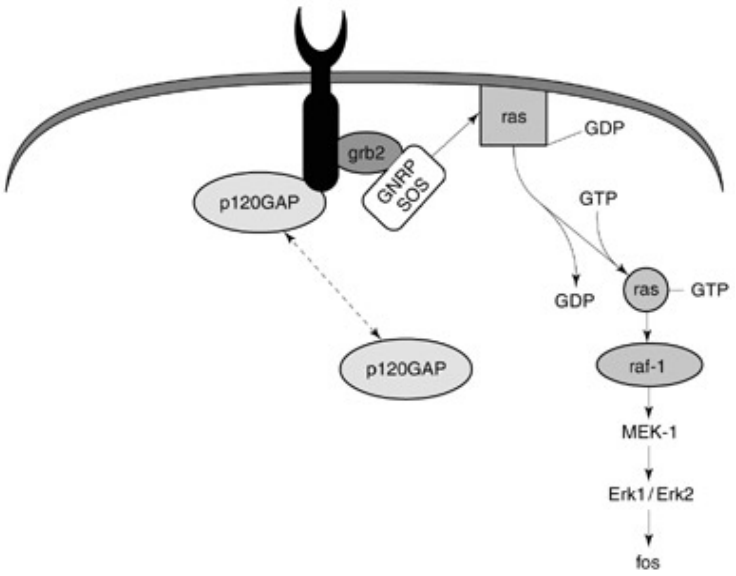


Figure 5 HSV-2 RR1 PK activates the RAS signaling pathway. The constitutively activated oncoprotein binds the Grb_2–hSOS complex thereby bringing hSOS in the vicinity of RAS. This results in the conversion of RAS-GDP into RAS-GTP (RAS activation). HSV-2 RR1 PK also binds the downregulatory factor RAS-GAP (p120GAP) which it inactivates through phosphorylation. The activated RAS initiates the kinase cascade that leads to the nucleus and results in AP-1 (fos) activation. Reproduced by permission from Aurelian L, Herpes simplex virus type 2: unique biological properties include neoplastic potential mediated by the PK domain of the large subunit of ribonucleotide reductase. MS #97-190 published in *Frontiers in Bioscience* 3, D237–D249, 1998.

expression, thereby initiating viral DNA synthesis. The levels of viral DNA synthesis are further amplified by the ribonucleotide reductase activity. The outcome is initiation of the lytic cascade and production of infectious virus.

HSV Infection during Pregnancy and Neonatal Disease

Localized genital infection is the most common form of HSV infection during pregnancy. In a small number of patients multiple visceral sites are also involved. Mortality among these pregnant women was reported to be $\geq$50%, presumably due to altered cell-mediated immune responses. Fetal deaths occurred in 50% of cases, although mortality did not correlate with the death of the mother. Severe intrauterine fetal growth retardation was reported in women with primary HSV infection during pregnancy. Maternal primary infection before 20 weeks of gestation was associated with spontaneous abortion in as much as 25% of infected women. Fetal infection is generally due to virus shed at the time of delivery. Neonates have the highest frequency of visceral and CNS involvement of all HSV-infected patients. Skin lesions are the most commonly recognized features of the disease. However, at least 70% of untreated neonatal HSV cases will lead to disseminated disease. Infection of the neonate can be prevented by delivery by cesarean section.

Pathology

Histopathologic changes characteristic of HSV-1 or HSV-2 induced skin lesions represent a combination of virus-mediated cellular death and associated inflammatory responses. HSV-induced changes include ballooning of the infected cells, the appearance of condensed nuclear chromatin and subsequent degeneration of the nuclei, generally within the parabasal and intermediate cells of the epithelium. Multinucleated giant cells are also formed and a clear (vesicular) fluid containing large quantities of virus, cell debris and inflammatory cells, appears between the epidermis and the dermal layer. An intense inflammatory response is seen in dermal structures particularly in the primary infection. With healing, the vesicular fluid becomes pustular and then scabs. Scarring is uncommon. As host defenses are mounted, an influx of mononuclear cells can be detected in the infected tissue. Similar histopathologic findings are seen in mucous membranes. Vascular changes in the area of infection include perivascular cuffing and areas of hemorrhagic necrosis. These are particularly prominent in organs other than skin. In the brain

oligodendrocytic involvement, gliosis and astrocytosis are common.

Host Immune Responses

Immune responses induced by HSV infection include an early nonspecific containment phase and a later HSV-specific effector phase. The relative contribution of these two phases differs according to the experimental model, the route of virus inoculation and the HSV serotype. Natural killer (NK) cells and/or interferon (IFN) are the major factors involved in the nonspecific containment phase against HSV-1. Natural resistance of certain mouse strains to HSV infection was correlated with these factors. They act early in infection, thereby limiting HSV-1 growth and reducing the virus load. Intrinsic macrophage resistance, the mechanism of which is abortive infection, was implicated as the major determinant of the nonspecific containment phase against HSV-2. Langerhans cells are involved in the control of cutaneous HSV infections. Various reports have incriminated or refuted the association of HLA with HSV infections. A1, A2, A9, BW16 and CW2 were associated with recurrent oral lesions; A1, A2, A9 and DR3 with recurrent ocular infections.

Humoral and cell-mediated responses are involved in the specific effector phase. HSV specific antibodies are first observed on days 5–10 postinfection and they persist indefinitely. Antibody titers are higher in subjects with, than those without, a history of recurrent disease, reflecting the propensity of the latent virus to reactivate. The response is heterogeneous with respect to protein specificity. Soon after onset of infection, antibodies to structural proteins are followed by those directed against gD, gB, ICP4, gE, gG-1/gG-2 and gC. Both IgM and IgG antibodies can be demonstrated depending on the method and time of assesment relative to disease onset. In children, antibodies to ICP4 may be predictive of long-term neurologic outcome. Infected newborns produce IgM antibodies (particularly against gD) within the first 3 weeks of infection. Transplacentally acquired antibodies do not protect the newborn.

Recovery from HSV infection, primarily involves the T-cell system. Newborns have delayed (2–4 weeks) HSV-specific T cell responses and decreased IFN-α and IFN-γ production in response to HSV antigen as compared to older individuals. This may be responsible for a higher frequency of disease progression. Effector mechanisms include: (1) delayed-type hypersensitivity (DTH), an early (days 5–10 postinfection) HSV-type common response that remains inducible for at least 2 years after infection; (2) effector lymphokines such as IFN-γ, interleukin 2 (IL-2) and other soluble factors, and (3) CTL. In contrast to other viruses, CTL to HSV are generally difficult to induce. In mice the activity is present only in draining lymph nodes, it is detected only in mice pretreated with cyclophosphamide or after extended (3 days) *in vitro* culture of the effector cells, and it is no longer seen by day 12 postinfection. HSV-1 specific CTL clones are directed against cell surface glycoproteins, primarily gB, gD or gE, and intracellular IE proteins; HSV-2-specific clones are against gC, gD, gE or gG. Naturally acquired HSV immunity reduces the severity of the disease but it does not prevent the establishment of latency, development of recurrent disease or reinfection, even by autoinoculation with the same HSV strain. This is due to transient downregulation of the HSV-specific T cell responses on re-exposure to the virus, resulting from increased production of downregulatory factors such as prostaglandin E_2 (PGE_2) and transforming growth factor β (TGFβ).

Neoplastic Transformation

Numerous seroepidemiologic studies have associated HSV-2 infection with an increased risk of human squamous cervical cancer. The exact role of HSV-2 in cervical carcinogenesis is unclear. Current opinion is that HSV-2 is a cofactor, or infection reflects the increased sexual activity of cervical cancer patients. However, HSV-2 is a tumor virus. It causes neoplastic transformation, also of human cells, and tumor formation in animals. Both are achieved with inactivated virus, temperature sensitive mutants, sub- or supraoptimal infection temperatures, sheared viral DNA and subgenomic fragments. Depending on conditions, the transformed cells retain and express the viral DNA or not, suggesting that viral genes cause increased cell proliferation or they initiate the transformation process which can subsequently progress independently. HSV-2 DNA sequences have both immortalizing and neoplastic functions, the latter mediated by the unique PK-encoding 5′ terminal of RR1 (HSV-2 oncogene). Transformed human cells cause tumors that express the viral oncoprotein when injected in nude mice. Mutations that inactivate the PK activity of the oncoprotein abrogate neoplastic potential, and both cell proliferation and tumor growth are inhibited by antisense oligonucleotides which inhibit oncoprotein synthesis. The HSV-1 RR1 PK does not have oncogenic potential. However, HSV-1 causes mutagenesis and gene amplification, activates cellular genes and causes morphologic transformation.

Prevention and Therapy

Neonatal infection is decreased by surgical abdominal delivery when membranes are ruptured for less than 4 h. For women with a past history of genital infection, HSV culture at the time of delivery can establish whether virus shedding can lead to fetal infection. Detection of viral DNA by PCR does not establish infectivity. Detection of HSV-2 specific antibodies could be valuable in identifying those women at greatest risk.

Vaccination remains the ideal method for prevention of infection. Recombinant and subunit vaccines have been engineered during the past 15 years in an attempt to remove transforming HSV DNA and enhance antigenicity. At Biocine/Chiron, a gD-2 gene was truncated to yield a mature protein containing 348 residues of gD-2. When administered together with adjuvants, this, as well as a similar gB-2 preparation induced neutralizing antibodies and caused amelioration of morbidity and reduction of mortality in immunized animals. However, disease was not prevented, and the preparations offered very little benefit in humans. Vaccinia/gD recombinants protected from HSV-2 recurrent disease in the guinea-pig model. However, not all constructs were equally protective. Information gained from these studies is that protection is contingent upon: (1) regulation of gD expression as an early gene, (2) optimal gD post-translational modification in epidermal cells and (3) antigen presentation by Langerhans cells to T cell subpopulations involved in protective immunity. Live genetically engineered HSV vaccines are predicated on their ability to fulfill these requirements. Two such vaccines, currently under development, are respectively deleted in $\gamma_1$34.5 or glycoprotein gH but retain oncogenic DNA. The third vaccine currently under development is an oncogene-deleted and growth-attenuated virus.

The ideal HSV therapy would both reduce the severity of the primary disease and prevent the establishment of latency. However, as there is no known agent that can prevent latency, the major goals for the treatment of HSV disease are to reduce: (1) the time to resolution of clinical symptoms; (2) the likelihood of complications and, should they occur, their severity; and (3) the time of virus shedding and therefore the likelihood of transmission. Synthetic nucleoside analogues licensed for the treatment of HSV infections include idoxuridine for HSV keratitis and vidarabine for HSV encephalitis, neonatal herpes and HSV keratitis. Agents currently used for the treatment of HSV infections are acyclovir, available in topical, intravenous and oral formulations, famciclovir, valaciclovir, foscarnet and cidofovir, the latter two available in intravenous formulation for use in infections caused by acyclovir-resistant HSV. Acyclovir is phosphorylated by HSV thymidine kinase at a rate 10^6-fold faster than by the cellular enzyme. Acyclovir triphosphate, the active form of the drug, is recognized by HSV DNA polymerase much more readily than by the host cell DNA polymerase. Acyclovir triphosphate blocks DNA synthesis by acting as an inhibitor of polymerase activity as well as its substrate, becoming incorporated into the growing DNA chain and causing termination of chain growth. As DNA synthesis is required for acyclovir to function, the drug cannot destroy the virus during the latent period.

Oral acyclovir speeds the healing and resolution of symptoms of primary and recurrent HSV infection. However, the subsequent recurrence rates are not affected. Routine use for recurrent disease, especially for mild episodes, is not recommended. Long-term daily suppressive therapy may be useful in reducing the frequency of reactivation in patients with very frequent recurrent episodes. Daily administration of two to five 200 mg capsules of oral acyclovir for 4–6 months appears to be safe. Long-term suppressive therapy with oral acyclovir does not eliminate ganglionic latency, and reactivation of disease occurs after therapy is discontinued. A recently voiced concern about potential consequences of long-term suppressive treatment is that nonreplicating HSV-2 is oncogenic. Acyclovir-resistant HSV strains emerge with moderate frequency. These strains can become sensitive after treatment with cidofovir, suggesting that alternating acyclovir and cidofovir therapies may provide a strategy for managing the emergence of alternating acyclovir-resistant and sensitive infections. A topical cidofovir formulation is currently under review by the FDA.

Future Perspectives

Better understanding of the molecular pathogenesis of HSV infections along with the role of HSV immunity in protection remain the principal goals for future research efforts. Vaccines that prevent infection and recurrent disease and are free of oncogenic DNA are currently under development. Further research must also focus on the mechanism of latency establishment and virus reactivation to develop improved antiviral chemotherapy and overcome problems of drug resistance. Antisense technology permits the design of drugs that can specifically inhibit critical HSV genes and can be modified to overcome resistance causing mutations. Significant progress may be achieved by developing novel delivery vectors that increase the intracellular levels of antisense oligo-

nucleotides and optimize their cellular localization. HSV is an attractive vehicle for delivery of therapeutic genes to the nervous system. The viral genome persists in the trigeminal and sacral ganglia and in brain neurons without apparent pathologic consequences and transgenes can be expressed from the viral genome. Further research is needed to elucidate the potential of HSV as a vector for gene therapy of brain tumors and certain neuropathies.

See also: **Antivirals; Herpes simplex viruses (*Herpesviridae*): Molecular biology; Immune response: Cell mediated immune response, General features; Latency; Persistent viral infection; Vaccines and immune response; Vectors: Animal viruses.**

Further Reading

Aurelian L (1989) Herpes simplex. In: Specter S, Bendinelli M and Friedman H (eds) *Virus-Induced Immunosuppression*, p. 73. New York: Plenum.

Aurelian L (ed.) (1990) *Herpesviruses, the Immune System and AIDS*. Norwalk, MA: Kluwer Academic.

Glorioso JC, Goins WF, Schmidt MC *et al* (1997) Engineering herpes simplex virus vectors for human gene therapy. *Adv. Pharmacol.* 40: 103.

Martinez CM and Luks-Golger DB (1997) Cidofovir use in acyclovir-resistant herpes infection. *Ann. Pharmacother.* 31: 15119.

Steiner I (1996) Human herpes viruses latent infection in the nervous system. *Immunol. Rev.* 152: 157.

Whitley RJ (1996) Herpes simplex viruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 2297. Philadelphia: Lippincott–Raven.

Molecular Biology

Edward K Wagner, Department of Molecular Biology and Biochemistry and Virus Research Program, University of California, Irvine, California, USA

The Virion

The capsid

Like all herpesviruses, HSV-1 and the closely related HSV-2 are enveloped icosahedral virions. The 152 000 bp (152 kbp) genome is densely packaged in a liquid–crystalline, phage-like manner within a 100 nm capsid. The capsid comprises 162 capsomeres of 150 hexons and 12 pentons. Hexons contain six molecules of the 155 kDa major capsid protein (VP5 or U_L19) with six copies of the vertex protein VP26 (U_L35) at the tips. Pentons contain five copies of VP5. Hexons are coordinated in three-fold symmetry with a triplex structure made up of two other proteins – one copy of VP19c (U_L38) and two copies of VP23 (U_L18) per triplex. Small amounts of other viral proteins are also associated with the capsid; these include VP24 (derived from U_L26), a maturational protease, and the U_L6 gene product.

The tegument

The capsid is surrounded with the 20 or so tegument or matrix proteins. These include the *α-trans*-inducing factor (αTIF, VP16 or U_L48) and a virion-associated host shut-off function (U_L41).

The envelope

The trilaminar viral lipid envelope forms the outer surface of the virion. This envelope has a nominal diameter of 170–200 nm, although exact dimensions will vary depending upon the method of visualization. The envelope is derived from the host cell nuclear membrane and contains at least ten viral encoded glycoproteins. One glycoprotein (gG–U_S4) has sufficient differences in amino acid sequence to serve as a type-specific immune reagent to differentiate HSV-1 and -2. At least four (gL–U_L1, gH–U_L22, gB–U_L26, gD–U_S6) are involved in virus penetration into the host cell; of these, the sequence of gB is conserved among a broad range of different herpesviruses. Another glycoprotein, gC (U_L44) facilitates the initial attachment of the virion to glycosaminoglycans on the cell surface, but is not essential for virus infectivity. Two others, U_S7–gI and U_S8–gE function together as a hetero-oligomer which binds the Fc region of IgG and influences cell-to-cell spread of virus.

The functions of the others are not fully known but presumably are involved in mediating host response to infection and (perhaps) certain levels of cell specificity.

The HSV Genome

General genome arrangement

Genome size and sequence. The complete sequences of prototype strains of both HSV-1 and HSV-2 are available in standard databases. Homology between HSV-1 strains is >99% for most regions of the genome, while overall homology between HSV-1 and HSV-2 approximates 85% within translational reading frames and significantly less outside them. The HSV-1 genome is schematically shown in **Fig. 1**, it is a linear, double-stranded DNA duplex 152 000 bp in length, with a base composition of 67% G+C. The genome circularizes upon infection. Because the

nucleotides and optimize their cellular localization. HSV is an attractive vehicle for delivery of therapeutic genes to the nervous system. The viral genome persists in the trigeminal and sacral ganglia and in brain neurons without apparent pathologic consequences and transgenes can be expressed from the viral genome. Further research is needed to elucidate the potential of HSV as a vector for gene therapy of brain tumors and certain neuropathies.

See also: **Antivirals; Herpes simplex viruses (*Herpesviridae*): Molecular biology; Immune response: Cell mediated immune response, General features; Latency; Persistent viral infection; Vaccines and immune response; Vectors: Animal viruses.**

Further Reading

Aurelian L (1989) Herpes simplex. In: Specter S, Bendinelli M and Friedman H (eds) *Virus-Induced Immunosuppression*, p. 73. New York: Plenum.

Aurelian L (ed.) (1990) *Herpesviruses, the Immune System and AIDS*. Norwalk, MA: Kluwer Academic.

Glorioso JC, Goins WF, Schmidt MC *et al* (1997) Engineering herpes simplex virus vectors for human gene therapy. *Adv. Pharmacol.* 40: 103.

Martinez CM and Luks-Golger DB (1997) Cidofovir use in acyclovir-resistant herpes infection. *Ann. Pharmacother.* 31: 15119.

Steiner I (1996) Human herpes viruses latent infection in the nervous system. *Immunol. Rev.* 152: 157.

Whitley RJ (1996) Herpes simplex viruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 2297. Philadelphia: Lippincott–Raven.

Molecular Biology

Edward K Wagner, Department of Molecular Biology and Biochemistry and Virus Research Program, University of California, Irvine, California, USA

The Virion

The capsid

Like all herpesviruses, HSV-1 and the closely related HSV-2 are enveloped icosahedral virions. The 152 000 bp (152 kbp) genome is densely packaged in a liquid–crystalline, phage-like manner within a 100 nm capsid. The capsid comprises 162 capsomeres of 150 hexons and 12 pentons. Hexons contain six molecules of the 155 kDa major capsid protein (VP5 or U_L19) with six copies of the vertex protein VP26 (U_L35) at the tips. Pentons contain five copies of VP5. Hexons are coordinated in three-fold symmetry with a triplex structure made up of two other proteins – one copy of VP19c (U_L38) and two copies of VP23 (U_L18) per triplex. Small amounts of other viral proteins are also associated with the capsid; these include VP24 (derived from U_L26), a maturational protease, and the U_L6 gene product.

The tegument

The capsid is surrounded with the 20 or so tegument or matrix proteins. These include the *α-trans*-inducing factor (αTIF, VP16 or U_L48) and a virion-associated host shut-off function (U_L41).

The envelope

The trilaminar viral lipid envelope forms the outer surface of the virion. This envelope has a nominal diameter of 170–200 nm, although exact dimensions will vary depending upon the method of visualization. The envelope is derived from the host cell nuclear membrane and contains at least ten viral encoded glycoproteins. One glycoprotein (gG–U_S4) has sufficient differences in amino acid sequence to serve as a type-specific immune reagent to differentiate HSV-1 and -2. At least four (gL–U_L1, gH–U_L22, gB–U_L26, gD–U_S6) are involved in virus penetration into the host cell; of these, the sequence of gB is conserved among a broad range of different herpesviruses. Another glycoprotein, gC (U_L44) facilitates the initial attachment of the virion to glycosaminoglycans on the cell surface, but is not essential for virus infectivity. Two others, U_S7–gI and U_S8–gE function together as a hetero-oligomer which binds the Fc region of IgG and influences cell-to-cell spread of virus.

The functions of the others are not fully known but presumably are involved in mediating host response to infection and (perhaps) certain levels of cell specificity.

The HSV Genome

General genome arrangement

Genome size and sequence. The complete sequences of prototype strains of both HSV-1 and HSV-2 are available in standard databases. Homology between HSV-1 strains is >99% for most regions of the genome, while overall homology between HSV-1 and HSV-2 approximates 85% within translational reading frames and significantly less outside them. The HSV-1 genome is schematically shown in **Fig. 1**, it is a linear, double-stranded DNA duplex 152 000 bp in length, with a base composition of 67% G+C. The genome circularizes upon infection. Because the

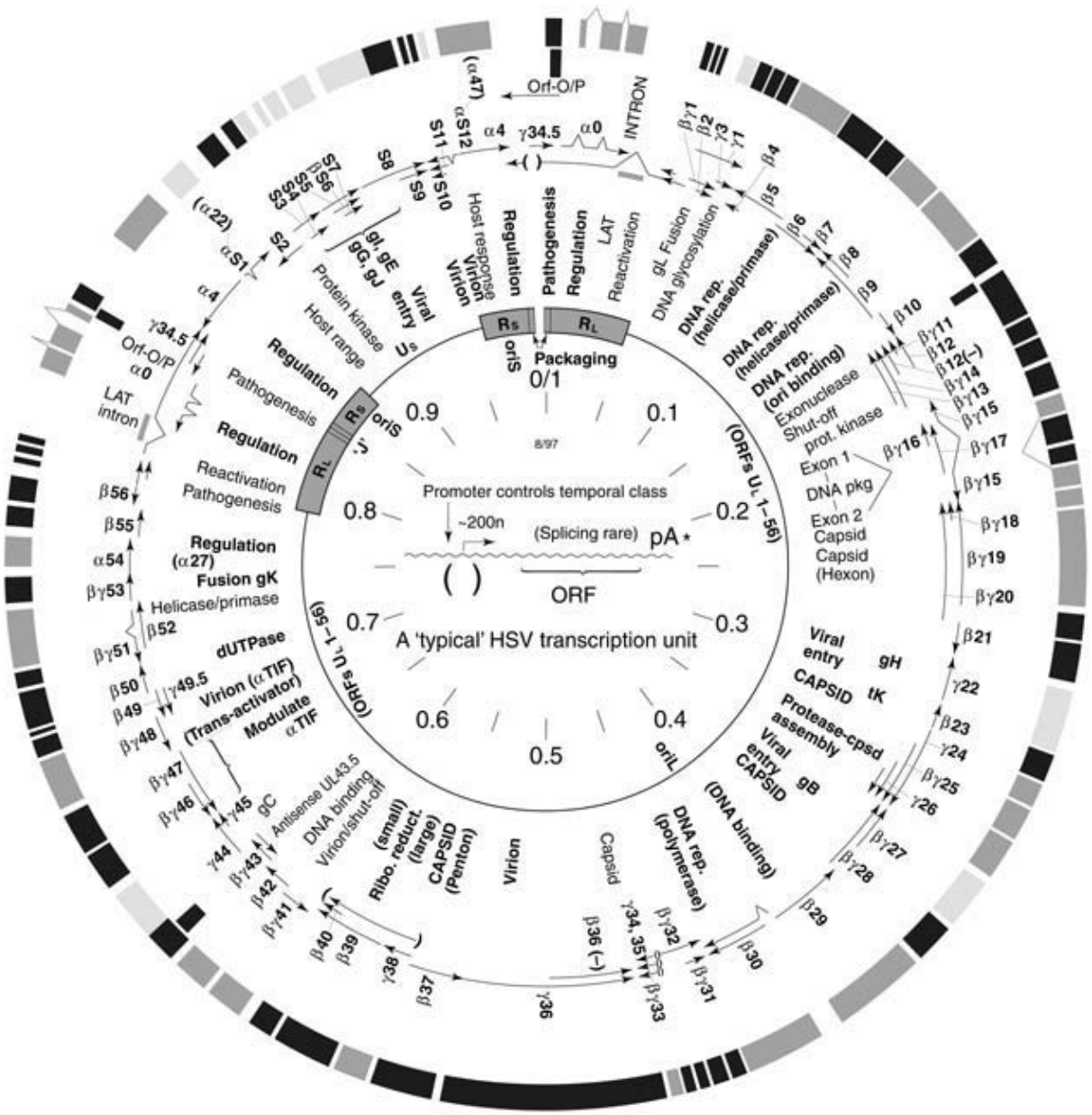


Figure 1 The genetic/transcription map of the HSV-1 (strain 17*syn*$^+$). The map is shown as a modified circle to indicate the fact that the linear genome circularizes upon infection. Open reading frames predicted from sequence data are named as described in the text, and transcripts expressing all or portions of them are indicated with the arrow heads showing the transcription termination/polyadenylation sites. Known kinetic classes of transcripts are indicated; the latency associated transcripts (LATs) in the R_L regions are the only transcripts expressed during latent infection. The functions of the two partially overlapping transcripts occurring in the R_L 5′ of the LAT start site are unknown. Also shown are the location of the origins of replication and the 'a' sequences at the genome ends which are involved in encapsidation. In addition, and where known, the functions of individual genes are shown.

genome circularizes, the genetic/transcription map is conveniently shown as a circle. Since the virus encodes nearly 100 transcripts and more than 80 open translational reading frames (ORFs), the map is complex. Still, general lack of splicing of HSV transcripts means that most ORFs are expressed by a single transcript.

The organization of the genome can be represented as $a_nbU_Lb'a_mcU_Sc'a$. It is made up of five important regions. These are:

1. The ends of the linear molecules made up of multiple repeats of 'a' sequences. These are important in both circularization of the viral

DNA upon infection, and in packaging the DNA in the virion.

2. The 9000 bp long repeat (R_L–'b' region). It encodes an important immediate early regulatory protein ($\alpha 0$), the promoter of and most of the 'gene' for the latency-associated transcript (LAT). It also encodes the translational reading frame for the ICP34.5 gene important in neurovirulence, and two partially overlapping ORFs antisense to that of ICP34.5, ORF-O and ORF-P. Preliminary reports suggest that these two ORFs encode proteins that may downregulate immediate early gene expression. Two other short translational reading frames 5′ of the LAT cap, ORF-X and ORF-Y, are also present but have unknown function.
3. The long unique region (U_L), which is 108 000 bp long, encodes more than 60 distinct proteins. It contains an origin or replication (the ori_L) and genes encoding DNA replication enzymes as well as capsid and many other proteins.
4. The 6600 bp short repeats (R_S–'c' region) encode the very important $\alpha 4$ immediate early transcriptional activator. It also encodes the polyadenylation signal for the 9 kb primary LAT, the major origin of replication (ori_s) and the promoter for two other immediate early proteins.
5. The 13 000 bp unique short region (U_S) encodes 12 ORFs, a number of which are glycoproteins important in viral host range and response to host defense.

Sequence inversions. The arrangement of the inverse repeat elements allows the viral genome to 'isomerize' or invert relative to each other into four sequence arrangements: prototype (P), inversion of the long segment relative to the short (I_L), inversion of the short relative to the long (I_S), and inversion of both relative to the P (I_{LS}). Inversion is facilitated by the 'a' sequence elements noted above.

HSV ORFs and transcripts

Number and genomic location. Superficial nucleotide sequence analysis reveals 70 'reasonable-sized' translational reading frames, but, when shorter, overlapping and complementary reading frames are included, this number increases markedly. Each viral protein is expressed from its own independently regulated transcript. The viral transcription map is included in **Fig. 1**, and correlation between transcripts and expressed proteins is evident from this map as well as the data in **Table 1.** Independent starts of overlapping transcripts, (rare) splicing, and temporally differentiated polyadenylation site utilization result in the number of independent transcripts expressed to over 100.

General properties of HSV transcripts and their promoters Generally, the translational reading frames of HSV-1 are expressed as unique, unspliced mRNA molecules which are controlled by temporal class-specific promoters. The functional architecture of a 'typical' transcript is shown in **Fig. 1**, and HSV-1 promoters representing the four temporal classes of productive infection are shown in **Fig. 2.** The average size of HSV mRNAs ranges between 1500 and 2000 bases, but transcripts as long 9000 bases (U_L36) are expressed. Nested, partially overlapping transcripts, each encoding a unique ORF but utilizing a common polyadenylation site are common (cf. transcripts encoding U_L24, 25 and 26 or those encoding U_S5, 6 and 7). Some complementary reading frames also exist; e.g. the mRNAs encoding ORFs-P and -O with that encoding ICP34.5, and transcripts encoding reading frames complementary to U_L43.

Most HSV promoters are recognizable as eukaryotic polII promoters with obvious 'TATA' box homologies 20–30 bases 5′ of the mRNA cap sites. The promoter for the early (β) HSV thymidine kinase gene (U_L23) has served as a prototype for eukaryotic polII promoters in general. Promoters generally occur outside of ORFs but examples exist of promoters lying within ORFs encoding upstream genes.

The *cis*-acting elements mediating transcription of early (β) promoters include several transcription factor binding sites located within the region from −120 to −50. These and the TATA box at −30 comprise the functional promoter; no elements important in activating transcription have been identified downstream of the TATA box. This pattern appears to be maintained with the promoters for immediate early (α) transcripts which also feature the presence of upstream enhancer elements at sites distal to and extending to several hundred bases upstream of the cap sites. These enhancers contain multiple copies of a nominal 'TATGARAT' sequence which interact with cellular transcription factors of the POU family (notably, Oct-1). Enhancement of transcription occurs through the action of virion-associated (tegument) αTIF (U_L48) protein, which is a powerful transcriptional activator. Some immediate-early promoters contain a strong binding site for the $\alpha 4$ protein near the cap site that represses transcription.

Leaky late ($\beta\gamma$ or γ_1) and strict late transcript (γ or γ_2) promoters characterized to date contain critical sequence elements mediating full levels of transcription at or near the cap site, and at least some contain transcription factor binding sites in the proximal part

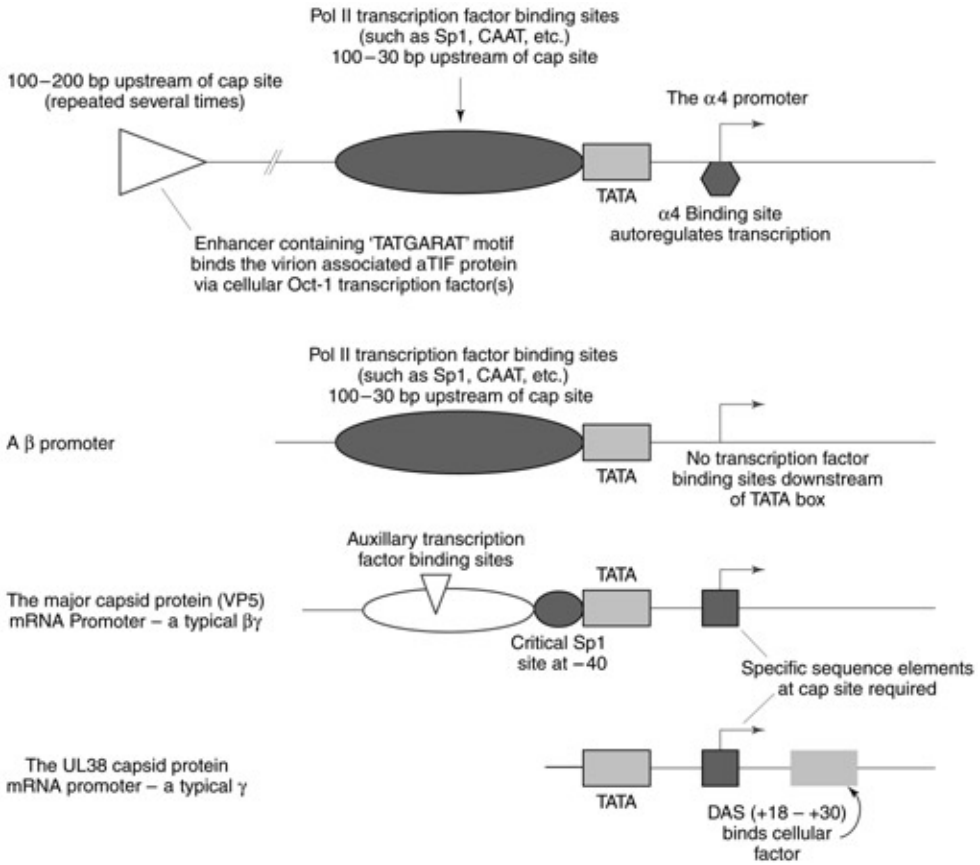


Figure 2 Architecture of productive cycle HSV promoters. Features of the promoters controlling representative members of the four kinetic classes of viral transcripts expressed during productive infection are shown.

of the leader. The promoter for the LAT (**Fig. 3**) has not been characterized in quite as much detail as those promoters controlling productive cycle transcripts. It appears to have a number of regulatory elements, some important in neuronal expression and some downstream of the cap site important for expression over extended periods of time.

Splicing The immediate-early α ICP27 protein actively inhibits splicing of cellular and viral transcripts, especially late in infection. Inhibition of splicing has also been reported to be a function of the ORF-O protein. For this and other reasons, the complex splicing patterns such as seen with adenovirus late transcripts or with the latent phase transcripts expressed by the γ herpesvirus Epstein–Barr virus do not occur. A few relatively abundant spliced transcripts are expressed: the $\alpha 0$, $\alpha 22$ (U_S1) and $\alpha 47$ (U_S12) transcripts; the latency-associated transcript family (LATs) in the long repeat which spans the joint region; and the transcript encoding the U_L15 ORF in the long unique region. This last transcription unit and the protein it encodes is conserved in all herpesviruses sequenced to date, and it may be related to a protein encoded by T_4 bacteriophage important in packaging newly synthesized viral genomes. Other spliced transcripts occur as low abundance spliced mRNAs encoding a portion of the major ORFs of unspliced mRNAs; notable examples are minor spliced variants of the transcript encoding gC (U_L44) – the function of these transcripts is unknown.

HSV replication origins

The HSV genome contains three *cis*-acting origins of replication 800–1000 bp in extent, with a much smaller (100–200 bp) critical 'core' region. They exhibit a high degree of dyad symmetry and palindromic sequences. At least one of the three copies of the ori is required for replication, and the ori in the long region (ori_L) has been suggested as having a role in neuronal replication and/or reactivation from latency, although there are no direct data to support this claim. As with bacteriophage T4, transcription through the origin occurs during virus replication,

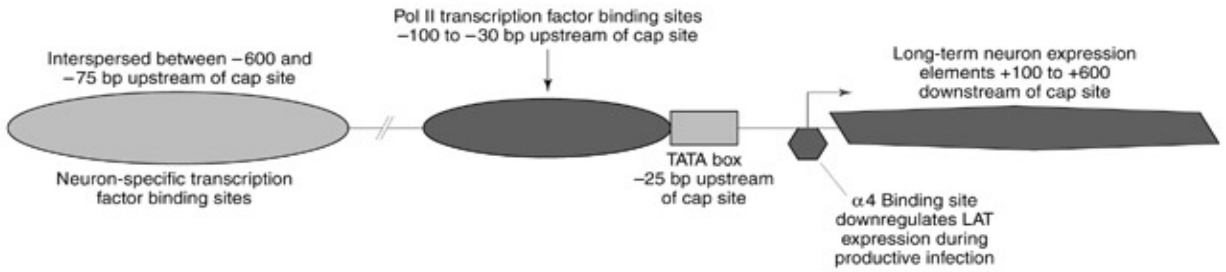


Figure 3 Architecture of the HSV latency associated transcript (LAT) promoter. Salient features of the latent phase promoter important in expression are indicated.

and a portion possesses some properties of a promoter sequence.

HSV Proteins

Nomenclature and general properties

The proteins encoded by HSV-1, their functions (if known), and their location on the genome are given in **Table 1.** Only 25 or so of the more than 80 HSV proteins expressed during productive replication are absolutely essential for virus replication in cultured cells. The remainder function in a number of ways to increase host range and tissue tropism, replication and/or plaquing efficiency, and to control or subvert the host response to infection.

Currently accepted protein nomenclature is a mix of trivial and systematic systems. Proteins encoded in the repeats and a number of other proteins of well-established function are referred to by their trivial or functional names. Proteins encoded by ORFs in the U_L and U_S regions are often referred to by the location of the ORF. Both nomenclatures are used in **Table 1.**

As predicted by the sizes of the ORFs encoding them, HSV proteins expressed during productive infection range in size from less than 10 000 M_r to more than 300 000 M_r for the tegument-associated U_L36 protein. Viral proteins tend to be quite proline rich (typically 10–14%) because of the high G+C content of the HSV genome, and thus they generally migrate anomalously slowly in denaturing SDS-acrylamide gels (i.e. the $\alpha4$ protein has a M_r of 140 000 Da but migrates at a rate expected for a protein of 170 000 Da). In addition, many HSV proteins tend to be rather rich in basic amino acids due to the preponderance of GC-rich codons encoded. It is not known whether either of these general properties has any role in specific aspects of HSV pathogenesis.

Functional classification of HSV proteins

Regulatory proteins The cascade of viral gene expression culminating in productive infection begins with the expression of α or immediate early proteins. This expression involves an interaction between the 50 000 M_r virion-associated αTIF (U_L48) protein and the cellular transcription factors, Oct-1 and CCF, mediated by the consensus 'TATGARAT' sequences upstream of α-gene promoters. The C-terminal 'acid blob' transcription activator of the αTIF protein is responsible for the enhanced expression of the immediate early transcripts.

Three of the five immediate early genes ($\alpha4$, $\alpha0$, $\alpha27$–U_L54) have roles in regulating viral gene expression at the level of transcription. Two of these ($\alpha4$ and $\alpha27$) have extensive areas of sequence similarity among a large number of α herpesviruses, but only $\alpha27$ appears to be recognizably conserved among the more distantly related β and γ herpesviruses.

The protein encoded by the $\alpha4$ transcript is a 140 000 M_r transcriptional activator with auto-regulatory functions. It is strictly required for virus replication and is continuously required for the transcription of all save the α kinetic class of viral mRNAs. It can be located in the nucleus of infected cells by immunofluorescence. Multiple phosphorylated forms of the protein have been identified in the infected cell, and it has been recently reported that the protein can also be post-translationally modified by uridine ribosylation. It has an extremely broad specificity in transient expression assay and apparently stabilizes the formation of the transcription initiation complex at and near the TATA box of viral and other promoters. This stabilization involves weak binding that does not require a highly conserved consensus sequence; however, weak binding sites occur near promoter/regulatory regions as well as many other regions in the genome. Its transcription-repressive function requires the strong association of

Table 1 Genetic functions encoded by herpes simplex virus type 1

Location	*Name*	*Function*
R_L	'a'	*cis* genome cleavage, packaging signal
	ICP34.5	RL1, neurovirulence, inhibits cellular apoptosis
	ORF-O	Modulates ICP0, ICP22 – inhibits splicing
	ORF-P	Modulates ICP4
	ICP0	(α0, IE1) immediate early *trans*-activator, disrupts nuclear ND10 structures, modulates early/late switch
	LAT-intron	Stable accumulation in nucleus of latently infected neurons, unknown function
	LAT	ca. 600 bases in 5′ region facilitates reactivation. No protein involved
	ORF-X	Unknown
	ORF-Y	Unknown
U_L	U_L1	gL, essential glycoprotein which functions as a heteromer with gH – involved with viral entry
	U_L2	Uracil DNA glycosylase, DNA repair
	U_L3	Nonvirion membrane-associated protein(?), dispensable in cell culture
	U_L4	Tegument protein, unknown function, dispensable in cell culture
	U_L5	Part of helicase/primase complex, essential for DNA replication
	U_L6	Capsid protein, capsid maturation, DNA packaging in capsid
	U_L7	Unknown, dispensable in cell culture
	U_L8	Part of helicase/primase complex, essential for DNA replication
	U_L9	ori binding protein, essential for DNA replication
	U_L10	gM, glycoprotein of unknown function, dispensable in cell culture
	U_L11	Tegument protein, capsid egress and envelopment
	U_L12	Alkaline exonuclease, DNA packaging (?), capsid egress
	$U_L12.5$	C-terminal 2/3 of U_L12, expressed by separate mRNA – specific function unknown
	U_L13	VP18.8, tegument-associated protein kinase
	U_L14	Unknown
	U_L15	DNA packaging, cleavage of replicating DNA(?), spliced mRNA, exons span U_L16 and U_L17
	U_L16	Unknown, dispensable in cell culture
	U_L17	Cleavage and packaging of DNA
	U_L18	VP23, capsid protein, triplex
	U_L19	VP5, major capsid protein, hexon
	U_L20	Membrane associated, virion egress
	U_L21	Tegument protein, dispensable in cell culture, auxillary virion maturation function (?)
	U_L22	gH, glycoprotein involved in viral entry as heteromer with gL
	U_L23	TK, thymidine kinase
	U_L24	Unknown
	U_L25	Tegument protein, capsid maturation, DNA packaging
	U_L26	VP24, maturational protease
	$U_L26.5$	Scaffolding protein
	U_L27	gB, glycoprotein required for virus entry, mediates binding through interaction with GAGs in plasma membrane
	U_L28	ICP18.5, capsid maturation, DNA packaging
	U_L29	ICP8, single-stranded DNA binding protein, essential for DNA replication
	ori_L	Origin of replication
	U_L30	DNA polymerase
	U_L31	Nuclear phosphoprotein, unknown function
	U_L32	Capsid maturation, DNA packaging
	U_L33	Capsid maturation, DNA packaging
	U_L34	Membrane phosphoprotein, unknown function
	U_L35	VP26, capsid protein, capsomer tips
	U_L36	ICP1/2, tegument protein
	U_L37	Tegument phosphoprotein
	U_L38	VP19c, capsid protein, triplex

Table 1 Continued

Location	*Name*	*Function*
	U_L39	Large subunit ribonucleotide reductase
	U_L40	Small subunit ribonucleotide reductase
	U_L41	*vhs*, virion-associated host shut-off protein, destabilizes mRNA
	U_L42	Polymerase accessory protein, essential for DNA replication
	U_L43	Unkown
	$U_L43.5$	Antisense to U_L43
	U_L44	gC, glycoprotein involved in initial stages of virion-cell association with GAGs, complement binding protein, dispensable in cell culture
	U_L45	Membrane associated, dispensable in cell culture
	U_L46	VP11/12, tegument associated, modulate αTIF
	U_L47	VP13/14, tegument associated, modulate αTIF
	U_L48	α-TIF, VP16, VSP, virion-associated acidic blob transcriptional activator, enhances immediate early transcription through cellular Oct-1 and CTF binding at 'TATGARAT' sites
	U_L49	VP22, tegument protein
	$U_L49.5$	Unknown
	U_L50	dUTPase, nucleotide pool metabolism
	U_L51	Unknown
	U_L52	Part of helicase/primase complex, essential for DNA replication
	U_L53	gK, glycoprotein involved in virion egress
	U_L54	α27, ICP27, IE2, Vmw63, immediate early regulatory protein, RNA transport, inhibits splicing, mediates poly(A) signal utilization
	U_L55	Unknown, dispensable in cell culture
	U_L56	Tegument protein, dispensable in cell culture, affects pathogenesis
R_L	R_L	See R_L above
	R_L/R_S junction	Joint region, site of inversions of long and short segments, contains 'a' sequences
R_S	LAT-poly(A) site	Polyadenylation site for the primary LAT and the transcripts encoding ORF-O and -P (only transcripts through the joint region)
	ICP4	α4, IE3, essential immediate early *trans*-activator, also represses the transcription of itself, α4, and latency associated, and some other transcripts by strong binding to cap site at consensus sequence 'ATCGTN$_5$YSG'
	ori_S	Origin of replication
	Promoter for ICP22	Promoter and splice donor for immediate early transcripts ICP22 and ICP47
U_S	ICP22	α22, U_S1, IE4, splice acceptor and ORF for nonessential spliced immediate early protein, affects ability of virus to replicate in certain cells
	U_S2	Unknown
	U_S3	Tegument-associated protein kinase, phosphorylates U_L34 and U_S9
	U_S4	gG, glycoprotein of unknown function
	U_S5	gJ, glycoprotein of unknown function
	U_S6	gD, glycoprotein involved with virus infectivity and entry, binds HVEM
	U_S7	gI, glycoprotein that, as heterodimer with gE, binds IgG Fc and influences cell-to-cell spread of virus
	U_S8	gE, glycoprotein that, as hetero-oligomer with gI, binds IgG-Fc and influences cell-to-cell spread of infection
	U_S9	Tegument-associated phosphoprotein
	U_S10	Tegument-associated protein
	U_S11	Tegument-associated protein phosphoprotein, RNA binding, post-transcriptional regulation
	U_S12	α47 ORF and splice acceptor for spliced immediate early protein, nonessential in cell culture, inhibits MHC I antigen presentation in human and primate cells
R_S	R_S	See R_s above
	'a'	*cis* genome cleavage, packaging signal

the $\alpha 4$ protein with the consensus sequence 'ATCGTC' at or near the transcript start site. This sequence is found at this location in the $\alpha 4$, $\alpha 0$ and LAT promoters.

The essential $\alpha 27$ protein (M_r 51 000) has sequence elements consistent with its formation of zinc fingers. It also binds DNA and RNA. This multifunctional protein accumulates in the nucleus of infected cells, and is absolutely required for the expression of a number of γ (γ_2) transcripts. Different functional domains of the protein are involved with inhibition of cellular splicing through specific association with splicosomes, inhibition of transport of spliced cellular transcripts mediated by its RNA binding activity, and in utilization of specific cleavage polyadenylation sites for the expression of some late mRNAs. The protein also exhibits transcriptional regulatory functions.

The non-essential $\alpha 0$ protein (M_r 79 000) enhances the transcriptional activation of the $\alpha 4$ protein in transient assays, and has been reported to act as a transcriptional activator on some promoters by itself. Interaction with the $\alpha 4$ protein is mediated by domains in the C-terminal region of the $\alpha 0$ protein. The protein is localized in the nucleus in infected cells, and while its function is dispensable for virus replication in cell culture, $\alpha 0$ null mutants have very poor plaquing efficiency. This implies an important role in virus replication in low-multiplicity infections and explains the observation that $\alpha 0$ negative viruses reactivate poorly from latent infections. Its mode of action remains obscure. While the protein binds to DNA, specific base sequence elements important in this binding have not been identified. The protein causes disruption of PML-containing subnuclear structures, and is thought to be a major factor in the changes in nuclear structure observed as viral infection proceeds. The $\alpha 0$ protein also interacts with the novel cellular herpesvirus-associated ubiquitin specific protease (HAUSP), but the result of this interaction is currently unclear. Finally, the $\alpha 0$ protein has been shown to be involved with the turnover of the catalytic subunit of cellular DNA-dependent protein kinase; again, the functional manifestation of this activity is unclear.

Other HSV regulatory proteins include three $\beta\gamma$ (γ_1) proteins: the U_L46 and 47 proteins, which modulate the activity of αTIF by unknown mechanisms; and the virion-associated host shut-off protein (U_L41), which functions by destabilizing all mRNA molecules in the infected cell. The major β DNA binding protein (U_L29), which has an essential role in viral DNA replication (see below), also appears to function in gene regulation by mediating the shut-off of transcription of at least some β genes. Finally, the ORF-O and ORF-P proteins have regulatory functions that may have a role in the latent phase of infection.

DNA replication Seven β proteins are strictly required for the formation of the viral DNA replication complex. The 123 000 M_r DNA polymerase (U_L30) exhibits regions of significant sequence similarity with other eukaryotic, viral and phage DNA polymerases. It functions in a complex with U_L42, the double-stranded DNA binding protein. The ori binding protein (U_L9) binds to the critical core sequence 'GTTCGCAC' in the ori_S and may function as a dimer. Equimolar amounts of proteins U_L5, 8 and 52 make up the helicase/primase complex; and, finally, the 120 000 M_r major DNA binding protein has been hypothesized to function in a manner analogous to phage T4 gene 32 single-stranded DNA binding protein in keeping the replication fork open.

A number of other β proteins are involved in genome replication by altering the pool of deoxyribonucleotide precursors and for repair and proofreading functions. While none is strictly essential, they increase the range of potential cells available for HSV replication in the host. Viral encoded pool enzymes include: thymidine kinase (U_L23); the large and small subunits of ribonucleotide reductase (U_L39 and 40), the former having significant areas of sequence similarity with the human enzyme; and deoxyuridine triphosphatase (U_L50). Enzymes with potential repair function include uracil-DNA glycosylase (U_L2).

Structural proteins More than 30 HSV proteins, all $\beta\gamma$ (γ_1) or γ (γ_2), are found associated with mature virions. These include the five capsid proteins described above, a large number of tegument proteins, and ten or more glycoproteins. Some of the tegument proteins, such as the host shut-off function (*vhs*–U_L41), are not absolutely required for viral replication in cell culture, although they increase plaquing efficiency and/or host range. While the transcriptional stimulatory function of αTIF (U_L48) is not strictly required for viral viability, this protein also has an important role in morphogenesis. Four glycoproteins have been shown to be strictly required for HSV viability – gB (U_L27), gD (U_S6), gL (U_L1) and gH (U_L22) – all of which are involved in the internalization of the virion in the initial stages of infection.

Proteins involved in capsid formation and maturation Capsid assembly can be readily accomplished both *in vitro* and in Sf9 cells with proteins expressed from a panel of recombinant baculoviruses expressing the four principal capsid proteins (U_L19-VP5, U_L38-

VP19c, U_L18-VP23, and U_L35-VP26), the scaffolding protein (encoded by ORF U_L26.5), and the maturational protease (U_L26). Other proteins have been implicated genetically in the capsid assembly process, but are dispensable in minimal *in vitro* systems.

The packaging of the viral DNA in the mature capsid requires a large number of viral proteins including U_L6, 15, 17, 25, 28, 32 and 33. The protein encoded from the spliced mRNA transcribed from the U_L15 gene in conjunction with U_L26 may function as a terminase, cleaving genome-sized fragments from the growing concatomeric replication complex. The U_L15–U_L26 complex also appears to mediate the initial entry of viral DNA into the capsid, i.e. it serves a 'docking' function. The alkaline exonuclease gene (U_L12) also plays a role in DNA packaging.

This latter enzyme is expressed in two forms via two partially overlapping mRNAs, the larger having a M_r of 63 000 and the smaller about 40 000. Interestingly, comparative sequence analysis suggests that in the γ herpesvirus Epstein–Barr virus homologues of these two proteins are encoded in two distinct genomic locations; therefore, two copies of this enzyme seem to have a significant general role in some aspect of herpesvirus replication or pathogenesis.

Proteins involved in pathogenesis and cytopathology Based upon dispensibility in productive infection in cultured cells, many, if not the majority, of HSV proteins are involved in aspects of pathogenesis and dealing with host defenses. A striking example is the ICP34.5 gene product which mediates many aspects of the ability of HSV-1 to replicate effectively in neural tissue, perhaps by inhibition of apoptosis in productively infected neural cells. The α47 protein appears to have a role in modulating host response to infection by specifically interfering with the presentation of viral antigens on the surface of infected cells by major histocompatibility complex (MHC) class I. While this function may be modulated by the neighboring U_S11 protein, the immediate expression of the α47 gene product appears to preclude immune surveillance by CD8+ T lymphocytes at the very onset of infection. Interestingly, it has recently been established that this activity is confined to cells of human and primate origins.

A number of the envelope glycoproteins found in the intact virion appear to have a significant role in controlling host response. The complex of glycoproteins gE (U_S9) and gI(U_S8) function as immunoglobulin Fc receptor, blocking the presentation of this region of the antibody molecule and precluding activation of the complement cascade. In addition, gC (U_L44) binds several components of the complement complex, and viruses with mutations in gD (U_S6) display altered pathogenesis in mice. Viral glycoproteins and other gene products also have a role in cytopathology. For example, gL (U_L53) is involved in the ability of many strains of HSV-1 to form syncytia among infected cultured cells, and a virion component (U_L41) mediates the disruption of host polysomes and destabilizes host (and viral) mRNA following infection.

Other viral gene products also have a role in cytopathology and pathogenesis. While dispensable for productive infection in some cells, the immediate early protein encoded by the α22 gene is required for HSV replication in others – perhaps by mediating the expression of a set of late transcripts. Such transcripts presumably encode other viral proteins important in full productive cytopathology. Viruses lacking thymidine kinase (U_L23) have significantly altered pathogenic patterns in experimental animals, while certain mutations in the DNA polymerase gene (U_L30) have altered patterns of neurovirulence.

Physical Properties of HSV

Being an enveloped virus, HSV is relatively stable in aqueous solution in the pH range from 6 to 8 and is resistant to gentle protease treatment. Its infectivity is rapidly lost following treatment with lipid solvents and lipases. The capsid is relatively unstable in CsCl; therefore banding is difficult. Specific capsid proteins can be extracted with mild guanidinium treatment without causing the gross morphology of the capsid to collapse; more rigorous treatment results in the release of viral DNA through the penton vertices of the capsid. Viral infectivity is rapidly lost with UV irradiation, but individual viral genes can be expressed from heavily irradiated virions following infection of cultured cells. This is consistent with both the fact that individual viral genes are expressed via individual promoters, and with the fact the target size for viral transcriptional regulatory functions is much smaller than that for the essential genes *in toto*.

Viral Replication: Viral Entry

Initial association of HSV with a host cell is mediated by the association with the envelope gC (U_L44) and glycosaminoglycans such as heparin sulfate on the cell surface. Clearly, this is not an obligatory route of initial interaction, as gC null mutants of the virus replicate efficiently in both cultured cells and in animal model systems. Virus internalization involves the mediation of four essential glycoproteins, gB (U_L27), gH (U_L22), gD (U_S6), and gL (U_L1). In many cells, this is mediated by interaction of gB, gD, and

other glycoproteins with one of at least three cellular surface proteins named the herpes virus entry mediator proteins (HVE – A, B, and C). HVEs are members of the TNF/NGF family of proteins. Other cellular surface proteins may also function in a similar capacity as some cells lacking this receptor still allow efficient viral entry. The viral genome is accompanied into the nucleus by the αTIF protein (U_L48) which functions in enhancing immediate early viral transcription via cellular transcription factors. The virion-associated host shut-off protein (*vhs*–U_L41) appears to remain in the cytoplasm, where it causes the disaggregation of polyribosomes and degradation of cellular and viral RNA. Infecting DNA may remain associated with one or several structural components of the virion since it does not interact with cellular histones in a manner analogous to infecting papovavirus DNA.

Viral Replication: Translation and Post-translational Processing

HSV mRNA is translated on polyribosome structures that are grossly identical to those seen in the uninfected host. The half-life of all mRNA is reduced in infected cells by the action of the virus-encoded *vhs* (U_L41); this, coupled with the high transcriptional activity from the viral genome, rapidly results in virtually all protein synthesis in the infected cell being virus specific. The protein encoded by the γ (γ_2) ICP34.5 mRNA has been shown to have an inhibitory effect upon the ability of ribosomes from infected cells to translate cellular mRNAs; thus, translational control of gene expression appears to have a role in viral pathogenesis if not viral replication.

Extensive proteolytic processing of HSV proteins does not occur, but the maturation of the capsid absolutely requires the activity of the maturational protease encoded by the U_L26 gene product. Many viral proteins are extensively phosphorylated, including the α4 and α27 transcriptional activators and a number of structural proteins. The precise role of such processing upon the function of these proteins is not well characterized. Other modifications such as the uridine-ribosylation of the α4 protein have also been described.

Replication: Virus-induced Modification of Nuclear Compartmentalization and the Role of Viral DNA Replication in the Productive Cycle Transcription Program

The temporally controlled transcriptional regulation program exhibited during productive infection by HSV is accomplished with class-specific viral promoters that are ostensibly cellular. There is no presently convincing evidence that the virus-encoded regulatory proteins alter cellular transcriptional machinery by changing its basic specificity. Rather, the viral transcription program appears to be mediated by virus-induced modifications of the nuclear organization of the infected cell. Although yet to be fully characterized, the virus-induced modifications are well illustrated by the disruption of the nuclear 'pod' structures (ND10) by the action of the immediate early α0 protein followed by a reassembly of viral replication/transcription compartments later in the replication cycle. This change involves interaction with α0 and α27.

These observations strongly suggest that transcription of early and late genes is likely to occur in two distinct environments. The other major factor in the restructuring of the nucleus during infection is the replication of viral DNA. The specific role of viral DNA replication enzymes was described above, and the best model for HSV DNA replication is probably exhibited by T4 bacteriophage. The vegetative replication of viral DNA represents a critical and central event in the viral replication cycle. High levels of DNA replication irreversibly commit a cell to producing virus, which eventually results in cell destruction.

DNA replication has a significant influence on viral gene expression. Early expression is significantly reduced or shut off following the start of DNA replication, while late genes begin to be expressed at high levels. Immunofluorescence studies show that DNA replication occurs at discrete sites, or 'replication compartments' in the nucleus. Prior to DNA replication, the α4 protein and the single-stranded DNA binding protein ICP8 (U_L29) are distributed diffusely throughout the nucleus; concomitant with viral DNA replication, the distribution of these proteins changes to a punctate pattern. The transcriptional environment within replication compartments seems critical in the preferential transcription of late genes, although the precise role of the many viral proteins involved, as well as the role of cellular proteins, is yet to be determined.

Viral Replication: Viral Assembly and Release

Viral capsids form in the nucleus in association with the scaffolding protein (U_L26). A vertex of the empty capsid then interacts with the 'a' sequence at a free end of replicating DNA and a genome equivalent of DNA is packaged and cleaved. As noted, this requires the function of the U_L15 and 28 proteins. The

packaging process also involves the 'a' sequence at the other end of the genome-sized DNA fragment, leading to maturational cleavage of the growing DNA chain, resulting in encapsidation of a full-length genome.

Presumably, full capsids associate with tegument proteins near the nuclear membrane which has become modified by inclusion of viral glycoproteins that have been glycosylated in the cellular Golgi apparatus. Two models have been proposed for what happens then: either the virion retains its nuclear membrane upon egress, or this nuclear membrane is lost by fusion with the cytoplasmic membrane prior to exocytosis. Experimental evidence has been variously interpreted to support either one or the other. Whatever the exact mechanism, full infectious virus can be recovered from cells many hours before cellular disintegration and release of virions into the extracellular medium. Indeed, virus spread by cell-to-cell contact and/or cell fusion is probably an important feature of the pathogenesis of infection in the host and can be readily observed with infections in cultured cells.

Viral Replication: Cytopathology

Classic features of the gross cytopathology of cells infected with HSV have long been used diagnostically. A very noticeable modification of the nucleus of the host cell with the condensation and margination of host chromatin at the nuclear membrane can be observed within a few hours after infection. This is followed by the accumulation of semicrystalline arrays of empty viral capsids in the nucleus leading to the formation of macroscopic inclusion bodies. Such nuclear changes are seen with most human and animal herpesvirus, and served for an early basis of classification of herpesviruses. Other 'classic' features of virus-induced cytopathology include extensive reduplication of the nuclear membrane as viral capsids associate with it, and the formation of extensive syncytia, which occurs with many (but not) all strains of the virus.

Latent Infection by HSV

Latent infection with HSV can be viewed as having three separable phases: establishment, maintenance and reactivation. In the establishment phase, the virus must enter a sensory neuron; and, following entry, there must be a profound restriction of viral gene expression so that the cytopathic results of productive infection do not occur. Thus, productive cycle genes are transcriptionally and functionally quiescent and only the 8.5 kb LAT is expressed. The expression of LAT is controlled by the latency specific promoter, which has a number of regulatory elements important in neuronal expression over extended periods of time (**Fig. 3**). The establishment of latent herpesvirus infections can be essentially viewed as a passive phenomenon, as replication-defective virus can establish latent infections. This has been taken as support for the model that latency is just the result of the failure of the productive cascade, perhaps due to the specific population of transcription factors in the cell in which the latent infection is established. Some recent analysis of the functions of the proteins encoded by ORF-O and ORF-P in the long repeats suggests, however, that these proteins may serve to modulate immediate early gene expression. Viral genes may therefore function in optimizing the establishment of latent infections.

The maintenance of the HSV genome in latently infected neurons appears passive, i.e. it requires no viral gene expression or gene product at all. This is in keeping with the fact that latent HSV genomes are harbored within the nucleus of a nondividing sensory neuron and do not need to replicate; indeed the challenge arises from the need for the virus to reactivate from a transcriptionally quiescent, non-replicating cell. HSV DNA is maintained as a nucleosomal, circular episome in latent infections, and low levels of genome replication might occur or be necessary for the establishment or maintenance of a latent infection from which virus can be *efficiently* reactivated.

Successful reactivation of HSV results in the appearance of infectious virus at the site of entry in an immune host. Expression of only a 350 bp region of LAT near its 5′ end is both necessary and sufficient to facilitate reactivation in several animal models. Whatever the specific mechanism for this facilitation, it does not involve expression of a protein; rather, the active region appears to function by a *cis*-acting mechanism, the details of which are currently obscure.

The process of reactivation from latency is triggered by stress as well as other signals which may transiently lead to increased transcriptional activity in the harboring neuron. A possible scenario is that one or several latently infected neurons replicate only a few viral genomes and generate only a few infectious virions during the initial reactivation event. This might then lead to infection of critical peripheral tissue when the overall immune capacity of the host is temporarily reduced by stress or other factors. The process may be augmented by viral genes shown to interfere with apoptosis, such as ICP34.5, which act to prevent neuronal death during reactivation where limited replication occurs.

Latent-phase transcripts

Latently infected neurons represent 1–10% of the total neurons in the ganglia, and infected cells contain as few as 5–10 copies of viral DNA. In some neurons, an 8.5 kb polyadenylated transcript whose promoter lies in the long repeat sequences about 1800 bases 3′ of the 3′ end of the $\alpha 0$ mRNA is expressed as the LAT (**Fig. 1**). This primary transcript extends through the joint region to a polyadenylation signal next to that of the $\alpha 4$ transcript in the short repeat.

The primary LAT transcript is extensively processed, forming an unknown number of unstable, spliced, polyadenylated mRNAs, and a stable 2 kb poly(A-)intron in lariat form which accumulates in the latently infected neuronal nucleus. A second, spliced variant of this LAT-intron can also be observed. The function of this stable intron is unclear; it is not involved in the facilitation of reactivation mediated by LAT expression.

Future Trends in Research on HSV

HSV possesses a number of features that will make it a continuing research model in the future. Among the most obvious are the following. (1) The neurotropism of the virus, along with the relative convenience of generating recombinants containing foreign genes expressed either under homologous or heterologous promoters, recommends HSV as a vector for introducing specific genes into neural tissue. This facility is increased by the fact that the viral genome can enter and be maintained in neural tissue in the complete absence of lytic phase viral gene expression. (2) The fact that HSV's replication is restricted in terminally differentiated neurons suggests its potential as a therapeutic agent against neural tumors, provided appropriate mutants can be constructed. (3) The benign nature of normal infection with HSV also suggests its potential use as a vaccine vector by incorporation of specific antigens into the viral genome, although the promise of this application may be clouded somewhat by the ability of replication-defective viruses to persist in the host. (4) The abundance of viral genes dedicated to the efficient replication of viral DNA in the productive cycle provides a number of potential targets for antiviral chemotherapy. (5) The interactive cascade of viral proteins involved in regulation at a number of levels in the infected cell makes HSV a useful model for the detailed analysis of specific aspects of control of eukaryotic gene expression.

See also: **Genetics of animal viruses; Herpes simplex viruses (*Herpesviridae*): General features; Latency; Pathogenesis: Animal viruses; Epstein-Barr virus (*Herpesviridae*): General features; Persistent viral infection; T4-like phages (*Myoviridae*); Varicella-Zoster virus (*Herpesviridae*): General features; Vectors: Animal viruses, Plant viruses; Viral membranes; Virus–host cell interactions; Virus structure: Atomic structure, Principles of virus structure.**

Further Reading

Roizman B and Sears AE (1996) Herpes simplex viruses and their replication. In: Fields BN, Kripe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd Edn, p. 2231. New York: Lippincott–Raven.

Steven AC and Spear PG (1997) Herpesvirus capsid assembly and envelopment. In: Chiu W, Burnett RM and Garcea RL (eds) *Structural Biology of Viruses*, p. 352. New York: Oxford University Press.

Wagner EK and Bloom DC (1997). The experimental investigation of herpes simplex virus latency. *Clin. Microbiol. Rev.* 10: 419.

Wagner EK, Guzowski JF and Singh J (1995) Transcription of the herpes simplex virus genome during productive and latent infection. In: Cohn WH and Moldave K (eds) *Progress in Nucleic Acid Research and Molecular Biology*, p. 123. San Diego: Academic Press.

HERPESVIRUSES 6 AND 7 – HUMAN (*HERPESVIRIDAE*)

Takuya Shimamoto and **Koichi Yamanishi**, Department of Microbiology, Osaka University Medical School, Osaka, Japan

History

Human herpesviruses 6 and 7 (HHV-6 and HHV-7) are ubiquitous agents of humans. HHV-6 was first isolated by Salahuddin *et al* in 1986 as a novel herpesvirus from patients with lymphoproliferative disorders, and initially named human B lymphotropic virus (HBLV). Subsequently, several laboratories

Prevention and Control

There is no prevention or therapy for the diseases caused by HHV-6 and HHV-7; however, ganciclovir and foscarnet are effective in suppressing replication of HHV-6 *in vitro*. Antiviral therapy might be appropriate in individual cases of severe HHV-6 disease.

Future Perspectives

The functions of most viral gene products of HHV-6 and HHV-7 in the lytic and latent infections have not yet been elucidated. Recently, the entire sequences of both viruses have been determined, accelerating the research of their molecular biology. The development of a recombinant virus system is awaited for the detailed examination of the function(s) of the viral genes in the context of the viral genomes. Since the only known host for HHV-6 and HHV-7 is humans, animal models are also a prerequisite for understanding the functions of the viral genes *in vivo*.

See also: **Epstein-Barr virus (*Herpesviridae*): General features, Molecular biology; Cytomegaloviruses (*Herpesviridae*): Animal cytomegaloviruses, General features (human), Molecular biology (human); Herpes simplex viruses (*Herpesviridae*): General features, Molecular biology; Varicella-Zoster virus (*Herpesviridae*): General features, Molecular biology; Herpesvirus 8 – Human (*Herpesviridae*).**

Further Reading

Frenkel N, Schirmer EC, Wyatt LS *et al* (1990) Isolation of a new herpesvirus from human CD4+ T cells. *Proc. Natl Acad. Sci. USA* 87: 748.

Frenkel N and Roffman E (1996) Human herpesvirus 7. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 2609. New York: Lippincott-Raven

Pellet PE and Black JB (1996) Human herpesvirus 6. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 2587. New York: Lippincott-Raven Press.

Salahuddin SZ, Ablashi DV, Markham PD *et al* (1986) Isolation of a new virus, HBLV, in patients with lymphoproliferative disorders. *Science* 234: 596.

Yamanishi K *et al* (1988) Identification of human herpesvirus 6 as a causal agent for exanthem subitum. *Lancet* i: 1065.

HERPESVIRUS SYLVILAGUS (*HERPESVIRIDAE*)

Peter G Medveczky, University of South Florida College of Medicine, Tampa, Florida, USA

History

Herpesvirus sylvilagus is a model for lymphoproliferative disorders and viral oncogenesis because it induces a lymphoma-like illness in rabbits. It was first isolated by Hinze in 1968 from kidney cell cultures of apparently healthy cottontail rabbits (*Sylvilagus floridanus*) in southern Wisconsin, USA. Morphological and molecular biological analysis proved that herpesvirus sylvilagus is a typical herpesvirus. The viral particle is enveloped and its capsid contains 162 capsomers as described for other herpesviruses. Neutralization assays and partial sequencing of the viral genome also demonstrated that herpesvirus sylvilagus is a unique herpesvirus. A virus referred to as cottontail rabbit herpesvirus (CTHV) has been isolated by Cebrian. This cottontail rabbit virus is unrelated to herpesvirus sylvilagus, as determined by Southern blot hybridization.

Classification

Herpesviruses have been classified into three subfamilies of the *Herpesviridae: Alpha-, Beta-* and *Gammaherpesvirinae*. The gamma or lymphotropic viruses infect lymphocytes and are further divided into two genera, *Lymphocryptovirus* and *Rhadinovirus*, on the basis of biological data, pathogenesis, sequence homology and latency genes (**Table 1**). There is little doubt that herpesvirus sylvilagus is a member of the gammaherpesvirus group but it is not clear whether it belongs to either of the two subgroups. While several investigators concluded that herpesvirus sylvilagus-induced pathology resembles

Table 1 Comparison of genera *Lymphocryptovirus* and *Rhadinovirus* herpesviruses

Virus species and common abbreviation	*Genus*	*Latency in lymphocytes*
Epstein-Barr virus (EBV)	*Lymphocryptovirus*	B
Herpesvirus papio (HVP)	*Lymphocryptovirus*	B
Kaposi's sarcoma associated herpesvirus (KSHV or HHV-8)	*Rhadinovirus*	B(?)
Herpesvirus saimiri (H. saimiri or HVS)	*Rhadinovirus*	T
Herpesvirus ateles (HVA)	*Rhadinovirus*	T
Murine herpesvirus 68 (MHV-68)	*Rhadinovirus*	B
Herpesvirus sylvilagus (H. sylvilagus)	*Rhadinovirus*	B and T

Epstein–Barr (EBV) infection of humans, analysis of the genomic structure suggests that herpesvirus sylvilagus belongs to the gamma-2 subgroup of herpesviruses (EBV is a gamma-1 virus). Typical members of the gamma-2 subgroup include the human Kaposi's sarcoma-associated herpesvirus (KSHV, also referred to as human herpesvirus 8 or HHV-8), herpesvirus saimiri, herpesvirus ateles and murine herpesvirus 68.

Genome Structure

The herpesvirus sylvilagus genome is a linear double-stranded DNA with an estimated size of 158 kbp (**Fig. 1**) and consists of a unique, approximately 120 kbp stretch of DNA flanked by a variable number of tandem repeats. Repeat units are 553 bp in length with an extremely high G + C content (83%). This arrangement of the repeats with high G + C content is typically seen in other gamma-2 herpesviruses. Sequence analysis of the unique central region of the genome should greatly help to determine whether herpesvirus sylvilagus is a member of the gamma-1 or gamma-2 subgroup.

Viral Proteins

Several studies have been conducted to identify and catalogue viral proteins. These studies were based on fractionation of purified virion proteins according to size, isoelectric point, glycosylation and phosphorylation. Pulse-labeling with radiolabeled amino acids during the course of infection was also performed. These early studies show that purified herpesvirus sylvilagus virions contain about 45 virion protein bands ranging from 18 to 230 kDa. There are nine glycoproteins, 13 phosphoproteins and four species that are both glycosylated and phosphorylated. Unfortunately, there are no data available as to how many of these protein bands represent distinct gene products, how many are various modified forms of the same polypeptide, and whether any of these bands are encoded by the host cell. DNA sequencing, transcript mapping in combination with genetic approaches and protein chemistry will be required to identify and match specific viral genes and protein products expressed during latency and various stages of lytic infection.

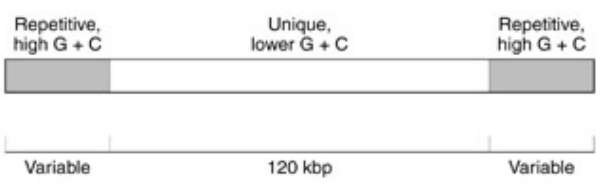


Figure 1 Organization of the herpesvirus sylvilagus genome.

Geographic Distribution

Little information is available on the geographic distribution of herpesvirus sylvilagus. Most surveys examined small numbers of animals from confined small areas of the USA. Prevalence was based on virus isolation assays and on detection of herpesvirus sylvilagus-specific antibodies against late (probably virion) proteins. Some studies found that less than 4% of wild cottontail rabbits are infected, which is an unusually low figure for herpesvirus infections occurring in mammals. It is possible that immune responses against late proteins may represent a recent or persistent infection, and after acute infection the titer of antibodies against virion proteins decreases. Also, viremia may not be detectable after acute infection in wild animals. Therefore, the prevalence of infection of herpesvirus sylvilagus in wild cottontail rabbits may be much higher and needs to be re-examined. Information on putative latent genes (such as the EBNA proteins of EBV) and immunogenicity of recombinant proteins would be required to conduct larger studies. Latent and/or recombinant proteins could be more sensitive and specific markers for estimating prevalence of infection.

Transmission

The mechanism of transmission of the virus is also uncertain. Herpesvirus sylvilagus can be isolated from the nasopharynx, suggesting that natural infection occurs through contact with oral/nasal secretions. According to the available data, herpesvirus sylvilagus infection does not appear to be transmitted through mosquitoes (*Aedes triseriatus*) or rabbit fleas (*Cediopsylla simplex*). Also, transplacental transmission may not occur at high frequency. All of these transmission studies were conducted by evaluating the presence of infectious virus in various tissues and blood of prospective recipient animals. Infectious virus in the blood and tissues is readily detected after experimental inoculation of large doses of the virus. However, it is possible that during natural infection only very low amounts of virus are transmitted. As a result, production of virus in tissues or viremia is much lower during natural infection and may not be detectable by the present assays.

Host Range

The host range of herpesvirus sylvilagus is very narrow and infection appears to be restricted to cottontail rabbits. New Zealand white rabbits (*Oryctologus cuniculus*), mice, hamsters and guinea pigs are resistant to experimental infection.

Virus Propagation

Herpesvirus sylvilagus can be cultured *in vitro* in monolayers derived from kidneys of cottontail rabbits. New Zealand white rabbit kidney cell cultures also support replication of the virus but yield is less than those obtained in cottontail rabbit kidney cultures. No lytic virus replication occurs in primate, mouse, hamster or chicken cells.

Clinical Features

No major studies have been published on the clinical features and pathology of natural infection occurring in wild animals. Most studies have been conducted either by housing infected and uninfected animals in common cages or by injection of herpesvirus sylvilagus grown in tissue culture into naive animals.

Pathogenicity

Experimentally infected cottontail rabbits develop a lymphoproliferative disease which usually spontaneously resolves after a few months. Animals show general lymphadenopathy and enlargement of lymph nodes. Splenomegaly with grayish nodules with petechias is also typically seen after infection. Kidneys are slightly enlarged with lymphoid infiltrates.

A lymphoma-like disease can develop in some experimentally infected animals; it is characterized by a severe and generalized infiltration of lymphoid organs, kidneys, liver and submaxillary glands by atypical lymphocytes. The lymphoproliferative syndrome/lymphoma induced by herpesvirus sylvilagus is more severe in young animals and can lead to death.

Low titers of the virus can be detected in whole blood 1 week after infection of cottontail rabbits inoculated intraperitoneally or subcutaneously with herpesvirus sylvilagus. It is unclear whether this represents viremia or virus production by infected white blood cells. After the second week of infection, total leukocyte count in the blood shows a moderate increase, accompanied by a large rise in the relative percentage of lymphocytes. Lymphocytosis can last for 10–18 weeks.

While the viral genome is a linear molecule in the virion, infected lymphocytes contain predominantly circular episomal forms. Herpesvirus sylvilagus infects both B and T lymhocytes of cottontail rabbits *in vivo* and the viral genome is a circular episome in these cells. Several copies per cell have been detected in the peripheral blood or spleen tissues, which suggests that a fairly large proportion of lymphocytes are infected. These data also show that the overwhelming majority of infected cells harbor only latent virus. These observations are also consistent with very low titers of virus in the plasma produced by a very low percentage of lymphoid or other cells. Whether the latent episomal form of the genome persists in lymphoid cells for the life of infected animals, as described for EBV infection, remains to be determined.

Taken together, clinical and molecular studies show that herpesvirus sylvilagus infection resembles acute lymphoproliferative disease associated with EBV infection. It is a massive and subacute infection that involves the lymphoid system and the virus undergoes latency in lymphocytes. It is also clear that the rabbit model is different because EBV only infects B cells, whereas herpesvirus sylvilagus was found in both B and T cells.

Immortalization of Macrophage-like Cells *In Vitro*

As herpesvirus sylvilagus induces a lymphoma-like disease, it infects both B and T cells *in vivo*, and these cells contain episomal viral genomes typically seen in cells immortalized with herpesviruses. Several laboratories investigated immortalization of lymphoid cells with the virus. All of the *in vitro* and *in vivo* attempts

(published or unpublished) failed, except for a report on a 'macrophage-like' rabbit cell line established by *in vitro* infection of splenocytes with herpesvirus sylvilagus. This cell line contained integrated herpesvirus sylvilagus DNA. Lymphoid cell lines immortalized with other herpesviruses such as EBV or herpesvirus saimiri typically carry multiple copies of episomal viral genomes and integration seldom occurs.

These basically negative results do not rule out the possibility that herpesvirus sylvilagus can immortalize lymphocytes; for example, infected cells may require special growth factors and lymphokines for *in vitro* culturing. To resolve the issue of the transforming ability of this virus, development of reagents and more systematic studies are required. Lymphocytes at various stages of differentiation as well as macrophages could be targets of immortalization by herpesvirus sylvilagus. Most studies have been conducted by using the original virus isolate of Hinze and this strain could have suffered spontaneous mutations; therefore, freshly isolated viruses may prove to be transforming.

Immune Response and Antiviral Drugs

The kinetics of virus-specific antibody development was determined using indirect fluorescent antibody (IFA) technique on herpesvirus sylvilagus-infected newborn cottontail rabbit kidney (NCRK) cells. Antibodies were detected by the IFA test as early as 5 days after experimental infection. All animals seroconverted to herpesvirus sylvilagus by 10 days after infection and antibodies could be observed in serum dilutions of 1:2000 or higher.

Neutralizing antibodies were also detected in experimentally infected cottontail rabbits standard plaque reduction assays. Neutralizing antibodies developed as early as 7 days after infection. The peak of neutralizing antibodies was at 2–4 weeks after infection, decreasing to nonmeasurable levels at 8–10 weeks after infection.

Significant major histocompatibility complex (MHC)-restricted virus-specific cytotoxic T lymphocyte (CTL) responses have been described in spleen cells of infected animals. The peak of CTL activity was observed after 7 days of infection. The level of virus-specific CTLs was much lower in peripheral blood lymphocytes.

No extensive studies on antiviral drugs have been reported. Replication of herpesvirus sylvilagus is significantly inhibited by phosphonoacetate (PAA) and phosphonoformate (PFA). These drugs also inhibit most other herpesviruses.

Future Perspectives

The initial enthusiasm for the study of herpesvirus sylvilagus in the 1970s and 1980s was based on the apparent resemblance of the cottontail rabbit model to EBV pathology in humans. During subsequent decades progress has significantly slowed because of unusual difficulties in handling wild cottontail rabbits. Another factor contributing to loss of interest was that most investigators were unable to establish tumor cell lines from animals infected with herpesvirus sylvilagus or by *in vitro* immortalization; however, renewed interest is anticipated in this rabbit model because of the discovery of the human gamma-2 herpesvirus KSHV. It is possible that herpesvirus sylvilagus is closely related to KSHV but this hypothesis could be only tested by sequencing of the herpesvirus sylvilagus genome. In summary, establishment of latently infected cell cultures and sequencing of the genome appear to be essential for further exploration of this interesting herpesvirus of cottontail rabbits.

See also: **Epstein-Barr virus (*Herpesviridae*): General features, Molecular biology; Latency; Persistent viral infection.**

Further Reading

Kieff E (1996) Epstein–Barr virus and its replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology* 3rd edn, p. 2343. New York: Lippincott-Raven.

Rickinson AB and Kieff E (1996) Epstein–Barr virus. In: Fields BN, Knipe DM, Howley PM et al (eds) *Fields Virology* 3rd edn, p. 2397. New York: Lippincott-Raven.

HERPESVIRUSES – BABOON AND CHIMPANZEE (*HERPESVIRIDAE*)

SS Kalter and **RL Heberling**, Virus Reference Laboratories, San Antonio, Texas, USA

History

Herpesviruses are ubiquitous, infecting a wide variety of animals, including humans. Because herpesviruses share many biological features in common, availability of nonhuman strains permits experimental studies that would be restricted if only human strains were available. Herpesviruses are capable of manifesting many different clinical syndromes. In their natural host, herpesviruses cause a mild or inapparent form of disease, and rarely give rise to an excessive fatal illness. When crossing species barriers, however, herpesviruses are frequently responsible for a highly virulent form of disease. Following infection, a feature common to all herpesviruses is their ability to persist for the remainder of the host's life as a latent infection.

Despite their sharing of many biologic characteristics, herpesviruses are diverse in their clinical capabilities, widespread in their geographic distribution and generally have their own natural host range. A number of different herpesviruses may be present in nonhuman primates, including baboons and chimpanzees. Depending upon their biologic characteristics each of the herpesviruses have been included among three subfamilies of the family *Herpesviridae*:

- *Alphaherpesvirinae* Herpes simplex virus group
- *Betaherpesvirinae* Cytomegalovirus group
- *Gammaherpesvirinae* Lymphoproliferative virus group

In this entry, consideration will be principally given to two related, but distinct, lymphoproliferative (*Gammaherpesvirinae*) herpesviruses recovered from the baboon and chimpanzee, herpesvirus papio and herpesvirus pan respectively. Other herpesviruses also present in baboons and chimpanzees will be briefly discussed.

The relatedness of lymphoproliferative viruses to Epstein–Barr virus (EBV) is to be noted. Alpha and beta herpesviruses associated with baboons and chimpanzees include: herpesvirus cercopithecus 2 (SA8) and closely related herpesvirus papio 2 (HVP-2) in baboons; cytomegalovirus (CMV) strains, widespread among Old and New World primate species (with their antigenic relative SA6 restricted to African primates) and VZ or varicella-zoster (simian VZ?) found in various species of monkeys and apes. Herpes simplex virus infections may be seen in chimpanzees and other apes.

Both herpesvirus papio and herpesvirus pan, along with the human (gamma) herpesvirus 4 (EBV), have a biologic characteristic that separates them from other herpesviruses and are classified in the lymphoproliferative virus group, subfamily *Gammaherpesvirinae*, genus *Lymphocryptovirus*. This characteristic, consisting principally of invasion and persistence in lymphocytes, results in a proliferative form of disease associated with oncogenicity. EBV, originally isolated from humans, is the prototype species of the *Gammaherpesvirinae*. A major clinical manifestation of EBV is tumor production. EBV-like isolates have been recovered from suspensions of continuous cell lines from both Old and New World simian species. Among Old World simians, isotypes have been obtained from baboons, chimpanzees, orangutans, gorillas and several species of macaques. Similar, but antigenically unrelated, viruses have also been isolated from several New World monkey species. The Old World isolates are B-cell tropic, whereas the New World lymphoproliferative isolates are T-cell tropic. All these isolates are capable of producing lymphoproliferative disease in several animal species.

Other herpesviruses recognized as baboon and chimpanzee associated are not lymphoproliferative and are classified in either *Alphaherpesvirinae* or *Betaherpesvirinae* subfamilies. SA8/HVP-2 is found in African monkeys and has shown pathogenicity in baboons. CMVs, like EBV strains, are worldwide in distribution among animal species. VZ is considered to be a human virus; the origin of the simian strains is not clear. Herpes simplex virus is also regarded as a human virus. **Table 1** lists herpesvirus antibody present in baboons and chimpanzees.

Baboon herpesviruses

In 1975, Agrba *et al* and Lapin *et al* in the former USSR reported the establishment of two continuous lymphoblastoid cell cultures (SPG-1 and KMPG-1) containing herpes-like viral particles from bone marrow and spleen of hamadryas baboons with malignant lymphoma. These investigators had previously reported an outbreak of leukemia among the

Table 1 Herpesvirus antibody detected in baboons and chimpanzees

Antibody	*Baboon*	*Chimpanzee*
EBV	97/102 (95)	47/47 (100)
HSV-1	8/105 (8)	13/56 (23)
HSV-2	0/76	6/51 (12)
SA-8	179/311 (58)	2/56 (4)
B virus	2/225 (0.9)	0/10
CMV	222/276 (80)	54/70 (77)
VZ	19/68 (28)	21/50 (42)
H. papio	+ (?)	?
H. pan	?	+(?)

Values in parentheses are percentages. +, antibody present, but unknown percentage.

baboons of the Sukhumi colony in the USSR. The cell lines were shown to contain morphological evidence of herpesviruses and, upon identification, the isolates were found to be distinct from herpes simplex 1 and 2. Confirmation was rapid as a result of a collaborative research effort between Soviet and American investigators. Independently, in 1977 Gerber *et al* also isolated a herpes-like virus from baboon lymphoid cell lines grown in long-term culture. Several investigators had previously demonstrated the presence of antibody to EBV capsid antigen in all Old and several New World monkeys. Moreover, EBV-related viruses have been isolated from a number of primate species.

lpha herpesviruses recovered from baboons have resulted in some confusion in classification and terminology. SA8, closely related to herpes B, was originally isolated from an African green monkey. More recently, related isolates of SA8 have been obtained from baboons. Molecular analyses of these isolates indicate that they are distinct and a separate provisional designation: herpesvirus papio 2 (HVP-2) was suggested. These isolates, capable of causing disease and death among baboons, are not lymphoproliferative, and have the properties of alpha herpesviruses (*Alphaherpesvirinae*). Thus far, there has been no evidence of human involvement.

The presence of antibody to such herpesviruses as human CMV (*Betaherpesvirinae*) and its related monkey virus, SA6, indicates their infectious capability in baboons. Antibody to a simian VZ isolate also suggests baboon infection with this group of agents. Herpes simplex virus antibody is rarely seen in baboons.

Pongine herpesviruses

Herpes-like viruses (herpesvirus pan) were first recovered from leukocytes of chimpanzees previously inoculated with cells or virus derived from human cell lines containing herpesvirus-like particles. Herpesvirus particles were also recovered from leukocytes of normal uninoculated chimpanzees as well as from the oropharynx of immunosuppressed chimpanzees.

Other herpesviruses of chimpanzees, as determined by the presence of antibody, recovery of virus or clinical disease include: EBV, CMV, simian VZ virus and herpes simplex (**Table 1**). Various monkeys and apes (including the chimpanzee) have shown clinical evidence of infection with VZ virus (*Alphaherpesvirinae*) or closely related viruses. Initially considered to be solely a human agent, subsequent outbreaks in nonhuman primates indicate otherwise. The origin and disposition of this 'human' virus is not clear. Another member of the *Alphaherpesvirinae* generally not considered among nonhuman primates is herpes simplex virus, which is frequently transmitted among captive apes.

Taxonomy and Classification

The Herpesvirus Study Group of the International Committee on Taxonomy of Viruses suggested that the family *Herpesviridae*, subfamily *Gammaherpesvirinae* and genus *Lymphocryptovirus* include as species both herpesvirus papio (designated as cercopithecine herpesvirus 2) and herpesvirus pan (as pongine herpesvirus 1) along with EBV (human herpesvirus 4). As a group, these are double-stranded DNA viruses with molecular weights of approximately 110×10^6, virion diameters of 118–220 nm, and capsomeres arranged in an icosahedron.

Herpesvirus papio and herpesvirus pan show a specificity for B lymphocytes and their host range in nature is limited to the same order as the natural host. These viruses all share various degrees of DNA sequence homology (approximately 40%), produce crossreactive viral proteins and immortalize B lymphocytes. EBV, herpesvirus papio and herpesvirus pan DNA all show extensive collinear homology with each other.

Final classification of SA8/HVP-2 must await more definitive analyses. However, because of neurotropic tendencies and antigenic relatedness to herpes B virus (cercopithecid herpesvirus 1), inclusion among the *Alphaherpesvirinae* is suggested. Molecular analysis of SA8 and HVP-2 strains indicate that baboon isolates are similar to, but distinct from, SA8, and should be distinctly designated. Similarly, as the VZ virus is apparently of human origin, it and its closely related simian viruses should continue among the *Alphaherpesvirinae*.

Human CMV is included with the *Betaherpesvirinae*, as SA6, a related CMV, should be. These are all

DNA viruses varying from 120–200 kbp. DNA sequences are present internally in inverted form.

Geographic and Seasonal Distribution

Herpesvirus papio, herpesvirus pan, SA8 (and related strains) are all natural to African species and in nature are restricted to that continent. However, baboons and chimpanzees have been transported throughout the world and as herpesviruses have a propensity for latency, they may be found wherever baboons and chimpanzees are located and without any seasonal distribution. In the Sukhumi colony, however, peak infection with herpesvirus papio occurred in the autumn and spring.

EBV and CMV are worldwide in distribution among primates and not seasonal. Although both EBV and CMV are considered as common human viruses, antibody to these viruses or related viruses are found among Old and New World simians. Little is known about the epizootiology of VZ and related simian viruses. Nonhuman primate infection with VZ probably occurs following contact with an infected human or a simian carrier.

Host Range and Viral Propagation

Gammaherpesvirinae, in general, have a host range limited to the family or order of the natural host. Cultivation of both herpesvirus papio and herpesvirus pan is restricted to B cell lymphocytes. Originally the baboon isolates were obtained by cultivation of bone marrow and splenic cells from a lymphomatous hamadryas baboon. Cells were grown in RPMI 1640 supplemented with 20% heat-inactivated bovine embryo serum and maintained at 37°C in a 5% CO_2 atmosphere. The appearance of cell clumps along with enhanced metabolic activity and cell growth are the criteria of successful cell cultivation. Viral presence is determined by observing typical herpes-like viral particles within the cells and/or the transformation (immortalization) of infected lymphocytes. Serological tests for the presence of viral (V) and soluble (S) antigens, using known positive sera, confirm the presence of virus.

Host range of herpesvirus papio and herpesvirus pan may be determined by the presence of transformed B cells along with development of viral-associated antigens. Transformation by herpesvirus papio is observed in lymphocytes derived from the baboon, gibbon, cynomolgus monkey, rhesus monkey and stump-tailed macaque monkey. Human newborn lymphocytes are inconsistently transformed. Lymphocytes from New World monkeys, such as the cotton top (*Saguinus oedipus*) and other marmosets (*S. fuscicollis, S. nigricollis*), are also transformed by the baboon virus.

In general, the chimpanzee isolates parallel the baboon viruses in their host range and lymphocyte invasiveness. However, herpesvirus pan has not been as extensively studied as the baboon virus so there may be some variation in host cell susceptibility.

Evidently EBV and CMV (or closely related antigenic relatives) are not host restricted, as infection evidenced by antibody is universal. Details of their host range and propagation may be found elsewhere in this encyclopedia. SA8 was originally isolated from the spinal cord of an African green monkey developing paralysis following inoculation with poliovirus vaccine. HVP-2 was isolated from baboons. The full extent of the SA8/HVP-2 host range has not been determined, but SA8/HVP-2 antibodies have been found in various primate species. With the exception of baboon outbreaks, overt disease with SA8/HVP-2 has not been recognized. Apparently invasiveness of HVP-2 may differ from that of SA8. SA8 and related strains may be isolated and propagated in a variety of cells: rabbit kidney, Vero, HEp-2 and a number of other primate cell cultures. Herpes simplex is also readily isolated in a number of cell types.

It is generally considered that VZ infection is restricted to humans. Experimental subclinical infections were produced in a number of laboratory animals with little evidence of clinical disease. In 1966 an outbreak of a fatal exanthematous herpesvirus infection occurred among African green (*Cercopithecus aethiops*) monkeys. Subsequently several other similar episodes occurred in macaques, patas monkeys and apes. Antigenic analyses of these isolates demonstrated a close relationship to VZ virus.

Genetics

The close homology (40%) of herpesvirus papio and herpesvirus pan with EBV suggests that these viruses all evolved from a common ancestor. In addition, extensive crossreactions exist between the viral capsid (VCA) and early antigens (EA) of these three viruses. Lymphotropic herpesvirus-induced EBNA (Epstein–Barr nuclear antigen)-related proteins contain an epitope that has been conserved during the evolution of the EBV family of viruses. Further evidence for a genetic relationship was the identification of a pair of small RNA segments from herpesvirus papio which crosshybridize with two low molecular weight RNAs encoded by EBV. Herpesvirus papio is able to transform B lymphocytes in culture as well as to superinfect EBV-negative or nonproducer B cells inducing viral antigens. Herpesvirus papio also has a nuclear

antigen distinct from that found in EBV. These differences should be valuable for genetic studies on this group of lymphotropic viruses. Although there appear to be some genomic alterations resulting from infection of different simian hosts, the rapid evolutionary changes seen with viruses such as influenza apparently do not occur here.

Although a close antigenic relationship exists between SA8/HPV-2 and herpes B virus, SA8 does not appear to be as pathogenic in laboratory animals as herpes B virus. Clarification is needed on the relationship of SA8 to HVP-2 viruses and their pathogenicity. Eberle and coworkers demonstrated strain variation among the different HVP-2 isolates. As with other *Alphaherpesvirinae*, there is a close genetic and antigenic relationship among members of this virus group. HVP-2 is closely related to SA8, but closer to B virus than is SA8. Questions exist on the genetic relationship of the VZ group of viruses. Many similarities are recognized between the human strains of VZ and the simian isolates. However there are distinct differences not only among the simian and human isolates, but with the simian isolates as well.

Evolution

On the basis of limited information, apparently all Old World B cell tropic primate *Gammaherpesvirinae* evolved from a common ancestor. Some simian EBNA-related proteins contain an epitope that has been conserved during the evolution of the EBV family of viruses.

Common antigens and other biologic characteristics would suggest that diverse baboon and chimpanzee herpesviruses may have evolved from infections following contact with humans or other primates.

Serologic Relationships and Variability

Lymphotropic herpesviruses papio and pan are antigenically closely related to isolates obtained from the gorilla (herpesvirus gorilla) and orangutan (herpesvirus pongo), as well as other EBV-like isolates, although there are antigenic differences. As a group, they all share DNA homology and antigenicity as well as other biologic activities with EBV.

EBV and the EBV-like viruses all have associated antigens: VCA, EA, membrane antigen (MA) and EBNA. Herpesvirus papio and herpesvirus pan will cross react with EBV when sera are tested for anti-VCA, anti-EA and anti-MA. However, the baboon virus does not crossreact with EBV when tested for anti-EBNA. Herpesvirus papio does not induce nuclear antigens in infected cells, as does EBV, but does produce an analogous antigen. All three viruses crossreact when tested by serum neutralization. Depending upon the source of antibody, differences in titer differentiate these viruses. The EA is composed of both R (restricted) and D (diffuse) components recognized by immunofluorescence and, while related, are distinguishable.

It is apparent that the more recently described HVP-2 isolates, while closely related to SA8, are distinct entities. Eberle *et al* even suggest that the recognized baboon infections were due to HVP-2 rather than SA8. A subset of HVP-2 antigens that is consistently present in HVP-2 viruses and able to act as immunogens is: gB and gD glycoprotein homologues, p40 antigen homologues and an unidentified glycoprotein doublet of 80–100 kDa. The first three of these proteins are essential in other *Alphaherpesvirinae* for replication and production of infective viruses.

Analysis of the simian VZ-like herpesviruses indicates properties similar to those of VZ. It is highly probable that all these viruses evolved from the same source. Enveloped virion size, buoyant density, polypeptide composition and immunogenic glycoproteins are consistent with those obtained for VZ.

Epidemiology

Herpesvirus papio was first isolated from a group of hamadryas baboons, previously inoculated with human leukemic blood, in the colony of the Institute of Experimental Pathology and Therapy, Sukhumi. Since 1967, more than 300 cases of lymphoma have been recognized in the main baboon colony. A separate baboon colony in a forest reserve some distance from the main colony has not shown any evidence of lymphoid disease, but the animals do contain herpesvirus papio VCA antibody. A C-type virus (simian T-cell leukemia virus, STLV-1?) along with a high incidence of STLV-1 antibody has also been observed in the Sukhumi baboons with lymphomas. It is not clear what the relationship between the presence of herpesvirus papio and C-type retroviruses (STLV-1) and leukemia is in these animals.

Serological surveys of the main baboon colony have shown an age-related increase in the prevalence of antibodies to herpesvirus papio and STLV-1. Herpesvirus papio is released into the environment by way of nasopharyngeal mucosa and lacrimal gland secretions, which appears to be the main route of horizontal transmission. The exact mechanism of STLV-1 transmission in the baboon is not clear, but evidently parallels that of infection with other retroviruses, i.e. a result of grooming, fighting, biting, sexual activity. The leukemic blood used to inoculate the baboons may also be the source of this virus.

STLV-1, which is closely related antigenically to human HTLV-1, is not to be confused with the vertically transmitted endogenous type C retroviruses present in primates.

The first isolation of herpesvirus pan was a serendipitous finding. Herpesvirus pan was isolated by cultivating chimpanzee leukocytes obtained from uninoculated chimpanzees as well as from chimpanzees previously inoculated with human derived cell lines containing herpes-type particles. Other isolates were obtained from oral secretions of immunosuppressed chimpanzees. Although no definitive data exist relative to the mechanism of viral transfer, it is highly probable that herpesvirus pan is spread via oral secretions or sexual intercourse.

EBV antibody is frequently observed in nonhuman primate sera, reflecting primary infection or cross-reactions of the indigenous EBV-like viruses present in different seropositive species. CMV infection probably parallels that of EBV.

SA8/HVP-2 infection needs additional study, although recent data suggest that the baboon infections were due to HVP-2. Most HVP-2 lesions in the baboons were venereal, implying that the epidemiology is sexual. The few chimpanzee herpes simplex infections are apparently of human origin.

Transmission and Tissue Tropism

Transmitting vehicles of herpesviruses may include such materials as respiratory secretions, blood, saliva, contact with infected lesions and sexual intercourse. Both herpesvirus papio and herpesvirus pan are readily transmitted in nature to their respective natural host animals via respiratory secretions. Experimental production of tumors, however, has been limited to the inoculation of marmosets with large numbers of herpesvirus papio-producing baboon cells. Cotton top marmosets develop an acute, widely disseminated lymphoproliferative disease. In view of the possible need for another virus (STLV-1, other ?) to help in the production of baboon tumors, it would be of interest to determine whether herpesvirus papio alone is responsible for tumor production in marmosets.

Although herpesvirus pan is not known to cause disease, experimental infection occurs in various species of animals, as measured by antibody development.

Lymphocyte infection by herpesvirus papio or herpesvirus pan is prelytic or lytic; at the prelytic stage infection is arrested, with persistence and minimum expression of viral genome. At the lytic stage, cell death results without production of complete virions.

EBV also shows a predilection for B lymphocytes. Antibody surveys demonstrate that it or related viruses are widespread among human and nonhuman primates. EBV-like viruses from both the baboon and chimpanzee are capable of transforming cells from other primates, including humans. Transmission is horizontal by oral secretions with a high frequency of occurrence.

The other herpesviruses, in general, demonstrate a preference for specific cell types, but many exceptions exist. Tissue tropism varies extensively. The *Alphaherpesvirinae* usually relate to mucosal epithelial cells, invade sensory neurons with neurological symptoms in alien hosts and with latent infection centered in ganglia. The major mechanism of SA8/HVP-2 transmission is apparently venereal. *Betaherpesvirinae* favor epithelial and mononucleated blood cells; transmission is principally by direct and indirect contact.

Pathogenicity

Herpesviruses all have their own natural host range which in general is narrow and restrictive. Experimentally there is a greater latitude which may be wide, depending upon the strains of virus. Infection may range from subclinical to that of tumor development or highly fatal neurologic involvement.

Although some variation exists among the herpesvirus papio and herpesvirus pan strains, their infectivity patterns are similar. The latter is apparently nonpathogenic, and infection is limited to antibody production and virus shedding. Infection by either virus is followed by localization of the virus in lymphocytes where a persistent infection results.

The relationship of herpesvirus papio to lymphoproliferative disease in the baboon must await further study. The precise roles of herpesvirus papio and STLV-1 or another virus in the production of tumors remains to be elucidated. However, experimental inoculation of adult marmosets (*S. oedipus, S. nigrifrons*) with herpesvirus papio isolated from diseased baboons results in a mild to severe, and sometimes fatal, lymphoproliferative disease. Such findings do not rule out the presence of endogenous viruses. It does not produce disease when inoculated into the same species newborn marmoset.

In addition to the possible role of herpesvirus papio and STLV-1 in the production of lymphoproliferative disease, a similar role may exist with STLV-1 or other viruses in conjunction with EBV or EBV-like virus infections.

SA8/HPV-2 infection and fatalities in baboons have been alluded to above. VZ infection of apes has also been indicated.

Clinical Features of Infection

In the original studies by Lapin and his collaborators, the observed clinical disease associated with herpesvirus papio infection included a wide variety of lymphomas: non-Hodgkin's lymphoma of the lymphoid type (predominantly); lymphosarcoma; prolymphocytic lymphosarcoma; reticulosarcoma; lymphoplasmacytic, immunoblastic lymphoma; and lymphogranulomatosis.

Advanced disease is associated with immunosuppression. Disease occurs over a long period of time (3–5 years) with cycles of relapse and remission. B, T and null cell type lymphomas have been observed in the 300+ cases reported since 1966. Initial clinical features include conjunctivitis, stomatitis, gingivitis, skin pigmentation and enlarged lymph nodes.

Herpesvirus pan is endemic in its natural host, the chimpanzee, without any evidence of clinical disease. Limited experimental infection with this virus has also shown no capacity to produce disease.

EBV is considered to be widely distributed in humans, but sufficient evidence exists that it, or closely related strains, is present in a variety of primates and other animals. New World primates do not carry EBV homologues. EBV is associated with human diseases (infectious mononucleosis) and oncogenicity (Burkitt's lymphoma, nasopharyngeal carcinoma) and a similar potential is suspected for the simian EBV-like viruses in nonhuman primates. Transforming capabilities of human EBV and simian EBV strains needs clarification. Both transforming and nontransforming strains are recognized.

Similarly, the pathogenic potential of simian CMVs requires further study. Antibody is well distributed among all simian species. Clinical disease has not been reported in nonhuman primates, with the possible exception of a combined infection resulting from a retrovirus and CMV in macaques.

The SA8/HPV-2 epizootic in baboons was manifested by severe ulcerative and/or papillomatous lesions on the penis and scrotum as well as vulvar and perivulvar lesions in the females. Oral lesions were observed primarily in juveniles. Fatal neonatal infections do occur.

Pathology and Histopathology

The pathologic changes induced by herpesvirus papio are those associated with malignant lymphomas. Examination of lymphomatous tissues shows numerous chromosome breaks and structural rearrangements. In advanced disease, the lymphocytes are suppressed. Lymph nodes and spleen are most frequently affected; as the disease progresses, other tissues become involved – lungs, liver, kidneys, bone marrow. Both diffuse and nodular growth are seen.

Pathology due to herpesvirus pan is restricted to the B cell lymphocytes in which the virus induces growth transformation and expression of an intranuclear antigen. Long-term cultivation of lymphoid cell lines will show the morphologic presence of herpes-like virus.

EBV agents from both baboons and chimpanzees transform B lymphocytes of most Old World simians, in which they are modest virus producers. Virus particles are present in about 1% of the producer cells. Overt disease is not seen.

SA8/HVP-2 lesions in the male baboons were observed as erythematous patches which progress to papules or vesicles that coalesce, forming an ulcerative hemorrhagic lesion. Lesions in the females were exclusively ulcerative.

VZ infection of chimpanzees and other apes parallels that of human infection. The zoster-like syndrome has not been reported in any of the simian species. Lesions have not been observed in any of the dorsal root ganglia that have been examined. However, as zoster does not manifest itself for many years post primary infection, necropsies on appropriate animals have not been done. All simian varicella herpesvirus infections have produced cutaneous, oral and visceral lesions similar to human varicella.

Immune Response

Four major classes of antigens are associated with EBV and EBV-like primate viruses: VCA, EA, MA and EBNA (in EBV). Sera from a wide assortment of nonhuman primates show antibody to these viruses and extensive crossreactivities with all of these antigens, although differences have been observed. The serological response of baboons with lymphoid disease due to herpesvirus papio is similar to that of humans with Burkitt's lymphoma and EBV, particularly the response to the VCA. As a group, lymphomatous animals have an increased frequency of, and higher titers to, herpesvirus papio antigens than contact animals or noncontact animals. The elevated antibody responses are specific for the antigens, as shown by lower titers to both CMV and a baboon foamyvirus. These CMV and foamyvirus titers may reflect the immunosuppression occurring as a result of herpesvirus infection. A decrease in the relative percentage of T cells has been noted. Immunosuppression may also be an indication of the accompanying STLV-1 infection. The development of antibodies is not age or sex dependent. Although herpesvirus papio shows considerable relatedness to EBV, the two

viruses may be distinguished on the basis of their EBNA and early antigens.

The immune response of an individual to infection with herpesvirus pan resembles that of infection with EBV. A close antigenic relationship between EBV and herpesvirus papio exists, as demonstrated by the crossreactions seen when testing human or baboon serum for antibody with herpesvirus pan. The EBNA and EA of the chimpanzee virus are distinguishable from those of EBV, but strong crossreactions may be observed with the VCA.

Surveys of baboon and chimpanzee sera for antibodies to other herpesviruses generally indicate widespread infection (**Table 1**). Baboon sera indicate SA8/HVP-2 antibody present in more than 50% of the sera examined. The incidence of SA8/HVP-2 antibody in chimpanzees is very low, suggesting limited infection. Although there is strain variation among the SA8/HVP-2 isolates, this antigenic variation does not appear to induce substantial differences in antibody response. The subset of virus antigens that induce antibodies in all infected baboons was identified as the gB and gD glycoprotein homologues, an unidentified glycoprotein doublet of 80–100 kDa and the p40 antigen homologues. Insufficient chimpanzee sera have been examined for detailed antibody evaluation.

All simian varicella herpesviruses show antigenic relationships to VZ virus; however, the chimpanzee isolate shared an antigen with VZ virus lacking in the other simian isolates. Conversely, these other simian isolates had a common shared antigen with VZ virus that was lacking in the chimpanzee isolate. Antigenic analyses indicate that the chimpanzee virus, the delta herpesvirus and the remaining isolates are three distinct viruses.

Prevention and Control

As with diseases due to EBV, little is available in the way of prevention and control of herpesvirus papio or herpesvirus pan infections. Infection is best prevented by isolation of the animals. The elevated anti-EA titers, as seen in prelymphoma baboons that subsequently die of malignant lymphoma, may be used as a high-risk marker for determining development of malignant lymphoma.

As a generalization, prevention and control of herpesvirus infections are now based on chemotherapy or prevention. Therapy is dependent upon the use of the human antiviral compounds: acyclovir and related agents as well as several newer compounds. Toxicity, resistance and lack of detailed study in the use of these agents in veterinary medicine makes selection and usage questionable.

Future Perspectives

This revised second edition article includes previously omitted herpesviruses of the baboon and chimpanzee. Certain of these viruses are described in detail elsewhere in the encyclopedia, however such viral entities as the SA8/HVP-2 complex, simian EBV and CMV as well as simian VZ, that involve the baboon and chimpanzee should be noted. Herpes simplex infection is seen in chimpanzees, but is rare and probably results from human contact.

Herpesvirus papio and herpesvirus pan have little significance in human disease, other than they may serve as models for those investigators who may wish to research the relationship between host and lymphoproliferative viruses. Herpesvirus papio and herpesvirus pan infections in their natural hosts mimic that seen in the human infected with EBV, however, only restricted use may be made of the chimpanzee as a model. Chimpanzees are both expensive and in limited supply. The baboon, on the other hand, which shows evidence of lymphoproliferative disease, is still readily available, even being bred in captivity, and would be an appropriate model for future studies.

The possible interaction of EBV-like viruses with type C viruses (STLV-1, others ?) found associated with lymphoma in the Sukhumi baboons is a further avenue for investigation. A recent study has raised the question of the association of these two agents in causing lymphomatous disease. Endogenous and exogenous type C viruses are found in a wide variety of animals, particularly in nonhuman primates. It would be of interest to ascertain whether or not herpesvirus papio alone is responsible for tumor production in baboons, as observed in the Sukhumi primate center or experimentally in marmosets. Causation of disease in the baboon by herpesvirus papio may also be questioned in terms of infection of a natural host by herpesviruses. Infection of the natural host by these viruses is usually mild or inapparent, rather than a highly fatal disease. Although a number of studies with a baboon endogenous virus isolate have failed to demonstrate oncogenicity, a pseudotype containing a murine sarcoma virus gene did produce tumors in several species of primates, including chimpanzees, and in other animals. These original findings in the baboon suggest further studies on the association of herpesviruses and various retroviruses as a possible tumor producing mechanism. HTLV-1, a type C virus related to STLV-1, has been implicated in the etiology of a human leukemia.

Other investigative avenues should delineate the pathogenic relationship between SA8 and the HVP-2

isolates. Which of these viruses produces a highly fatal disease in baboons needs clarification.

Recent concerns with regard to xenotransplantation, particularly as it relates to the use of baboon organs, suggests continued investigation into the problem of biohazards and emerging diseases. Of great anxiety is the transfer and activation of a donor's latent infection in association with host immunosuppression. Although the number of human transplant needs far outweigh the baboon capacity (currently the leading contender for the provision of organs), the need for baboon organs will probably continue. Transgenic studies suggest an animal source other than the baboon will undoubtedly be more appropriate in xenotransplantation.

See also: **Cytomegaloviruses (*Herpesviridae*): Animal cytomegaloviruses, General features (human), Molecular biology (human), Murine cytomegaloviruses; Epstein-Barr virus (*Herpesviridae*): General features, Molecular biology; Herpes simplex viruses (*Herpesviridae*): General features, Molecular biology; Varicella-Zoster virus (*Herpesviridae*): General features, Molecular biology.**

Further Reading

Agrba VZ, Yakovleva LA, Lapin BA *et al* (1975) The establishment of continuous lymphoblastoid suspension cell cultures from hematopoietic organs of baboons (*Papio hamadryas*) with malignant lymphoma. *Exp. Pathol. Bd.* 10: 318.

Eberle R, Black DH, Blewett EL and White GL (1997) Prevalence of *Herpesvirus papio* 2 in baboons and identification of immunogenic polypeptides. *Lab. Anim. Sci.* 47: 256.

Gerber P, Kalter SS, Schidlovsky G, Peterson WD Jr and Daniel MD (1977) Biologic and antigenic characteristics of Epstein–Barr virus-related herpesvirus of chimpanzees and baboons. *Int. J. Cancer* 20: 448.

Kalter SS and Heberling RL (1977) Primate endogenous viruses: their role in oncogenesis and as biohazards. *Dev. Biol. Stand.* 37: 219.

Landon JC, Ellis LB, Zeve VH and Fabrizio DPA (1968) Herpes-type virus in cultured leukocytes from chimpanzees. *J. Natl Cancer Inst.* 40: 181.

Roizman B, Carmichael LE, Deinhardt F *et al* (1981) *Herpesviridae. Intervirology* 16: 201.

HERPESVIRUSES SAIMIRI AND ATELES (*HERPESVIRIDAE*)

Julia Hilliard, Viral Immunology Center, Department of Biology, Georgia State University, Atlanta, Georgia, USA

History

Herpesvirus saimiri and herpesvirus ateles include four different viruses within the family *Herpesviridae*. This entry focuses upon two of those four: herpesvirus saimiri 2 (HVS-2) and herpesvirus ateles 2 (HVA-2), each classified in the genus *Rhadinovirus* within the subfamily *Gammaherpesvirinae*. Each virus is easily confused with its counterpart herpesvirus saimiri 1 and herpesvirus ateles 1, which are distinct viruses of squirrel and spider monkeys, respectively, and both members in the *Simplexvirus* genus of the subfamily *Alphaherpesvirinae*. All four viruses that are called saimiri and ateles are herpesviruses associated with members of New World monkey species, those originating from Central and South America.

HVS-2 is endemic in the squirrel monkey (*Saimiri sciureus*), while HVA-2 is found most frequently in the spider monkey (*Ateles geoffroyii*), the monkey species from which it was initially isolated. HVS-2 was reported upon following isolation from a owl monkey kidney tumor, and was also found in association with a fatal disease in a marmoset, but additionally from primary kidney cell cultures derived from a squirrel monkey. In time, only the squirrel monkeys were identified to be the natural hosts for the virus. In early reports, it is often difficult to determine whether investigators are referring to HVS-1 versus HVS-2 when reporting or publishing results, or HVA-1 versus HVA-2. Nomenclature for each of these viruses has often been confusing because each virus has had a variety of different common names, some applying to the neurotropic counterparts HVS-1 and HVA-1, and others to HVS-2 and HVA-2, including marmoset herpesvirus (MHV), herpesvirus tamarinus (HVT), herpesvirus platyrrhinae (HVP)

Latent-phase transcripts

Latently infected neurons represent 1–10% of the total neurons in the ganglia, and infected cells contain as few as 5–10 copies of viral DNA. In some neurons, an 8.5 kb polyadenylated transcript whose promoter lies in the long repeat sequences about 1800 bases 3′ of the 3′ end of the $\alpha 0$ mRNA is expressed as the LAT (**Fig. 1**). This primary transcript extends through the joint region to a polyadenylation signal next to that of the $\alpha 4$ transcript in the short repeat.

The primary LAT transcript is extensively processed, forming an unknown number of unstable, spliced, polyadenylated mRNAs, and a stable 2 kb poly(A-)intron in lariat form which accumulates in the latently infected neuronal nucleus. A second, spliced variant of this LAT-intron can also be observed. The function of this stable intron is unclear; it is not involved in the facilitation of reactivation mediated by LAT expression.

Future Trends in Research on HSV

HSV possesses a number of features that will make it a continuing research model in the future. Among the most obvious are the following. (1) The neurotropism of the virus, along with the relative convenience of generating recombinants containing foreign genes expressed either under homologous or heterologous promoters, recommends HSV as a vector for introducing specific genes into neural tissue. This facility is increased by the fact that the viral genome can enter and be maintained in neural tissue in the complete absence of lytic phase viral gene expression. (2) The fact that HSV's replication is restricted in terminally differentiated neurons suggests its potential as a therapeutic agent against neural tumors, provided appropriate mutants can be constructed. (3) The benign nature of normal infection with HSV also suggests its potential use as a vaccine vector by incorporation of specific antigens into the viral genome, although the promise of this application may be clouded somewhat by the ability of replication-defective viruses to persist in the host. (4) The abundance of viral genes dedicated to the efficient replication of viral DNA in the productive cycle provides a number of potential targets for antiviral chemotherapy. (5) The interactive cascade of viral proteins involved in regulation at a number of levels in the infected cell makes HSV a useful model for the detailed analysis of specific aspects of control of eukaryotic gene expression.

See also: **Genetics of animal viruses; Herpes simplex viruses (*Herpesviridae*): General features; Latency; Pathogenesis: Animal viruses; Epstein-Barr virus (*Herpesviridae*): General features; Persistent viral infection; T4-like phages (*Myoviridae*); Varicella-Zoster virus (*Herpesviridae*): General features; Vectors: Animal viruses, Plant viruses; Viral membranes; Virus–host cell interactions; Virus structure: Atomic structure, Principles of virus structure.**

Further Reading

Roizman B and Sears AE (1996) Herpes simplex viruses and their replication. In: Fields BN, Kripe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd Edn, p. 2231. New York: Lippincott–Raven.

Steven AC and Spear PG (1997) Herpesvirus capsid assembly and envelopment. In: Chiu W, Burnett RM and Garcea RL (eds) *Structural Biology of Viruses*, p. 352. New York: Oxford University Press.

Wagner EK and Bloom DC (1997). The experimental investigation of herpes simplex virus latency. *Clin. Microbiol. Rev.* 10: 419.

Wagner EK, Guzowski JF and Singh J (1995) Transcription of the herpes simplex virus genome during productive and latent infection. In: Cohn WH and Moldave K (eds) *Progress in Nucleic Acid Research and Molecular Biology*, p. 123. San Diego: Academic Press.

HERPESVIRUSES 6 AND 7 – HUMAN (*HERPESVIRIDAE*)

Takuya Shimamoto and **Koichi Yamanishi**, Department of Microbiology, Osaka University Medical School, Osaka, Japan

History

Human herpesviruses 6 and 7 (HHV-6 and HHV-7) are ubiquitous agents of humans. HHV-6 was first isolated by Salahuddin *et al* in 1986 as a novel herpesvirus from patients with lymphoproliferative disorders, and initially named human B lymphotropic virus (HBLV). Subsequently, several laboratories

isolated similar viruses from patients with acquired immune deficiency syndrome (AIDS) and children with exanthem subitum, and revealed that the virus is predominantly T lymphotropic. The name of the virus was changed to human herpesvirus 6 because it was a newly discovered one distinguished from other five known human herpesviruses. HHV-6 isolates are classified into two groups as variants A (HHV-6A) and B (HHV-6B). The two variants are closely related but show consistent differences in biological, immunological, epidemiological and molecular properties. HHV-6B is the major causative agent of exanthem subitum, but no disease has yet been associated with HHV-6A.

HHV-7 was isolated by Frenkel *et al* in 1990, as the seventh human herpesvirus, from CD4+ lymphocytes of a healthy adult. The virus also causes exanthem subitum.

Taxonomy and Classification

HHV-6 and HHV-7 are members of the genus *Roseolovirus*, which belongs to the *Betaherpesvirinae* subfamily, along with human cytomegalovirus (HCMV), of the family *Herpesviridae*. HHV-6 and HHV-7 share many characteristics with other herpesviruses, including typical herpesvirus morphology and large DNA genome, and the ability to establish persistent and latent infections.

Host Range and Viral Propagation

Humans are the natural host for HHV-6 and HHV-7. It was reported that some species of monkeys have antibody to HHV-6. HHV-6 and HHV-7 replicate most efficiently *in vitro* in activated primary T cells and T cell lines. HHV-6 and HHV-7 can be isolated by cocultivation of phytohemaglutinin-stimulated umbilical cord blood lymphocytes with peripheral blood mononuclear cells (PBMCs) from patients and saliva from individuals, respectively. The rate of HHV-6B isolation from PBMCs is nearly 100% on days 0–2 after the onset of exanthem subitum, and gradually decreases to zero during the convalescent phase.

Properties of the Virion

Virions of HHV-6 and HHV-7, like all other herpesviruses, have four main structural elements. The electron-dense core encompasses viral DNA genome and associated proteins. The icosahedral nucleocapsid contains 162 capsomeres and surrounds the core. The tegument occupies the space between the nucleocapsid and the envelope. The envelope is derived from cellular membrane and contains virally encoded glycoproteins and integral membrane proteins. The most characteristic feature of the virions of HHV-6 and HHV-7 is the very distinct tegument layer.

Properties of the Genome

The genomes of HHV-6 and HHV-7 are linear double-stranded DNA molecules of approximately 159 kb and 145 kb, respectively. The gene organization of each is illustrated in **Fig. 1**. The complete genomic sequences were determined for HHV-6A strain U1102 and HHV-7 strain JI, and partial sequences were reported for other strains, including HHV-6B strain HST.HHV-6A and HHV-6B are very similar genetically because their nucleotide sequences differ by 4–10% between the variants, depending on the gene being compared. This similarity, which is higher than that for any other independently recognized herpesvirus species, led to the decision to adopt the variants A and B nomenclature, rather than name the variants as distinct species. Sequence comparison among herpesvirus species revealed the highest conservation of genetic content and encoded protein products between HHV-6 and HHV-7, ranging from 41 to 75% amino acid sequence identity for the core herpesvirus gene products. HHV-6A strain U1102 DNA consists of a single unique component (U) of 143 kb, flanked by two direct terminal repeats DRL and DRR, each of which contains about 8 kb. The overall GC content is 43% but is lower in U (41%) and higher in DR (58%). The genome is considered to contain more than 100 separate genes likely to encode protein. HHV-7 genome has almost the same configuration with that of HHV-6. The relative arrangements of the HHV-6 and HHV-7 herpesvirus-conserved genes are identical to those of homologous genes in HCMV, as expected for betaherpesvirus genomes. At the left and right termini of the DRs are *cis*-acting sequences involved in DNA packaging, pac-1 and pac-2, respectively. The DRs encompass repetitive (TTAGGG)n motifs, identical to human telomeric repeat sequences. The motifs have also been found in Marek's disease virus, which is a lymphotropic alphaherpesvirus of chicken, but not in other human herpesviruses. The function of the telomeric sequences is not known.

HHV-6 encodes adeno-associated virus type 2 (AAV-2) *rep* gene homologue. The rep protein is essential for AAV-2 DNA replication and possesses site-specific ATP-dependent endonuclease and helicase activities. The functions of the *rep* gene and its product in the life cycle of HHV-6 are unknown, although rep from HHV-6 can provide helper function for AAV-2 DNA replication *in vitro*. Additionally, rep from HHV-6A can complement the replica-

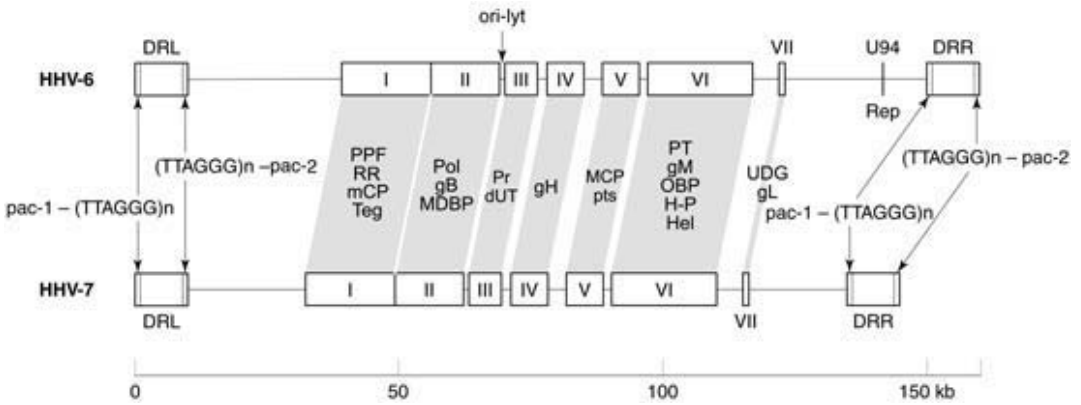


Figure 1 Gene organization of HHV-6 and HHV-7. Gene blocks I–VII containing genes conserved in all herpesviruses are marked in relative positions in HHV-6 and HHV-7. The ORFs encompassed in each block are as follows: I, U27–U37; II, U38–U41; III, U42–U45; IV, U48–U53; V, U56–U58; VI, U60–U77; VII, U81–U82. HHV-6 ori-lyt is indicated by an arrowhead. Representative genes encoded in the conserved and unique regions are listed. Abbreviations are: PPF, DNA polymerase processivity factor; RR, ribonucleotide reductase; mCP, minor capsid protein; Teg, large tegument protein; Pol, DNA polymerase; gB, glycoprotein B; MDBP, major single-stranded DNA-binding protein; Pr, primase; dUT, dUTPase; gH, glycoprotein H; pts, protease/assembly protein; MCP, major capsid protein; PT, phosphotransferase; gM, glycoprotein M; OBP, origin-binding protein; H-P, helicase–primase associated factor; Hel, helicase; UDG, uracil-DNA glycosylase; gL, glycoprotein L; Rep, AAV-2 *rep* gene homologue.

tion of a rep-deficient AAV-2 genome. There is no *rep* homologue in HHV-7, suggesting the acquisition of the gene by HHV-6 after the evolutionary divergence between HHV-6 and HHV-7.

Proteins of HHV-6 and HHV-7

In HHV-6, potentially protein-coding open reading frames (ORFs) are numbered by the order occurring in the U and DR regions of the genome from left to right. Since almost all ORFs of HHV-7 encode homologues of HHV-6 and are present in the corresponding location in the genome, common numbers have been adopted for naming the homologous HHV-7 ORFs. For example, an ORF encoding the DNA polymerase of HHV-6 is the 38th, numbering from the left of the U, and therefore designated ORF U38, which is also used for indicating that of the DNA polymerase of HHV-7.

Proteins involved in DNA replication

The elements essential and sufficient for lytic HSV-1 DNA replication are an origin for lytic phase replication (ori-lyt) and seven viral gene products: the major single-stranded DNA-binding protein (MDBP), the DNA polymerase, a DNA polymerase processivity factor and an origin binding protein (OBP), as well as a helicase–primase complex composed of three proteins (helicase, primase, helicase–primase associated factor). HHV-6 and HHV-7 encode homologues to all of the seven gene products. In HHV-6 and HHV-7, these genes correspond to ORFs U41, U38, U27, U73, U77, U43 and U74, respectively. An interesting feature is that HHV-6 and HHV-7 encode a homologue of herpes simplex virus type 1 (HSV-1) OBP, which has been found only in alphaherpesviruses. HHV-6 has an origin for lytic-phase DNA replication that, as in HSV-1, varicella-zoster virus (VSV) and HCMV, is located upstream of the gene encoding the MDBP homologue (U41). The minimal active region of HHV-6B ori-Lyt is about 400 bp. The HHV-6B ori-lyt includes two binding sites, OBP-1 and OBP-2, for the HHV-6B OBP. The OBP sites are arranged with dyad symmetry, flank an AT-rich spacer, and are within the minimum essential origin region. This region is adjacent to a larger AT-rich imperfect direct repeat of about 195 bp that has been proposed to act as a DNA-unwinding element. Both OBP sites are required for DNA replication. Thus, the HHV-6 ori-lyt has features of both beta- and alphaherpesvirus origins, in having a larger set of repeat motifs (beta, 'complex' ori) and sequences which bind the OBP (alpha ori).

HHV-6 and HHV-7 homologues of herpesvirus gene products that are involved indirectly in DNA replication, by providing nucleotide substrates and DNA repair functions, include ribonucleotide reductase (U28), dUTPase (U45), phosphotransferase (U69), alkaline exonuclease (U70) and uracil-DNA glycosylase (U81). Homologues of the thymidine kinase genes found in the alpha- and gammaherpesviruses are absent in HHV-6 and HHV-7, as well as HCMV. The phosphotransferase encoded by UL97 in HCMV is capable of phosphorylating nucleosides, an

activity that accounts for the efficacy of ganciclovir in suppressing HCMV infections. The *in vitro* susceptibility of HHV-6 to ganciclovir may also be attributed to the function of the phosphotransferase (U69). The dUTPase and uracil-DNA glycosylase homologues of HHV-6 and HHV-7 presumably specify enzymatic activities involved in excision of uridine residues from DNA, by analogy to bacterial and eukaryotic counterparts, although there is notable sequence divergence between the HHV-6 or HHV-7, cellular and other herpesvirus dUTPase homologues.

Glycoproteins

There are several glycoprotein genes conserved in all sequenced herpesviruses. These genes are glycoprotein B (gB), glycoprotein H (gH), glycoprotein M (gM) and glycoprotein L (gL), and also are conserved in HHV-6 and HHV-7. These genes correspond to U39, U48, U72 and U82, respectively. gB and gH are structurally highly conserved between herpesviruses, are membrane-bound glycoproteins, and appear to play roles in virus cell fusion and cellular spread of virus infection. gL forms a physical association with gH precursor protein and may be required for gH transport and/or processing. The gM is an integral membrane protein containing multiple hydrophobic, putative membrane-spanning domains and fairly well conserved between herpesviruses. It has been shown to be a component of the virus particle in HSV-1.

In HHV-6, a highly spliced gene at the right end of the U component of the genome has recently been characterized and shown to encode a glycoprotein species, gp82/105. In strain GS, this gene contains 13 exons. The role of gp82/105 is not known but is likely to be important in the biology of HHV-6, as neutralizing epitopes are present. Three HHV-7 ORFs (U98, U99 and U100) show homology to regions of the gp82/105 amino acid sequence.

Glycoprotein U18 appears to be betaherpesvirus-specific. In HCMV this gene is spliced and encodes an immediate early glycoprotein.

HHV-6 U20 exhibits significant sequence similarity to the immunoglobulin E (IgE) C chain and therefore is a member of the Ig superfamily. The U85 genes of HHV-6 and HHV-7 encode homologues of OX-2 membrane antigen, which is also a member of the Ig family. Members of the Ig family have roles in cell-to-cell contact via protein–protein interactions and it is postulated that expression of these glycoproteins will function in infected cell adhesion to uninfected lymphocytes or monocytes.

Glycoproteins U21, U23, U24 appear to be unique to HHV-6 and HHV-7. There are glycoproteins encoded in a similar location in HCMV, but they share no detectable similarity.

Capsid, tegument and virus assembly proteins

Several of the ORFs identified in HHV-6 and HHV-7 are homologous to herpesvirus genes encoding characterized and candidate structural proteins. These include homologues of the major capsid protein (MCP; U57), minor capsid protein (mCP; U29), large tegument protein (U31) and virion proteins specified by U33, U34, U36, U50, U56 and U76. Betaherpesvirus-specific and conserved structural proteins are homologues of HCMV UL32 (antigenic phosphoprotein, pp150) and UL82/83 (tegument transactivator, pp65/72K), which correspond to U11 and U54, respectively. The HHV-6 U11 gene product is called pp100 and has been characterized as a major antigenic phosphoprotein and a component of the virion. The homologues of herpesvirus gene products involved in DNA packaging and capsid assembly have also been identified in HHV-6 and HHV-7. These correspond to U29 (mCP), U30, U53 and U60/U66. These genes appear to be conserved (at least positionally) in all of the sequenced herpesviruses. The U60/U66 ORFs correspond to the two exons of the late spliced gene characterized initially in HSV-1, which is likely to play a role in DNA packaging and capsid assembly. U53 sequences code for the protease/assembly protein (assemblin) and the scaffolding protein. These proteins are derived through proteolytic cleavage (assemblin) and internal initiation (scaffolding protein). The sequences encoding the scaffolding protein are referred to as U53a and correspond to HSV-1 UL26.5. From studies on HSV-1 UL26/26.5 viral mutants, it appears that proteolytic activity is essential for DNA packaging and virus assembly.

Chemokine receptors

HHV-6 and HHV-7 contains two genes (U12 and U51) that encode putative homologues of cellular G-protein-coupled receptors (GCR). GCR homologues have also been identified in HCMV, as well as in the gammaherpesviruses herpesvirus saimiri (HVS) and Kaposi's sarcoma-associated herpesvirus (KHSV or HHV-8). The U12 gene of HHV-6 is expressed late in infection from a spliced mRNA. U12 functionally encodes a calcium-mobilizing receptor for β-chemokines, such as regulated upon activation, normal T expressed and secreted (RANTES), macrophage inflammatory proteins 1α and 1β (MIP-1α and MIP-1β) and monocyte chemoattractant protein 1, but not for the α-chemokine interleukin 8, suggesting that the chemokine selectivity of the U12 product is distinct from that of the known mammalian chemokine

receptors. These findings suggest that the product of U12 may play an important role in the pathogenesis of HHV-6 through transmembrane signaling by binding with β-chemokines.

Transmission and Tissue Tropism

The *in vivo* host tissue range of HHV-6 includes lymph nodes, lymphocytes, macrophages and monocytes, kidney tubule endothelial cells, salivary glands and central nervous system (CNS) tissues, where viral gene products have been localized to neurons and oligodendrocytes. The human salivary system is the only known source for persistent production of infectious HHV-7. HHV-6 and HHV-7 show predominant CD4 T cell tropism. CD4 is a critical component of the cellular receptor for HHV-7, but not for HHV-6. The membrane receptor for HHV-6 has not yet been elucidated. HHV-6B and HHV-7 are present in saliva, which is considered to be a most common source of transmission from mothers to infants. No HHV-6A isolates from saliva have been described.

Several studies suggest that intrauterine or perinatal transmission of HHV-6B can occur, but the possibility is very low. The detection of HHV-6B DNA by polymerase chain reaction (PCR) in the cervices of approximately 20% of women in late pregnancy, compared with 6% of nonpregnant controls, suggests reactivation of HHV-6 infection during pregnancy, with the possibility of perinatal transmission of the virus. A report of two cases of fulminant hepatitis-associated HHV-6B infection in neonates 3 and 5 days old is also suggestive of pre- or perinatal transmission.

Breast-feeding does not appear to be the route of transmission of HHV-6. HHV-6 DNA has not been detected in breast milk, and no difference was found in the prevalence between breast- and bottle-fed infants.

Epidemiology

HHV-6 and HHV-7 infections are widespread throughout the world.

About 90% of people worldwide over the age of 2 years are HHV-6 seropositive. Antibody against HHV-6 can be detected in more than 90% of infants' serum at birth, most likely due to maternally derived transplacental antibody. The positivity rate decreases during the first 5 months of life to as low as 5% and then rapidly increases between 6 months and 5 years, with 80% of children developing an antibody response by 13 months of age. A number of investigators have reported a decline in seropositivity with age among adults, but others have found no significant differences or even an increase after age 62. Although 95% of children seroconvert to HHV-6 before age 3, approximately 30% in the USA and 60% in Japan experience clinical exanthem subitum.

Like HHV-6, HHV-7 is a prevalent virus to which the great majority of the population become seropositive by adulthood. HHV-7 infection occurs somewhat later than HHV-6B.

Clinical Features of Infection

The primary infection of HHV-6B typically occurs in early infancy and causes exanthem subitum, also known as roseola infantum or sixth disease. The high frequency of the virus isolation from patients with the disease, along with seroconversion during convalescence, were reported by Yamanishi *et al* and established HHV-6B as the etiologic agent responsible for most cases of exanthem subitum. It is a generally benign illness of infants and young children. The incubation period is estimated to be about 10 days. The illness is characterized by 3–5 days of fever, sometimes accompanied by mild upper respiratory infection symptoms and cervical lymphadenopathy. Characteristically, a maculopapular rash on the trunk and neck appears within 48 h following decline of fever. Although roseola is the major manifestation of primary infection in Japan, in the large prospective studies of American children the classical manifestations of roseola are present in about 15–20% of primary cases evaluated in clinics and the emergency room. In most cases, children can recover from this disease after a few days without any complications. As rare events, HHV-6B has also been identified as the causative agent of the other related primary illnesses in young children. These include cases of liver dysfunction, hepatosplenomegaly, fatal and nonfatal fulminant hepatitis, intussusception, thrombocytopenia, thrombocytopenic purpura, fatal and nonfatal hemophagocytic syndrome, and disseminated disease resulting in death.

Neurologic complications are also observed during primary infection. The most frequent complication of acute infection is febrile seizure. Often the seizures appear late and are prolonged or recurrent. HHV-6 infections account for one-third of all febrile seizures in children up to the age of 2 years. In follow-up studies over a period of 1–2 years the HHV-6 genome persisted in blood mononuclear cells after primary infection. Reactivation, sometimes with febrile illnesses, was suggested by subsequent increases in antibody titers and by detection of viral DNA. Other rare complications include encephalitis, meningoencephalitis and aseptic meningitis. Although the

neurologic involvement is usually self-limited, in some cases HHV-6-associated encephalitis resulted in death or was followed by the development of epilepsy.

HHV-6 may be one of the agents of sinus histiocytosis with massive lymphadenopathy (SHML). SHML, also known as Rosai–Dorfman disease, is a rare, usually benign disease of children and young adults, primarily manifested by painless cervical lymphadenopathy.

As described above, most persons are infected with HHV-6B at an early age. Consequently, primary infection of adults is encountered infrequently. Such infections appear to have greater severity than do most primary infections in children. Mononucleosis-like syndrome, prolonged lymphadenopathy and fulminant hepatitis have been described.

There is a possibility that HHV-6 is an important pathogen in organ transplant recipients. Serologic reactivation was reported in recipients of bone marrow, liver and kidney transplants. Specific illnesses associated with HHV-6 seroconversion or reactivation in these patients include fever, rash, hepatitis, pneumonitis, neurologic dysfunction and bone marrow suppression.

HHV-6 has been implicated as the etiologic agent or as a component of pathogenesis for a number of clinical disorders, including chronic fatigue syndrome and multiple sclerosis. It is essential that further studies be performed to determine the association between HHV-6 and these diseases.

HHV-7 primary infection was reported to cause a typical exanthem subitum, confirmed by the isolation of HHV-7 from typical patients with exanthem subitum who had no antibody to HHV-6 and seroconversion to HHV-7 during convalescent phase. Incomplete clinical features of the disease, such as fever without rash and the common cold, were observed in the infection with HHV-7 as in that of HHV-6. Most cases of HHV-7 primary infection seem to show atypical clinical manifestations of exanthem subitum, making it difficult to relate the concerned illnesses to HHV-7.

Latent and/or Persistent Infection

The primary infections of HHV-6 and HHV-7 are followed by latent infection throughout life. HHV-6 can often be reactivated under conditions of immunosuppression, such as AIDS and organ transplantation, at times leading to severe to fatal diseases. Several lines of evidence indicate that PBMCs are one of the sites of latent infection with these viruses. When PBMCs from patients with exanthem subitum during acute and convalescent phases and from healthy adults were separated into two fractions of lymphocytes and monocytes, HHV-6B DNA was detected by PCR in both fractions during the acute phase of the disease, whereas mainly in the monocyte fraction in patients in the convalescent phase and of healthy adults. The virus could not be isolated from cells of convalescent patients or healthy adults, suggesting the latent infection of HHV-6B in these cells. HHV-6B may persist for a long time in monocytes/macrophages in a latent state, although it is not clear whether these cells are the main site of latent infection *in vivo*. HHV-7 can be reactivated from PBMCs of healthy individuals by T cell activation.

HHV-7 has been isolated from saliva and HHV-7 DNA detected in salivary glands by PCR, indicating the possibility that salivary glands are reservoirs of persistently infected HHV-7.

Host Immune Response

Humoral immunity to HHV-6B in exanthem subitum patients is as follows. Virus-neutralizing IgM is not detected in the sera until day 4 after the onset of disease, but is detectable on day 7 and persists for 3 weeks. On the other hand, IgG is first detected on day 7, increasing and persisting for a long time.

Very little is known about immunological defense by cellular immunity against HHV-6. The presence of HHV-6-specific T cell clones in seropositive individuals has been reported. Natural killer (NK) cells, a key component of the innate immune system, are known to play an important role against viral infections. Infections of PBMCs with HHV-6 and HHV-7 lead to upregulation of their NK cell cytotoxicity by inducing IL-15, which is one of the NK activity-enhancing cytokines. The cell type in PBMCs that has been identified as the source of IL-15 is the monocyte/macrophage. In fact, it has been reported that HHV-6 interacts directly with monocytes and induces IL-15 in them. This enhancement of NK cell cytotoxicity following infection is consistent with the previous observation that NK activity increased during the acute phase of exanthem subitum. IL-15 production following viral infections may therefore represent a powerful host defense mechanism involved in restricting viral growth and in limiting the spread of the infectious agents.

HHV-6 is a potent inducer of interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α) in cultures of PBMCs. In contradistinction, HHV-6 has no effect on IL-6 synthesis. HHV-6 infection also induces the production of interferon α, IL-10 and IL-12 in monocytes. The effects of these cytokines may contribute to our understanding of the pathogenesis of HHV-6 during primary infection and reactivation.

Prevention and Control

There is no prevention or therapy for the diseases caused by HHV-6 and HHV-7; however, ganciclovir and foscarnet are effective in suppressing replication of HHV-6 *in vitro*. Antiviral therapy might be appropriate in individual cases of severe HHV-6 disease.

Future Perspectives

The functions of most viral gene products of HHV-6 and HHV-7 in the lytic and latent infections have not yet been elucidated. Recently, the entire sequences of both viruses have been determined, accelerating the research of their molecular biology. The development of a recombinant virus system is awaited for the detailed examination of the function(s) of the viral genes in the context of the viral genomes. Since the only known host for HHV-6 and HHV-7 is humans, animal models are also a prerequisite for understanding the functions of the viral genes *in vivo*.

See also: **Epstein-Barr virus (*Herpesviridae*): General features, Molecular biology; Cytomegaloviruses (*Herpesviridae*): Animal cytomegaloviruses, General features (human), Molecular biology (human); Herpes simplex viruses (*Herpesviridae*): General features, Molecular biology; Varicella-Zoster virus (*Herpesviridae*): General features, Molecular biology; Herpesvirus 8 – Human (*Herpesviridae*).**

Further Reading

Frenkel N, Schirmer EC, Wyatt LS *et al* (1990) Isolation of a new herpesvirus from human CD4+ T cells. *Proc. Natl Acad. Sci. USA* 87: 748.

Frenkel N and Roffman E (1996) Human herpesvirus 7. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 2609. New York: Lippincott-Raven

Pellet PE and Black JB (1996) Human herpesvirus 6. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 2587. New York: Lippincott-Raven Press.

Salahuddin SZ, Ablashi DV, Markham PD *et al* (1986) Isolation of a new virus, HBLV, in patients with lymphoproliferative disorders. *Science* 234: 596.

Yamanishi K *et al* (1988) Identification of human herpesvirus 6 as a causal agent for exanthem subitum. *Lancet* i: 1065.

HERPESVIRUS SYLVILAGUS (*HERPESVIRIDAE*)

Peter G Medveczky, University of South Florida College of Medicine, Tampa, Florida, USA

History

Herpesvirus sylvilagus is a model for lymphoproliferative disorders and viral oncogenesis because it induces a lymphoma-like illness in rabbits. It was first isolated by Hinze in 1968 from kidney cell cultures of apparently healthy cottontail rabbits (*Sylvilagus floridanus*) in southern Wisconsin, USA. Morphological and molecular biological analysis proved that herpesvirus sylvilagus is a typical herpesvirus. The viral particle is enveloped and its capsid contains 162 capsomers as described for other herpesviruses. Neutralization assays and partial sequencing of the viral genome also demonstrated that herpesvirus sylvilagus is a unique herpesvirus. A virus referred to as cottontail rabbit herpesvirus (CTHV) has been isolated by Cebrian. This cottontail rabbit virus is unrelated to herpesvirus sylvilagus, as determined by Southern blot hybridization.

Classification

Herpesviruses have been classified into three subfamilies of the *Herpesviridae: Alpha-, Beta-* and *Gammaherpesvirinae*. The gamma or lymphotropic viruses infect lymphocytes and are further divided into two genera, *Lymphocryptovirus* and *Rhadinovirus*, on the basis of biological data, pathogenesis, sequence homology and latency genes (**Table 1**). There is little doubt that herpesvirus sylvilagus is a member of the gammaherpesvirus group but it is not clear whether it belongs to either of the two subgroups. While several investigators concluded that herpesvirus sylvilagus-induced pathology resembles

isolates. Which of these viruses produces a highly fatal disease in baboons needs clarification.

Recent concerns with regard to xenotransplantation, particularly as it relates to the use of baboon organs, suggests continued investigation into the problem of biohazards and emerging diseases. Of great anxiety is the transfer and activation of a donor's latent infection in association with host immunosuppression. Although the number of human transplant needs far outweigh the baboon capacity (currently the leading contender for the provision of organs), the need for baboon organs will probably continue. Transgenic studies suggest an animal source other than the baboon will undoubtedly be more appropriate in xenotransplantation.

See also: **Cytomegaloviruses (*Herpesviridae*): Animal cytomegaloviruses, General features (human), Molecular biology (human), Murine cytomegaloviruses; Epstein-Barr virus (*Herpesviridae*): General features, Molecular biology; Herpes simplex viruses (*Herpesviridae*): General features, Molecular biology; Varicella-Zoster virus (*Herpesviridae*): General features, Molecular biology.**

Further Reading

Agrba VZ, Yakovleva LA, Lapin BA *et al* (1975) The establishment of continuous lymphoblastoid suspension cell cultures from hematopoietic organs of baboons (*Papio hamadryas*) with malignant lymphoma. *Exp. Pathol. Bd.* 10: 318.

Eberle R, Black DH, Blewett EL and White GL (1997) Prevalence of *Herpesvirus papio* 2 in baboons and identification of immunogenic polypeptides. *Lab. Anim. Sci.* 47: 256.

Gerber P, Kalter SS, Schidlovsky G, Peterson WD Jr and Daniel MD (1977) Biologic and antigenic characteristics of Epstein–Barr virus-related herpesvirus of chimpanzees and baboons. *Int. J. Cancer* 20: 448.

Kalter SS and Heberling RL (1977) Primate endogenous viruses: their role in oncogenesis and as biohazards. *Dev. Biol. Stand.* 37: 219.

Landon JC, Ellis LB, Zeve VH and Fabrizio DPA (1968) Herpes-type virus in cultured leukocytes from chimpanzees. *J. Natl Cancer Inst.* 40: 181.

Roizman B, Carmichael LE, Deinhardt F *et al* (1981) *Herpesviridae. Intervirology* 16: 201.

HERPESVIRUSES SAIMIRI AND ATELES (*HERPESVIRIDAE*)

Julia Hilliard, Viral Immunology Center, Department of Biology, Georgia State University, Atlanta, Georgia, USA

History

Herpesvirus saimiri and herpesvirus ateles include four different viruses within the family *Herpesviridae*. This entry focuses upon two of those four: herpesvirus saimiri 2 (HVS-2) and herpesvirus ateles 2 (HVA-2), each classified in the genus *Rhadinovirus* within the subfamily *Gammaherpesvirinae*. Each virus is easily confused with its counterpart herpesvirus saimiri 1 and herpesvirus ateles 1, which are distinct viruses of squirrel and spider monkeys, respectively, and both members in the *Simplexvirus* genus of the subfamily *Alphaherpesvirinae*. All four viruses that are called saimiri and ateles are herpesviruses associated with members of New World monkey species, those originating from Central and South America.

HVS-2 is endemic in the squirrel monkey (*Saimiri sciureus*), while HVA-2 is found most frequently in the spider monkey (*Ateles geoffroyii*), the monkey species from which it was initially isolated. HVS-2 was reported upon following isolation from a owl monkey kidney tumor, and was also found in association with a fatal disease in a marmoset, but additionally from primary kidney cell cultures derived from a squirrel monkey. In time, only the squirrel monkeys were identified to be the natural hosts for the virus. In early reports, it is often difficult to determine whether investigators are referring to HVS-1 versus HVS-2 when reporting or publishing results, or HVA-1 versus HVA-2. Nomenclature for each of these viruses has often been confusing because each virus has had a variety of different common names, some applying to the neurotropic counterparts HVS-1 and HVA-1, and others to HVS-2 and HVA-2, including marmoset herpesvirus (MHV), herpesvirus tamarinus (HVT), herpesvirus platyrrhinae (HVP)

and others. Additionally confounding the nomenclature is the identification of the natural host of each virus. Crossreactive antibodies found outside the natural hosts in which the viruses are thought to be endemic provide a challenge to investigators identifying the natural versus the foreign host repertoires of each agent. Squirrel monkeys live in the South American rain forests and are the endemic host to HVS-2. The spider monkey is also a South American resident and host to HVA-2. HVA-1 was reported in 1972 in the spider monkey, while HVA-2 was reported also in 1972 and the pathologic features of this lymphoma-causing virus in cotton-top marmosets (*Callithrixjacchus*) were described, but subsequently the spider monkey was identified as the natural host of both HVA-1 and HVA-2.

HVS-2 and HVA-2 are closely related but antigenically differentiable viruses in the laboratory. These agents have been studied primarily with respect to oncogenic potential in hosts, particularly in marmosets. HVS-1 and HVA-1 share little to no crossreactivity with HVS-2 and HVA-2, as is common when comparing alphaherpesviruses with gammaherpesviruses of humans. Recent data have extended knowledge with regard to these agents' (HVS-1 and HVA-1) relatedness to Old World nonhuman primate herpesviruses, particularly with regard to the counterparts, HSV-1 and HSV-2, as each of these four viruses are alphaherpesviruses. To clarify the evolutionary relationship of all of these viruses with each other, both alpha- and gammaherpesviruses, and to specific nonhuman primate hosts, **Fig. 1** illustrates the phylogenetic tree of the New World monkeys and indicates the viruses that have been found in each.

Taxonomy and Classification/Host Range

HVS-2, saimiriine herpesvirus 2, is classified as a gammaherpesvirus, based upon virus molecular organization and growth properties, including cell tropism. This agent is not to be confused with saimiriine herpesvirus 1 (HVS-1) which is an alphaherpesvirus in marmosets. Similar to other herpesviruses, HVS-2 can establish latency within the natural hosts' target cells. HVS-2 DNA sequence analysis has demonstrated that, genetically, this virus is organized most like Epstein–Barr virus (EBV), when compared with viruses within the broad subfamilies of alpha-, beta-, and gammaherpesviruses. However, distinctive features are apparent, including overall arrangement of the genome of the virus. Because of these differences, along with the relatively distant relationships of each of these New World monkey viruses to EBV, HVS-2 is considered a gamma-2 herpesvirus within the subfamily *Gammaherpesvirinae*, genus *Rhadinovirus*, rather than gamma-1 into which EBV (lymphocrytptovirus) is classified.

Interestingly, gamma-2 herpesviruses are T lymphocyte tropic, as opposed to gamma-1 herpesviruses which are B lymphocyte tropic. At least one variant strain of HVS-2 has been found to be especially effective in nonhuman primate T cell transformation, a property that has made this agent an invaluable tool in acquired immune deficiency syndrome (AIDS)-related studies. Differences identified in the genome structure of this variant compared to prototype HVS-2 have provided investigators with information regarding transformation-inducing genes.

HVA-2 is classified in the subfamily *Gammaherpesvirinae*, similar to HVS-2, and also has strong oncogenic capabilities. HVA-1 remains unclassified within the subfamily of *Alphaherpesvirinae*, genus *Simplexvirus*, along with SA8 virus and HVS-1 or MHV.

Properties of the Virion

HVS-2 contains double-stranded DNA that is 67% guanosine plus cytosine (GC), with high intramolecular heterogeneity in G:C content, which results in a unique genomic organization. On analysis by isopyknic density gradient centrifugation, two bands of DNA are found when manipulations of the DNA are performed carefully to avoid shear. These genomes are classified as M and H genomes, the former which are linear double-stranded DNA ranging in size from 145 to 165 kb with a buoyant density of 1.705 g ml^{-1}, or approximately 36% G:C content, characterized by a long unique segment (L-DNA) which is 112 kb with direct repeats of 1.4 kb of H-DNA on either side (DNA with 72% GC content). There is a variable number of these repeats of H-DNA, from 1 to 40 times. The H-DNA, which has a buoyant density of 1.729 g ml^{-1}, can comprise up to 30% of the total M-genome size. Some virions have only tandem repeats of H-DNA linked together to approximate the size of the intact genome, i.e., 145–165 kb; these virions are characterized as particles containing only H genomes. H-DNA, whether in the H genome or M genome, appears to be identical; however, it is H-DNA where evolutionary change occurs more often, as demonstrated by the fact that this DNA shows sequence variation divergence when analysis of DNA by restriction endonucleases, as well as measurement of DNA thermal melting temperature is performed. The M genomes are infectious, whereas the H genomes are highly defective, lacking L-DNA. H genomes are comprised of variable numbers of tandem repeats of H-DNA. The overall genomic organization of HVS-2 resembles that found in EBV,

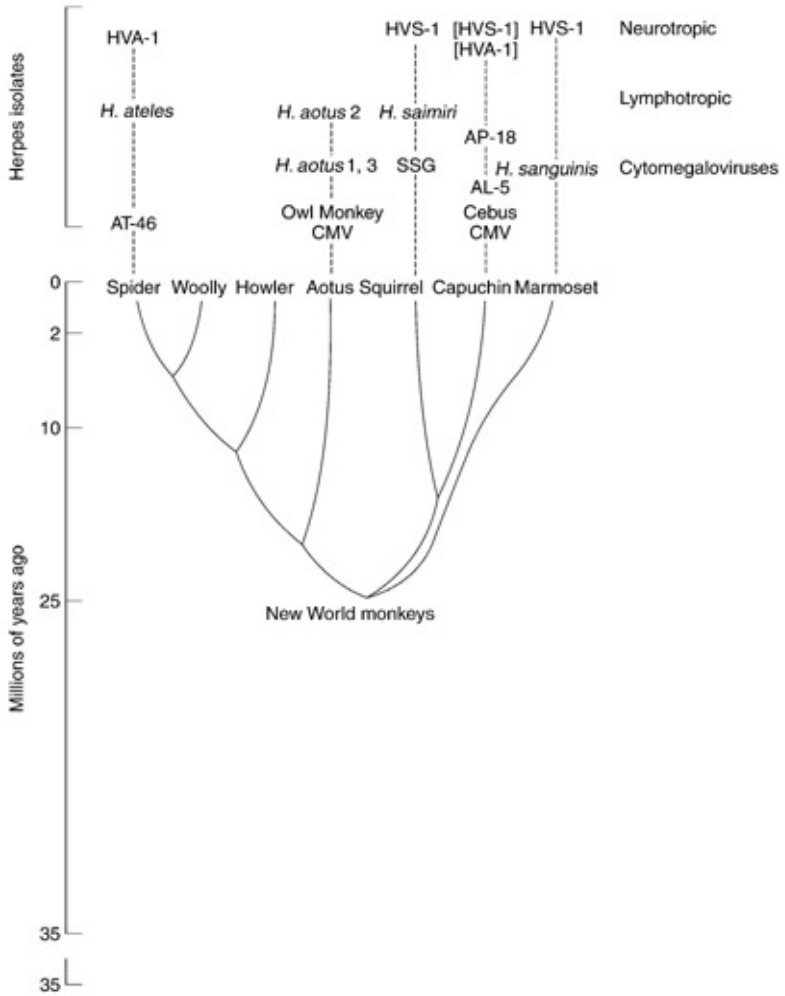


Figure 1 Phylogenetic summary of New World monkey hosts of herpesvirus agents, including the viruses found in each species (shown above each monkey). Those viruses in square brackets indicate that the species of monkey indicated with the virus is not the 'natural' host or endemic carrier of the agent. Viruses that have been identified are categorized into alpha-, beta- and gammaherpesviruses.

although the two are clearly not identical. Variability among HVS-2 isolates often shows heterogeneity in the left region of the unique L-DNA of the M genome, and, as such, isolates are further categorized into A, B and C groups.

HVS-2 DNA is capable of causing transformation of cells in marmosets. The DNA can subsequently be retrieved from the transformed cells, indicating its persistence within the transformed target.

The herpesvirus ateles genome is organized similarly to herpesvirus saimiri; however, the GC content is greater, with a lower number of H-DNA repeats.

Transmission

HVS-2 and HVA-2 can be transmitted horizontally, most often from adult animals to juveniles within the New World monkey populations. Subsequently, juvenile animals can transmit the virus to each other. Transmission of squirrel monkey herpesvirus, HVS-2, is reportedly facilitated by oral contact with virus-contaminated saliva or exposure of respiratory or conjunctival mucous membranes to virus-containing fluids. Virus can be demonstrated in saliva for prolonged periods of time after primary infection. Infection transmission can apparently be facilitated with either cell-associated or cell-free virus. In this regard, HVS-2 is similar to EBV, with the notable exception that the former seems to persist in T lymphocytes, while EBV is primarily B cell tropic. Virus can persist for long periods of time in the host lymphocytes, even for the life of the monitored animal, with as little as 1 copy per 1×10^6 T lymphocytes from normal squirrel monkeys.

Interestingly, longitudinal monitoring of infected animals has shown that different strains of HVS-2 and HVA-2 can be recovered at different time periods from individual monkeys. Intrastrain variation in HVS-2 has been documented by restriction endonuclease cleavage of viral DNA. Whether all strains, or only one, are equally capable of establishing persistence and/or latency is unknown at present. Although vertical transmission of either HVS-1 or HVA-1 is possible based upon knowledge of this family of viruses, it has rarely if ever been observed in hosts for either virus.

Virus cannot be recovered from homogenized lymphocyte populations collected from infected animals, indicating that the virus probably persists in cells as a nonencapsidated form, or even as DNA either in plasmid form or inserted into host DNA. Is virus present in kidney tissue because of contaminating lymphocytes or because the kidney tissue provides a site for virus replication or persistence? The answers to these, as well as many questions regarding these interesting agents, remain unknown at present and await further study.

It is noteworthy that HVS-2 was found initially in marmosets and owl monkeys rather than in squirrel monkeys. It was some time before the natural hosts' identity was recognized as the squirrel monkey. This has resulted in the assignment of a variety of names for HVS-2, names denoting other species of hosts which have now been identified as foreign hosts. Similarly, the existence of HVA-1 has complicated the nomenclature for the ateline herpesviruses, as there are both neurotropic and lymphotropic isolates, which are classified under separate genus names.

Immunological Response in Infected Nonhuman Primates

Comparative analysis of serum antibodies from different nonhuman primate hosts eventually led to the identification of the natural versus foreign hosts for each of these virus members of the genus *Rhadinovirus*. Initially, maternal antibodies induced by HVS-2 can be seen in infants, declining within the first 3–6 months of age. After primary infection, monkeys demonstrate antibody levels to late antigens which rise during the first several months of infection and then subsequently decline, suggesting the persistent state that this virus assumes within the infected host. Early and immediate early polypeptides translated during infection induce antibody which is transient and undetectable within a few months postinfection. Early antibody studies were mostly performed using immunofluorescence assays.

Antibody levels to each virus within their respective hosts were found to be related to age, with older animals showing higher levels of antibodies. Primary infection of squirrel monkeys with HVS-2 follows an age distribution, with onset of infection often seen by the time the monkey is 2 years of age. Antibodies induced by HVS-2 are differentiable from those produced by HVA-2 by application of indirect immunofluorescence as well as by western blot and radioimmunoprecipitation methodologies. The pathogenesis of HSV-2 and HVA-2 are remarkably similar with respect to the specificities and levels of antibodies induced in each host with increasing time postinfection.

Growth and Propagation of Virus in the Laboratory

Each of these New World monkey herpesviruses has a morphology consistent among the agents classified as herpesviruses. Nucleocapsids are formed in the nuclei of infected cells and are approximately 100 nm in diameter, with dense centers. The mature, enveloped particles are slightly larger and located in cytoplasmic vacuoles of infected cells. Glycosylation of envelope proteins occurs within the endoplasmic reticulum and Golgi apparatus to form mature virions. HVS-2 grows well in primary cells derived from New World monkeys but less efficiently in those derived from Old World monkeys, while HVA-2 grows well in a variety of epithelial and fibroblast cells from New World monkeys as well as African green monkeys (Old World monkeys). The spider monkey virus generally replicates to a lower titer in cell culture than the squirrel monkey herpesvirus. *In vitro*, HVA-2 is capable of immortalizing a variety of lymphoid cells from New World primates, whereas there has been more difficulty using prototype HVS-2, which imbues the cells it transforms with natural killer (NK) cell function and phenotypic markers of CD12+ and CD8+. HVA-2 immortalizes cells resulting in CD4+ and CD8+ phenotypes.

The first isolate of HVS-2 is designated SMKI-83, prime strain, or prototype strain, identified as S295C. This virus was isolated from a primary kidney cell culture established from a healthy squirrel monkey. Initial cell culture changes observed were characterized by small clumps of swollen, refractile cells. The monolayer of cells was completely destroyed within 1 month postculture. Intranuclear, acidophilic inclusions of different sizes were seen. Both primary epithelial cells as well as fibroblastic cells from New World monkeys were found to be permissive for virus replication. Owl monkey kidney cells were reported

to be the best choice for a productive system of virus propagation.

HVS-2 transformed can be subpassaged easily and a number of permanent cell lines have been established. The virus causes a lytic infection of the susceptible cells, with complete destruction of the monolayers spanning 2–20 days postinfection and a high titer output of virus. Old World monkey cells in culture are notably less susceptible to HVS-2 infection, although virus will replicate in Vero and CV-1 cell lines. In these cases, yield is significantly reduced compared with that measured from cell cultures derived from New World monkeys. Human cells that are susceptible are fibroblastic in origin, virus yield is low, and persistently infected cells can be maintained.

Summary and Future Perspectives

Each of these *Rhadinovirus* members of the family *Herpesviridae* has immense potential for use as a tool to transform/immortalize target cells of interest, particularly with respect to studies of human immunodeficiency virus (HIV) and AIDS. Gene-specific transformation or immortalization of cells is also possible, reducing the input of the nonessential viral genes against the background of the transfected cells. Transformation-associated genes represent a novel class of viral oncogenes which warrant further investigation. Furthermore, each of these virus–host systems continues to offer investigators a unique and invaluable avenue for epidemiological studies of virus transmission in nature, both within natural and foreign hosts. Last, but not least, due to the capacity of these viruses for carrying large amounts of DNA inserts, the use of HVS-2 and HVA-2 as novel gene vectors will most surely be continuously explored by investigators until ideal vectors are identified.

See also: **Herpesviruses – baboon and chimpanzee (*Herpesviridae*); Herpes simplex viruses (*Herpesviridae*): General features, Molecular biology; Herpesvirus sylvilagus (Herpesviridae); Epstein-Barr virus (*Herpesviridae*): Molecular biology; Immune response: Cell mediated immune response, General features; Transformation: Animal viruses.**

Further Reading

Biesinger B, Trimble JJ, Desrosiers RC and Fleckenstein B (1990) The divergence between two oncogenic herpesvirus saimiri strains in a genomic region related to the transforming phenotype. *Virology* 176: 505.

Daniels MD, Karpas A, Melendez LV, King NW and Hunt RD (1969) Isolation of herpes-T virus from a spontaneous disease in squirrel monkeys (*Saimiri sciureus*). *Arch. Gesamte Virus-Forsch.* 22: 324.

Eberle R and Hilliard JK (1995) The simian herpesviruses. *Infect. Agents Dis.* 4: 55.

Fleckenstein B and Desrosiers RC (1982) Herpesvirus saimiri and Herpesvirus ateles. In: Roizman B (ed.) *The Herpesviruses,* vol. 1, pp. 253–332. New York: Plenum Press.

Jung JU *et al* (1991) Identification of transforming genes of subgroup A and C strains of herpesvirus saimiri. *Proc. Natl Acad. Sci.* USA 88: 7051.

Murphy F (1996) Virus taxonomy. In: Fields BN, Knipe, DM, Howley PM *et al* (eds) *Fields Virology*, pp. 45–51. Philadelphia: Lippincott-Raven.

Trimble JJ and Desrosiers RC (1991) Transformation by herpesvirus saimiri. *Adv. Cancer Res.* 56: 335.

HISTORY OF VIROLOGY

Contents

General

Frank Fenner, John Curtin School of Medical Research, Australian National University, Canberra, Australia

Introduction

As in other branches of experimental science, the development of our knowledge of viruses has depended on the techniques available. Initially viruses, like other microbes, were studied by pathologists interested in the causes of the infectious

applied to problems of the pathogenesis and epidemiology of animal and plant viral diseases, and the expansion in knowledge of these subjects in the next decade can be expected to exceed that of the previous century.

See also: **Tobamoviruses.**

Further Reading

Bos L (1995) The embryonic beginning of virology: unbiased thinking and dogmatic stagnation. *Arch. Virol.* 140: 613.

Fenner F and Gibbs AJ (eds) (1988) *Portraits of Viruses. A History of Virology*. Basel: Karger.

Hughes SS (1977) *The Virus. A History of the Concept*. London: Heinemann.

Joklik WK (1996) Famous institutions in virology. The Department of Microbiology, Australian National University and the Laboratory of Cell Biology, National Institute of Allergy and Infectious Diseases. *Arch. Virol.* 141: 969.

Matthews REF (ed.) (1983) *A Critical Appraisal of Viral Taxonomy*. Boca Raton: CRC Press.

van Helvort T (1994) History of virus research in the twentieth century: the problem of conceptual continuity. *Hist. Sci.* 32: 185.

Bacteriophages

Donna H Duckworth, Department of Molecular Genetics and Microbiology, College of Medicine, University of Florida, Gainesville, Florida, USA

With the recent establishment in the United States of a commercial enterprise whose sole purpose is to isolate bacteriophages for therapeutic purposes, the history of phage research has come full circle. Bacteriophages were first discovered at a time when bacterial disease was rampant and antibiotics were no more than a gleam in the eyes of several visionaries. And although it has never been definitively determined who first discovered phage, at least one of the individuals involved was determined to make that gleam a reality by utilizing the killing power of these viruses in the treatment and prevention of disease. That individual was the French-Canadian, Dr Felix d'Herelle.

In August 1916, Dr d'Herelle was asked to investigate an epidemic of dysentery in a Paris hospital. He had come to Paris after an illustrious career as a bacteriologist which had taken him literally around the world. At various times, he had observed a mysterious killing activity in some of his bacterial cultures, something that made clear spots on his agar plates, and decided to look for this activity in a patient with dysentery to see if it correlated with an amelioration of the symptoms of the disease. So he filtered the feces of a patient with dysentery daily for several days and incubated the filtrate with a culture of the dysentery bacillus. In one of those rare 'Eureka!' moments in the history of science, he found that on the fourth day of the man's disease, a filterable substance from his feces completely killed the dysentery pathogen. He says,

> The next morning, on opening the incubator, I experienced one of those rare moments of intense emotion which reward the research worker for all his pains: at the first glance I saw that the broth culture, which the night before had been very turbid, was perfectly clear: all the bacteria had vanished, they had dissolved away like sugar in water. As for the agar spread, it was devoid of all growth and what caused my emotion was that in a flash I had understood: what causes my clear spots was in fact an invisible microbe, a filterable virus, but a virus parasitic on bacteria. Another thought came to me also: 'If this is true, the same thing has probably occurred during the night in the sick man, who yesterday was in serious condition. In his intestine, as in my test-tube, the dysentery bacilli will have dissolved away under the action of their parasite. He should now be cured.' I dashed to the hospital. In fact, during the night, his general condition had greatly improved and convalescence was beginning.

D'Herelle showed that the agent that appeared in the intestines of individuals recovering from dysentery was not found in normal individuals or in individuals suffering from the acute stage of the disease, that it could be filtered and still retain its ability to kill the dysentery bacillus and that it would not grow on any artificial medium. And, because the killing or lytic activity could be serially transmitted, he concluded that it must be a 'living germ'. These observations were published in 1917 in the *Compte Rendus Academie Science* (Paris).

The paper excited considerable attention. Imagine if, at the height of the acquired immune deficiency syndrome (AIDS) epidemic, someone had announced that they had isolated a natural substance that would destroy the AIDS virus. The situation in 1917 was similar. Infected war wounds and dysenteric diseases were taking an enormous toll, and people everywhere cried out for a cure for these ills – for a magic bullet, as it were. After d'Herelle's report, it seemed to many that this had finally materialized, and people all over the world tried to isolate agents of transmissible lysis

in order to use them in the treatment of infections. Years later d'Herelle described the observation that led to his discovery of phage, the clear spots on a lawn of bacteria on an agar plate, as 'ordinary enough, so banal indeed that many bacteriologists had certainly made it before on a variety of cultures'. Why was it, then, that d'Herelle pursued the observation, whereas others hadn't?

D'Herelle had been working in the Yucatán in 1910 when there was an invasion of locusts. The native Indians reported to d'Herelle that in a particular place the locusts were sick and dying from a severe diarrheal disease. D'Herelle investigated and determined that the locust diarrhea was caused by a bacterium which he described as a coccobacillus, 'le coccobacille de sauterelles'. And in what surely must be one of the earliest attempts at biological control of insects, he found that he could kill the locusts by spreading cultures of the bacteria on plants in front of advancing locust columns, starting epidemics of the diarrheal disease among the insects. He later took this 'technology' to Africa, and when the bacteria were used in Tunisia, a large invasion of locusts that threatened to destroy the harvest was apparently stopped. Even more interesting, Tunisia remained locust-free the following year. So d'Herelle had been primed for the idea of biological control (science *does* favor the prepared mind), and when he found a natural agent that could kill bacteria, as he did in Paris in the dysentery patient, he went after it with a vengeance. His book, *The Bacteriophage and its Behavior*, published in 1925, contains almost two hundred pages detailing the use of bacteriophage in both the prevention and treatment of disease. He describes the behavior of bacteriophage in dysentery, streptococcal and staphylococcal infections, typhoid fever, and other 'colon bacillus infections'. He talks about how he stopped epidemics by using phage – epidemics of avian typhosis, hemorrhagic septicemia of the buffalo, bubonic plague, dysentery, and flacherie of the silk worm. He describes how 'immunization' against a number of diseases was accomplished with phage, and how a formidable list of bacterial diseases – dysentery, typhoid, plague, wound infections and streptococcal and staphylococcal infections – could be cured with phage. Typical of his observations is the following dysentery case:

> Robert D. ... (twelve years). This patient had a very severe dysentery, with vomiting, cold sweats, chilling of the extremities, and involuntary and uncountable stools. September 8. The stools could not be counted. They were fetid, purulent, and streaked with blood. Examination showed: *B. dysenteriae Shiga* present; about 1 out of every 10 colonies on the plates was the dysentery bacillus. The intestinal bacteriophage showed no virulence for *B. coli*, or for the Shiga, Flexner, or Hiss organisms. September 9. Two cubic centimers of a suspension of Shiga-bacteriophage were ingested at 11 o'clock. This suspension was three and one-half months old. During the afternoon and the night, the stools became less numerous but continued bloody. September 10. There were 6 fluid stools, without blood. Examination showed: *B. dysenteriae Shiga*, not present. Intestinal bacteriophage active as follows: *B. coli* ++++, Shiga ++++, Flexner ++++, Hiss +++. September 11. There were 2 normal, formed stools. September 20. The patient was discharged from the hospital.

No controls in which patients were given inactive phage preparations were done, although it could be argued that this would not be ethically appropriate in people. But he also discussed a number of experiments with chickens, and again control experiments were not performed. The following experiments are typical:

> *Experiment III*. This experiment was conducted at Provins, with the aid of M. Sorriau, D.V.M., in an important poultry-yard where typhosis was present in endemic form. For several months the daily mortality had been 2 or 3 fowls. On January 25, the 225 survivors were immunized [author's note: this refers to the use of phage to prevent disease]. The epizootic immediately and permanently disappeared from the date of the immunization.
>
> *Experiment IV*. Performed at Rouillac, Charente, with the assistance of M. Chollet, D.V.M. On December 15, 100 fowls were immunized [author's note: this again refers to use of bacteriophage] in a poultry-yard where typhosis had appeared about ten days previously. The daily mortality had been from 4 to 6 animals. With the immunization there was an immediate and permanent cessation of the epizootic. Typhosis continued to prevail on all the neighboring farms. Among the 100 chickens inoculated, about 12 were already affected. Of these, only 2 died, 2 and 3 hours after the injection.

He does mention that the disease continued on neighboring farms. Presumably, this is evidence that his 'immunization' using phage was working. But injecting only half the chickens on a single farm would have provided more convincing evidence.

But controls or not, d'Herelle's work resulted in trials of phage therapy at a number of places throughout the world. The Department of Bacteriology and Experimental Pathology at Stanford University set up a laboratory to determine whether or

not phage might be used therapeutically. They advertised to physicians that the laboratory would try to adapt a bacteriophage – of which there was a large collection – to any bacterium isolated from a patient. This was done at a cost to the laboratory, then about $2. Eli Lilly, Squibb & Sons, and Abbott Laboratories, all sold bacteriophage to physicians, and spectacular results – especially with staphylococcal infection – were reported up until the mid-1930s. A patient with a ruptured appendix made an uneventful recovery after being instilled with 15 ml of *E.-coli*-specific phage from Eli Lilly. In Brazil, antidysentery phage was widely distributed with apparent success. One clinic reported their results as follows:

> The therapeutic effect – the sudden change in the condition of the patient – consequent to the administration of the bacteriophage has astonished all of the physicians who have worked with it. A few hours after the administration of the first dose improvement has been noted, and the rapidity of the recovery has been a cause of amazement. In examining the collected data pertaining to the patients treated, it appears that the majority of them had first received all known treatments, and had been given the bacteriophage as a last resort. We are, therefore, absolutely convinced that it has saved the lives of a great many patients.

One would think that d'Herelle might have won a Nobel Prize for his seminal discovery and that he would have been a revered scientist. But in fact, he died with questions about his integrity – and about the usefulness of phage therapy – never having been resolved.

In 1921, Jules Bordet and one of his collaborators published a paper entitled, 'Remarks on the History of Research Concerning Transmissible Bacterial Lysis.' They pointed out that someone prior to d'Herelle may have reported on the existence of phage. Said they, 'The burden of an exact history makes it necessary for us to cite a previous work which d'Herelle has not known and that we ourselves have been ignorant of until now that contains the observations that d'Herelle has made. This remarkable work ... appeared in *The Lancet* in 1915, that is to say, two years before the research of d'Herelle'.

The 1915 communication referred to was authored by F. W. Twort, a bacteriologist and the last superintendent of the Brown Institute, a primarily veterinary institution in a rundown section of London, founded by an eccentric Irishman for 'investigations, studying and without charge beyond immediate expenses, endeavoring to cure maladies, distempers and injuries to Quadrupeds or Birds useful to Man'. In his article, Twort describes what he called a 'glassy transformation' of bacteria, discovered while he was performing experiments designed to grow viruses in culture. Twort had performed many such experiments. He would prepare a variety of media: 'generally agar, egg, or serum was used as a basis, and to these varying quantities of certain chemicals or extracts of fungi, seeds, and etc., were added.' He would then inoculate these media with extracts of various soils, dung, or sera, which had been filtered to remove all bacteria. In one of these experiments he inoculated his various media with vaccinia calf lymph – the material used at the time for vaccinating humans against smallpox. Apparently the vaccinia had been contaminated by some harmless bacteria Twort called micrococci, and although the vaccinia virus did not grow in the inoculated agar tubes, the micrococci did. Twort observed what he considered 'interesting results' concerning the growth of these micrococci. After 24 h in the incubator, instead of colonies, 'watery-looking areas' appeared, and some of the micrococci would not grow again if transferred to fresh media. If the cultures were kept on the shelf, some parts of the growth also became 'glassy and transparent'.

> ... This action, unlike an ordinary degenerative process, started from the edge of the colonies, and further experiments showed that when a pure culture of the white or yellow micrococcus isolated from vaccinia is touched with a small portion of one of the glassy colonies, the growth at the point touched soon started to become transparent or glassy, and this gradually spread over the whole growth, sometimes killing all the micrococci and replacing these by fine granules ... This condition or disease of the micrococcus can be conveyed to fresh cultures for an indefinite number of generations; but the transparent material will not grow by itself on any medium. Although the transparent material shows no evidence of growth when placed on a fresh agar tube without micrococci it will retain its powers of activity for over six months.

Twort found it difficult to interpret what he had seen, and thought it might be 'an ultramicroscopic virus, a tiny amoeba which thrives on living microorganisms, living protoplasm that forms no definite individuals, or an enzyme with power of growth'.

In the 1920s, Twort's 'enzyme with the power of growth' idea gained a number of very devoted followers, and it is noteworthy that their description of the way this would work is very similar to the proposed mechanism of prion action. But the idea was not given much credence or even attention in 1915. In fact, no one apparently even noticed Twort's paper

until 1921, when Bordet pointed out that it was Twort who should be called the discoverer of bacteriophage, not d'Herelle, by virtue of his earlier publication, which Bordet thought described bacteriophage. Never mind that Twort's description of the glassy transformation was so unusual that no one even recognized it as the same phenomenon as the bacterial viruses that could cause clear spots on a lawn of bacteria. D'Herelle certainly didn't think it was the same thing and, at a meeting of the Society of Biology in Paris in May 1921, indicated that what Twort had discovered was definitely not bacteriophage! D'Herelle spent much of his energy during the ensuing years proclaining that he, d'Herelle, was the sole discoverer of phage. In fact, he maintained, he really had discovered phage in 1910 while he was doing the research on the disease of locusts. His papers from 1910 do not refer to the phenomenon that he later said were bacterial viruses, however. This led people to become suspicious that perhaps d'Herelle really had known of Twort's 1915 work when he published his 1917 paper. Bordet, in particular, was insistent that Twort had discovered bacteriophage and that d'Herelle had stolen the idea from him. It is an issue that was not, and never will be, resolved.

It is interesting, though, to speculate as to why Bordet was so adamant in his support of Twort. Bordet was an immunologist and a Nobel Prize winning one at that. What could he possibly have cared about bacteriophage? I think it was precisely because Bordet was an immunologist that he got involved in the fight over who discovered phage. It is my feeling that Bordet actually thought d'Herelle's phenomenon really might be an agent of antibacterial immunity in infected hosts. D'Herelle said that the agent appeared in individuals recovering from dysentery and he further claimed that he could actually immunize people by infecting them with phage. By supporting Twort's theories, Bordet could at least feel that he had a hand in the discovery of antibacterial immunity, even though, in fact, he had not had anything to do with it, and phage have yet to be shown to be agents of 'immunity'. Twort, himself, never followed up on his finding or tried to do experiments that would have elucidated the nature of the agent causing the 'glassy transformation'. Had he shown convincingly that it was actually phage that were the agents of the change in his colonies, claims that he was the true discoverer of phage would have been stronger.

Who really first discovered phage and why Bordet became so involved are only two of the unanswered questions about the early history of phage. It would probably raise more than a few eyebrows to ask whether Twort had discovered a bacterial prion. But if not, just what did cause his glassy transformation? A lysogenic phage is the most likely, but who could say for sure? Perhaps of greatest relevance today, as the specter of bacteria resistant to all known antibiotics looms very large, is whether phage really were of any use as antibacterial agents. Reports in the 1930s documented commercial phage preparations with weak or no activity, and the wisdom of uncontrolled use of phage 'at the present state of our knowledge' was questioned. Certainly, as soon as the first antibiotics were discovered, research on the therapeutic use of phage dropped to the level of background noise. It remained a viable topic in Russia (in fact, an institute that d'Herelle founded is still functioning, albeit on a shoestring, in Tbilisi) and Poland and a few other places, but in the mainstream it was not considered worth pursuing until very recently. Still, phage research has contributed to human's ability to cure disease in ways that d'Herelle never could have imagined, in that basic research with bacteriophages has been responsible for almost every discovery underlying modern biological science. This mass of research stemmed from the idea that how an organism decodes its genetic program could be learned by studying phage.

Max Delbruck and Salvador Luria were largely responsible for the idea that phage could be used to understand the basic properties of genes and to determine how they functioned. They formed the nucleus of the 'phage group', scientists from all over the world, most of whom came together each summer at the Cold Spring Harbor Laboratories on Long Island during the 1940s, 1950s, and 1960s, and all of whom were dedicated to using phage to discover how life 'worked'. The group included, in addition to Luria and Delbruck, Thomas Anderson, Seymour Benzer, A. H. Doerman, Emory Ellis, Roger Herriott, Alfred Hershey, Edward Kellenberger, Lloyd Kozloff, Andre Lwoff, Gunther Stent, and many other dedicated scientists. Members of the group unraveled the process of phage replication, showing that it was characterized by a latent period, a rise period and a sudden burst. This pattern of growth was later confirmed for all lytic viruses. In addition, Hershey and his assistant, Martha Chase, showed that it was only the phage nucleic acid that was needed for the virus to reproduce itself, providing the final proof that nucleic acid and not protein was the genetic substance. Seymour Cohen, who was more interested in the biochemistry of the infected cell than the genetic program of the phage, discovered that the DNA of the T-even phages was glucosylated and that a new enzyme was synthesized in the infected cells that carried out the glucosylation. This led to an explosion of work on the enzymology of infected cells and the

discovery of dozens of phage-coded proteins and the genes that controlled them. The power of using conditional lethal mutants to study gene–protein relationships was shown by many studies of these phage-induced enzymes. Other historic discoveries made with phage include the existence of messenger RNA, shown by Hall and Spiegelman, and the colinearity of DNA and protein confirmed in Sidney Brenner's lab. Francis Crick used the strange rII mutants of T4 to show that the genetic code is a triplet code read from a fixed starting point. Phage also proved to be very useful in elucidating the mechanism of action of many mutagens. DNA synthesis was studied in phage-infected cells and it was found that DNA is synthesized discontinuously in 'Okazaki' fragments and that these are primed by RNA. Studies of phage infection in sporulating cells led to the discovery of sigma factors, subunits of RNA polymerase that act to initiate RNA synthesis at certain sequences, and a strange phage called Mu (because it caused infected cells to mutate) led to the discovery of transposition or the ability of certain DNA elements to insert themselves randomly in any DNA. The fact that the diphtheria toxin gene is a phage gene led, eventually, to the realization that many virulence factors are coded for by extrachromosomal elements. In addition, a mortal blow to vitalism was struck when it was shown that biologically-active Q-beta phage DNA could be synthesized in the test tube. No other discovery, however, has had more effect on the future course of research in the biological sciences than another discovery made with bacteriophage. This is, of course, the discovery of restriction endonucleases. This discovery occurred as a result of the study of a fairly arcane phenomenon concerning the behavior of bacteriophage grown on two different strains of bacteria. It was observed that phage grown on one strain of bacteria grew very poorly (i.e. were restricted) on a second strain and vice versa. The few phage that invariably escaped restriction on the second host could subsequently grow very well on this host but grew poorly on the first host. It was later shown that this occurred because the phage DNA acquired host-specific markings or modifications and these protected the DNA from being degraded by restriction enzymes in that host. If unmarked or inappropriately marked DNA entered a bacterium, it would be broken down. Hence, each bacterium appeared to have a way to protect itself from foreign 'invading' DNA. It was later found that many of the enzymes involved in this attack on foreign DNA (restriction) were site-specific endonucleases that generated overlapping ends. Thus any two pieces of DNA cut by the same restriction enzyme could be hybridized and ligated to make a spliced DNA molecule. This ability to join different DNAs together has led to previously unimaginable technologies of which gene therapy is but one. It should be noted particularly that the initial observation that led to the discovery of restriction enzymes – poor growth of a bacterial virus on certain hosts – was in no way remarkable and would not have generated a great deal of public excitement and support, yet it led to one of the most significant biological discoveries of this century.

Many scientists felt that the depth of Nature's secrets to be uncovered with phage had not been fully plumbed, but with the many advances in knowledge about viruses, genes and the production of proteins that phage had made possible, basic research using phage gave way to other fields of endeavor and by the 1980s was relegated to the back burner by the scientific establishment. But just as basic phage research was going out of style, there was some interest being rekindled in phage therapy. Early in the 1980s, the Wellcome Trust gave a small grant to H. W. Smith and his collaborators to study phage therapy in animals. They found phage to be very effective in controlling systemic *E. coli* infections in mice and experimental diarrhea in calves. This led to the opinion expressed in the *British Medical Journal* that 'phages may be poised to make an important entry into veterinary medicine and ... their exploitation in human clinical practice cannot be far behind.' It was thought that phage would be particularly useful in 'side-stepping the tiresome problem of antibiotic resistance'. This promise for human clinical medicine seemed to have been substantiated by a paper from Poland in 1987 that reported positive results in 508 of 550 cases of suppurative bacterial infections treated with bacteriophage.

In 1996 the credibility of phage therapy was given a little boost by a commentary in *Proceedings of the National Academy of Sciences* by Joshua Lederberg. His interest was spurred by the isolation of phage variants that are not rapidly cleared from the circulation by spleen cells – the removal of phage by the immune system being, of course, one obstacle to their usefulness. Lederberg suggested that a study of phage ecology – 'bacteriophage as smaller fleas on their bacterial hosts' – might lead to the knowledge necessary to use these viruses as therapeutic agents. He thought that highly antibiotic-resistant organisms would be a good target, as would agricultural diseases such as citrus canker. He also suggested that perhaps refugee camps could be made less terrifying if the 'transmission of cholera or dysentery could but be mitigated'. This same hope had been expressed by d'Herelle some seventy-five years before! And so the echoes of the past are being heard. Will the venture,

this time around, be more successful? We can but hope.

See also: **Antivirals; Phage ecology, evolution and speciation; Phage Homologous Recombination; Phage toxins and disease; Phage transduction; Phages as cloning vehicles; Phages in industrial fermentations; Phages in soil.**

Further Reading

Cairns J, Stent GS, Watson JD (eds) (1966) *Phage and the Origins of Molecular Biology*. New York: Cold Spring Harbor Laboratory of Quantitative Biology.

d'Herelle F (1926) *The Bacteriophage and its Behavior*. Williams and Wilkins, Baltimore.

d'Herelle F (1949) The bacteriophage. *Sci. News* 14: 44.

Duckworth DH (1987) History and basic properties of bacterial viruses. In: Goyal SM, Gerba CP and Bitton G (eds) *Phage Ecology*. New York: John Wiley.

Duckworth DH (1995) Discovery of bacterial viruses. In Webster RG and Granoff A (eds) *Encyclopedia of Virology*, First Edition. Orlando: W. B. Saunders.

Twort, A. (1993) In focus, out of step: a biography of Frederick William Twort, FRS. Dover, NH: Alan Sutton Publishing.

Polio, Coxsackie, Echo and Other Enteroviruses

Joseph L Melnick, Baylor College of Medicine, Houston, Texas, USA

Introduction

The enteroviruses constitute one of the genera of the picornavirus family. The genus includes the polioviruses, the coxsackieviruses and the echoviruses of humans, plus a number of enteroviruses of lower animals (e.g. monkeys, cattle, pigs, mice). Over 100 serotypes are recognized, the first to be discovered being the polioviruses.

Early Developments

Reports on clinical and epidemiological aspects of poliomyelitis came in the late 19th and early 20th centuries from von Heine and Medin in Germany, Wickman in Sweden and Caverly in the USA, but the virological study of polioviruses only began in 1908 when Landsteiner, Popper and Levaditi transmitted the disease to monkeys, inducing typical histopathological lesions in the animals' spinal cords. Shortly thereafter, Flexner and Wollstein achieved monkey-to-monkey passage. As it turned out, however, their use of rhesus monkeys contributed to serious problems in the development of polio research. Flexner was director of the Rockefeller Institute for Medical Research in New York, and a highly respected advocate of scientific medicine. Unfortunately, he assumed that the disease in man paralleled precisely what he was observing in the rhesus monkeys infected in his laboratory. He influenced many of his colleagues through his belief that polioviruses were strictly neurotropic, because of the way they behaved in his rhesus monkeys. He believed that poliovirus entered the human body through the nasal route and proceeded directly to the central nervous system (CNS). Besides delaying progress in unraveling the pathogenesis of poliomyelitis, this conviction also led to a misguided and unfortunate attempt at prophylaxis in human beings. When zinc sulfate was administered to Flexner's rhesus monkeys to coat the lining of their noses, the animals were protected against development of paralytic polio when challenged by intranasal inoculation. Flexner entirely overlooked Kling's work in Sweden; as early as 1912, Kling and his associates reported isolation of poliovirus not only from the throat but also from the feces of both paralytic and nonparalytic patients. Meanwhile, on the basis of belief in the nasal portal of entry in the human infection and in the efficacy of zinc sulfate to protect that portal, many persons during a 1927 epidemic underwent what was, at least, considerable discomfort produced by administration of zinc sulfate, but received no protection against poliomyelitis.

In 1936, Sabin and Olitsky reported that rhesus monkeys succumbing to polio had olfactory lesions only when infected by intranasal instillation of virus, and not when virus was administered by other routes. Five years later, Sabin and Ward showed that olfactory bulbs from 65 fatal human cases were all negative for virus. This underscored the previous observations by pathologists of the rarity with which olfactory lesions had been observed in autopsy studies of human polio cases – a surprising observation if the infection route had been nasal.

In the late 1930s, American investigators had begun turning toward the possibility of an oral–alimentary route of infection. Investigators at the Pasteur Institute had successfully infected cynomolgus monkeys by the oral route. Impetus was added when Howe and Bodian came across a report by Muller describing spontaneously acquired polio in chimpanzees in a children's zoo in Cologne. They followed up this clue with a series of experiments establishing that chimpanzees were far more susceptible to infection by the oral route than other experimental primates. This

to be the best choice for a productive system of virus propagation.

HVS-2 transformed can be subpassaged easily and a number of permanent cell lines have been established. The virus causes a lytic infection of the susceptible cells, with complete destruction of the monolayers spanning 2–20 days postinfection and a high titer output of virus. Old World monkey cells in culture are notably less susceptible to HVS-2 infection, although virus will replicate in Vero and CV-1 cell lines. In these cases, yield is significantly reduced compared with that measured from cell cultures derived from New World monkeys. Human cells that are susceptible are fibroblastic in origin, virus yield is low, and persistently infected cells can be maintained.

Summary and Future Perspectives

Each of these *Rhadinovirus* members of the family *Herpesviridae* has immense potential for use as a tool to transform/immortalize target cells of interest, particularly with respect to studies of human immunodeficiency virus (HIV) and AIDS. Gene-specific transformation or immortalization of cells is also possible, reducing the input of the nonessential viral genes against the background of the transfected cells. Transformation-associated genes represent a novel class of viral oncogenes which warrant further investigation. Furthermore, each of these virus–host systems continues to offer investigators a unique and invaluable avenue for epidemiological studies of virus transmission in nature, both within natural and foreign hosts. Last, but not least, due to the capacity of these viruses for carrying large amounts of DNA inserts, the use of HVS-2 and HVA-2 as novel gene vectors will most surely be continuously explored by investigators until ideal vectors are identified.

See also: **Herpesviruses – baboon and chimpanzee (*Herpesviridae*); Herpes simplex viruses (*Herpesviridae*): General features, Molecular biology; Herpesvirus sylvilagus (Herpesviridae); Epstein-Barr virus (*Herpesviridae*): Molecular biology; Immune response: Cell mediated immune response, General features; Transformation: Animal viruses.**

Further Reading

Biesinger B, Trimble JJ, Desrosiers RC and Fleckenstein B (1990) The divergence between two oncogenic herpesvirus saimiri strains in a genomic region related to the transforming phenotype. *Virology* 176: 505.

Daniels MD, Karpas A, Melendez LV, King NW and Hunt RD (1969) Isolation of herpes-T virus from a spontaneous disease in squirrel monkeys (*Saimiri sciureus*). *Arch. Gesamte Virus-Forsch.* 22: 324.

Eberle R and Hilliard JK (1995) The simian herpesviruses. *Infect. Agents Dis.* 4: 55.

Fleckenstein B and Desrosiers RC (1982) Herpesvirus saimiri and Herpesvirus ateles. In: Roizman B (ed.) *The Herpesviruses,* vol. 1, pp. 253–332. New York: Plenum Press.

Jung JU *et al* (1991) Identification of transforming genes of subgroup A and C strains of herpesvirus saimiri. *Proc. Natl Acad. Sci.* USA 88: 7051.

Murphy F (1996) Virus taxonomy. In: Fields BN, Knipe, DM, Howley PM *et al* (eds) *Fields Virology*, pp. 45–51. Philadelphia: Lippincott-Raven.

Trimble JJ and Desrosiers RC (1991) Transformation by herpesvirus saimiri. *Adv. Cancer Res.* 56: 335.

HISTORY OF VIROLOGY

Contents

General

Frank Fenner, John Curtin School of Medical Research, Australian National University, Canberra, Australia

Introduction

As in other branches of experimental science, the development of our knowledge of viruses has depended on the techniques available. Initially viruses, like other microbes, were studied by pathologists interested in the causes of the infectious

diseases of humans and their domesticated animals and plants. These concerns remain the main force advancing the subject. When bacterial viruses were discovered in 1915–1917, it was the hope that they might control bacterial diseases, such as cholera and dysentery, that stimulated their investigation. The idea that viruses might be used to probe fundamental problems of biology arose in the early 1940s, with the development of the Phage Group. These studies helped establish the new field of molecular biology in the period 1950–1970, and molecular biology has in turn revolutionized the study of viruses and led to an explosion of knowledge of them. To keep the subject in perspective, this brief review of the history of virology will be restricted to the period before 1980.

The Word 'Virus'

Since antiquity the term 'virus' had been synonymous with 'poison', but during the nineteenth century it became a synonym for microbe, Pasteur's word for an infectious agent. It did not acquire its present connotation until the 1890s, when the bacterial or fungal causes of many infectious diseases were discovered, using the agar plate, effective staining methods and efficient microscopes. It then became apparent that there were a number of infectious diseases of animals and plants from which no bacterium or fungus could be isolated or visualized with the microscope. After the introduction in 1884 of the Chamberland filter, which held back bacteria, Ivanowski and Beijerinck independently showed that the cause of tobacco mosaic disease was a filterable, infectious agent, in that its infectivity passed through a bacteria-proof filter. Loeffler and Frosch demonstrated that the cause of foot-and-mouth disease was also a 'filterable (or ultramicroscopic) virus'. The first compendium of all then known of viruses was edited by Rivers of the Rockefeller Institute and published in 1928. Entitled *Filterable Viruses*, this emphasized that viruses required living cells for their multiplication. In the 1930s chemical studies of the particles of tobacco mosaic virus and of bacteriophages showed that they differed from all cells, in that at their simplest they consisted of protein and nucleic acid, which was always of one kind, DNA or RNA. Gradually the adjectives 'filterable' and 'ultramicroscopic' were dropped and the word viruses developed its present connotation.

Early Investigations

Tobacco mosaic virus (TMV)

From 1879 there had been an interest in Europe in a mosaic disease of tobacco plants from which no bacterium could be recovered. In 1892 Ivanowski, in St Petersburg, found that the disease could be transmitted with filtered sap and concluded that the most likely cause was a bacterial toxin. Unaware of Ivanowski's work, in 1897 Beijerinck, in Delft, also found that sap from an infected plant remained virulent after filtration, although bacteriologically sterile. Further, he found that the mosaic disease could be transmitted successively, by filtered sap, through an 'unlimited' number of plants. Beijerinck concluded that the cause of tobacco mosaic disease was a living infectious agent in fluid form for which he coined the term *'contagium vivum fluidum'*.

Foot-and-mouth disease virus

While Beijerinck was defining his concept of a *contagium vivum fluidum*, Loeffler and Frosch were making equally revolutionary discoveries with an important disease of cattle. In 1898 they described the filterability of an animal virus for the first time, noting that 'the filtered material contained a dissolved poison of extraordinary power or that the as yet undiscovered agents of an infectious disease were so small that they were able to pass through the pores of a filter definitely capable of retaining the smallest known bacteria'. On the basis of its virulence after successive dilution in experimental animals, they concluded that the causal agent of foot-and-mouth disease was not soluble but 'corpuscular'. Loeffler and Frosch gave filtration a new emphasis by focusing attention on what passed through the filter rather than what was retained, and established an experimental methodology which was widely adopted in the early twentieth century in research on viral diseases.

Yellow fever virus

Following the acceptance of the notion of filterable infectious agents, pathologists investigated diseases from which no bacteria could be isolated and several were soon shown to be caused by viruses. One of the most fruitful investigations, in terms of new concepts, was the work of the United States Army Yellow Fever Commission headed by Walter Reed in 1900–1901. Using human volunteers, they demonstrated that yellow fever was caused by a filterable virus which was transmitted by mosquitoes and that the principal vector was *Aedes aegypti*. They also showed that infected persons were infectious for mosquitoes only during the first few days of the disease and that mosquitoes were not infectious until 7–10 days after imbibing infectious blood, thus defining the extrinsic incubation period and establishing essentially all of the basic principles of the epidemiology of what came to be called 'arboviruses'.

Discovery of Bacterial Viruses

In 1915 Twort, in London, described a filterable principle that caused lysis of bacterial colonies and could be transmitted to fresh cultures for an indefinite number of generations. His paper was virtually unnoticed, and 2 years later d'Herelle, a Canadian working at the Pasteur Institute in Paris, rediscovered Twort's lytic principle, noted that it was an obligate parasite of various species of bacteria, gave it the name 'bacteriophage' (bacteria-eater), and classified it as a filterable virus.

Physical Studies of Viruses

Further advances in understanding of the nature of viruses depended on physical and chemical studies. As early as 1907 Bechhold in Germany developed filters of graded permeabilities and a method of determining their pore size. Subsequently Elford, in London, used such membranes for determining the size of bacteriophage and animal virus particles with remarkable accuracy.

In Germany, on the eve of World War II, Ruska and his colleagues had produced electron microscopic photographs of the particles of TMV, bacteriophages and poxviruses, noting the extraordinary 'sperm-shaped' particles of bacteriophage. Technical improvements in instrumentation after the war, and the introduction of negative staining for studying the structure of TMV and other viruses by Cambridge scientists Huxley in 1957 and Brenner and Horne in 1959 resulted in photographs of the particles of virtually every known kind of virus. These demonstrated the variety of their size, shape and structure, and the presence of common features such as the helical particles of many plant viruses and the icosahedral symmetry of many other viruses.

Following a perceptive paper on the structure of small virus particles by Crick and Watson in 1956, in 1962 Caspar of Boston and Klug of Cambridge, England, produced a general theory of the structure of regular virus particles of helical and icosahedral symmetry. Structure of the virus particle became one of the three major criteria in the system of classification of viruses that was introduced in 1966.

Chemical Composition of Viruses

If the ultramicroscopic particles found in virus-infected hosts were the pathogens, what did these particles consist of? In 1935, stimulated by work on the crystallization of enzymes, Stanley, at the Rockefeller Institute for Medical Research, announced that he had isolated TMV as a crystalline protein. Contemporaneously, Bawden and Pirie at Cambridge University examined the TMV particles that they had purified and showed that their product was a nucleoprotein, containing RNA. At about the same time Schlesinger, working in exile in London, had shown that a purified suspension of bacteriophage particles consisted of approximately equal amounts of protein and DNA, and in 1940 Hoagland and his colleagues at the Rockefeller Institute found that vaccinia virus particles contained DNA but no RNA. Thus evidence was accumulating that viruses differed from bacteria not only in size and their inability to grow in lifeless media, but in that they contained only one kind of nucleic acid, which could be either DNA or RNA.

The development of restriction endonuclease digestion of DNA, based on studies of phage restriction by Arber of Basel in the 1960s, and then elaborated by biochemist Smith of Baltimore in 1970, has simplified the mapping of the genomes of DNA viruses, a study initiated by Nathans, also of Baltimore, using the papovavirus SV40. Improvements in sequencing methods for both DNA and RNA have now led to the publication of the complete genomic nucleotide sequences for representatives of most families of viruses.

Bacterial Viruses

d'Herelle brought wide attention to bacteriophages because of his belief, in the event never realized, in their potential for the treatment of bacterial diseases. The next major figure to elucidate the nature of bacteriophage was Burnet. Working in Melbourne over the period 1924–1934, Burnet showed that there was not a single virus, 'the bacteriophage', as d'Herelle had thought, but a wide variety of bacterial viruses with very different biologic and physical properties. He devised the single-burst experiment, demonstrated the difference between resistance of bacteria at the level of adsorption and the immunity of lysogenic bacteria to the phage already carried, and started phage genetics with the discovery of a mutant virus that inherited the inability to be carried in the lysogenic state.

The Phage Group

In the late 1930s three men working in the United States, physicist Delbrück, medical bacteriologist Luria and bacteriologist Hershey, took up the study of bacteriophages. Their meeting in 1940 led to the establishment of the Phage Group, which was united by the desire to understand how, in the half-hour latent period revealed by the one-step growth experi-

ment, a phage particle achieves its hundredfold multiplication within the bacterial cell. The Phage Group expanded in 1945, when Delbrück organized an annual summer phage course at Cold Spring Harbor. An early and important recruit was Anderson, who used electron microscopy to explore the attachment of phage particles to bacteria by their tails, but was unable to find any particles in the cell during the eclipse period, indicating that there is more to a virus than its particles.

A major advance came with the demonstration in 1952 by Hershey and Chase that phage infection proceeded by injection of DNA, the protein shell being left outside the bacterium. A year later Watson (who had received his PhD degree for work on phage with Luria) and Crick, working in Cambridge, published their classic papers on the structure of DNA.

Phage genetics and molecular biology

During the 1950s and 1960s work with phages advanced the study of viral genetics far beyond what had been established for animal or plant viruses and provided a model for later studies with these agents. In 1946, Hershey had demonstrated that bacterial viruses can undergo genetic recombination, and, by 1955, Benzer had produced a very detailed fine structure genetic map of the T4 bacteriophage. Although the Phage Group included few biochemists, it was the combination of genetics and biochemistry in their studies that led to the birth of molecular biology.

Lysogeny

In the 1930s Burnet had suggested a solution to one of d'Herelle's greatest problems, lysogeny, by postulating that lysogenic bacteria carried a precursor or 'anlage' that multiplied within the bacterium, but could by unknown stimuli be induced to replicate independently and produce virus particles. Because the T-even phages with which they worked were virulent, this problem was never taken up by the Phage Group, but after attending a meeting at Cold Spring Harbor in 1946, Lwoff and his colleagues at the Pasteur Institute in Paris applied themselves to solving the mechanism of lysogeny. By 1950 they had shown that x-rays induced lysogenic bacteria to release phage and, using an elegant single-cell technique, demonstrated that what is reproduced by a lysogenic bacterium during its replication is the genetic material of the phage, which Lwoff called prophage.

Other Discoveries with Bacterial Viruses

All earlier phage work had been carried out with viruses with DNA genomes, but in 1961 Loeb and Zinder, working at the Rockefeller Institute, discovered the first example of an RNA phage. Other seminal discoveries in molecular biology that arose out of studies with phages were the demonstration of transduction by Zinder and Lederberg in 1952, the discovery of messenger RNA by Brenner, Jacob and Meselson in 1961 and the report in 1965 by Spiegelman and his colleagues of the *in vitro* replication of infectious viral (Qβ) RNA.

Animal Virology

Whereas investigations by the Phage Group were motivated mainly by scientific curiosity, animal virology was developed by pathologists studying the large number of diseases of humans and livestock caused by viruses, and it has retained this practical bias. Over the first two decades of the twentieth century, testing of filtrates of material from a number of diseases of humans and animals confirmed that they were caused by viruses. Among the most important was the demonstration in 1911 by Rous, at the Rockefeller Institute, that a sarcoma of fowls could be transmitted by a bacteria-free filtrate.

Cultivation of animal viruses

The first systematic use of small animals for virus research was Pasteur's use of rabbits, which he inoculated intracerebrally with rabies virus in 1881. It was not until 1930 that mice were used for virus research, with intracerebral inoculations of rabies and yellow fever viruses. By using graded dilutions and large numbers of mice, quantitative animal virology had begun. The next important step was the use of chick embryos for growing poxviruses by Goodpasture in 1931–1932, followed a few years later by the demonstration by Burnet that many viruses could be titrated by counting the pocks that they produced on the chorioallantoic membrane, whereas others grew well in certain embryonic cavities of developing chick embryos.

Tissue cultures had first been used for cultivating vaccinia virus in 1928, but it was the discovery by Enders, Weller and Robbins of Harvard University in 1949 that poliovirus would grow in nonneural cells that gave a tremendous stimulus to the use of cultured cells in virology. Over the next few years their use led to the cultivation of medically important viruses, such as those causing measles, by Enders and Peebles in 1954, and rubella, by Weller and Neva in 1962. Even

more dramatic was the isolation of a wide variety of 'new' viruses, belonging to many different families. Also the different cytopathic effects produced by different viruses in monolayer cell cultures were found to be diagnostic.

The next great advance, which greatly increased the accuracy of quantitative animal virology, occurred in 1952, when the plaque assay method for counting phage was adapted to animal virology by Dulbecco, using a monolayer of chick embryo cells in a Petri dish. In 1958 Temin and Rubin applied Dulbecco's method to Rous sarcoma virus, initiating quantitative studies of tumor viruses. Biochemical studies of animal virus replication were simplified by using continuous cell lines and by growing the cells in suspension.

Biochemistry

During the 1950s virus particles were thought to be 'inert' packages of nucleic acid and proteins, although in 1942 Hirst of the Rockefeller Institute had shown that particles of influenza virus contained an enzyme, later identified by Gottschalk of Melbourne as a neuraminidase.

Further advances in viral biochemistry depended on methods of purification of virus particles, especially the technique of density gradient centrifugation. In 1967 Kates and McAuslan demonstrated the presence of a DNA-dependent RNA polymerase in purified vaccinia virus virions, a discovery that was followed the next year by the demonstration of a double-stranded (ds) RNA-dependent RNA polymerase in reovirus virions and then of single-stranded (ss) RNA-dependent RNA polymerases in those of paramyxoviruses and rhabdoviruses. In 1970 came the revolutionary discovery by Baltimore of Boston and Temin of Wisconsin, independently, of the RNA-dependent DNA polymerase, or reverse transcriptase, of Rous sarcoma virus. Many other kinds of enzymes were later identified in the larger viruses; for example, no fewer than 16 enzymes have now been identified in vaccinia virus virions.

Just as investigations with bacteriophages were of critical importance in the development of molecular biology, studies with animal viruses have led to the discovery of several processes that have proved to be important in the molecular biology of eukaryotic cells, although many of these discoveries are too recent for serious historical appraisal. Thus in 1968 Jacobson and Baltimore of Boston showed that the genomic RNA of poliovirus was translated as a very large protein, which was then cleaved by a protease that was subsequently found in the viral replicase gene. RNA splicing was discovered in 1977, independently by Sharp and Chow and their respective colleagues during studies of adenovirus replication. Work with the same virus by Rekosh and colleagues in London in 1977 resulted in the definition of viral and cell factors for initiation of new DNA strands using a novel protein priming mechanism. Capping of mRNA by m^7G and its role in translation was discovered in 1974, during work with reoviruses by Shatkin. Other processes first observed with animal viruses and now known to be important in eukaryotic cells were the existence of 3′-(poly)A tracts on mRNAs, the pathway for synthesis of cell surface proteins and the role of enhancer elements in transcription.

Analysis of viral nucleic acids progressed in parallel with that of the viral proteins. Animal viruses were found with genomes of ssRNA, either as a single molecule, two identical molecules (diploid retroviruses), or segmented; dsRNA; ssDNA, dsDNA and partially dsDNA. The so-called 'unconventional viruses' or prions of scrapie, kuru and Creutzfeld–Jacob disease, which have been extensively investigated since 1957 by Gajdusek of the US National Institutes of Health and Prusiner of the University of California at San Francisco, appear to be infectious proteins. Soon after the discovery that the isolated nucleic acid of TMV was infectious (see below), it was shown that the genomic RNAs of viruses belonging to several families of animal viruses were infectious, namely those with single, positive-sense RNA molecules. Then in 1972 came the discovery of recombinant DNA by Berg of Stanford University who inserted genes from bacteriophage λ into the genome of SV40.

Structure of the virion

Using more sophisticated methods of electron microscopy, the vast majority of animal virus virions have been shown to be isometric icosahedral structures, or to be nucleoprotein tubes of helical symmetry surrounded by a lipid-containing shell, the envelope, which contains a number of virus-coded glycoprotein spikes. X-ray crystallography of crystals of purified isometric viruses has revealed the molecular structure of their icosahedra; similar revealing detail has been obtained for the neuraminidase and hemagglutinin spikes of influenza virus.

Tumor virology

After the discovery of Rous sarcoma virus in 1911 there was a long interval before the second virus to cause tumors, rabbit papilloma virus, was discovered by Shope in 1933. However, after that viruses were found to cause various neoplasms in mice, and in 1962

Trentin of Yale University showed that a human adenovirus would produce malignant tumors in hamsters. Since then, direct proof has been obtained that several DNA viruses (but not adenoviruses) and certain retroviruses can cause tumors in humans. Molecular biological studies have shown that oncogenicity is largely caused by proteins they produce that are encoded by viral oncogenes, a concept introduced by Huebner and Todaro of the US National Institutes of Health in 1969 and corrected, refined and greatly expanded since the mid-1970s by Bishop, Varmus (both of San Francisco) and Weinberg of Boston.

Only one group of RNA viruses, the retroviruses, which replicate through an integrated DNA provirus, cause neoplasms, whereas viruses of five groups of DNA viruses are tumorigenic. Study of these oncogenic viruses has shed a great deal of light on the mechanisms of carcinogenesis. Oncogenic DNA viruses contain oncogenes as an essential part of their genome, which when integrated into the host cell DNA may promote cell transformation, whereas the oncogenes of retroviruses are derived from proto-oncogenes of the cell.

Impact on immunology

Immunology arose as a branch of microbiology and several discoveries with animal viruses were important in the development of important concepts in immunology. It was the discovery in the 1930s, by Traub in Tübingen, of persistent infection of mice with lymphocytic choriomeningitis virus that led to the development by Burnet of the concept of immunological tolerance in 1949. It was work with the same virus that led to the discovery of major histocompatibility complex (MHC) restriction by Doherty and Zinkernagel in Canberra in 1974.

Vaccines and disease control

From the time of Pasteur's use of rabies vaccine in 1885, a major concern of medical and veterinary virologists has been the development of vaccines. Highlights in this process have been the development of the 17D strain of yellow fever virus by Theiler of the Rockefeller Foundation laboratories in 1937, the introduction of influenza vaccine in 1942, based on Burnet's 1941 discovery that the virus would grow in the allantois, the licensing in 1954 of inactivated poliovaccine developed by Salk and in 1961 of the live poliovaccine introduced by Sabin, both based on the cultivation of the virus in 1949 by Enders, and development of the first genetically engineered human vaccine with the licensing of yeast-grown hepatitis B vaccine in 1986. Finally, 1977 saw the last case of natural smallpox in the world and thus the first example of the global eradication of a major human infectious disease, the result of a 10 year campaign conducted by the World Health Organization with a vaccine directly derived from Jenner's vaccine, first used in 1796.

Viruses of Invertebrates

Study of the viruses of invertebrates has lagged a long way behind that of vertebrate viruses. The first virus of insects to be identified was that of silkworm jaundice. In 1907 von Prowazek of Hamburg showed that filtered material, free of the polyhedra he had found in diseased silkworms, would nevertheless transmit the disease, but it was not until 1947 that the German scientist Bergold showed that the polyhedra consisted of a matrix of noninfective protein in which were embedded rod-shaped particles of a virus with a DNA genome. Over the succeeding years a large number of other viruses of insects, some clearly resembling viruses of vertebrates, have been discovered and more recently viruses have been found that affect many other kinds of invertebrates. The development of methods of growing insect cells in culture by Grace in Canberra in 1962 opened the way for the molecular biological investigation of the replication of insect viruses and arboviruses in invertebrate cells.

Arthropods, mainly insects and ticks, were early shown to be important as vectors of virus diseases of vertebrates (the arboviruses) and of plants. Sometimes carriage was found to be mechanical but in most cases the virus was found to multiply in the vector as well as the vertebrate or plant concerned.

Plant Virology

Like animal virology, plant virology was at first dominated by plant pathologists, but subsequently certain plant viruses have been intensively studied by molecular biologists. Because its stable particles occur in such large amounts in infected plants, TMV has been a most important model virus for plant virologists and its study has contributed greatly to virology in general. In 1929 Beale of the USA showed that plants infected with TMV contained a novel antigen, suggesting that the virus contained protein and that serologic methods could be used to estimate its concentration. In the same year Holmes, also working in the USA, discovered that local lesions on plant leaves could be used to assay infectivity in much the same way that plaques were used in phage assays.

The next major dicovery with TMV came in 1956, when Gierer and Schramm, in Tübingen, and Fraenkel-Conrat at Berkeley, demonstrated that, if

protected from inactivation, the RNA isolated and purified from TMV particles was infectious. Two years later Gierer showed that mutants of the virus could be obtained by reacting inocula of its RNA with nitrous acid, a treatment which changes the bases in the RNA. Although it was not the first virus whose RNA was completely sequenced, TMV RNA was the first mRNA used in the establishment of a reliable RNA-requiring translation system, by Nirenberg in 1961.

TMV was also important in the development of ideas about viral structure. Studies in the 1930s–1940s, including x-ray diffraction of preparations of the virus by Bernal and Fanfuchen of Cambridge, suggested that the viral particles were long, thin rods. This was confirmed by electron micrographs taken in Germany in 1939. Then, during the 1950s, Watson and Crick, working in Cambridge University, recognized that the x-ray pictures made in 1941 suggested that the particles were helical, with a turn every 23 Å along the helical axis, and suggested that the protein shell consisted of a large number of identical subunits of molecular weight about 20 000, a view supported by biochemical studies by Knight in California in 1956. Fraenkel-Conrat and his colleagues then developed ways to reconstitute virions from purified viral RNA and viral protein, and showed that the protein tended to aggregate to double disks, each of 17 molecules, and that these disks had a high affinity for a particular site on the RNA. Sequential addition of disks led to assembly of the complete virion. The fact that TMV RNA was infectious showed that it functioned as an mRNA, but experimenters were unable to make the coat protein with this RNA, a result that led to the discovery of subgenomic mRNA.

Kinds of plant viruses

A large number of plant viruses belonging to many different groups have now been characterized. Most contain ssRNA and have particles that are long, nonenveloped, tubular structures, but some are icosahedral. Plant representatives of the dsRNA reoviruses were discovered by Black in 1963, and in 1968 Shepherd showed that cauliflower mosaic virus contained dsDNA; later this virus, like hepatitis B virus, was found to use a reverse transcriptase in the replication of its genome. In addition, plant viruses have been found with several unusual genome structures. Tobacco rattle virus is multiparticulate, with the RNA for its replicative functions in one rod and that for its coat protein in another; the satellite virus described by Kassanis of Rothamsted in 1962 is dependent on tobacco necrosis virus for essential replicative functions. Then in 1971 potato spindle tuber virus was shown by Diener of the US Department of Agriculture to be a replicating, 359 nucleotide circular ssRNA, devoid of a protein coat; it is one of several plant 'viroids'.

Transmission of plant viruses

Unlike animals, plants are not mobile, so that a great many transmission modes are used by plant viruses. Some are passed vegetatively or through seed, others by contact or in soil water, but most are transmitted by vector organisms that feed on plants. In the 1890s, before viruses had been characterized, Japanese workers showed that leafhoppers transmit rice dwarf disease; the virus has since been shown to multiply in both plant and leafhopper. Aphids carry a great variety of plant viruses; more unusual vectors are soil nematodes, first incriminated by Hewitt of the USA in 1958, and soil fungi, incriminated by Teakle of Brisbane in 1960.

Taxonomy and Nomenclature

After earlier tentative efforts with particular groups of viruses, viral nomenclature took off in 1948, with the production of a system of latinized nomenclature for all viruses by the plant virologist Holmes. This stimulated others, in particular Andrewes of London and Lwoff of Paris, to actions which resulted in the setting up of an International Committee on Nomenclature (later Taxonomy) of Viruses in 1966. Adopting the kind of viral nucleic acid, the strategy of replication and the morphology of the virion as its primary criteria, this Committee has now achieved acceptance of its decisions by the great majority of virologists. One interesting feature of its activities is that its rules avoid the controversies about priorities that plague taxonomists working with other groups of living things.

The Future

This brief historical survey stops in about 1980, just as virology began to experience an explosive expansion with the application of techniques of genetic engineering, nucleic acid sequencing, the polymerase chain reaction and the use of monoclonal antibodies. The last decade has also seen the emergence of the acquired immunodeficiency disease syndrome (AIDS), the most important new human viral disease this century, the isolation of the causative human immunodeficiency viruses and their dramatically rapid molecular characterization. All these discoveries are addressed in the appropriate sections of this encyclopedia. Molecular biology, which was initially conceived during studies of bacterial viruses, is now being

applied to problems of the pathogenesis and epidemiology of animal and plant viral diseases, and the expansion in knowledge of these subjects in the next decade can be expected to exceed that of the previous century.

See also: **Tobamoviruses.**

Further Reading

Bos L (1995) The embryonic beginning of virology: unbiased thinking and dogmatic stagnation. *Arch. Virol.* 140: 613.

Fenner F and Gibbs AJ (eds) (1988) *Portraits of Viruses. A History of Virology*. Basel: Karger.

Hughes SS (1977) *The Virus. A History of the Concept*. London: Heinemann.

Joklik WK (1996) Famous institutions in virology. The Department of Microbiology, Australian National University and the Laboratory of Cell Biology, National Institute of Allergy and Infectious Diseases. *Arch. Virol.* 141: 969.

Matthews REF (ed.) (1983) *A Critical Appraisal of Viral Taxonomy*. Boca Raton: CRC Press.

van Helvort T (1994) History of virus research in the twentieth century: the problem of conceptual continuity. *Hist. Sci.* 32: 185.

Bacteriophages

Donna H Duckworth, Department of Molecular Genetics and Microbiology, College of Medicine, University of Florida, Gainesville, Florida, USA

With the recent establishment in the United States of a commercial enterprise whose sole purpose is to isolate bacteriophages for therapeutic purposes, the history of phage research has come full circle. Bacteriophages were first discovered at a time when bacterial disease was rampant and antibiotics were no more than a gleam in the eyes of several visionaries. And although it has never been definitively determined who first discovered phage, at least one of the individuals involved was determined to make that gleam a reality by utilizing the killing power of these viruses in the treatment and prevention of disease. That individual was the French-Canadian, Dr Felix d'Herelle.

In August 1916, Dr d'Herelle was asked to investigate an epidemic of dysentery in a Paris hospital. He had come to Paris after an illustrious career as a bacteriologist which had taken him literally around the world. At various times, he had observed a mysterious killing activity in some of his bacterial cultures, something that made clear spots on his agar plates, and decided to look for this activity in a patient with dysentery to see if it correlated with an amelioration of the symptoms of the disease. So he filtered the feces of a patient with dysentery daily for several days and incubated the filtrate with a culture of the dysentery bacillus. In one of those rare 'Eureka!' moments in the history of science, he found that on the fourth day of the man's disease, a filterable substance from his feces completely killed the dysentery pathogen. He says,

> The next morning, on opening the incubator, I experienced one of those rare moments of intense emotion which reward the research worker for all his pains: at the first glance I saw that the broth culture, which the night before had been very turbid, was perfectly clear: all the bacteria had vanished, they had dissolved away like sugar in water. As for the agar spread, it was devoid of all growth and what caused my emotion was that in a flash I had understood: what causes my clear spots was in fact an invisible microbe, a filterable virus, but a virus parasitic on bacteria. Another thought came to me also: 'If this is true, the same thing has probably occurred during the night in the sick man, who yesterday was in serious condition. In his intestine, as in my test-tube, the dysentery bacilli will have dissolved away under the action of their parasite. He should now be cured.' I dashed to the hospital. In fact, during the night, his general condition had greatly improved and convalescence was beginning.

D'Herelle showed that the agent that appeared in the intestines of individuals recovering from dysentery was not found in normal individuals or in individuals suffering from the acute stage of the disease, that it could be filtered and still retain its ability to kill the dysentery bacillus and that it would not grow on any artificial medium. And, because the killing or lytic activity could be serially transmitted, he concluded that it must be a 'living germ'. These observations were published in 1917 in the *Compte Rendus Academie Science* (Paris).

The paper excited considerable attention. Imagine if, at the height of the acquired immune deficiency syndrome (AIDS) epidemic, someone had announced that they had isolated a natural substance that would destroy the AIDS virus. The situation in 1917 was similar. Infected war wounds and dysenteric diseases were taking an enormous toll, and people everywhere cried out for a cure for these ills – for a magic bullet, as it were. After d'Herelle's report, it seemed to many that this had finally materialized, and people all over the world tried to isolate agents of transmissible lysis

this time around, be more successful? We can but hope.

See also: **Antivirals; Phage ecology, evolution and speciation; Phage Homologous Recombination; Phage toxins and disease; Phage transduction; Phages as cloning vehicles; Phages in industrial fermentations; Phages in soil.**

Further Reading

Cairns J, Stent GS, Watson JD (eds) (1966) *Phage and the Origins of Molecular Biology*. New York: Cold Spring Harbor Laboratory of Quantitative Biology.

d'Herelle F (1926) *The Bacteriophage and its Behavior*. Williams and Wilkins, Baltimore.

d'Herelle F (1949) The bacteriophage. *Sci. News* 14: 44.

Duckworth DH (1987) History and basic properties of bacterial viruses. In: Goyal SM, Gerba CP and Bitton G (eds) *Phage Ecology*. New York: John Wiley.

Duckworth DH (1995) Discovery of bacterial viruses. In Webster RG and Granoff A (eds) *Encyclopedia of Virology*, First Edition. Orlando: W. B. Saunders.

Twort, A. (1993) In focus, out of step: a biography of Frederick William Twort, FRS. Dover, NH: Alan Sutton Publishing.

Polio, Coxsackie, Echo and Other Enteroviruses

Joseph L Melnick, Baylor College of Medicine, Houston, Texas, USA

Introduction

The enteroviruses constitute one of the genera of the picornavirus family. The genus includes the polioviruses, the coxsackieviruses and the echoviruses of humans, plus a number of enteroviruses of lower animals (e.g. monkeys, cattle, pigs, mice). Over 100 serotypes are recognized, the first to be discovered being the polioviruses.

Early Developments

Reports on clinical and epidemiological aspects of poliomyelitis came in the late 19th and early 20th centuries from von Heine and Medin in Germany, Wickman in Sweden and Caverly in the USA, but the virological study of polioviruses only began in 1908 when Landsteiner, Popper and Levaditi transmitted the disease to monkeys, inducing typical histopathological lesions in the animals' spinal cords. Shortly thereafter, Flexner and Wollstein achieved monkey-to-monkey passage. As it turned out, however, their use of rhesus monkeys contributed to serious problems in the development of polio research. Flexner was director of the Rockefeller Institute for Medical Research in New York, and a highly respected advocate of scientific medicine. Unfortunately, he assumed that the disease in man paralleled precisely what he was observing in the rhesus monkeys infected in his laboratory. He influenced many of his colleagues through his belief that polioviruses were strictly neurotropic, because of the way they behaved in his rhesus monkeys. He believed that poliovirus entered the human body through the nasal route and proceeded directly to the central nervous system (CNS). Besides delaying progress in unraveling the pathogenesis of poliomyelitis, this conviction also led to a misguided and unfortunate attempt at prophylaxis in human beings. When zinc sulfate was administered to Flexner's rhesus monkeys to coat the lining of their noses, the animals were protected against development of paralytic polio when challenged by intranasal inoculation. Flexner entirely overlooked Kling's work in Sweden; as early as 1912, Kling and his associates reported isolation of poliovirus not only from the throat but also from the feces of both paralytic and nonparalytic patients. Meanwhile, on the basis of belief in the nasal portal of entry in the human infection and in the efficacy of zinc sulfate to protect that portal, many persons during a 1927 epidemic underwent what was, at least, considerable discomfort produced by administration of zinc sulfate, but received no protection against poliomyelitis.

In 1936, Sabin and Olitsky reported that rhesus monkeys succumbing to polio had olfactory lesions only when infected by intranasal instillation of virus, and not when virus was administered by other routes. Five years later, Sabin and Ward showed that olfactory bulbs from 65 fatal human cases were all negative for virus. This underscored the previous observations by pathologists of the rarity with which olfactory lesions had been observed in autopsy studies of human polio cases – a surprising observation if the infection route had been nasal.

In the late 1930s, American investigators had begun turning toward the possibility of an oral–alimentary route of infection. Investigators at the Pasteur Institute had successfully infected cynomolgus monkeys by the oral route. Impetus was added when Howe and Bodian came across a report by Muller describing spontaneously acquired polio in chimpanzees in a children's zoo in Cologne. They followed up this clue with a series of experiments establishing that chimpanzees were far more susceptible to infection by the oral route than other experimental primates. This

work excluded the olfactory portal and also showed that the disease of chimpanzees after oral administration of virus closely resembled that of human beings, both clinically and pathologically.

Evidence from human cases also began to accumulate; some 25 years after the almost-ignored reports of Kling and his associates, investigators again began looking for poliovirus in the alimentary tract. At this point it was not understood how poliomyelitis was spread. Although Wickman had postulated spread through inapparently infected persons, the circumstances under which the paralytic disease occurred, or whether the virus was in the infected or diseased individual, were unknown. The manner of transmission was puzzling: cases in epidemics seemed to move out from a focus, but in erratic patterns, for those afflicted usually had no direct contact with an earlier case. Even within a family where paralytic polio occurred, the disease was hardly ever seen in more than a single member.

At Yale University, the search for virus included specimens other than the spinal cord, and in 1938 John Paul made a 'heretical' report to the American Epidemiological Society on some of the findings: not only was virus found in large quantities in feces, but it was also recovered repeatedly over a period of weeks, from both patients and 'healthy carriers'. This report was met with skepticism by many in the audience, but it was soon confirmed and extended. The shift to the concept of poliomyelitis as an enteric infection was under way. Wickman's observations on the spread of virus by persons with inapparent infections could now be confirmed by laboratory documentation. The new concept also had direct implications for measures to control fecal contamination in hospitals and in households.

Still another series of questions was brought into focus: If polio was an enteric infection, could it be isolated from urban sewage? One of my first tasks upon joining the Yale Poliomyelitis Study Unit in 1940 was to devise methods for testing effluents from one of the large sewage treatment plants of New York City. During periods when paralytic polio was prevalent, I found poliovirus to be present in New York sewage in huge quantities. Knowing the quantity of virus in sewage, the amount excreted by a carrier each day, and the case rate in the area from which the sewage came, I could establish a ratio of inapparent infection to paralytic cases. This turned out to be more than 100 inapparent or subclinical infections for each paralytic case. I also learned that, although purified poliovirus could be readily inactivated by low doses of chlorine, virus in sewage or feces is resistant to extraordinarily high doses of chlorine.

Despite the increasing knowledge about polio that was gained through investigations that had to depend on large and expensive laboratory animals (monkeys and chimpanzees), it was clear that simpler systems were badly needed. Following original efforts by Levaditi in 1913, there had been two decades of repeated attempts to grow polioviruses in various kinds of culture substrates and conditions, with little success. Sabin and Olitsky in 1936 reported the successful growth of poliovirus in tissue culture. However, their study was done with the MV strain of poliovirus, which had gone through 20 years of intracerebral passage from monkey to monkey at the Rockefeller Institute. When these investigators attempted to grow this highly neurotropic strain in human embryonic nervous tissue or in nonnervous tissue from the viscera of the same embryos, it grew only in the nervous tissue. Thus it became accepted doctrine that poliovirus could be grown only in human nervous-system tissue. Such tissue is difficult to obtain, and furthermore its use as a substrate for growing antigenic material for a possible vaccine was precluded because of the known encephalitogenic potential of administering tissue of nervous system origin (as in the case of the early rabies vaccine). Moreover, proof that the poliovirus had indeed multiplied in these cultures still depended upon inoculating monkeys with the tissue culture fluid and duplicating the disease in them. Another 13 years elapsed before the landmark report by Enders, Weller and Robbins, who used different strains of poliovirus and showed that poliovirus could indeed be grown in a variety of tissue cultures and could be assayed by observing its cytopathic effect *in vitro*.

Meanwhile, the search for a susceptible laboratory animal more convenient than monkeys and chimpanzees continued. Armstrong at the National Institutes of Health in 1937 began inoculating several rat species with the Lansing strain of poliovirus. After some partial but erratic success with the cotton rat, he eventually succeeded in producing experimental poliomyelitis in a series of these rodents, and soon thereafter adapted the virus strain from the cotton rat to mice. Other strains of poliovirus were not as readily adapted to rodents, but the mouse model with Lansing strain (subsequently identified as type 2) contributed immensely to studies of poliovirus. With this test animal, quantitative techniques were established for titrating the virus, and a neutralization test in mice was developed. The distribution and duration of type 2 neutralizing antibody in human populations was determined. Thus, we began to have access more readily to the serological history of poliomyelitis in different communities, to correlate the development of antibody with the ages at which cases occur, and to

find that early, and immunizing, exposure to this virus took place in less developed nations, as we found in Egypt in 1952. Within industralized nations, such early immunizing infections occurred in crowded urban slums, but, in affluent segments of industrialized societies, exposure to poliovirus was postponed.

Many new avenues of investigation were opened by the findings that the chimpanzee could be readily infected by oral administration of poliovirus, could become a transient intestinal carrier of the virus but seldom developed clinical signs, and did indeed show many parallels to human beings similarly infected. Some of the unanswered questions that could now be pursued with new insights included the transmission of poliovirus in nature, the patterns of pathogenesis, and the site and course of the infection that results in the development of antibody in the human being. Thus we could approach questions of how efforts toward the development of vaccines might best proceed.

In the 1930s, Burnet, looking at the studies of neutralization with epidemic strains and at the fact that antibody was already present in patients' blood at the onset of CNS-related symptoms, suggested that if antibodies were already present at the 'beginning' of the illness, then such antibodies were not able to protect against the disease. However, Herdis von Magnus and I, in studies with rhesus, cynomolgus and vervet monkeys, induced paralytic disease by feeding them virus. Paralysis occurred within 1–2 weeks, but the monkeys already had neutralizing antibodies in their blood by the first day of paralysis. These findings reopened the question of whether, in the human disease, antibodies found this early were indeed irrelevant to current disease, as Burnet thought, or were a response to a current pathogenic process that included growth of the virus in nonnervous tissue. A number of the monkeys in our study had remained asymptomatic but they also developed antibody, yet histologic examination showed them to be free of CNS lesions. Thus, not only the early antibody production in the paralyzed monkeys but also the development of antibodies in those without CNS lesions suggested that antibody development was an early response to infection of tissue outside the CNS, and that such antibody might actually protect the CNS from virus invasion.

In 1952, Dorothy Horstmann at Yale and David Bodian at Johns Hopkins reported the isolation of virus from the blood of cynomolgus monkeys a few days after oral administration of virus but several days before the appearance of symptoms. When these findings were followed by observations of a similar pattern in humans, hope for immunization increased: if viremia was the route by which virus entered the CNS, then the virus might be blocked by circulating antibody and thus be prevented from invading the human CNS. One result of these developments was a trial to determine whether γ-globulin could be effective in preventing paralytic polio. A large and well-conducted field trial under Hammon's direction in 1952 showed that passive antibody could indeed protect against paralytic polio, although only for about 2–5 weeks. This trial also showed that the quantity of antibody need not be large, and it favored the hope that an effective method of active immunization could be developed.

Application of Laboratory Findings

We had investigated a polio outbreak in North Carolina in 1944, but returned in 1948 when a much larger one occurred. There were more than 2200 cases in the state, an attack rate of about 65 cases per 100 000 population. At the beginning of the summer 'polio season', when we learned that four cases had been reported, we had thought that an epidemic might be in the making, and in early June we obtained blood samples from children in Winston-Salem, along with placental sera which were used to determine the proportion of adults (or newborns) possessing antibodies at that time. These 'pre-epidemic' specimens were then matched by second bleedings obtained in November for testing as 'post-epidemic' specimens. This was the first time that pre- and post-epidemic sera had been studied for polio antibodies, even though, among the 248 children contributing paired specimens, no polio-like illnesses were reported during 1948.

Nada Ledinko and I found that the antibody patterns in the spring of 1948 were similar for all three types of poliovirus; they indicated a low incidence of infection during the previous 4-year period. During the 1948 epidemic, antibodies against type 1 and type 2 were acquired; the antibody conversions indicated infection rates of 23% with type 1 and 17% with type 2. Six children acquired antibodies against both type 1 and type 2 viruses. The development of antibodies took place to a greater extent in children under the age of 4 years than in those 4 years and older. No child in the study produced type 3 antibodies that summer.

The age-specific rates of inapparent infection by type 1 were compared with age-specific attack rates for polio in the epidemic. The number of subclinical type 1 infections per case was found to be about 100.

Previous infection of individuals with type 2 or type 3 virus did not prevent their infection with type 1 virus in 1948. In the spring of 1948, antibody to more than one type of poliovirus was a rare occurrence in

children of 4 years or under, whereas 41% of those over 4 years of age had antibodies against two or three types of poliovirus. This correlated with the fact that in this area the last outbreak prior to that of 1948 occurred in 1944, when we had isolated type 1 and type 3 viruses.

In the lower socioeconomic group of Winston-Salem, antibodies to all three types of poliovirus were present in a larger sector of the population, both in spring and in fall, than in the upper economic group. In this regard, poliomyelitis antibody patterns differed from those for influenza and mumps, for these two viruses, which are spread by respiratory pathways, the patterns were the same in both socioeconomic groups.

The studies in North Carolina also contributed to another series of investigations we were pursuing at the time on polio-related viruses: previously unknown mouse-pathogenic agents were being found in the human enteric tract. A virus that we named High Point circulated widely in Winston-Salem during the summer of 1948, but we could not associate any disease with infections by this virus. Soon thereafter the agent was identified as one of the new coxsackieviruses and became the A4 prototype. This leads me to a discussion of classification and nomenclature of polio and related viruses.

Classification of Polioviruses

> It might be mentioned that although foreseen and unforeseen problems inherent in the classification of any biologic species have existed since the days of Linnaeus, the main reason for laboring the point ... is that the manner in which a given entity is classified usually reflects the contemporary ideas concerning it ...

So wrote John Paul in 1971, looking back on changes that had taken place over the years in the classification and nomenclature of polioviruses.

Serious questions of picornavirus classification and nomenclature were first made a specific issue at the time of the 4th International Congress for Microbiology held in Copenhagen in 1947. There was debate about distinguishing poliovirus isolates not only from each other but also from human encephalitis viruses and from several mouse agents that had properties similar to those of the human polioviruses. Many virologists felt that the problem had not been adequately met at the Congress, and this led Paul and me to circularize virologists in the USA with alternative suggestions for the definition and classification of poliomyelitis virus. A related concern was that, at that time, no-one knew how many serological types of human poliomyelitis existed, clearly a crucial point for many reasons, including our ability to assess the possibility for future immunization.

In the 1930s many investigators already suspected that more than one antigenic type of poliovirus existed, and in fact a report had been published by Burnet and Macnamara in 1931 describing antigenic differences between two Australian strains of poliovirus. Although a number of respected scientists could not accept the idea of diversity among the poliovirus strains, references to antigenic differences among them continued to be made from time to time. Robert Ward and I, in reporting adaptation of two additional human polio strains to rodents, wrote

> Both strains cross immunologically with each other, and with the Lansing strain, but are immunologically distinct from two other 'non-adaptable' strains, the 1944 North Carolina strain and the 1945 Philippine Islands strain. Although ready means of designating strains immunologically as poliomyelitis A, B, or C are not yet available, nomenclature and classification will be discussed.

One year later, Bodian and his colleagues reported definitive results that three antigenic types existed.

With a considerable amount of effort involving the facilities of several laboratories and the use of many monkeys, it was clearly proven that three, and only three, serologic types of poliovirus exist. Furthermore, it was shown that type 1 was by far the most frequent type being recovered from paralytic polio cases.

An International Committee on Nomenclature in 1948 took on the task of establishing a provisional system for nomenclature and classification of poliomyelitis virus. This Committee reported to the First International Polio Conference held in New York later that year, and there followed the establishment of a study group of three members (H. von Magnus, J. H. S. Gear and J. R. Paul), who were appointed by the Virus Subcommittee (headed by Sir Christopher Andrewes) of the International Nomenclature Committee, International Association of Microbiological Societies. This study group incorporated the suggestion, made originally by Burnet of Australia, that the name poliomyelitis virus should be shortened so as to have some uniformity with the provisional names arrived at by other nomenclature groups. Thus the term poliovirus was coined, in line with already accepted terms such as poxvirus, herpesvirus and myxovirus. Paul later wrote

> In retrospect, the only lasting feature of this committee's suggestions was that the name polio-

virus became universally adopted. For within two years the family of polioviruses was embraced by a far larger group. What was more extraordinary was that the uniqueness of polioviruses in inducing characteristic lesions in the central nervous system was given up. Now it was the intestinal tract with which taxonomists were concerned. Viruses that had similar physical and chemical properties, i.e. small size and resistance to ether, and behaved in a similar fashion biologically with regard to the human intestinal tract as their special, though transient, habitat were all placed together in a new category regardless of whether or not they affected the central nervous system. Thus the large family of enteroviruses was created as a subgroup of the newly designated picornaviruses. Included besides polioviruses in the human enterovirus group were the coxsackie and echoviruses. This complete change in the image of polioviruses coincided with the general decline in the incidence of paralytic poliomyelitis. As vaccination became more and more effective the tragic paralytic results of the disease began to fade, and the virus acquired the more earthy name of its main anatomical site of predilection, which had so long been denied it. Polioviruses thus became members of the enterovirus family, incredible as this would have sounded to past generations of investigators.

Coxsackieviruses

The discovery of the large group of viruses to which the polioviruses belong came about in 1948 as follows. Dalldorf and Sickles of the New York State Health Department Laboratories, in their attempts to find an animal more readily available than monkeys for poliomyelitis studies, tried an animal that had been used successfully some years earlier with yellow fever virus. This new animal was the newborn (suckling) mouse. When these investigators inoculated suckling mice with fecal suspensions from two suspected polio patients from the town of Coxsackie, New York, the mice became paralyzed, but not from a poliovirus infection. A new virus had been discovered. The virus differed from polioviruses and from several other neurotropic viruses. It induced fatal disease with paralysis in baby mice, but not in adult mice, adult hamsters or rhesus monkeys. The lesions were located in the skeletal muscles rather than in the CNS, that is, the disease of the suckling mice was a myositis rather than an encephalomyelitis. The isolates subsequently became the first prototypes of the group A coxsackieviruses.

The use of newborn mice helped solve a problem that had been puzzling in some poliomyelitis outbreaks, in which the paralytic cases and the 'nonparalytic polio' (meningitis) cases peaked at different times. This did not make sense in light of what was known about the relationship of meningitis and paralysis as differing manifestations of infection with the same virus. We had been trying to find explanations by studying viruses from polio epidemics that showed this unusual pattern, and we turned up several new viruses in specimens from patients in the atypical polio epidemics. In 1949, together with Curnen and Shaw, I reported on a number of these mouse-pathogenic viruses. Many of our strains not only differed antigenically from the New York viruses, but also differed markedly in the pathologic picture that they produced in suckling mice. With Dalldorf's isolates, the principal damage to the baby mice was generalized and widespread myositis, affecting chiefly the striated muscles. The viruses that we discovered caused only focal and limited myositis in striated muscles, but produced degeneration of the brain, pancreas, heart muscle and embryonic fat pads under the skin. Clearly different from the New York isolates, they became the prototypes of the group B coxsackieviruses.

As more of these agents were found, it was recognized that, unlike most of the viral agents known up to that time, the new coxsackieviruses were associated not with one but with several different kinds of illnesses: one such was 'nonparalytic poliomyelitis' (aseptic meningitis). Thus we came to realize that not all of the 'nonparalytic polio' cases were due to poliovirus infections; this fact accounted for the differences sometimes observed in the epidemic peaks of the paralytic and nonparalytic illnesses. Further, we discovered that dual infections with poliovirus and coxsackieviruses could occur; hence, a patient's disease might be caused by a poliovirus but he could at the same time be excreting a coxsackievirus as a fellow traveler, or vice versa! Illnesses caused by the new viruses were herpangina, myocarditis, rash and pleurodynia, also known as epidemic myalgia or devil's grip. This illness had also been termed Bornholm disease, which some European workers had considered to be limited to the Danish island of that name.

We used every possible lead to gain insight into these new viruses, including some unhappy personal experiences. An isolate from my 3-year-old daughter, Nancy, became the prototype for coxsackievirus B3. And I, together with other members of our laboratory group, involuntarily demonstrated another way in which virologists sometimes learn about their viruses. From a number of infections that occurred among workers in the laboratory, we developed a clearer picture of the course of coxsackievirus infections and

the development of antibodies. We had convincing personal evidence that symptoms of coxsackievirus infections could rightly be said to include 'devil's grip'.

In working with coxsackieviruses in suckling mice, which already became insusceptible to the virus by 1 week of age, the ordinary method for testing antibody development and immunity could not be used: if the animals were given an immunizing dose at birth, they could not be challenged after developing antibodies, because by the time antibodies and immunities appeared, the animals would have been too old to be susceptible anyway. So we devised a method to fit this need: mother mice were vaccinated, and then both homotypic and heterotypic challenges were administered to the newborns. This was the *in vivo* means by which we unraveled the separate serotypes of the group B coxsackieviruses.

Chimpanzees were readily infected, but asymptomatically, following oral administration of various strains of the newly discovered coxsackieviruses, and we were already beginning to see some important similarities between these viruses and the polioviruses. This gave special impetus to extensive studies on the natural history of the coxsackieviruses.

Neutralizing antibodies and cross-protection were found to be specific for each of the different coxsackievirus types and to be maintained for at least 2 years in these chimpanzees. Complement-fixing (CF) antibodies, however, showed marked cross-reactions among coxsackieviruses. Although the animals usually responded with rises in homologous CF antibodies after feeding or inoculation with one serotype, heterologous CF antibodies to a variable number of other coxsackieviruses also increased significantly at the same time.

Tissue Culture

Just as we began to get a grasp on how to work with the coxsackieviruses that were turning up in such variety and quantity from isolates in laboratory mice, another world was opened up. For much of the work discussed above, the presence of poliovirus in a specimen obtained from patients or from their environment could be demonstrated only by producing the disease in monkeys and examining their spinal cords for the characteristic histological lesions of neuronal damage and perivascular infiltrations. Monkeys had to be trapped in Asia or Africa and transported to the laboratory. The supply was often irregular, and research sometimes was delayed for many weeks awaiting a new shipment. Poliomyelitis laboratories had to be equipped with extensive quarters and a large staff to care for large numbers of monkeys; valuable research time was used in dealing with the monkey's special nutritional needs and their susceptibility to diseases acquired in nature, such as tuberculosis, dysentery and parasitic infections.

Then came cell cultures. Cell cultures of nerve tissue had been successfully prepared in 1907 by Harrison at Yale, and there followed some efforts to cultivate polioviruses in such cultures, but there was no way of knowing whether the viruses multiplied except by inoculating monkeys. In 1942 an important new dimension was added by a young research fellow from China, Chen-hsiang Huang, working at Columbia University. He showed that a neurotropic virus, western equine encephalitis virus, when cultivated in cultures prepared from minced muscle of chick embryos, produced characteristic cell damage that could be seen by microscopic examination. By measuring the effects of the virus on cells, Huang was able to calculate the quantities of virus present in the culture, and also to test serum for the presence of antibodies against the virus. In addition, he developed a virus test based on metabolic (color) changes in the culture fluid that could be observed with the naked eye.

Both of these discoveries foreshadowed by several years the work of others that became much better recognized in the development of virology. At the time, the significance of Huang's discoveries may have been missed, partly because he soon returned to his native China and did not pursue his findings. However, he remained active and productive in China's biomedical community, where he became the Director of the Institute of Virology, Chinese Academy of Medical Sciences, Beijing.

A few years later, Enders, Weller and Robbins demonstrated that poliovirus could be grown in cultures of human cells derived from tissues other than those of the nervous system, and produced visible cell damage. The characteristic cytopathic effect in these cultures could be assigned scores, and thus could serve as a basis for quantification of the virus and of its antibody. The important work of these investigators was recognized by their receipt of the Nobel Prize in 1954. With monkey or human cell cultures, relatively simple and rapid tests were soon devised to detect the polioviruses, to measure them quantitatively and to determine the levels of antibodies that developed after infection.

Now the virus could be readily purified and visualized in the electron microscope. Investigators could analyze the biochemical and biophysical characteristics of the virus, trace the details of its multiplication, and explore the mechanisms by which it damages individual cells and the human host. Of

immediate importance, this new technology led to the production of the huge quantities of uniform poliovirus stocks that were required for preparation of killed vaccines. Furthermore, it opened the way to the development of live poliovirus vaccines, since pathogenic poliovirus, by propagation under specified conditions through many generations in cells widely different from human spinal cord cells, could be deliberately attenuated so as to become no longer neurovirulent for man. Less well recognized, but at least as important, were the contributions of the tissue culture methods to the development of cell and molecular biology and to cancer research.

Echoviruses and the Concept of Enteroviruses

The development in Enders' laboratory also allowed virologists working with polioviruses and coxsackieviruses at that time to detect a whole new assortment of previously unknown viruses. These viruses were abundant in human stools and grew in tissue cultures, but did not cause disease in laboratory animals. Moreover, at the time of their discovery they could not be clearly associated with specific human diseases. During 1950 we recovered hundreds of isolates from stools of patients with 'nonparalytic poliomyelitis'. As we looked further, these viruses were also found in healthy individuals in various areas of the world. By 1953 these agents were beginning to be called 'orphan' viruses, that is, viruses in search of a parent disease. A more explicit name was agreed upon, enteric cytopathogenic human orphan (or ECHO) viruses – a name that shortly became echoviruses. By 1955 it was recognized that some order had to be brought into the field, and, under the sponsorship of the National Foundation for Infantile Paralysis (NFIP) (which since has become the National Foundation), a Committee on the ECHO viruses was established, charged with continuing to explore these viruses, their interrelationships and their possible relation to human disease. The committee consisted of G. Dalldorf, J. F. Enders, W. M. Hammon, A. B. Sabin, J. T. Syverton and myself (chairman). A cooperative study on the antigenic types then available (eight from our laboratory, five from Sabin's laboratory and three from Hammon's laboratory) resulted in the differentiation of 13 distinct viruses.

We had thought at first that as soon as an 'orphan' virus could be attached to a parent disease, it would be removed from the echovirus group, but things did not turn out that way. The echoviruses and the coxsackieviruses were shown to cause various illnesses, but a direct and regular linkage of any one serotype with any one disease was (and is) the exception rather than the rule. Any one type may cause any of several syndromes, and a typical 'enteroviral' syndrome may be caused by any of many serotypes. Besides the well-known associations of enteroviruses with diseases such as poliomyelitis, meningitis, encephalitis, epidemic pleurodynia, rashes, hand-foot-and-mouth disease, myocarditis, herpangina and acute hemorrhagic conjunctivitis, as well as minor malaises and undifferentiated febrile illnesses, these viruses are also associated with diseases of the respiratory tract more frequently than is usually recognized. Enterovirus-associated respiratory diseases include pneumonitis of infants, pneumonia and bronchiolitis, as well as the common cold and other upper respiratory illnesses. It should be mentioned that not only polioviruses, but also a number of other enteroviruses may at times be etiologically associated with paralytic disease. Fortunately such cases are rare.

As studies of polioviruses, coxsackieviruses and echoviruses continued, it became more and more clear that these viruses shared far more than a human enteric habitat, and they were placed in one group called the enteroviruses [Committee on the enteroviruses, G. Dalldorf, J. F. Enders, W. M. Hammon, A. B. Sabin, J. T. Syverton, H. A. Wenner, and myself (chairman)].

New Enterovirus Types

As more enterovirus strains were isolated and studied, difficulties were sometimes encountered in attempting to assign them to the coxsackievirus and echovirus subgroups on the basis of the distinctions initially made between these groups (mouse pathogenicity for coxsackieviruses and exclusive growth in tissue cultures for echoviruses). The mouse-pathogenic prototype for coxsackievirus A23 turned out to be a host-range variant of a virus already established as echovirus type 9. The confusions of classification can be seen in the title of a paper published in 1957, 'Association of mouse pathogenic strains of echovirus type 9 with aseptic meningitis', when the cardinal property of an echovirus was its failure to produce disease in mice! In efforts to deal with these problems, the Committee on Enteroviruses in 1962 had suggested that all previously recognized polio-, coxsackie- and echoviruses be designated simply as enteroviruses and that each be given a serial number. One of the major objections of the International Enterovirus Study Group to such sequential numbering was that the new numbers would 'minimize much of the benefit gained from the rich virological and clinical literature in this field', and suggested instead that the designation of 'unclassified picornavirus' be used

when only a single strain of a virus was known, or when a virus did not fall clearly into one or another subgroup. However, neither recommendation gained wide acceptance.

The problem was highlighted once more by experience with the DN19 virus, an agent isolated by Alan Phillips in my laboratory. The virus was accepted by the Directors of the World Health Organization (WHO) Enterovirus Reference Laboratories as a previously unrecognized enterovirus and was designated as echovirus type 34. Data obtained subsequently, however, showed that DN19 virus should be considered an antigenic variant of a previously recognized enterovirus, coxsackievirus A type 24 (prototype strain, Joseph). In contrast to coxsackievirus A23, which was an example of a mouse-pathogenic echovirus, DN19 became an example of a coxsackievirus which was nonpathogenic for mice. But had DN19 been discovered before the coxsackievirus A24 prototype Joseph, then it would have been accepted as an authentic echovirus. If the Joseph strain had been discovered subsequently, it would have been considered a mouse-pathogenic echovirus. A further problem was presented by the Fermon enterovirus, which in 1969 had not yet been given a subgroup or a numerical designation because one strain of this virus produced lesions in suckling mice characteristic of the group A coxsackieviruses, but three other strains antigenically related to Fermon behaved as true echoviruses.

In view of these and other intratypic divergences, the previous proposal for enterovirus terminology was modified. Instead of sequential numbering of all agents in the group, we proposed that current subgroup names of all serotypes of the polio-, coxsackie- and echoviruses recognized through 1970 be retained, but that enterovirus serotypes recognized after that year not be assigned to subgroups. To avoid confusion with the enterovirus numbers proposed previously, we suggested that the new series begin with enterovirus type 68, and that this designation be given to the Fermon virus. This allowed for the three polioviruses, the 24 group A coxsackieviruses, the six group B coxsackieviruses and the 34 echoviruses reported previously in the literature. We did not imply, however, that each of these 67 viruses is antigenically distinct. Early on, echovirus 10 had been reclassified as the prototype reovirus, and echovirus 28 as the prototype rhinovirus. Echovirus 9 was found to be related to coxsackievirus A23, echovirus 34 to coxsackievirus A24, echovirus 1 to echovirus 8 and coxsackievirus A13 to coxsackievirus A18. Nevertheless, we felt that it would be the better part of wisdom to start the new classification with enterovirus 68.

Among the new enterovirus types, enterovirus 68 has caused lower respiratory tract illnesses, enterovirus 70 is the agent of widespread epidemics of acute hemorrhagic conjunctivitis and enterovirus 71 has caused outbreaks of aseptic meningitis and encephalitis, as well as hand-foot-and-mouth disease, in a number of countries.

An important feature of infections with enterovirus 71 has been meningitis. This virus has exhibited a variety of clinical manifestations in different regions of the world and at different times. In the California outbreak from which the prototype strain was reported, meningitis predominated but there were other CNS manifestations including a fatal encephalitis case. Meningitis also predominated in outbreaks in Europe, but there were also some cases of hand-foot-and-mouth disease in some of these outbreaks, and this latter syndrome predominated in Japan. In some parts of Japan a number of the patients had concomitant hand-foot-and-mouth symptoms and meningitis symptoms. This agent received more attention when it was found to cause a severe outbreak of poliomyelitis-like disease in 1975 in Bulgaria: of 700 patients, about 21% developed poliomyelitis-like paralysis and 44 died. A large outbreak in Hungary included chiefly meningitis cases, but fatal encephalitis was also reported.

Hepatitis A virus was provisionally classified as enterovirus 72. After decades of investigation, this virus, which inhabits the enteric tract, had been shown clearly to have many of the physicochemical properties of an enterovirus. However, it has been found to have a genetic composition sufficiently distinct to warrant its being classified as the prototype of a separate picornavirus genus. The name, heparnavirus, has been suggested, to indicate tropism for hepatocytes and an RNA genome.

Future Perspectives

It is interesting to note that the enteroviruses, originally established on provisional bases, now appear to form a 'real' genus, in which the members are indeed related in fundamental ways. The original grouping was based on small size (about 27 nm), an RNA genome, resistance to ether and mild acid and an enteric habitat. The grouping has been validated by studies utilizing sophisticated techniques of modern molecular virology that allow us to compare the genetic makeup of these viruses and details of their structure and their mode of replication.

Regarding the enteroviruses and their relatives within the picornavirus family, new methods have yielded many details. A high degree of homology exists between the genomes of the various members of

the enterovirus genus. The virion consists of a capsid shell of 60 subunits, each of four proteins (Vp1–Vp4), arranged with icosahedral symmetry around a genome made up of a single strand of positive-sense RNA.

By means of X-ray diffraction studies, the molecular structures of poliovirus and rhinovirus have been determined. The three largest viral proteins, Vp1–Vp3, have a very similar core structure in which the peptide backbone of the protein loops back on itself to form a barrel of eight strands held together by hydrogen bonds (the β-barrel). The amino acid chain between the β-barrel and the N- and C-terminal portions of the protein contains a series of loops. These loops include the main antigenic sites that are found on the surface of the virion and are involved in the neutralization of viral infection. There is a prominent cleft or canyon around each pentameric vertex on the surface of the viral particle. The receptor-binding site used to attach the virion to a host cell is thought to be located near the floor of the canyon. This location would protect the crucial cell attachment site from structural variation influenced by antibody selection in hosts, as the canyon is too narrow to permit deep penetration of antibody molecules.

We anticipate many new developments as investigators delve deeper into the structure of the enteroviruses and their mode of replication and survival in nature.

See also: **Coxsackieviruses (*Picornaviridae*); Echoviruses (*Picornaviridae*); Enteric viruses; Enteroviruses (*Picornaviridae*): Human enteroviruses (serotypes 68–71); Hepatitis A virus (*Picornaviridae*); Polioviruses (*Picornaviridae*): General features.**

[This article is reproduced from the 1st edn (1994).]

HOG CHOLERA VIRUS (*FLAVIVIRIDAE*)

Jan T van Oirschot, Institute for Animal Science and Health, Lelystad, The Netherlands

History

Hog cholera (synonym: classical swine fever) is a highly contagious virus disease of swine. The infection can run an acute, subacute, chronic or inapparent course, mainly depending on the virulence of the virus. High-virulent virus causes acute disease and high mortality, whereas infections with low-virulent virus often go unnoticed.

The first description of a cholera-like disease among pigs that was later considered to have been hog cholera was from Tennessee, about 1810. Other outbreaks were reported from Ohio in the early 1830s. Hog cholera may possibly have occurred in France in 1822 and in Germany in 1833, but other reports suggest the disease first appeared in the UK in 1862 and subsequently spread to the European continent. The infection was reported from South America in 1899 and from South Africa in 1900.

In 1903, De Schweinetz and Dorset proved the disease was caused by a virus. During the epizootic in the USA in 1914, approximately 90% of the pigs in infected herds died, which was estimated to be a loss of 100 million dollars. Hog cholera was the most devastating disease of pigs and consequently eradication programs were implemented, which have been successful in many countries.

Taxonomy and Classification

Hog cholera virus (HCV) belongs to the family *Flaviviridae*. Together with bovine virus diarrhea virus (BVDV) and border disease virus (BVD), it constitutes the genus *Pestivirus*.

Properties of the Virion

It is among the smallest enveloped animal RNA viruses known, measuring between 40 and 50 nm in diameter; the envelope surrounding the isometric nucleocapsid core measures 27–29 nm in diameter. Fringe-like projections of 6–8 nm have been demonstrated on the surface of the virion.

The buoyant density has been reported to be between 1.12 and 1.17 g ml^{-1} and sedimentation coefficient values of 140–180 S have been found. The large variations reported in size, shape and density of the virion may be due to: (1) the fact that the virus is

the enterovirus genus. The virion consists of a capsid shell of 60 subunits, each of four proteins (Vp1–Vp4), arranged with icosahedral symmetry around a genome made up of a single strand of positive-sense RNA.

By means of X-ray diffraction studies, the molecular structures of poliovirus and rhinovirus have been determined. The three largest viral proteins, Vp1–Vp3, have a very similar core structure in which the peptide backbone of the protein loops back on itself to form a barrel of eight strands held together by hydrogen bonds (the β-barrel). The amino acid chain between the β-barrel and the N- and C-terminal portions of the protein contains a series of loops. These loops include the main antigenic sites that are found on the surface of the virion and are involved in the neutralization of viral infection. There is a prominent cleft or canyon around each pentameric vertex on the surface of the viral particle. The receptor-binding site used to attach the virion to a host cell is thought to be located near the floor of the canyon. This location would protect the crucial cell attachment site from structural variation influenced by antibody selection in hosts, as the canyon is too narrow to permit deep penetration of antibody molecules.

We anticipate many new developments as investigators delve deeper into the structure of the enteroviruses and their mode of replication and survival in nature.

See also: **Coxsackieviruses (*Picornaviridae*); Echoviruses (*Picornaviridae*); Enteric viruses; Enteroviruses (*Picornaviridae*): Human enteroviruses (serotypes 68–71); Hepatitis A virus (*Picornaviridae*); Polioviruses (*Picornaviridae*): General features.**

[This article is reproduced from the 1st edn (1994).]

HOG CHOLERA VIRUS (*FLAVIVIRIDAE*)

Jan T van Oirschot, Institute for Animal Science and Health, Lelystad, The Netherlands

History

Hog cholera (synonym: classical swine fever) is a highly contagious virus disease of swine. The infection can run an acute, subacute, chronic or inapparent course, mainly depending on the virulence of the virus. High-virulent virus causes acute disease and high mortality, whereas infections with low-virulent virus often go unnoticed.

The first description of a cholera-like disease among pigs that was later considered to have been hog cholera was from Tennessee, about 1810. Other outbreaks were reported from Ohio in the early 1830s. Hog cholera may possibly have occurred in France in 1822 and in Germany in 1833, but other reports suggest the disease first appeared in the UK in 1862 and subsequently spread to the European continent. The infection was reported from South America in 1899 and from South Africa in 1900.

In 1903, De Schweinetz and Dorset proved the disease was caused by a virus. During the epizootic in the USA in 1914, approximately 90% of the pigs in infected herds died, which was estimated to be a loss of 100 million dollars. Hog cholera was the most devastating disease of pigs and consequently eradication programs were implemented, which have been successful in many countries.

Taxonomy and Classification

Hog cholera virus (HCV) belongs to the family *Flaviviridae*. Together with bovine virus diarrhea virus (BVDV) and border disease virus (BVD), it constitutes the genus *Pestivirus*.

Properties of the Virion

It is among the smallest enveloped animal RNA viruses known, measuring between 40 and 50 nm in diameter; the envelope surrounding the isometric nucleocapsid core measures 27–29 nm in diameter. Fringe-like projections of 6–8 nm have been demonstrated on the surface of the virion.

The buoyant density has been reported to be between 1.12 and 1.17 g ml^{-1} and sedimentation coefficient values of 140–180 S have been found. The large variations reported in size, shape and density of the virion may be due to: (1) the fact that the virus is

heavily cell-associated and therefore difficult to purify from host cell membranes; and (2) the host cell system used for viral propagation.

Properties of the Genome

The virus has a single-stranded linear RNA genome of positive polarity of about 12.3 kb. The genome contains one large open reading frame that codes for a hypothetical large polyprotein of 3898 amino acids, that is cleaved, co- and post-translationally, by host cell- and virus-encoded proteases to yield mature viral proteins. The open reading frame is flanked by a 5′ noncoding region of almost 400 nucleotides and a 3′ noncoding region of approximately 200 nucleotides, which lacks significant polyadenylation. The order of the gene products along the open reading frame is as follows:

NH_2-(N^{pro}-C-E^{rns}-E1-E2-p7-NS2.3-NS4A-NS4B-NS5A-NS5B)-COOH

Properties of the Viral Proteins

The nonstructural N^{pro} is a protease with autoproteolytic activity; it has no counterpart in flaviviruses. The nucleocapsid protein (C) precedes the three envelope glycoproteins. The E^{rns} protein (formerly called gp42) forms a disulfide-linked homodimer. The mechanisms by which it is associated with the virion is unknown; it is also secreted from infected cells. A remarkable feature of E^{rns} is its RNase activity, which is unique among viruses. The function of this enzymatic activity is as yet unclear. The E^{rns} protein exhibits a strong immunosuppressive effect *in vitro*, and elimination of the RNase activity results in a cytopathogenic virus (HCV is normally noncytopathogenic). The E1 protein (gp31) is, as a disulfide-linked E1–E2 heterodimer, present in the viral envelope, and the E2 (gp53) as a disulfide-linked homodimer and as a heterodimer with E1. The E2 protein is composed of at least four antigenic domains, three of which give rise to neutralizing antibodies. The E2 protein expressed in a replicating vector (porcine herpesvirus) or produced in baculovirus and used as a subunit vaccine confers strong protection in pigs against a lethal challenge with HCV.

The p7 protein is probably not incorporated in the virion. The remaining part of the open reading frame is coding solely for nonstructural proteins. The C-terminal two-thirds of NS2.3 contains sequence motifs like NS3 in other flaviruses. This protein has protease and nucleoside triphosphatase (NTPase) activity. The role of NS4A and NS4B in virus replication is not known. The NS5B protein exhibits characteristic sequence motifs suggestive of an RNA polymerase.

Physical Properties

The resistance of HCV to physical and chemical treatment is partly dependent on the physical state of the material containing the virus. For instance, in cell culture fluid the virus is inactivated more rapidly than in defribinated blood. The mean inactivation rate at 56°C was found to be between 1.6 and 2.7 log units in 30 min. Below pH 4 and above pH 11, viral infectivity is quickly lost. Because the virion envelope contains lipids, solvents such as chloroform and ether easily inactivate HCV. In pork, the virus remains infectious for months, whereas in the environment outside the host the virus is usually inactivated in a couple of days; however, in liquid pig manure, HCV may survive for 2 weeks at 20°C and more than 6 weeks at 4°C. For disinfection, 1–2% sodium hydroxide is most suitable.

Geographic and Seasonal Distribution

HCV is distributed worldwide; it is regularly diagnosed in Africa, Asia, South and Latin America and in Europe. The virus has been eradicated from Australia, Canada, the USA, the Scandinavian countries, Ireland, the UK and Switzerland, but it still circulates in areas of Europe with a dense pig population. Between 1991 and 1996 the European Union was plagued by 530 outbreaks. In 1997 a devastating epidemic swept pig-dense regions in The Netherlands. The number of outbreaks was between 400 and 500. Consequently, 10 million pigs were destroyed and the total losses of the eradication program amounted to 2 billion US dollars. The virus has infected wild boar populations in various European countries, but not those in the USA. In Western European countries there are seasonal peaks in spring and autumn and a 3–4 year periodicity, associated with the so-called 'pig cycle'.

Host Range and Viral Propagation

The pig is the sole natural host of HCV. Experimental hosts include goats, sheep, calves, deer and rabbits. Although HCV can replicate in nonporcine cells, porcine kidney or testicle cells, be it primary cells or established cell lines, are normally used for propagation. Under single growth-cycle conditions, the first progeny virus is detected at 5–7 h after infection, followed by an exponential growth that peaks about 15 h after infection, with yields not exceeding 50 infectious particles per cell. The major portion of infectious virus remains cell associated. The virus replicates in the cytoplasm and induces no or minimal

cytopathology *in vitro*. Infected cells have a slightly extended period of DNA synthesis and may show some chromosomal alterations. Virus spreads from cell to cell and from mother to daughter cells in the presence of immune serum. The virions appear to assemble and to mature at intracytoplasmic membranes and may be released via exocytosis. Viral antigens are either absent or present in low density on the cell surface.

The growth of Newcastle disease virus is enhanced in porcine testis cells infected with HCV.

Genetics and Evolution

HCV shares a common evolutionary history with BVDV and BDV. Sequence analysis revealed that amino acid similarity between HCV and BVDV is around 70%. The lowest degree of homology exists between E2 sequences, the highest between the NS3 part of the NS2.3 protein. The percentage of homology between two HCV strains was 93%. On the basis of nucleotide sequences of the 5′ noncoding region, the N-terminal part of E2 and a region of NSB5, HCV strains may be divided into two major groups.

Serological Relationships and Variability

Although there is antigenic variation among HCV strains, they belong to one antigenic group, as shown by extensive crossneutralization with polyclonal antisera. The antigenic variation appears to reside on the antigenic unit on the N-terminal half of E2, as well as on E1. Within strains, with the use of monoclonal antibodies directed against E2, antigenic heterogeneity is observed as well.

There is a clear-cut distinction between HCV and BVDV, but these viruses are antigenically closely related, as shown by various serological techniques, including immunodiffusion and neutralizing antibody tests. In addition, there is a certain degree of cross-protection, i.e. pigs given a BVDV strain can be protected against mortality, but not disease, due to HCV. The common antigens among pestiviruses reside largely on the nonstructural protein NS2.3.

Epidemiology

The pig is the sole animal that is naturally infected with HCV, and thus the sole source of virus spread. Pigs are mostly infected via the oronasal route, and virus is shed in highest quantities in saliva, then in lacrimal secretions, urine and feces. Pigs infected with high-virulent strains and developing acute to subacute hog cholera, excrete the virus until death, whereas pigs with chronic disease excrete the virus intermittently. Postnatal infections with low-virulent strains are characterized by short periods of virus excretion, until antibodies appear in the blood. A congenital infection may result in the birth of persistently infected healthy pigs that shed virus in high titers continuously until they eventually die. Such pigs constitute a difficult recognizable source of virus transmission. Thus, low-virulent HCV infection may smolder in a herd for several months before it is diagnosed. Virulent HCV strains will usually spread faster in an infected herd and induce a higher morbidity than low-virulent strains.

Direct contact between infected and susceptible pigs is the most common mode of virus transmission. Herd-to-herd spread is commonly effected by pig movements. The risk of infection increases with the size of the herds. Humans and their contaminated equipment contribute considerably to virus spread between herds. The virus can also be transmitted by artificial insemination with infected semen. Aerogenic transmission may be a mode of spread between herds at close proximity. Wild boar are often, directly or indirectly, the source of new outbreaks in Europe.

Because HCV can survive in pork and pork products beyond processing, and for years when the meat is frozen, it can be transported over long distances. An important source of spread is virus-containing kitchen leftovers or garbage that has not been properly sterilized. Because of its potential danger, several countries prohibit the feeding of garbage to pigs.

Transmission and Tissue Tropism

Pigs are infected via the oronasal route. The tonsil is the primary target tissue for virus replication. The spread of high-virulent HCV through the pig is characterized by lymphatic, viremic and visceral phases. The virus primarily replicates in epithelial cells of tonsillar crypts and then invades the underlying lymphoreticular tissues. Through the lymphatic capillaries the virus is carried to lymph nodes and, after replication in these nodes, it enters the efferent blood capillaries, giving rise to a high-titered viremia. It subsequently replicates to high titers in secondary target organs, e.g. spleen, visceral lymph nodes, lymphoid structures lining the intestine and in bone marrow. It is presumably late in the viremic phase, which persists till death, that HCV invades the parenchymatous organs. Generally, the virus titers are higher in lymphoid tissues than in parenchymatous organs. The virus has an affinity for vascular

endothelium and cells of the immune system. The infection induces a severe leukopenia, which persists until death.

Intermediate-virulent virus strains generally induce subacute to chronic infections, during which virus persists in epithelial and lymphoreticular tissue. Infections with low-virulent virus tend to be confined only to the lymphatic phase.

Sows usually transmit the virus to their fetuses, irrespective of the stage of pregnancy. Infected fetuses harbor virus throughout their epithelial, reticulo-endothelial and lymphoidal tissues and a high-titered viremia persists until death, which may be months after birth. Immune exhaustion may develop in chronic and late-onset hog cholera.

Pathogenicity and Virulence

Although there is a continuous spectrum of virulence of HCV strains, one can distinguish strains of high, intermediate and low virulence, whereas the HCV vaccines are avirulent. It is not known which viral genes are involved in the expression of virulence. The severity and outcome of infection is the result of the composite interaction of viral virulence and host factors, such as breed, age, nutritional condition and immune competence. An association may exist between virulence and antigenicity: strains that are antigenically more related to BVDV seem to be less virulent. Low-virulent strains may grow optimally at 33–34°C, whereas virulent strains do so at about 39–40°C. The growth of virus in porcine alveolar macrophage cultures has been used as a marker for virulence. Virulent virus may grow to higher titers *in vivo* and *in vitro* than low-virulent strains. Whereas virulent virus thoroughly infects epithelial cells, reticular cells and macrophages in the tonsil, growth of virus of lowered virulence is mainly restricted to cells of the epithelial crypts. Virulence of CSFV seems to be an unstable property, because enhancement of virulence after one or multiple passages in pigs has repeatedly been reported.

Clinical Features of Infection

The infection can range from an acute disease with high mortality to a harmless subclinical course. Pigs with acute hog cholera show, after an incubation period of 2–6 days, high fever, with peaks above 42°C, anorexia, depression, conjunctivitis, nasal discharge, constipation preceding diarrhea, vomiting, weak hind legs and purplish discoloration of the skin. Concurrent with fever, a leukopenia and thrombocytopenia develop. Most pigs that suffer from acute classical swine fever die between 8 and 20 days postinfection.

Pigs infected with strains of intermediate virulence show a great variation in clinical responses. They may die from (sub)acute disease, they may recover or may survive the acute phase only to succumb later from chronic HCV, or they may develop only mild signs of disease. Chronic HCV is characterized by intermittent disease periods with anorexia, fever, leukopenia, diarrhea and dermatitis. Runt pigs can develop during chronic classical swine fever. Such pigs are severely retarded in growth, have skin lesions and often stand with arched backs. Secondary bacterial infections are common during chronic disease. Pigs infected with low-virulent strains show mild signs of disease or remain healthy.

Depending on the developmental stage of the fetus, a congenital infection can result in prenatal death, followed by mummification or stillbirth, malformation or neonatal death, congenital tremor, or the birth of healthy offspring that develop late-onset disease weeks or months after birth. Late-onset disease is characterized by runting, often associated with leukopenia, and pigs eventually die weeks or months after birth.

Pathology and Histopathology

The pathological picture of (sub)acute HCV is that of a septicemic disease with widespread multiple hemorrhages of various sizes, mainly in lymph nodes and kidneys. Infarction of the spleen is considered almost pathognomonic. There is disseminated intravascular coagulation with the occurrence of microthrombi in small blood vessels. In addition, various inflammatory reactions develop in respiratory, digestive and urogenital tracts. Encephalitis with perivascular cuffing is often observed. The infection results in degeneration and necrosis of capillary endothelial cells and in proliferation followed by necrosis and depletion of lymphocytes along with hyperplasia of histiocytes and reticular cells.

The most outstanding lesion in pigs that die from persistent HCV is a general exhaustion of the lymphoid system, characterized by a complete atrophy of the thymus and lymphocyte depletion of peripheral lymphoid tissues. A consistent finding is degeneration of endothelial cells. Button-ulcers in cecum and colon and rib lesions are frequently seen in subacute and chronic cases.

In stillborn pigs an excessive accumulation of fluid in body cavities is most prominent. The virus is teratogenic, and most commonly defects of the central nervous system, such as cerebellar hypoplasia, are induced.

Immune Response

Compared to other virus infections of pigs, antibodies to HCV appear late in the blood. In pigs that recover from the disease, antibodies first appear in the blood between 10 and 21 days after infection. Neutralization tests are commonly used to detect these antibodies but these tests can detect antibodies to BVDV as well. Recently, ELISAs utilizing HCV-specific monoclonal antibodies have become available that specifically detect antibodies to HCV. Antibodies probably persist during the pig's entire lifetime. An HCV-specific proliferation of T lymphocytes and a cytotoxic T-lymphocyte response have been described.

In chronically infected pigs, the neutralizing antibody response can remain completely absent, be severely impaired and delayed, or only be transiently detectable. Pigs with congenital persistent hog cholera do not mount an antibody response to the virus, but they respond normally to unrelated antigens. This state of immunotolerance is usually maintained until death.

After vaccination, neutralizing serum antibodies are first detectable around 2–3 weeks and persist lifelong. Vaccinated sows transmit antibodies to their offspring through colostrum. These antibodies enter directly into the blood circulation because the intestine of the neonate is permeable for immunoglobulins during the first 36–48 h of life. Piglets are thus passively protected against mortality due to hog cholera during the first 1–2 months after birth. Passively acquired antibodies strongly suppress the induction of active immunity after vaccination, so that pigs born from vaccinated sows cannot be successfully vaccinated before 6–9 weeks of age.

HCV has a distinct affinity for cells of the immune system and consequently can induce defects in immune functions. During acute hog cholera, the pig's immune reactivity is suppressed, e.g. peripheral blood and spleen lymphocytes hardly respond to mitogens, whereas tonsillar and lymph node lymphocytes have normal to enhanced responses. HCV infections seem to induce alterations in lymphocyte recirculation in pigs.

Prevention and Control

Countries that are free from hog cholera have measures in force, such as a ban on the import of pigs and pork from infected countries, to prevent introduction of virus. If, despite these measures, hog cholera is diagnosed, rigorous measures are taken to eradicate the virus. These eradication programs are based on notifying suspected cases to the veterinary authorities, a rapid laboratory diagnosis and an immediate destruction of infected herds. In order to diagnose hog cholera, recently dead or moribund pigs should undergo postmortem examination, and tonsil, spleen, kidney and ileum should be sent to the laboratory for the detection of antigen in frozen tissue sections by an immunofluorescence test. The results can be available in 2 h. Because pigs can also be naturally infected with BVDV, monoclonal antibodies that are directed against conserved epitopes of HCV and do not recognize BVDV are used for staining tissue sections, to distinguish between an HCV and a BVDV infection. The immunofluorescence test is slightly less sensitive than virus isolation in tissue culture. In the latter case, it takes 24–72 h before HCV is diagnosed. Antibody detection, in neutralization tests and ELISAs, is also used as a diagnostic tool to identify herds with convalescent pigs. Once the diagnosis has been made, standstill orders, closure of markets, intensive monitoring of adjacent farmers and immediate destruction of infected herds are implemented.

Systematic and strictly applied large-scale vaccination programs have been implemented to stop the uncontrollable perpetuation of the virus. Such programs, in conjunction with the usual sanitary measures, have contributed enormously to the eventual eradication of hog cholera from heavily infected areas; however, the European Union adopted a nonvaccination policy at the end of the 1980s. Vaccines of HCV are based on strains attenuated by serial passage in rabbits ('Chinese' strain) or in cell cultures. The vaccines are highly efficacious and safe, although they occasionally spread to contact pigs. Vaccination establishes immunity within 1 week and the immunity persists lifelong. Vaccination not only protects against disease, but also prevents transmission of field virus from vaccinated to contact pigs.

Future Perspectives

A wide spectrum of virulence exists among HCV strains: some strains induce almost 100% mortality, whereas others only give rise to inapparent infections. The virus is virtually noncytopathogenic *in vivo*. The viral genes involved, and the processes underlying the expression of virulence leading to severe disease, tissue damage and immune exhaustion, are virtually unknown. Research on the molecular pathogenesis of hog cholera might reveal hitherto unknown disease-making processes and may shed more light on the pathogenesis of related hemorrhagic fevers in humans.

In countries that are (almost) free of HCV, it is of the utmost importance to make an early and rapid

diagnosis once the virus is introduced. By means of reverse transcription–polymerase chain reaction, infected pigs may be detected earlier than with the commonly used immunofluorescence techniques. For pork-exporting countries, an early diagnosis may occasionally mean the prevention of losses of millions of dollars.

In heavily infected countries or in areas where there is an uncontrollable virus spread, a systematic and strict vaccination scheme should be implemented to curtail virus circulation. Vaccination, however, precludes the use of serology for diagnostic purposes, because the antibody response after vaccination cannot be distinguished from that after infection. Consequently, it is difficult to demonstrate convincingly the absence of HCV which jeopardizes the export of pork. Marker vaccines that allow the differentiation between infected and vaccinated pigs with the use of an epitope-specific serological test will soon become available. These vaccines contain the E2 protein, produced in baculovirus, and are formulated with an adjuvant.

See also: **Bovine diarrhea virus and Border disease virus (*Flaviviridae*); Diagnostic techniques: Detection of viral antigens, nucleic acids and specific antibodies, Isolation and identification by culture and microscopy; Immune response: Cell mediated immune response, General features; Vaccines and immune response.**

Further Reading

Liess B (ed.) (1988) Classical swine fever and related viral infections. *Developments in Veterinary Virology*. Boston: Martinus Nijhoff.

Terpstra C (1991) Hog cholera: an update of present knowledge. *Br. Vet. J.* 147: 397.

Thiel H-J, Plagemann PGW and Moennig V (1996) Pestivirus. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 1059. Philadelphia: Lippincoff-Raven.

van Oirschot JT (1992) Classical swine fever. In: Straw B *et al* (eds) *Diseases of Swine*, 7th edn. Iowa State University Press.

HONEY BEE VIRUSES

Brenda V Ball, IACR-Rothamsted, Harpenden, UK

Leslie Bailey, 20 West Common Grove, Harpenden, UK

History and General Characteristics

Isolation and characterization of bee viruses began in the 1960s, with work on two diseases, sacbrood and paralysis, which have striking symptoms. Sacbrood, although extremely common in Britain, was, until the virus was isolated, dismissed there as a genetic disorder. Similarly, paralysis was ascribed to independent, but accompanying and very common, well-known parasites, especially microsporidia and mites, which do not cause any overt symptoms.

Many, if not all, of the viruses of bees persist in the population as slight or inapparent infections, but under certain circumstances they begin to multiply within individuals and spread between them, leading to outbreaks of disease. These are sometimes spectacular, causing severe losses, but mortality due to virus infection, although significant, is usually less obvious, and is still commonly attributed to other factors. This, and the fact that there are no known direct means of controlling bee viruses may persuade beekeepers and their advisors that they are of little consequence and they continue to disregard them. However, past ignorance of the viruses of bees has frequently led to mistaken or seriously inadequate diagnoses of diseases and so to much wasted effort on inappropriate treatments.

Some bee viruses depend on, but do not invariably accompany, certain common parasites of adult bees. Further, at least two usually inapparent virus infections become activated to multiply to lethal levels by *Varroa jacobsoni*, which can also act as a vector of several other viruses.

Physicochemical properties

The principal physicochemical properties of the known bee viruses are summarized in **Table 1**. All except chronic paralysis virus and filamentous virus have isometric particles. Apart from *Apis* iridescent

diagnosis once the virus is introduced. By means of reverse transcription–polymerase chain reaction, infected pigs may be detected earlier than with the commonly used immunofluorescence techniques. For pork-exporting countries, an early diagnosis may occasionally mean the prevention of losses of millions of dollars.

In heavily infected countries or in areas where there is an uncontrollable virus spread, a systematic and strict vaccination scheme should be implemented to curtail virus circulation. Vaccination, however, precludes the use of serology for diagnostic purposes, because the antibody response after vaccination cannot be distinguished from that after infection. Consequently, it is difficult to demonstrate convincingly the absence of HCV which jeopardizes the export of pork. Marker vaccines that allow the differentiation between infected and vaccinated pigs with the use of an epitope-specific serological test will soon become available. These vaccines contain the E2 protein, produced in baculovirus, and are formulated with an adjuvant.

See also: **Bovine diarrhea virus and Border disease virus (*Flaviviridae*); Diagnostic techniques: Detection of viral antigens, nucleic acids and specific antibodies, Isolation and identification by culture and microscopy; Immune response: Cell mediated immune response, General features; Vaccines and immune response.**

Further Reading

Liess B (ed.) (1988) Classical swine fever and related viral infections. *Developments in Veterinary Virology*. Boston: Martinus Nijhoff.

Terpstra C (1991) Hog cholera: an update of present knowledge. *Br. Vet. J.* 147: 397.

Thiel H-J, Plagemann PGW and Moennig V (1996) Pestivirus. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 1059. Philadelphia: Lippincoff-Raven.

van Oirschot JT (1992) Classical swine fever. In: Straw B *et al* (eds) *Diseases of Swine*, 7th edn. Iowa State University Press.

HONEY BEE VIRUSES

Brenda V Ball, IACR-Rothamsted, Harpenden, UK

Leslie Bailey, 20 West Common Grove, Harpenden, UK

History and General Characteristics

Isolation and characterization of bee viruses began in the 1960s, with work on two diseases, sacbrood and paralysis, which have striking symptoms. Sacbrood, although extremely common in Britain, was, until the virus was isolated, dismissed there as a genetic disorder. Similarly, paralysis was ascribed to independent, but accompanying and very common, well-known parasites, especially microsporidia and mites, which do not cause any overt symptoms.

Many, if not all, of the viruses of bees persist in the population as slight or inapparent infections, but under certain circumstances they begin to multiply within individuals and spread between them, leading to outbreaks of disease. These are sometimes spectacular, causing severe losses, but mortality due to virus infection, although significant, is usually less obvious, and is still commonly attributed to other factors. This, and the fact that there are no known direct means of controlling bee viruses may persuade beekeepers and their advisors that they are of little consequence and they continue to disregard them. However, past ignorance of the viruses of bees has frequently led to mistaken or seriously inadequate diagnoses of diseases and so to much wasted effort on inappropriate treatments.

Some bee viruses depend on, but do not invariably accompany, certain common parasites of adult bees. Further, at least two usually inapparent virus infections become activated to multiply to lethal levels by *Varroa jacobsoni*, which can also act as a vector of several other viruses.

Physicochemical properties

The principal physicochemical properties of the known bee viruses are summarized in **Table 1**. All except chronic paralysis virus and filamentous virus have isometric particles. Apart from *Apis* iridescent

Table 1 Properties of honey bee viruses

Virus	Dimensions (nm)	S_{20w} (Svedbergs)	Buoyant density in CsCl (g ml^{-1})	Nucleic acid Type	Proteins Mol. wt (× 10^{-6})	Mol. wt (× 10^{-3})
Chronic paralysis	20 × 30–60	80–130	1.33	RNA	1.35, 0.9, 0.35	23.5
Chronic paralysis virus associate	17	41	1.38	RNA	0.35	15
Sacbrood (*Apis mellifera*)	30	160	1.35	RNA	2.8	26, 28, 31
Sacbrood (*Apis cerana*)	30	160	1.35	RNA	2.8	30, 34, 39
Acute paralysis	30	160	1.37	RNA	ND	24, 33, 35
Kashmir (*Apis mellifera*)	30	172	1.37	RNA	ND	25, 33–36, 40, 44–46
Kashmir (*Apis cerana*)	30	172	1.37	RNA	ND	25, 34, 35, 37, 46
Deformed wing	30	165	1.37	RNA	ND	26, 27, 30–32, 45, 46
Egypt	30	165	1.37	RNA	ND	27, 30, 31, 45
Slow paralysis	30	176	1.37	RNA	ND	27, 29, 46
Black queen cell	30	151	1.34	RNA	2.8	6, 29, 32, 34
X	35	187	1.35	RNA	ND	52
Y	35	187	1.35	RNA	ND	50
Cloudy wing	17	49	1.38	RNA	0.45	19
Filamentous	150 × 450	ND	1.28	DNA	12.0	13–70[a]
Iridescent (*Apis cerana*)	150	2216	1.32	DNA	ND	ND
Arkansas	30	128	1.37	RNA	1.8	41
Berkeley	30	170	ND	RNA	1.4	32, 35, 37

ND, not determined.
[a] About 12 proteins.

virus, which is only very distantly related to some other known insect iridoviruses, no virus from honey bees has been found to be serologically related to any other virus.

Chronic Paralysis Virus and Chronic Paralysis Virus Associate

Special features

Particles of chronic paralysis virus (CPV) are mostly ellipsoidal in outline, often with a small irregular protuberance at one end. A wide range of shapes and sizes of particles occur, but these are resolvable in sucrose gradients or by analytical centrifugation into three or four components with sedimentation coefficients of 82, 97–106, 110–124 and 125–136 S. These components contain particles that differ considerably in size but have modal lengths of about 30, 40, 55 and 65 nm respectively, and all have a modal width of about 20 nm.

CPV has five single-stranded RNA components: two larger RNAs designated 1 (M_r 1.35 × 10^6; 4200 nucleotides) and 2 (M_r 0.9 × 10^6; 2800 nucleotides) and three smaller RNAs each with M_r 0.35 × 10^6 (1100 nucleotides). CPV adopts a flexible packaging arrangement in which individual RNA species may be packaged separately or various combinations incorporated together.

Chronic paralysis virus associate (CPVA) has 17 nm isometric particles which are serologically unrelated to CPV but are frequently found associated with it in natural infections. CPVA does not multiply when injected alone into bees and it interferes with the multiplication of CPV, inhibiting particularly the relative amount of the longest, most infective particles. It is more evident in females of the reproductive caste (queens) than in workers and may be of some significance in, or a reflection of, the defence mechanisms of individuals against paralysis. Gel electrophoretic and T1 fingerprint analyses have shown that the three RNAs of CPVA are probably identical to the three smallest RNA components of CPV.

Pathology and epizootiology

CPV causes one of two distinct sets of symptoms or syndromes. One of these, described as 'paralysis' more than a hundred years ago, includes an abnormal trembling motion of the wings and bodies of the infected bees, which are unable to fly but often crawl on the ground beneath their colonies, sometimes in masses of thousands of individuals. The sick bees have a bloated abdomen, caused by distension of the honey sac with liquid, and they frequently have partially spread, dislocated wings. They die within a few days. Severely affected colonies suddenly collapse, leaving the queen with a few bees on neglected combs. All these signs are those attributed to the so-called 'Isle of Wight disease', which is alleged to have been prevalent in Britain early in the twentieth century, but was of unresolved cause.

Bees with the other syndrome become hairless, appearing dark or almost black and shiny. At first they can fly but they suffer nibbling attacks by healthy bees and are prevented from returning to their colony by guard bees. In a few days they become flightless, trembly and soon die. Both syndromes can occur in the same colony and the difference in expression of symptoms probably reflects genetic differences between the bees.

Many millions of particles of CPV can be extracted from one bee with paralysis and about half of these are in the head. Particles resembling those of CPV have been seen in densely but randomly packed groups, either free or within vesicles, in the cytoplasm of thoracic and abdominal ganglia of the gut and of the mandibular and hypopharyngeal glands of paralytic bees. They do not appear in the cytoplasm of fat or muscle tissue. Sections of the hindgut epithelium of paralytic bees show basophilic cytoplasmic bodies (Morison's cell inclusions) which seem specific to the disease.

The LD_{50} by injection into the hemolymph of adult bees is about 100 particles. Individuals are also readily infected by rubbing virus preparations on their bodies, but many millions of particles are required to cause paralysis when applied as a spray or in food. Bees infected by any method become flightless and paralytic between 5 and 7 days later and die soon after. Infected individuals live longer at 30°C than at 35°C, which is about the maximum temperature in a bee colony, yet more virus multiplies in bees at the lower temperature. The disease spreads rapidly between overcrowded adult bees, probably via the cytoplasm of broken cuticular hairs.

CPV occurs commonly throughout the world. Infectivity tests indicate that the virus is endemic in apparently healthy colonies in Britain but there is no seasonality in incidence or in disease outbreaks. Irregular factors that inhibit the normal activities of bees probably increase transmission of the virus. These factors include unseasonable weather, queenlessness, crop failures and high colony densities, each of which decreases the foraging activity of bees and hence increases their bodily contact within colonies during their usually active season. This may well explain the close correlation between the decline in the number of colonies in Britain and the decline in the incidence of outbreaks of paralysis. It contrasts

with the unchanged or somewhat increasing incidences, during the same period, of noncontagiously transmitted infections of bees.

Sacbrood

Special features

Sacbrood virus (SBV) from the European honey bee, *A. mellifera,* is serologically related to a strain identified in the Eastern hive bee, *A. cerana,* from Thailand (TSBV) but is distinguishable from it physicochemically.

Unlike SBV particles, those of TSBV aggregate in buffers of low (about 0.01) molarity, and in the absence of sodium ethylene diaminetetra-acetate TSBV particles appear empty when viewed by electron microscopy. Particles of both virus strains appear empty when Mg^{2+} is added but SBV is much less sensitive than TSBV.

TSBV sediments at 160 S in 0.1 M KCl but at only 150 S in 0.01 M phosphate. It produces three close but well-defined bands of protein on 5% sodium dodecyl sulfate (SDS) polyacrylamide gels. By contrast, SBV sediments at 160 S in 0.01 M phosphate and on 5% SDS polyacrylamide gels gives one band which separates into three only on gels of higher concentration.

Pathology and epizootiology

The symptoms of sacbrood are clear and specific in both *A. mellifera* and *A. cerana.* Infected larvae fail to pupate and remain stretched on their backs with their head towards the cell capping. Much ecdysial fluid, containing millions of virus particles, collects between the body and the tough unshed skin of the larva. The body colour changes from a pearly white to a pale yellow and after death begins to darken from the anterior end, drying down to a dark brown gondola-shaped scale. The remains are usually removed, mostly before they desiccate, by young adult bees, which ingest large quantities of virus in the process. Many particles, sometimes in crystalline array, have been seen in the cytoplasm of fat, muscle and tracheal end-cells of infected larvae.

Sacbrood can be readily caused experimentally by adding fresh suspensions of the virus to the food of healthy unsealed larvae in the brood cell. Larvae about 2 days old are the most susceptible.

SBV ingested by young adult bees multiplies readily without causing obvious disease. It accumulates in the hypopharyngeal glands, from which it probably joins the food of larvae with the gland secretions. However, bees are usually prevented from transmitting the virus by behavioural changes; infection accelerates the normal sequence of duties and shortens the life of individuals. Infected young bees cease to eat pollen; they cease to feed and tend larvae; and they fly and forage much earlier in life than usual. These effects are the same as the changes induced by aging or by brief anesthesia with carbon dioxide.

Outbreaks of sacbrood most commonly occur in the spring and early summer or when forage is limited, probably because, when young bees are few, the usual division of labor according to age is least well developed.

Sacbrood occurs worldwide and in some areas is considered to be the most common honey bee disease; however, this may reflect the ease and reliability of field diagnosis by symptoms. The mite *V. jacobsoni* is sometimes a vector of the virus.

Acute Paralysis Virus

Special features

Acute paralysis virus (APV) is stable below pH 4.0 but, unlike acid-stable mammalian picornaviruses, its buoyant density increases with increasing pH. APV has also been detected in *Bombus* species and is the only honey bee virus that appears to have an alternative host in nature.

Pathology and epizootiology

Bees injected with approximately 100 particles of APV develop trembling and semiparalytic symptoms after 2–3 days and die 1–2 days later. Inoculated bees are killed quickly at 30°C but they live longer, even though more virus accumulates within them, at 35°C. This is in direct contrast with the effect of temperature on bees infected with CPV. Crystalline arrays of particles have been seen in the cytoplasm of fat-body cells, the brain and hypopharyngeal glands of infected bees.

APV commonly persists as an inapparent infection in adult bees and, until recently, had never been associated with disease or mortality in nature. In continental Europe and North America, strains of APV have now been detected in dead adult bees and pupae from colonies infested with the parasitic mite *V. jacobsoni.* The mite induces the virus to multiply when it feeds on apparently healthy but virus-infected bees and the virus rapidly becomes systemic and lethal. Mites acquire virus by feeding on an overtly infected host and can then act as virus vectors, transmitting APV to other adult bees or to pupae within the brood cell.

APV has been detected in *A. mellifera* from most regions of the world. All strains of APV are

serologically closely related and distantly related to Kashmir bee virus.

Kashmir Bee Virus

Special features

Strains of Kashmir bee virus (KBV) from Canada and Spain differ from other strains of KBV in their tendency to aggregate in buffers of low (about 0.01) molarity and in containing only three capsid proteins when analyzed by SDS-polyacrylamide gel electrophoresis, compared with five proteins resolved in the type strain of KBV from *A. cerana* in India and six proteins in KBV strains from South Australia and New Zealand. Protein instability may account for one or two of the minor bands resolved in the latter three strains.

Pathology and epizootiology

KBV multiplies quickly and profusely when injected into adult bees or when suspensions are rubbed on their bodies, killing them within 3 days, but it causes no apparent harm when given in food to adult bees or larvae. The virus multiplies in most tissues of infected bees, apparently within cytoplasmic membrane-bound vesicles, but not in nerve cells. Infected cells show marked changes, including condensation of chromatin and disruption of cytoplasmic organelles.

KBV commonly persists as an inapparent infection in both adult bees and pupae, but it may spread contagiously, in the same way as CPV, to cause overt disease in nature.

KBV has been detected in *A. cerana* from India and Papua New Guinea and in *A. mellifera* from North America, Europe, Australasia and Oceania. It may be more widely distributed than previously thought but, like APV, has remained undetected until the spread of *V. jacobsoni* caused the activation and establishment of overt infection in infested colonies.

Deformed Wing and Egypt Bee Viruses

Special features

Semipurified preparations of the particles of these viruses are unstable, aggregating and disintegrating in buffers of low (about 0.01) molarity or below pH 5.0. Under these conditions, many capsids, typically with six clearly defined thickenings, are penetrated by neutral phosphotungstate stain. Deformed wing virus (DWV) particles are much more sensitive to these conditions than those of Egypt bee virus (EBV).

DWV strains show some differences in their capsid protein profiles but all are serologically closely related to each other and distantly related to EBV.

Pathology and epizootiology

DWV sometimes kills adult bees but infection is most evident in colonies infested with *V. jacobsoni* because the mite transmits the virus to honey bee pupae, causing wing malformation and early death of newly emerged adults. Injecting the virus into very young pupae causes the disorder after an incubation period of about 14 days.

DWV has been detected in *A. mellifera* from Japan, most European countries, Saudi Arabia, Iran, Vietnam and Argentina and in *A. cerana* from China.

EBV was isolated from samples of dead adult bees from Egypt, but nothing is known of its natural history.

Slow Paralysis Virus

Purified particles of slow paralysis virus (SPV) aggregate spontaneously in the agar of immunodiffusion plates when these contain 0.85% sodium or potassium chloride at pH 5.8–8.8.

Until recently, SPV had never been associated with disease in nature. The virus has now been identified as a cause of the death of both adult bees and pupae in honey bee colonies in Britain infested with the parasitic mite *V. jacobsoni*. SPV may be induced to multiply to lethal levels and be transmitted in a manner similar to that of APV in mite-infested colonies. Adult bees injected with SPV die after about 12 days, typically suffering paralysis of the first pair of legs shortly before death.

Black Queen Cell Virus

Black queen cell virus (BQCV) was first identified as the cause of death of queen larvae or prepupae after they had been sealed in their cells, which developed dark-brown to black cell walls. Diseased larvae are pale yellow and have a tough sac-like skin, resembling those killed by sacbrood virus, but unlike this virus, BQCV does not multiply readily when ingested by young worker larvae or young adult bees. It multiplies abundantly and fatally when injected into pupae, but infects adult bees only when these are also infected with the microsporidian *Nosema apis* and in nature it almost always accompanies this parasite. BQCV in association with *N. apis* shortens the lives of bees more than when these are infected with the microsporidian alone, but it causes no sign of disease.

BQCV has been identified in adult bees from every continent and the virus is sometimes transmitted to pupae by the mite *V. jacobsoni*.

Bee Viruses X and Y

Bee virus X (BVX) is distantly serologically related to bee virus Y (BVY) and is almost indistinguishable from it both physically and chemically. They multiply primarily in the gut epithelium when ingested by young adult bees that are maintained at 30°C but not at 35°C. There are no symptoms of infection but each shortens the life of adult bees, BVX being the more virulent. Neither virus multiplies when injected into adult bees or pupae.

Despite their many similarities, BVX and BVY differ strikingly in their epizootiology. In Britain, BVX is most prevalent during winter, whereas the peak incidence of BVY occurs in spring. In nature, infection with BVY is almost invariably associated with the microsporidian *N. apis,* but BVX shows no similar association with this parasite. BVX is, however, significantly associated with the protozoan *Malpighamoeba mellificae* in dead bees in late winter. This association is not due to a dependence of the virus on the protozoan but to the much greater virulence of the pathogens when they occur together in individuals than when they are alone.

BVX has not been detected in Asia, but with this exception both viruses have a worldwide distribution.

Cloudy Wing Virus

Although the particles of cloudy wing virus (CWV) are similar in size to CPVA they differ in their stability in dilute (0.01M) buffer, are serologically unrelated and, as far as is known, CWV multiplies independently of any other virus. The wings of severely infected individuals often lose transparency but this is not a reliable symptom. The lives of infected bees are shortened and colonies in which most individuals are infected soon become inactive and die.

In nature, infection may be airborne over short distances because it spreads between cages of bees that are incubated close together in the laboratory. Crystalline arrays of particles occur in the region of the tracheoles between the muscle fibrils of the thorax where the main inhalatory spiracles are located. Although the virus is primarily an infection of adult bees, it has recently been detected in dead pupae and newly emerging bees from colonies infested with *V. jacobsoni,* which indicates that it may be infective by injection.

There is no seasonal cycle of incidence, which suggests that irregular events determine the spread of CWV within colonies, similar to those controlling the spread of CPV and other contagiously transmitted pathogens.

About 15% of colonies have been found to be infected by CWV in Britain. The virus is widely distributed throughout mainland Europe and has been detected on every other continent.

Filamentous Virus

Special features

Filamentous virus (FV) particles each comprise a flexuous rod-shaped nucleocapsid, measuring 3000 nm × 40 nm, tightly coiled within an ellipsoidal envelope measuring 450 nm × 150 nm. The particles of virus preparations in solutions of low ionic strength appear intact when negatively stained with ammonium molybdate and viewed in the electron microscope, whereas the nucleocapsid emerges partially or entirely from particles in similar preparations stained with neutral phosphotungstate.

Pathology and epizootiology

FV particles have been seen in ultrathin sections of all tissues examined but virogenesis has been observed only in fat-body and ovarian cells. The hemolymph of severely infected individuals becomes milky white with particles but there are no other known symptoms and no unequivocal evidence that the lives of bees are shortened by infection.

The virus can multiply when injected into bees but it infects them most readily when fed to adults together with spores of the microsporidian *N. apis,* with which it is almost invariably associated in nature.

First reported from the USA, FV is probably the most common but least pathogenic of all honey bee viruses in Britain. It has been detected in adult bees from every continent except South America.

Apis Iridescent Virus

Apis iridescent virus (AIV) is only distantly serologically related to the other iridescent viruses isolated from insects and, curiously, unlike the majority of iridoviruses, it fails to multiply by injection into larvae of the greater wax moth *Galleria mellonella,* which is a specific pest of honey bee combs.

Natural infections of AIV in colonies of the Eastern hive bee, *A. cerana,* cause symptoms of 'clustering disease'; colonies become inactive and clusters of flightless bees form on the front of the hive or crawl on the ground. AIV will multiply in various tissues of the European honey bee, *A. mellifera,* forming crystals which appear a bright blue-violet colour when illuminated with white light.

AIV has been found only in *A. cerana* from Kashmir and northern India and is the only known iridovirus of Hymenoptera.

Other Honey Bee Viruses

These viruses or virus-like particles are not known to be related to any virus described above or to be associated with any particular disease or parasite of bees.

Arkansas bee virus

Arkansas bee virus (ABV) was originally detected in honey bees in Arkansas by injecting apparently healthy adult bees with extracts of locally gathered pollen loads of foraging bees. Individuals injected with the virus die after about 14 days but otherwise show no sign of disease.

ABV has been detected, together with chronic paralysis virus, in dead bees from dwindling colonies in California but has not been detected anywhere other than these two localities in the USA.

Berkeley bee virus

Virus-like particles, unrelated to ABV and named Berkeley bee virus, have been found associated with the Californian and Arkansas isolates of ABV, but nothing is known of their pathogenicity or natural history.

Future Perspectives

The recent recognition of virus infections as a primary cause of mortality in honey bee colonies infested with the parasitic mite *V. jacobsoni* has stimulated increased interest and awareness of honey bee viruses worldwide. A more extensive capability to detect and identify these pathogens by serological or molecular techniques will provide information on the natural history and prevalence of virus infections in honey bee populations in many areas of the world where little is currently known. The improved ability to accurately diagnose these infections will also help to clarify the role of viruses in outbreaks of mortality previously attributed to a few well-known groups of larger parasites or to noninfectious causes.

Although almost all of the viruses of bees have been shown to be widespread and to shorten the lives of individuals, their effects under most circumstances are not striking. Indeed, the persistence of a number of distinct and occasionally virulent viruses in apparently healthy, perennial colonies emphasizes the powerful innate ability of bees to resist their multiplication and spread. This natural propensity is most evident when colonies of bees are allowed to develop and undertake their normal activities unhindered – an ideal which is increasingly difficult to attain with the demands of modern intensive beekeeping, particularly migratory and pollination work. Some honey bee viruses may still be localized but their ability to persist as inapparent infections, together with the continued and increasingly rapid international trade in bees seems to make their ultimate distribution worldwide inevitable.

Further Reading

Allen MF and Ball BV (1996) The incidence and world distribution of honey bee viruses. *Bee World* 77(3): 141.

Bailey L and Ball BV (1991) *Honey Bee Pathology*. London: Academic Press.

Bailey L and Ball BV (1991) Viruses of honey bees. In: Adams JR and Bonami JR (eds) *Atlas of Invertebrate Viruses*, p. 525. Boca Raton: CRC Press.

HORDEIVIRUSES

Diane M Lawrence and **Andrew O Jackson**, University of California-Berkeley, Berkeley, California, USA

Taxonomy and Classification

The *Hordeivirus* genus of plant viruses includes three members: barley stripe mosaic virus (BSMV, type member), poa semilatent virus (PSLV) and lychnis ringspot virus (LRSV). The genus has not been assigned to a family. BSMV, PSLV and LRSV have rod-shaped virions that are shorter and are larger in diameter than those of the tobamoviruses and tobraviruses. The coat proteins of BSMV, PSLV and LRSV are the same size, but have different mobilities on polyacrylamide gels, and are distantly related by serotype. The particle morphology, serological relatedness of the coat proteins, and genome organization place these three viruses in the same group. Analyses of the coat protein sequences and the

AIV has been found only in *A. cerana* from Kashmir and northern India and is the only known iridovirus of Hymenoptera.

Other Honey Bee Viruses

These viruses or virus-like particles are not known to be related to any virus described above or to be associated with any particular disease or parasite of bees.

Arkansas bee virus

Arkansas bee virus (ABV) was originally detected in honey bees in Arkansas by injecting apparently healthy adult bees with extracts of locally gathered pollen loads of foraging bees. Individuals injected with the virus die after about 14 days but otherwise show no sign of disease.

ABV has been detected, together with chronic paralysis virus, in dead bees from dwindling colonies in California but has not been detected anywhere other than these two localities in the USA.

Berkeley bee virus

Virus-like particles, unrelated to ABV and named Berkeley bee virus, have been found associated with the Californian and Arkansas isolates of ABV, but nothing is known of their pathogenicity or natural history.

Future Perspectives

The recent recognition of virus infections as a primary cause of mortality in honey bee colonies infested with the parasitic mite *V. jacobsoni* has stimulated increased interest and awareness of honey bee viruses worldwide. A more extensive capability to detect and identify these pathogens by serological or molecular techniques will provide information on the natural history and prevalence of virus infections in honey bee populations in many areas of the world where little is currently known. The improved ability to accurately diagnose these infections will also help to clarify the role of viruses in outbreaks of mortality previously attributed to a few well-known groups of larger parasites or to noninfectious causes.

Although almost all of the viruses of bees have been shown to be widespread and to shorten the lives of individuals, their effects under most circumstances are not striking. Indeed, the persistence of a number of distinct and occasionally virulent viruses in apparently healthy, perennial colonies emphasizes the powerful innate ability of bees to resist their multiplication and spread. This natural propensity is most evident when colonies of bees are allowed to develop and undertake their normal activities unhindered – an ideal which is increasingly difficult to attain with the demands of modern intensive beekeeping, particularly migratory and pollination work. Some honey bee viruses may still be localized but their ability to persist as inapparent infections, together with the continued and increasingly rapid international trade in bees seems to make their ultimate distribution worldwide inevitable.

Further Reading

Allen MF and Ball BV (1996) The incidence and world distribution of honey bee viruses. *Bee World* 77(3): 141.

Bailey L and Ball BV (1991) *Honey Bee Pathology*. London: Academic Press.

Bailey L and Ball BV (1991) Viruses of honey bees. In: Adams JR and Bonami JR (eds) *Atlas of Invertebrate Viruses*, p. 525. Boca Raton: CRC Press.

HORDEIVIRUSES

Diane M Lawrence and **Andrew O Jackson**, University of California-Berkeley, Berkeley, California, USA

Taxonomy and Classification

The *Hordeivirus* genus of plant viruses includes three members: barley stripe mosaic virus (BSMV, type member), poa semilatent virus (PSLV) and lychnis ringspot virus (LRSV). The genus has not been assigned to a family. BSMV, PSLV and LRSV have rod-shaped virions that are shorter and are larger in diameter than those of the tobamoviruses and tobraviruses. The coat proteins of BSMV, PSLV and LRSV are the same size, but have different mobilities on polyacrylamide gels, and are distantly related by serotype. The particle morphology, serological relatedness of the coat proteins, and genome organization place these three viruses in the same group. Analyses of the coat protein sequences and the

serotype indicate that BSMV and PSLV are more closely related to each other than to LRSV. Anthoxanthum latent bleaching virus (ALBV) which has been detected in Great Britain has recently been assigned to the genus and serological analysis shows that ALBV is closely related to BSMV.

Host Range and Economic Importance

Hordeiviruses have been detected in nature in members of both monocot and dicot families. BSMV has primarily been found in barley and occasionally in wild oats and wheat. PSLV has been isolated from *Poa palustris* from two different locations in Western Canada. LRSV appears to be of European origin, and has been recovered from several members of the Caryophyllaceae and Labiatae families. Hordeiviruses also infect several experimental hosts. BSMV will form local lesions on *Chenopodium* species and causes a systemic infection in *Nicotiana benthamiana*. PSLV infects Graminae and also several dicots experimentally, whereas LRSV has been shown to infect several dicot plants. BSMV, LRSV and PSLV have no known vectors, and are all transmitted by mechanical means. BSMV and LRSV are seed transmitted, but as yet PSLV has not been shown to be seed transmitted.

BSMV appears to be the only hordeivirus of economic importance with serious yield losses due primarily to infection of barley. Losses result from poor seed set, shriveled seeds and decreased vigor of seedlings. However, BSMV has no known biological vector and requires efficient seed and mechanical transmission for maintenance in barley, so virus spread can be prevented by planting virus-free seed. As more sensitive detection methods have been developed, seed can be screened prior to planting and plants grown for certified seed production can be rogued prior to harvesting. In North America these practices have significantly reduced the yield losses due to BSMV infection of barley. In addition, investigation into resistance by genome mapping has revealed that in Morex barley, a single gene confers resistance to the BSMV strain CV42.

Physical Properties

Hordeivirus particles are rod shaped with a diameter of approximately 25 nm and lengths ranging from 100 to 160 nm. The particles are composed of a single coat protein species that encapsidates a positive sense single-stranded (ss) RNA molecule. The particles appear to contain three nucleotides per protein subunit, with approximately 24 protein subunits per turn of the helix. The hordeivirus genome is tripartite in nature with the complete sequences of BSMV, PSLV and LRSV RNAs having been determined.

Genome Structure and Organization

The hordeivirus genomes are composed of three RNAs designated α, β and γ. The sizes of the α, β and γ RNAs are 3.8, 3.2 and 2.8 kb (BSMV-ND18 strain), 3.9, 3.6 and 3.2 kb (PSLV) and 3.7, 3.1 and 2.6 kb (LRSV), respectively. The sizes of RNAs α and β are quite constant among the different strains of BSMV examined to date, however RNA γ varies in size between strains. The BSMV type strain is 3.2 kb compared to 2.8 kb in the ND18 strain, and the Argentina Mild strain contains mixtures of RNA γ species of 3.2, 2.8 and 2.6 kb. The 2.6 kb RNA is defective as the result of a deletion in the 3′ terminus of the γa gene. Each of the RNAs has a 7-methylguanosine cap at the 5′ terminus and a conserved 3′ terminal nontranslated sequence. The conserved 3′ termini are variable in length between the different viruses, ranging from 148 nt (LRSV) to 330 nt (PSLV). The sequences form tRNA-like structures which in BSMV are capable of binding tyrosine *in vitro*. Directly upstream of the conserved termini in the BSMV and LRSV genomes is an internal poly(A) sequence of varying length that is located directly following the stop codon of the 3′ proximal gene of each RNA. This poly(A) sequence is absent in the PSLV genome.

Hordeivirus genomes (**Fig. 1**, BSMV) have a number of structural differences that distinguish them from brome mosaic virus, alfalfa mosaic virus, cucumber mosaic virus, and other tricornaviruses. A major difference is that hordeiviruses encode seven rather than four proteins. The α RNAs of all three hordeiviruses encode a single protein (αa) that contains methyltransferase and helicase domains that are highly conserved between Sindbis-like viruses. The γ RNAs encode two proteins, the polymerase (γa) and a cysteine-rich protein (γb). The γa proteins contain the conserved GDD found in RNA-dependent RNA polymerase proteins of RNA viruses. The γa proteins are members of the tobamo-lineage of supergroup III of RNA-dependent RNA polymerases that also includes tricornaviruses, tobacco mosaic virus (TMV), and the furovirus, soil borne wheat mosaic virus (SBWMV). The γb proteins share some structural similarities, but show no direct amino acid sequence homology with the cysteine-rich proteins of tobra and carlaviruses. The organization of the genes on RNAβ are quite distinct from those of the tricornaviruses. The first open reading frame (ORF) encodes the coat protein, and this ORF is followed by a series of overlapping genes termed the 'triple gene

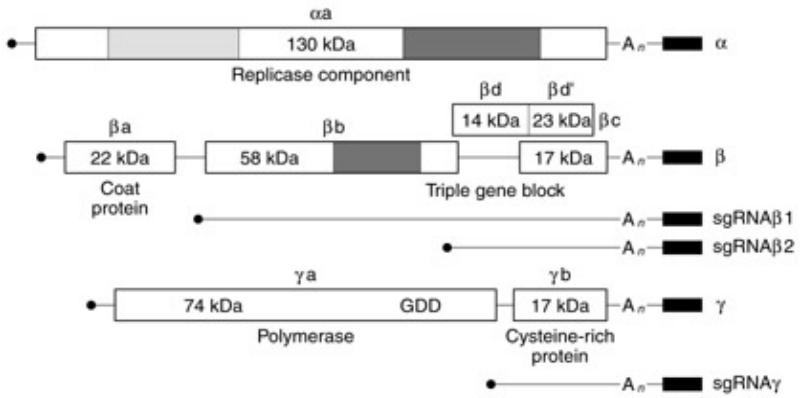


Figure 1 Genome organization of barley stripe mosaic virus. The filled circle, open rectangles and solid rectangles represent the 5′ cap structure, the open reading frames (ORFs) and the 3′ terminal 238 nt tRNA-like structure. The 3′ proximal ORFs of each RNA terminate with a UAA to initiate the short poly(A) sequence separating the 238 nt conserved 3′ termini from the coding region. RNAα encodes a single protein, αa, that is a replicase component and contains methyltransferase ( ) and helicase domains ( ). RNAβ encodes the coat protein (βa) and a series of overlapping genes (the triple gene block), that are separated by a 117 nt intergenic region. The βb and βc ORFs are separated by 173 nt and overlap the βd ORF. The βb protein is translated from a subgenomic (sg) RNA and contains a helicase domain ( ). The βd, βd', and βc proteins are all translated from one sgRNA. Translational readthrough of the βd stop codon occurs to yield the βd' protein. RNAγ encodes two proteins: the γa protein is the polymerase and contains the conserved GDD motif; the γb protein is translated from a sgRNA and is a cysteine-rich protein that has a role in gene expression and pathogenesis.

block' that are also present in carla-, furo-, and potexviruses. In hordeiviruses, the first ORF of the triple gene block encodes the βb protein. This protein contains a conserved helicase domain similar to the helicase domain present in the αa protein. The remaining two ORFs of the triple gene block encode the small hydrophobic proteins, βc and βd.

Molecular Biology

The availability of full-length cDNA clones and the subsequent generation of infectious transcripts has permitted analysis of the proteins encoded by the BSMV genome (**Fig. 1**). Only RNAs α and γ are required for replication in protoplasts, with the αa and γa proteins being essential subunits of the RNA-dependent RNA polymerase. The 17 kDa BSMV γb protein is a cysteine-rich protein that is expressed from a 737 nt subgenomic (sg) RNA. This protein is not strictly required for infectivity in plants, but it has an effect on pathogenicity and expression of the genes encoded by RNAβ. Null mutations in γb alter the symptom phenotype, and affect expression of both the βa and βb proteins.

The γb protein has been expressed and purified from *Escherichia coli* and shown to bind ssRNAs *in vitro*. By subcellular fractionation studies and localization of the γb protein as a fusion protein with the green fluorescence protein (GFP) in infected cells, the γb protein appears to be cytoplasmically localized. Genetic analyses show that changes in localization of the γb protein are correlated with distinct phenotypes in barley.

The 22 kDa BSMV coat protein (βa) is expressed by direct translation of the genomic RNA. BSMV is unusual in that the coat protein is not required for infectivity in barley (a systemic host) or *Chenopodium* species (local lesion hosts) suggesting that the coat protein is not a major determinant in either cell-to-cell or systemic movement. Moreover, the infections elicited by coat protein-deficient BSMV mutants are more aggressive, and the phenotype is more severe and protracted in barley than in the presence of the coat protein. The triple gene block is responsible for cell-to-cell and systemic movement of the virus. The 5′ proximal gene of the BSMV triple gene block encodes the 58 kDa βb protein which is expressed from a 2.45 kb sgRNA. Genetic analyses have shown that βb is strictly required for cell-to-cell movement and it appears to function in part by RNA binding activities. The βb protein has been purified from infected plants and shown to bind ss- and dsRNA, nucleotide triphosphates (NTPs), and to exhibit ATPase activity *in vitro*. Helicase activity has not been detected in association with this protein *in vitro*. However, mutations in any one of the six conserved helicase domains prohibit cell-to-cell movement, demonstrating that this domain has an important function *in planta*. The βb protein appears to associate with membranes in infected cells, as

assessed by examination of the localization of a GFP:βb fusion protein. Viral RNA also associates with βb *in vivo*, because a βb:RNA complex can be recovered from infected tissue. The RNAs in this complex are BSMV specific and of genomic sense. Additional virus- or host-encoded proteins have not been detected in association with the complex.

The remaining genes of the triple gene block are expressed from a 960 nt sgRNA. The βc (17 kDa) and βd (14 kDa) proteins both contain two hydrophobic regions of amino acids separated by a hydrophilic stretch suggesting the presence of transmembrane domains. Each of these proteins is strictly required for cell-to-cell movement of the virus. The βd protein has been shown to be present in membrane fractions using subcellular fractionation. BSMV is unique among the triple gene block-containing viruses in that a fourth protein, βd′ (23 kDa), is encoded by the triple gene block. The βd′ protein is expressed as the result of a translational readthrough of the βd amber stop codon and is present in infected plants, but it is not required for cell-to-cell movement of the virus in barley or *Chenopodium* species.

Replication of each of the BSMV RNAs requires the 5′ and 3′ terminal regions, but different *cis*-elements are required for replication in the internal sequences. RNAα replication is *cis*-preferential, with replication appearing to be coupled with translation of a functional αa protein. Each of the genes on RNAβ is dispensable for replication, however the 117 nt intergenic region separating βa and βb has a *cis*-acting function. In contrast, RNAγ replication is not dependent on the 42 nt intergenic region separating γa and γb, but essential *cis*-acting elements are present in the first 507 nt of the γa gene.

Cytopathology

The cytopathology of the BSMV infection in cells is dependent on many different factors including the virus strain, the host, the timing of the infection and the environmental conditions. During an infection BSMV virions accumulate to high levels in the cytoplasm with a low number being present in the nucleus. The cytoplasmically localized virions do not tend to form crystalline inclusions as observed in TMV infections, but membranes often proliferate and large globular inclusions of unknown function are observed. Most of the visible changes in leaf appearance can be attributed to the disruption of organelles, most notably chloroplasts, in the rapidly expanding cells of the growing leaf. The morphology and shape of chloroplasts are altered during the infection; the grana become disorganized and numerous small vesicles appear between the inner and outer membranes of the chloroplast envelope.

Pathogenicity

Determinants of BSMV pathogenicity have been mapped to different areas of the genome. In *N. benthamiana*, the ND18 strain is able to cause a systemic infection whereas the Type strain is able to replicate and move in the inoculated leaf but is unable to invade systemically. This aberrant phenotype appears to be caused by the presence of a short ORF in the leader sequence preceding γa that reduces translation of γa. Infections with the ND18 or Type strains also result in different local lesion phenotypes on *Chenopodium amaranticolor*. These differences are complex, but are primarily due to expression of γa, the presence of an amino-terminal 372 nt duplication in the Type strain, and differences in amino acids of the γb proteins. The ND18 strain is nonpathogenic on oats whereas the CV42 strain is pathogenic. The ND18 strain phenotype may be the result of relatively simple associations of αa with a host protein, because pathogenicity to oats can be engineered by altering a single nucleotide resulting in an amino acid change in the αa protein.

Future Perspectives

Future emphasis needs to be placed on biochemical and genetic experiments to elucidate the functions and properties of the virus-encoded proteins. The isolation and characterization of the replicase complex will provide us with important information on hordeivirus replication and interaction with the cell. Host components associated with these complexes need to be identified and characterized to define their interactions. Additional cell biological analyses of the triple gene block-encoded proteins and their interactions with the plant cell are required in order to obtain a clearer understanding on how viruses move from cell-to-cell and through vascular tissue to cause systemic infections. These analyses will not only increase our understanding of how viruses move to establish infections in vegetative tissue, seed and pollen, but they may also provide important insights into subcellular transport and plasmodesmatal functions. Dissection of the interactions between virus-encoded proteins and host components will therefore provide a more detailed understanding of disease development and the host cell processes exploited during infection.

See also: **Bromoviruses (*Bromoviridae*); Carlaviruses; Furoviruses; Potexviruses.**

Further Reading

Carroll TW (1986) Hordeiviruses: biology and pathology. In van Regenmortel MHV and Fraenkel-Conrat H (eds) *The Plant Viruses, The Rod-shaped Plant Viruses*, vol. 2, p. 373. New York: Plenum Press.

Donald RGK, Petty ITD, Zhou H and Jackson AO (1995) Properties of genes influencing barley stripe mosaic virus movement phenotypes. In Bills DD (ed.) *Biotechnology and Plant Protection: Viral Pathogenesis and Disease Resistance*. Proceedings of the Fifth International Symposium, p. 115. Singapore: World Scientific Publishing.

Donald RGK, Lawrence DM and Jackson AO (1997) The BSMV 58 kD βb protein is a multifunctional RNA binding protein. *J. Virol.* 71: 1538.

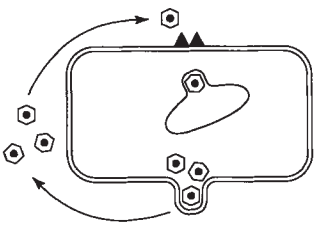
Jackson AO and Lane LC (1981) Hordeiviruses. In: Kurstak E (ed.) *Handbook of Plant Virus Infections and Comparative Diagnosis*, p. 565. Amsterdam: Elsevier.

Jackson AO, Petty ITD, Jones RW, Edwards MC and French R (1991) Analysis of barley stripe mosaic virus pathogenicity. *Semin. Virol.* 2: 107.

Savenkov E, Solovyev AG and Morozov SYu (1998) Genome sequences of poa semilatent virus and lychnis ringspot hordeiviruses. *Arch. Virol.* 143: 1379–1393.

HOST GENETIC RESISTANCE

David G Brownstein, Yale University School of Medicine, New Haven, Connecticut, USA



History

Genetic diversity is maintained among individuals of any noninbred species in order to provide the raw material to adapt to evolutionary forces. Although the species as a whole benefits from this diversity, individuals may not. Individuals may inherit certain genes or gene combinations that make them unusually susceptible to specific infectious or noninfectious diseases. In genetic terms, alleles, or alternative forms of the same gene, are the basis of variations in genetic resistance to diseases whether these variations are controlled by single or multiple loci. Developments in many fields but especially molecular genetics have facilitated the characterization of genes with multiple alleles that regulate disease resistance. Those polymorphous genes that regulate resistance to viral diseases are the subject of this entry.

In 1933, Webster was the first to observe that resistance to a disease caused by a virus could be inherited. The virus was yellow fever and the host was the laboratory mouse. He crossed strains of mice that were resistant and susceptible to the lethal effects of yellow fever virus and showed that segregant progeny expressed resistance according to predictions of Mendelian genetics. Other examples of genetic resistance in mice to what were then termed arboviruses were soon to follow. Today, polymorphous genes that regulate resistance to 11 genera of viruses have been reported.

Experimental results using laboratory mice needed corroboration in a natural population of another species. This came in the 1950s when the highly virulent myxoma virus was introduced into wild European rabbits in Australia to control burgeoning lagomorph populations. Although numerous factors conspired to attenuate initially high mortality rates, selection for resistant host genes was clearly an important factor. In humans, attempts to associate the severity of smallpox, poliomyelitis, congenital rubella and hepatitis B with various blood group and major histocompatibility antigens met with limited success. An impetus for this line of inquiry in humans came with Allison's discovery in 1964 of the relationship between resistance to falciparum malaria and heterozygosity of the sickle cell gene. Although this work did not deal with a viral infection, it established that genetic polymorphisms in humans regulate resistance to at least one microbial disease.

To date, the most systematic approach to the study of relationships between the severity of viral diseases and specific host genes remains comparisons of highly inbred strains of mice that vary in their susceptibility to a particular disease. Derivatives of resistant and susceptible strains such as bilineal congenic and recombinant inbred strains have been especially valuable in these studies. What has emerged from them is confirmation that polymorphous host genes are important regulators of the severity of many if not most viral infections, that single or multiple loci are involved, and that each locus appears to specify resistance to a particular genus of virus, or even a specific strain of virus within a genus. These studies have also shown that polymorphisms within the

Further Reading

Carroll TW (1986) Hordeiviruses: biology and pathology. In van Regenmortel MHV and Fraenkel-Conrat H (eds) *The Plant Viruses, The Rod-shaped Plant Viruses*, vol. 2, p. 373. New York: Plenum Press.

Donald RGK, Petty ITD, Zhou H and Jackson AO (1995) Properties of genes influencing barley stripe mosaic virus movement phenotypes. In Bills DD (ed.) *Biotechnology and Plant Protection: Viral Pathogenesis and Disease Resistance*. Proceedings of the Fifth International Symposium, p. 115. Singapore: World Scientific Publishing.

Donald RGK, Lawrence DM and Jackson AO (1997) The BSMV 58 kD βb protein is a multifunctional RNA binding protein. *J. Virol.* 71: 1538.

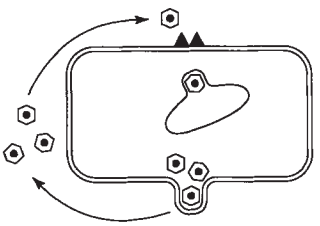
Jackson AO and Lane LC (1981) Hordeiviruses. In: Kurstak E (ed.) *Handbook of Plant Virus Infections and Comparative Diagnosis*, p. 565. Amsterdam: Elsevier.

Jackson AO, Petty ITD, Jones RW, Edwards MC and French R (1991) Analysis of barley stripe mosaic virus pathogenicity. *Semin. Virol.* 2: 107.

Savenkov E, Solovyev AG and Morozov SYu (1998) Genome sequences of poa semilatent virus and lychnis ringspot hordeiviruses. *Arch. Virol.* 143: 1379–1393.

HOST GENETIC RESISTANCE

David G Brownstein, Yale University School of Medicine, New Haven, Connecticut, USA



History

Genetic diversity is maintained among individuals of any noninbred species in order to provide the raw material to adapt to evolutionary forces. Although the species as a whole benefits from this diversity, individuals may not. Individuals may inherit certain genes or gene combinations that make them unusually susceptible to specific infectious or noninfectious diseases. In genetic terms, alleles, or alternative forms of the same gene, are the basis of variations in genetic resistance to diseases whether these variations are controlled by single or multiple loci. Developments in many fields but especially molecular genetics have facilitated the characterization of genes with multiple alleles that regulate disease resistance. Those polymorphous genes that regulate resistance to viral diseases are the subject of this entry.

In 1933, Webster was the first to observe that resistance to a disease caused by a virus could be inherited. The virus was yellow fever and the host was the laboratory mouse. He crossed strains of mice that were resistant and susceptible to the lethal effects of yellow fever virus and showed that segregant progeny expressed resistance according to predictions of Mendelian genetics. Other examples of genetic resistance in mice to what were then termed arboviruses were soon to follow. Today, polymorphous genes that regulate resistance to 11 genera of viruses have been reported.

Experimental results using laboratory mice needed corroboration in a natural population of another species. This came in the 1950s when the highly virulent myxoma virus was introduced into wild European rabbits in Australia to control burgeoning lagomorph populations. Although numerous factors conspired to attenuate initially high mortality rates, selection for resistant host genes was clearly an important factor. In humans, attempts to associate the severity of smallpox, poliomyelitis, congenital rubella and hepatitis B with various blood group and major histocompatibility antigens met with limited success. An impetus for this line of inquiry in humans came with Allison's discovery in 1964 of the relationship between resistance to falciparum malaria and heterozygosity of the sickle cell gene. Although this work did not deal with a viral infection, it established that genetic polymorphisms in humans regulate resistance to at least one microbial disease.

To date, the most systematic approach to the study of relationships between the severity of viral diseases and specific host genes remains comparisons of highly inbred strains of mice that vary in their susceptibility to a particular disease. Derivatives of resistant and susceptible strains such as bilineal congenic and recombinant inbred strains have been especially valuable in these studies. What has emerged from them is confirmation that polymorphous host genes are important regulators of the severity of many if not most viral infections, that single or multiple loci are involved, and that each locus appears to specify resistance to a particular genus of virus, or even a specific strain of virus within a genus. These studies have also shown that polymorphisms within the

major histocompatibility complex (MHC) may influence the course of viral infections, but that loci outside of the MHC are often more important.

During the past several years a number of viral disease resistance genes have been identified. In some cases their protein products or their mechanisms of action are known. In other cases their protein products and their functions are unknown but as they become more precisely mapped and eventually cloned 'reverse genetics' can be used to delineate their functions. Substantial homology between the murine and human genomes makes it possible to identify similar genetic resistance elements in humans. This has already been done for the *Mx1* locus on mouse chromosome 16 and human chromosome 21 which regulates susceptibility to influenza A and B viruses. The resistance loci that have been provisionally or definitively mapped are discussed here since this represents the most substantial progress in recent years (**Table 1**).

General Considerations

Viruses rely heavily upon products of host genes for their replication. Beginning with their interaction with host-encoded cell surface receptors and host factors involved in uncoating, continuing with their use of host transcription and translation factors, and ending with host-encoded post-translational enzymes, viruses require multiple products of host genes. The specific viral requirements differ depending on the replication strategy of the virus. In addition, viruses elicit protective responses in the host that are ultimately orchestrated by the host genome and these responses also vary depending on the virus. Any of these multiple points of interaction between the virus and products of the host genome have the potential to be sources of variation if alleles exist at these points within the host population and if the products of these alleles interact differently with the viral genome or its products.

Polymorphous resistance genes may therefore be expressed at the level of the target cells if they control proteins that are necessary for virus replication or at the organismal level if they regulate antiviral effector mechanisms. The effects of the former can usually be demonstrated in primary cultures of target cells from genetically resistant and susceptible hosts because they are expressed at the cellular level. The effects of the latter cannot usually be demonstrated in pure populations of target cells in culture because they involve cell interactions which are orchestrated at the organismal level. There is at least one exception to this organizational paradigm, however, and that is the antiviral state that is induced in target cells by interferons. In this case, target cells express proteins, now numbering about 24, that inhibit rather than promote virus replication. Polymorphous resistance genes can be classified as follows based on their organizational level of expression and whether they are induced by the infection or are constitutively expressed:

1. Genes expressed at the target cell level:
   a. constitutive expression – these genes regulate proteins that are necessary for virus replication;
   b. inducible expression – these genes regulate interferon-dependent proteins.
2. Genes expressed at the organismal level: these genes regulate antiviral effector mechanisms.

Resistance Genes Constitutively Expressed in Target Cells

Two resistance genes listed in **Table 1** are constitutively expressed in target cells. These are the *H-2D* gene that controls resistance to the lethal effects of mouse cytomegalovirus (MCMV) infection and the *Hv-2* locus that regulates susceptibility to acute mortality caused by mouse hepatitis virus type 4 (MHV4).

Inbred strains of mice that carry the MHC $H\text{-}2^k$ haplotype are among the most resistant to acute mortality caused by MCMV, a herpesvirus. Mice that carry the $H\text{-}2^d$ or $H\text{-}2^b$ haplotypes are 10 times more susceptible to lethal cytomegalovirus infection and permit significantly more virus replication than mice of the same genetic background that carry the $H\text{-}2^k$ haplotype. This difference is determined by the class I gene, *H-2D*. Products of class I MHC genes are important in cellular immune responses. Class I antigens are expressed by a wide variety of cells and serve as recognition molecules for specific classes of T cells. In the case of cytomegalovirus infection, however, the role of *H-2D* in genetic resistance is independent of its role in immune recognition. Cells from $H\text{-}2D^k$ mice are highly insensitive to infection with MCMV. Class I heavy-chain antigens are covalently bound to a second cell surface antigen, β_2-microglobulin, which is believed to serve as a cytomegalovirus receptor. The *H-2D* antigen is therefore directly involved with virus binding or it regulates the affinity of β_2-microglobulin for the virus antireceptor. In the case of $H\text{-}2D^k$ antigen the affinity of mouse cytomegalovirus for β_2-microglobulin is markedly reduced.

The *Hv-2* locus controls susceptibility of mice to the lethal effects of MHV4, a coronavirus. Like *H-2D* in the MCMV system, its effects are readily seen in

Table 1 Mapped or tentatively mapped polymorphous resistance genes

Virus	*Disease*	*Gene*	*Mouse chromosome*	*Linkage marker*	*Homologous human chromosome*	*Resistant genotype (carrier)*	*Susceptible genotype (carrier)*	*Dominant phenotype*	*Gene action*
Coxsackie-virus B3	Chronic myocarditis	*amd*	14	*Tcrα*	14q	amd^r (C57BL/6)	amd^s (A, DBA/2)	R	Effector
	Acute myocarditis	*H*-2-linked	17	*H-2*	6p	(A.CA)	(A.BY, A.SW)	Unknown	Unknown
		H-2-linked	17	*H-2*	6p	(B10, B10.A)	(B10.Q, B10.BR)	Unknown	Unknown
Ectromelia virus	Acute mortality	*Rmp*-2	2	*Hc*	9q	$Rmp\text{-}2^r$ (C57BL/6)	$Rmp\text{-}2^s$ (DBA/2)	R	Unknown
		Rmp-3	17	*H-2*	6p	$Rmp\text{-}3^r$ (C57BL/6)	$Rmp\text{-}3^s$ (DBA/2)	R	Unknown
Influenza A virus	Acute mortality	*Mx*1	16	*Ets*-2	21	$Mx\text{-}1^+$ (A2G)	$Mx\text{-}1^-$ (BALB/c)	R	Inducible
Mouse cytomegalo-virus	Acute mortality	*Cmv*-1	6	*Prp*	12p	$Cmv\text{-}1^l$ (C57BL/6)	$Cmv\text{-}1^h$ (BALB/c)	R	Unknown
		H-2D	17	*H-2*	6p	$H\text{-}2D^k$ (BALB.K)	$H\text{-}2D^d$ (BALB/c)	S	Constitutive
Mouse encephalomyelitis virus	Chronic demyelination	–	6	*Tcrβ*	7q	(BALB/c)	(SJL)	R/S	Effector
		–	3	*Car*-2	8q	(C57BL/c)	(DBA/2)	R	Effector
		H-2D	17	*H-2*	6p	$H\text{-}2D^b$ (C57BL/6)	$H\text{-}2D^d$ (DBA/2)	R	Effector
		H-2D	17	*H-2*	6p	$H\text{-}2D^b$ (C57BL/10)	$H\text{-}2D^s$ (SJL)	R	Effector
Mouse hepatitis virus	Acute mortality					$Hv\text{-}2^r$	$Hv\text{-}2^s$		
		Hv-2	7	*Svp*-2	19q	(SJL)	(C57BL/10)	S	Constitutive
		H-2-linked	17	*H-2*	6p	(A.CA)	(A/Sn)	R/S	Unknown
	Chronic demyelination	*H*-2-linked	17	*H-2*	6p	(A.CA)	(A.BY)	R	Unknown

infected cells in primary cultures. The mechanism of action of *Hv-2* is currently disputed. Only one strain of mouse, SJL, is known to carry the *Hv-2*^{r} allele. Mendelian genetics of resistance to the lethal effects of MHV4 in crosses between SJL and various susceptible strains indicate that one gene controls resistance. It has been shown that SJL mice fail to express a 110K cell surface glycoprotein that binds MHV4, whereas susceptible strains of mice do. These data suggest that *Hv-2* encodes the viral receptor. *Hv-2* was defined on the basis of differences in the replication of MHV4 in macrophages from resistant and susceptible mice. In macrophages from *Hv-2*^{r} and *Hv-2*^{s} mice, the virus replication diverges at a point after the virus is internalized and after transcription of viral genes. This suggests that *Hv-2* does not encode the virus receptor but a protein that functions subsequent to binding. There is evidence that *Hv-2* encodes the host protease that activates the viral S glycoprotein which serves as the antireceptor. This virus–host interaction is necessary for the infection to spread and could explain why the infection fails to spread in macrophage cultures from *Hv-2*^{r} mice.

Resistance Genes Inducible in Target Cells

The single example of this class of resistance gene is *Mx1* which regulates resistance to diseases caused by influenza A and B viruses. This is the most thoroughly examined resistance gene, having been mapped, cloned and sequenced. Its protein product is known although the exact mechanism whereby it inhibits influenza virus replication has not been determined. Its inhibitory action occurs after virus uncoating and before translation. *Mx1* is an interferon-inducible nuclear protein with an M_r of 72000. Interferons α and β but not γ induce the synthesis of *Mx1*$^+$ mRNA. Most laboratory strains of mice have deletion or point mutations in the *Mx1* gene resulting in a potential coding capacity for the inactive N-terminal half of the *Mx1* protein only.

Resistance Genes Expressed Through Host Antiviral Effectors

Five of the genes listed in **Table 1** are expressed at the organismal level through antiviral effectors. All of these examples are believed to regulate specific immune responses that are involved in virus clearance or in immunopathic injury. Four of these genes regulate susceptibility to Theiler's mouse encephalomyelitis virus (TMEV), a picornavirus. Mice that are inoculated intracerebrally with certain laboratory-adapted strains of TMEV may develop one of several diseases, depending on their genotype. The most intensively studied disease occurs after the acute phase of infection and is characterized by progressive paralysis in association with chronic demyelination of the central nervous system. This disease is immunologically mediated and is associated with viral persistence in the white matter oligodendrocytes and infiltrating macrophages. The genes that mediate susceptibility to viral persistence and myelin breakdown in this system vary with the strain of mouse. In susceptible DBA/2 mice, demyelination is mediated by the *H-2D*^{d} allele of the MHC and a second allele at a locus near the *Car-2* gene on chromosome 3. In susceptible SJL mice, disease is mediated by an allele on a gene located near the structural gene for the T cell receptor β-chain constant region on chromosome 6. The *H-2D*^{s} allele of the MHC of SJL mice also appears to mediate susceptibility in SJL mice but its effects have not been seen in all studies.

All mice that are susceptible to paralytic TMEV infection share augmented delayed-type hypersensitivity responses to the virus when compared to resistant mice. Their susceptibility is therefore believed to result from inappropriate cell-mediated immune responses to the virus which allow it to persist and which mediate tissue injury. Consistent with this interpretation is the fact that most of these loci map to regions that are known to regulate immune responses. The localization of one gene to the vicinity of the T cell receptor β-chain structural gene is of special interest because the T cell receptor repertoire has been implicated as an etiology in certain autoimmune diseases.

Another example of an inappropriate immune response underlying genetic susceptibility to a virus-induced disease is that which occurs after infection with coxsackievirus B3 (CVB3). In this case, however, the immune response that is triggered by the infection is not directed at the virus, but rather at a host protein in the tissue where the virus replicates.

CVB3 is a common cause of infectious myocarditis in humans. CVB3 causes acute and chronic myocarditis in inbred mice depending on the strain of mice. The chronic myocarditis results from an autoimmune response to cardiac myosin that is triggered by viral replication in and injury to the heart of susceptible mice. Both *H-2*-linked and non-*H-2*-linked genes control susceptibility to chronic myocarditis. One form of the disease is mediated by IgG antibodies specific for cardiac myosin. This form is seen in DBA/2 and A mice and is modulated by a gene that is located on chromosome 14 in the vicinity of structural loci for the T cell receptor α-chain and the cardiac myosin α-heavy chain. Associations between the T cell receptor repertoire and autoimmune

diseases has been previously mentioned in the context of TMEV-induced chronic demyelination. The possibility that allelic differences in cardiac myosin may account for the observed chromosomal localization of the susceptibility gene is also being considered. However, the susceptible phenotype has been shown to correlate with susceptibility to autoimmune myocarditis after immunization with cardiac myosin, suggesting that allelic differences in cardiac myosin are not the basis of susceptibility.

Resistance Genes Expressed Through Unknown Mechanisms

Seven of the genes listed in **Table 1** exert their effects through unknown mechanisms. Five of these are linked to the MHC and six regulate susceptibility to acute viral diseases. The associations with the MHC suggest that some of these genes regulate specific immune responses. However, the temporal divergence of phenotypes of mice with resistance and susceptibility alleles for some of these genes occurs before effects of specific immune mechanisms are likely. For this reason the *H-2*-linked genes that regulate susceptibility to acute effects of ectromelia virus (mouse-pox), CVB3 and MHV are more likely to be expressed through early, nonspecific effectors than through specific immune responses. These early effectors include activated natural killer cells, interferon and acute-phase reactants.

There is evidence that *Cmv-1,* which regulates resistance to the lethal effects of MCMV, is expressed through the early inflammatory response. This gene limits virus replication, prevents virus-induced splenic necrosis within 72h of infection, and appears to be expressed at the organismal level. Allelic differences at this locus are correlated with differences in the phenotypic character of the early inflammatory infiltrate. There is preliminary evidence that resistance to the lethal effects of ectromelia virus, an orthopoxvirus is regulated by a gene (not among the two listed in **Table 1**) that maps to the same region of mouse chromosome 6 as *Cmv-1*. Other similarities between these two loci are that they are expressed in the spleen, they regulate virus-induced splenic necrosis, they regulate the influx of cells into the spleen or the proliferation of cells already present, and their resistance alleles are expressed by the same strains of mice. Should these turn out to be the same locus, it would be the first example of a polymorphous gene active against more than one genus of virus.

Future Perspectives

Genetically determined variations in responses to viral infections are important determinants of disease severity in all species, including humans. The difficulty in identifying modifying genes directly in genetically heterogeneous populations has retarded efforts to identify such loci in humans. The continuing success in identifying these genes in mice and the extensive genetic homology between mice and humans provides a means for identifying resistance genes for specific viruses in humans. In some cases, allelic differences at these loci will modulate the severity of human viral illnesses as they do in mice. A thorough understanding of how these polymorphisms modulate viral infections will lead to more enlightened approaches to the therapy of viral diseases and the identification of individuals at risk to develop serious sequelae.

See also: **Genetics of animal viruses; Immune response: General features; Interferons: General features; Pathogenesis: Animal viruses; Replication of viruses; Viral receptors; Virus–host cell interactions.**

Further Reading

Brownstein DG, Bhatt PN, Gras L and Jacoby RO (1991) Chromosomal locations and gonadal dependence of genes that mediate resistance to ectromelia (mousepox) virus-induced mortality. *J. Virol.* 65: 1946.

Melvold RW, Jokinen DM, Miller SD, Dal Canto MC and Lipton HL (1990) Identification of a locus on mouse chromosome 3 involved in differential susceptibility to Theiler's murine encephalomyelitis virus-induced demyelinating disease. *J. Virol.* 64: 686.

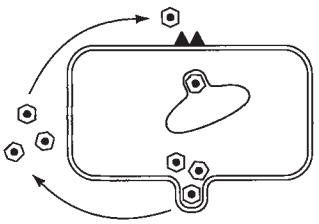
Price P, Winter JG, Nikoletti S, Hudson JB and Shellam GR (1987) Functional changes in murine macrophages infected with cytomegalovirus relate to *H-2*-determined sensitivity to infection. *J. Virol.* 61: 3602.

Reeves RH, O'Hara BF, Pavan WJ, Gearhart JD and Haller O (1988) Genetic mapping of the *Mx* influenza virus resistance gene within the region of mouse chromosome 16 that is homologous to human chromosome 21. *J. Virol.* 62: 4372.

Traystman MD *et al.* (1991) Susceptibility to coxsackievirus B3-induced chronic myocarditis maps near the murine *Tcrα* and *Myhcα* loci on chromosome 14. *Am. J. Pathol.* 138: 721.

[This article is reproduced from the 1st edn (1994).]

HOST-CONTROLLED MODIFICATION AND RESTRICTION



Detlev H Krüger and **Monika Reuter**, Humboldt University, Charité School of Medicine, Institute of Medical Virology, Berlin, Germany

Introduction

The application of restriction endonucleases has revolutionized molecular biological research. As so often was the case, studies on bacterial viruses led to the discovery of this class of enzymes. At the beginning of the 1950s, the observation was noted that phages 'remember' the last host strain in which they reproduced. Depending on the host cell, the virus carries a specific modification which usually improves its ability to grow on the same host in subsequent rounds of infection, while impairing its growth in others. Host-controlled modification results in a reversible phenotype, clearly distinguishable from irreversible mutational changes in host range (**Fig. 1**).

The molecular explanation of these phenomena (first demonstrated for phage lambda and P2) was elaborated in the 1960s and 1970s by groups of Arber, Smith, Meselson and others. Host-controlled modification consists of a specific DNA methylation at a defined recognition sequence of 4–8 bp. Restriction is caused by DNA cleavage occurring at an unmethylated recognition site. Pairs of corresponding DNA methylases and restriction endonucleases (i.e. recognizing the same DNA sequence) coded by *hsd* genes (*h*ost *s*pecificity of *D*NA) in bacterial cells are responsible for these activities. During DNA replication, recognition sequences in nascent daughter strands are transiently unmethylated. Such hemimethylated sites are preferential substrates for DNA methylases, while sites unmethylated in both strands are primary targets of restriction endonucleases and are very rarely methylated.

DNA modification consists of a C-5 or N-4 methylation of cytosine or in an N-6 methylation of adenine in the recognition sequence. *S*-Adenosylmethionine (AdoMet) functions as methyl donor. DNA restriction results in fragments with cohesive or blunt ends that are further degraded intracellularly by other nucleases, in particular by the *rec*BCD (=*exo*V) enzyme.

Classes of Modification/Restriction (M/R) Systems in Prokaryotes

M/R enzymes are coded by chromosomal, prophage or plasmid genes and are classified into at least three groups (type I, II and III) according to their properties. The types of M/R systems differ in the organization of the genes encoding them, the complexity of their supramolecular structure, their cofactor requirements, the symmetry of the recognized base sequence and in the location of the cleavage site within or outside of the recognition sequence (**Table 1**).

Type I enzymes are hetero-oligomers composed of three subunits (HsdR, endonuclease; HsdM, methylase; HsdS, DNA recognition). Restriction requires

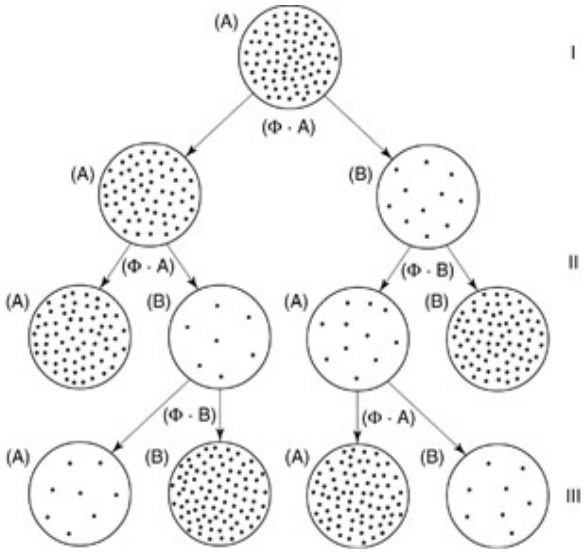


Figure 1 Scheme for the demonstration of host-controlled modification and restriction of a virus Φ. (I) Isolation of a single plaque from cell lawn A (Φ · A) and parallel infection of cell strains A and B with the same virus population. Virus Φ · A is restricted on B (less virus plaques than on strain A). (II) Passage of the virus from the cell strains A or B to both strains. (III) The reversibility of modification proves that Φ · B from the second transfer is not a host range mutant, but a modified virus. When one of the cell strains is restriction and modification negative, the reversibly changeable growth of the virus is only manifest on the other cell strain; plating on the first one is not altered. In the figure the efficiency of restriction is about tenfold; that is, the virus titer on the restriction-proficient host is about 10^{-1} of that on the nonrestricting host. Measured efficiencies vary with the phage and restriction system from barely detectable to about 10^{-5}. (Adapted from Krüger DH and Bickle TA (1983) *Microbiol. Rev.* 47: 345–360.)

Table 1 Distinguishing properties of different types of restriction enzymes

Type	*Structural features of proteins and genes*	*Cofactors*	*DNA cleavage*	*Example*	*DNA recognition sequence*[a]
I	Multifunctional, multisubunit; three structural genes	Mg^{2+}, ATP, AdoMet	Cut randomly, far from asymmetric recognition sites, need two copies of recognition sequence, ATP hydrolysis	*Eco*B	5′-TGA*(N$_8$)TGCT 3′-ACT(N$_8$)$_{\star}$ACGA
II	Separate enzymes for restriction and modification; two structural genes	Mg^{2+}	Cut at fixed positions within symmetric recognition sites	*Eco*RI	5′-GA*ATTC 3′-CTT$_{\star}$AAG
IIE	Like type II	Mg^{2+}	Like type II, but need two copies of recognition sequence for functional interaction, activable by effector oligonucleotides containing the site	*Eco*RII	5′-C*C(A/T)GG 3′-GG(T/A)$_{\star}$CC
IIS	Like type II (sometimes three structural genes)	Mg^{2+}	Cut at fixed distance from asymmetric recognition sites	*Fok*I	5′-GGA*TG (N$_9$) 3′-CCT$_{\star}$AC (N$_{13}$)
*Bcg*I-like	Two structural genes; bifunctional; multisubunit	Mg^{2+}, AdoMet	Cut DNA at defined distances, on both sides of the recognition sequence	*Bcg*I	(N$_{10}$)CGA*(N$_6$)TGC(N$_{12}$) (N$_{12}$)GCT(N$_6$)$_{\star}$ACG(N$_{10}$)
McrBC	Several subunits and genes, no modification enzymes	Mg^{2+}, GTP	Cut DNA with modified C residues at different positions between the two R^{m}C sites	McrBC	5′-R^{m}C(N$_{40-2000}$)R^{m}C
III	Multifunctional, multisubunit; two structural genes	Mg^{2+}, ATP (AdoMet)	As type IIS, need functional interaction of two inversely oriented recognition sites, ATP hydrolysis	*Eco*P15	5′-CAGCA*G(N$_{25}$) 3′-GTCGTC(N$_{27}$)

[a] Nucleotides methylated by the cognate DNA methylase are indicated by asterisks. Strings of unspecified nucleotides are designated (N$_x$), where x specifies the number of nucleotides. R is G or A, ^{m}C is 5-methylcytosine, N^4-methylcytosine or 5-hydroxymethylcytosine.

AdoMet as an allosteric cofactor, as well as ATP. The enzymes recognize asymmetrical sequences interrupted by unspecific spacers of 6–8 bp. DNA cleavage does not occur within the recognition sequence but at a (sometimes very great) distance from it, up to several kilobase-pairs away. After binding to a nonmethylated recognition sequence the enzyme complex starts to translocate DNA on both sides of the recognition site. As soon as it meets a second enzyme complex, the DNA is cut. DNA translocation and scission are accompanied by extensive ATP hydrolysis.

Type II restriction enzymes are extensively exploited in gene technology. Meanwhile more than 2500 enzymes have been isolated, exhibiting more than 200 different DNA sequence specificities. Their usually palindromic recognition sequences encompassing 4–8 bp may also include unspecific spacer sequences. Normally, recognition and cleavage sites are identical. The recognition sequences for subclass IIS enzymes are asymmetric and the cleavage occurs outside the site with a short defined distance of 1–20 nucleotides. The cofactor requirements of type II enzymes are limited to bivalent cations (mostly Mg^{2+}). Restriction endonuclease and modification methylase are distinct molecules.

Similar to type I, type III and McrBC enzymes (**Table 1**), there are a few type II restriction endonucleases (called IIE, where 'E' stands for effector) which need simultaneous recognition of two copies of the specific DNA sequence to form an endonucleolytically active enzyme–substrate complex. For IIE enzymes, however, the second (effector) DNA site can be supplied as an oligonucleotide duplex resulting in a functional interaction of the enzyme with two DNA sites 'in trans'. The requirement of type IIE enzymes for simultaneous interaction with two DNA sites resembles that of proteins mediating site-specific recombination and transposition. Future investigations will show whether these restriction endonucleases provide other biological functions besides restriction of foreign DNA. In addition, the special mode of action of type IIE enzymes may represent a safety mechanism to avoid suicidal restriction of cell-own DNA: necessity of two unmodified sites for DNA scission makes it less likely that the enzyme will attack the rare unmodified sites in the cellular DNA occasionally arising after DNA repair or incomplete methylation.

Only a handful of type III systems have been discovered. Their properties are somewhere in between those of type I and type II enzymes. The molecules are composed of two forms of subunits (endonuclease units and methylase-recognition units). AdoMet stimulates the ATP-dependent nuclease. Recognition sequences of type III enzymes are nonsymmetrical, consisting of 5–6 bp, only one strand of which can be methylated. While each individual unmethylated site represents a substrate for DNA methylation, for DNA restriction the presence of two inversely oriented unmethylated recognition sites is essential.

M/R systems of different DNA specificities have been found to coexist within individual cells of various bacterial species. On the other hand, enzymes acting on identical recognition sequences (iso- and neoschizomers) can be isolated from diverse species.

Biological Functions

The biological significance of M/R systems lies in the destruction of invading foreign DNA while providing protection of self-DNA against suicidal degradation, as has been proven for type I and III systems and for a few representatives of type II. Most type II enzymes have been characterized solely with regard to their use in genetic engineering, and the question of biological relevance has largely been ignored so far.

Obviously, DNA restriction reduces the efficiency with which new genetic material can be introduced into a bacterial cell. This does not only relate to bacteriophage infection but, in certain hosts, also to processes like DNA conjugation and transformation. This is why strains impaired in DNA restriction are the preferred recipients of gene transfer.

Bacteriophage pose the greatest threat to bacteria *in vivo*. Efficient though not absolute protection against viral infection provides the cells with an evolutionary advantage. The perpetual conflict between bacterial and phage populations probably produces the pressure for the development and maintenance of M/R systems. The specificity of individual M/R systems can be altered mutationally or by recombination, and new M/R genes can be acquired by conjugation, transformation or transduction.

Evolutionary and functional links between M/R and cellular recombination systems are suggested by similarities of some of the enzymes involved.

Antirestriction by Bacterial Viruses

Under the pressure of host cell M/R systems, bacterial viruses have evolved a multitude of mechanisms allowing them to escape DNA restriction. Most of these mechanisms act quite specifically against certain M/R systems.

Virus-encoded inhibitory proteins can bind to and inactivate restriction endonucleases. This has been

described for the Ocr protein of bacteriophage T3 and T7 (which can be exploited to prevent the restriction of foreign DNA in experimental gene transfer) and for certain proteins of bacillusphage.

Other bacillusphage and T-even phage encode their own methyltransferases with specificities corresponding to those of restriction enzymes of certain hosts. In addition, the incorporation of unusual bases into viral DNA (this occurs in several bacillusphage as well as in T-even phage containing hydroxymethylcytosine (HMC) instead of cytosine) or specific postsynthetic modifications of DNA bases (formation of N^6-1-acetimidoadenine in phage Mu) can mitigate the effects of certain restriction endonucleases.

Bacterial viruses like P1 protect their genomes by co-injecting internal phage proteins with their DNA. The degradation of cofactors of restriction (and modification) is a further mechanism, exemplified by phage T3 AdoMet hydrolase.

For some phage genomes, counterselection of recognition sequences of potential M/R systems has been demonstrated. The DNA is not merely protected by lack of sites but also by a disposition of sites that thwarts the reaction mechanism of the restriction endonuclease. The type II restriction enzyme *Eco*RII requires two functionally cooperating sites for restriction; T3 DNA is not cleaved because its *Eco*RII sites are too far apart. The type III endonuclease *Eco*P15 can only cleave at a pair of (nonsymmetrical) recognition sites inversely oriented in the DNA double strand; the genome of phage T7 contains all its 36 recognition sites in the same orientation and is therefore resistant to cleavage.

There are additional mechanisms that are not effective in protecting the primary infecting phage DNA but do prevent restriction of progeny and superinfecting phage respectively: the *ral* function of phage λ and the *arn* function of phage T4 (directed against Rgl, see below).

Restriction of Nonglucosylated HMC-containing DNA

It has been known for a long time that the DNA of T-even phage previously grown in host cells with impaired sugar metabolism is attacked by a specific restriction system. These viruses possess HMC instead of cytosine in their DNA. This unusual base protects the genome against virus-encoded nucleases that degrade the host chromosome during virus replication, as well as against some restriction enzymes of the host. However, certain *Escherichia coli* strains have developed nuclease systems specific for HMC-containing DNA (Rgl = *r*estriction of non*gl*ucosylated DNA). The phage, in turn, protects itself against Rgl by glucosylation of HMC, catalyzed by phage-encoded glucosyltransferases. When glucosylation is blocked, e.g. by lack of sugar precursors, the phage DNA in such cells (e.g. in the Gal^- strain *E. coli* $B/4_0$) remains unglucosylated and is degraded during the next round of infection of an Rgl^+ host.

Differences between the Hsd and the Rgl systems are also evident in the phenotypic behavior of phages on passage over different host strains (**Table 2**). The Hsd system relies on the 'classical' principle: one reproductive cycle on a restrictive strain (Res^+Mod^+) leads to adaptation (modification) of the few surviving phages, which are then capable of high replication rates on subsequent infection of this host. This does not in every case apply to the Rgl system; replication of T2 in Gal^- *E. coli* $B/4_0$ cells does not improve the growth of the virus in the next infectious cycle, since the HMC-containing DNA remains unglucosylated.

While the existence of Hsd restriction-proficient but modification-deficient hosts is ruled out due to suicidal degradation of the host chromosome, this constellation does occur in the Rgl system because the host chromosome does not contain HMC and is therefore susceptible to neither modification (glucosylation) nor restriction (**Table 2**).

Methylation-dependent DNA Restriction

Typically, methylation of a given DNA recognition sequence protects it against cleavage by the restriction enzyme of this specificity. But there are also restriction enzymes where cleavage is triggered by methylation. Their best known representative is *Dpn*I from *Streptococcus pneumoniae*, which only cleaves its recognition sequence 5′-GATC when the adenines in both strands are methylated.

Attention has recently been focused on defense systems of *E. coli* which attack incoming methylated DNA. The McrA and McrBC systems (*m*ethylated *c*ytosine *r*estriction) as well as the Mrr system (*m*ethylated adenine *r*ecognition and *r*estriction) interfere with efficient cloning and expression of certain DNA methylase genes. The Mcr systems also destroy cloned C-methylated DNA of eukaryotic origin.

Up to now little is known about the Mrr- and Mcr-dependent enzymes. They are apparently less sequence specific than the other M/R enzymes. However, it became clear that the Mcr systems are equivalent to the earlier described Rgl systems. These restriction systems are targeted to modified DNA cytosine, be it 5-hydroxymethylcytosine, 5-methylcytosine or N^4-methylcytosine (for McrBC, see also **Table 1**).

Table 2 Examples of phenotypical M/R of phage by the Hsd and Rgl restriction systems

Host system	*Phage last grown on*	*Relative efficiency of plating on host strain*[a,b]		
		E. coli B R.*Eco*B$^+$ M.*Eco*B$^+$	*E. coli* K-12 R.*Eco*K$^+$ M.*Eco*K$^+$	*E. coli* C R.*Eco*B$^-$K$^-$ M.*Eco*B$^-$K$^-$
Hsd	$\lambda \cdot$ B	1	5×10^{-4}	1
	$\lambda \cdot$ K	10^{-4}	1	1
	$\lambda \cdot$ C	10^{-4}	5×10^{-4}	1
	$\lambda \cdot$ C · B	1	5×10^{-4}	1
		E. coli B Rgl$^+$ Gal$^+$	*Shigella dysenteriae* SH Rgl$^-$ Gal$^+$	*E. coli* B/4$_0$ Rgl$^+$ Gal$^-$
Rgl	T2 · B	1	1	1
	T2 · SH	1	1	1
	T2 · B/4$_0$	10^{-3}	1	10^{-3}
	T2 · B/4$_0$ · SH	1	1	1

[a] Abbreviations: Hsd, host specificity of DNA; R, restriction endonuclease; M, modification methylase; Rgl, restriction of nonglucosylated DNA; Gal, DNA glucosylation capability.
[b] Plating data from S. E. Luria and the present authors.

Further Functions of DNA Methylation in Prokaryotic Cells

Not every methylation of prokaryotic DNA is functionally related to processes of DNA restriction, i.e. by either protecting against, or itself triggering, the cleavage activity of restriction endonucleases. The methylases M.dam (for *D*NA *a*denine *m*ethylation) and M.dcm (for *D*NA *c*ytosine *m*ethylation) occur in *E. coli*. They catalyze the conversion of adenine in the recognition sequence 5′-GATC to 6-methylaminopurine, or of the internal cytosine in the recognition sequence 5′-CC(A/T)GG to 5-methylcytosine, although there are no functionally corresponding restriction enzymes in these cells.

Meanwhile, several functions of M.dam- and M.dcm-specific methylations have been identified. Dam methylation plays a crucial role in DNA repair, specifically in proofreading of nascent DNA. Since methylation of daughter strands lags behind their synthesis, methylase recognition sequences are hemimethylated for a short time, allowing repair enzymes to distinguish between the two strands and correct errors guided by the parental DNA strand.

The methylation of promoter regions can influence gene expression, an effect first described for certain eukaryotic cells and their viruses (see below). The relation between Dam methylation and control of expression of a phage gene is exemplified by the *mom* gene of phage Mu. The gene is only expressed when its promoter is methylated. Since promoters of transposase genes can be methylation-dependent, DNA methylation may also influence DNA transposition.

In several cases where the *ori* region contains M.dam sites (e.g. in *E. coli*, various plasmids and phage P1), primary initiation of DNA replication is regulated via DNA methylation. Packaging of P1 DNA during morphogenesis is also supposed to be dependent on the methylation of certain sequences in the viral *pac* locus. In some cases, phase variation of pilus expression is controlled by Dam methylation in *E. coli* cells.

Just as sequence-specific DNA methylation is not necessarily connected to M/R systems, neither is sequence-specific DNA cleavage (e.g. integration and excision of the λ genome).

Nonclassical Modification and Restriction of Pro- and Eukaryotic Viruses

Phenotypic modification and restriction can also be the consequence of molecular processes not directly involving the DNA but rather certain viral proteins. They were termed 'nonclassical' to distinguish them from the 'classical', purely DNA-based mechanisms.

In nonclassical M/R, which was first described for phage T3, the ability of a phage to adsorb to a given host cell is affected by the prior host strain. Representatives of the genetically unaltered virus varying in their adsorption-protein modification also

differ serologically. The molecular mechanism has not been resolved yet.

In eukaryotic viruses so far all host cell adaptation is explained by the appearance and selection of viral mutants. It has, however, not been investigated whether phenotypic M/R processes of genetically unaltered viruses do also occur in the kingdom of eukaryotes, possibly explaining the occurrence of reversible changes in the host cell tropism of viruses.

DNA Methylation in Eukaryotic Viruses

There is no unequivocal evidence of the existence of DNA M/R systems in eukaryotic cells. Enzymes resembling pairs of DNA methyltransferases and restriction endonucleases are, however, encoded by viruses infecting eukaryotic algae (*Chlorella* spp.); their biological functions remain still unclear. Other viruses, like Frog virus 3 and lymphocystis disease virus (members of the family *Iridoviridae*), encode their own DNA methyltransferases, and their genomes carry high numbers of 5-methylcytosine residues. Functional consequences of the extensive methylation have to be further elucidated.

In the DNA of eukaryotic cells 5-methylcytosine is usually generated by cellular methyltransferase activities and mainly but not exclusively occurs in CpG dinucleotides. DNA methylation is known to be involved in processes like transcription inhibition, genomic imprinting, cell differentiation and dedifferentiation (carcinogenesis), and hypermutability. Furthermore, DNA methylation is assumed to silence transcription from integrated retroviral genomes, whereas demethylation results in the reactivation of the provirus. For some human pathogenic viruses, such as herpes simplex viruses, cytomegalovirus, Epstein–Barr virus or papillomaviruses, a role of DNA methylation in gene regulation has been demonstrated.

See also: **Adenoviruses (*Adenoviridae*): Animal viruses, General features, Malignant transformation and oncology, Molecular biology; Algal viruses (*Phycodnaviridae*); Frog virus 3 (*Iridoviridae*); Herpes simplex viruses (*Herpesviridae*): General features, Molecular biology; Human immunodeficiency viruses (*Retroviridae*): Molecular biology, Anti-retroviral agents, General features; Coliphage lambda (*Siphoviridae*); P4 phage (*Satellites*); T7-like phages (*Podoviridae*); P2, 186 and related phages (*Myoviridae*).**

Further Reading

Arber W (1965) Host-controlled modification of bacteriophage. *Annu. Rev. Microbiol.* 19: 365.

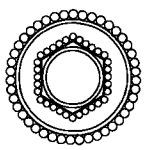
Bickle TA and Krüger DH (1993) The biology of DNA restriction. *Microbiol. Rev.* 57: 434.

Jost JP and Saluz HP (eds) (1993) *DNA Methylation: Molecular Biology and Biological Significance*. Basel: Birkhäuser.

Krüger DH, Kupper D, Meisel A, Reuter M and Schroeder C (1995) The significance of distance and orientation of restriction endonuclease recognition sites in viral DNA genomes. *FEMS Microbiol. Rev.* 17: 177.

McClelland M, Nelson M and Raschke E (1994) Effect of site-specific modification on restriction endonucleases and DNA modification methyltransferases. *Nucleic Acids Res.* 22: 3640.

HUMAN IMMUNODEFICIENCY VIRUSES (*RETROVIRIDAE*)



Contents

General Features

Luc Montagnier, Institut Pasteur, Paris, France and Queens College, NY, USA

History

The infectious origin of acquired immunodeficiency syndrome (AIDS) was recognized soon after the identification of the disease itself in 1981. The first AIDS cases diagnosed in 1982 in hemophiliacs

production or perhaps in blocking apoptosis. An amino-terminus domain of Vpr is important for virion targeting and nuclear localization. A carboxyl-terminal domain is important for G2 arrest. Vpx is an additional ORF present in HIV-2 and related simian immunodeficiency virus strains. Vpx is also a virion protein but it does not induce G2 arrest.

vpu

The *v*irus *p*rotein *U* ORF encodes an 81 amino acids protein (Vpu). Vpu is expressed as part of a bicistronic message that also encodes the Env protein and is regulated by Rev. Vpu is a type I membrane protein with two well-described functions: degradation of CD4 in the endoplasmic reticulum and enhancement of virus release from the plasma membrane. The coordinated expression of Vpu and Env is important because CD4 degradation results in the release of Env from a complex with CD4 which otherwise would be retained in the endoplasmic reticulum. Therefore, Vpu expression in CD4+ cells would result in increased levels of Env at the site of particle assembly. A third function has also been described for Vpu, the ability to form a cation-selective ion channel. The role of this ion channel in Vpu activity is presently unknown.

nef

The protein product of the *nef* gene (Nef) was originally described as having a negative effect on virus replication and thus being a *ne*gative *f*actor hence the acronym. Subsequent *in vivo* and *in vitro* studies have shown that Nef has a positive effect on virus replication. The *nef* ORF encodes a 27–29 kDa protein. Nef is expressed from multiply spliced messages that are Rev independent and expressed at high levels early and throughout infection. Nef is myristoylated and phosphorylated in HIV-infected cells. Both post-translational modifications are important for function. Analysis by electron microscopy or immunofluorescence analysis indicates that Nef is found predominantly in the perinuclear region with a small fraction of Nef associated with the inner portion of the cell membrane. Biochemical analysis shows that a large proportion of Nef is associated with membranes through its N-terminus myristyl group. *In vitro* analysis indicates that Nef is a multifunctional protein. There are at least four reproducible functions for Nef: cell surface CD4 and major histocompatability complex (MHC) I downregulation, enhancement of particle infectivity, association with cellular serine/threonine kinases, and cellular activation. These functions of Nef contribute to the high level and efficient replication of HIV *in vivo*, but it is clear that the mechanisms of Nef action remain unknown.

See also: **Antivirals; Human immunodeficiency viruses (*Retroviridae*): Anti-retroviral agents, General features; Human T-cell leukemia viruses (*Retroviridae*): HTLV-1, HTLV-2; Retroviruses – type D (*Retroviridae*); Retroviruses of drosophila: The gypsy paradigm; Simian immunodeficiency viruses (*Retroviridae*).**

Further Reading

Coffin JM, Hughes SH and Varmus HE (1997) *Retroviruses*. Plainview, NY: Cold Spring Harbor Laboratory Press.

Pavlakis GN (1997) In: The molecular biology of Human Immunodeficiency Virus Type 1. De Vita VT, Hellman S and Rosenberg SA (eds) *AIDS*, 4th edn, p. 45. Philadelphia: Lippincott–Raven.

Antiretroviral Agents

Ranga V Srinivas and **Arnold Fridland**, Department of Infectious Diseases, St Jude Children's Research Hospital, Memphis, Tennessee, USA

Introduction

This review attempts to give a brief overview of antiretroviral agents currently used in the control of HIV-1 infections. Compounds in development, as well as other viral targets for the development of potential new drugs are considered briefly. An overview of compounds targeted against various viral gene products is presented and issues that affect the pharmacological response of the various compounds are raised.

Human Immunodeficiency Virus (HIV) Genome and Viral Targets for Drug Development

The HIV genome encodes both structural genes, *gag*, *pol* and *env*, that are common to all retroviruses, as well as additional regulatory/accessory genes unique to lentiviruses. In HIV, these genes include *tat*, *rev*, *nef*, *vif*, *vpu* and *vpr*. *Nef*, *vif*, *vpu* and *vpr* are not essential for virus replication *in vitro* although they play a role in viral pathogenesis. The HIV *gag* gene encodes a precursor polyprotein, which is processed to form the viral capsid which encases the viral

genomic RNA. A properly folded mature viral capsid is obligatory for infectivity, and agents that disrupt any one step in the complex process of capsid assembly can serve as an antiviral agent. The *pol* gene codes for three virus specified enzymes: reverse transcriptase (RT), integrase and protease, all of which play an essential role in virus replication. RT is one of the viral encoded enzymes along with integrase and viral protease that are essential for viral replication. RT converts single-stranded viral RNA into the double-stranded proviral DNA. Proviral DNA is then incorporated into the host cell DNA via the integrase. Transcription and translation of integrated proviral DNA primarily synthesize HIV RNA and polyproteins. Because RT and integrase are not used by host cell metabolism and are specific for viral replication, they are natural targets for inhibitors. HIV protease plays an important role in the processing of viral gag and gag-pol polyprotein, a process essential for the formation of infectious virus. HIV protease inhibitors are by far the most potent HIV inhibitors, and exert their antiviral effects on both acutely and chronically infected cells. Because of their enzymatic nature, *pol* gene products are readily amenable targets for drug development. Furthermore, the three-dimensional x-ray crystal structure has been determined for RT, protease, as well as integrase, which provide an opportunity for rational drug design. These structures can be accessed and viewed on the web using Molecules R US resource from the Center of Molecular Modeling, NIH (*http://molbio.info.nih.gov/cgi-bin/pdb*).

HIV envelope protein is also an attractive target for drug development, since it is responsible for virus attachment and entry to the target cells. The CD4 molecule has been identified as the major receptor for HIV entry. A ganglioside molecule can also serve as a receptor for CD4-independent entry of HIV into certain cell types. More recently, various members of the CXC and CC chemokine receptor superfamily have been shown to function as coreceptors for HIV entry, and determine virus tropism. The molecular biology of various regulatory and accessory genes of HIV, and their role in viral replication and pathogenesis is well studied and identifies several potential targets. HIV *tat* gene transactivates viral RNA transcription, whereas the *ev* gene facilitates nuclear cytoplasmic export of unspliced viral RNAs. Both genes are synthesized early during HIV replication, and are essential for infectivity. The *nef* gene, although dispensable for *in vitro* replication, plays an important role in viral pathogenicity. The other accessory genes, *vif*, *vpu* and *vpr*, although not required for viral infectivity, do have specific effects on cells, that can modulate HIV pathogenicity, and agents that disrupt these functions can prove useful in preventing HIV disease progression.

Reverse Transcriptase Inhibitors

HIV RT is a multifunctional enzyme with RNA-dependent DNA polymerase, ribonuclease (RNase) H, and DNA-dependent DNA polymerase activities. The active protein is a heterodimer comprising p66 and p51 subunits. The N-terminal 440 amino acids of p66 contain polymerase activity and the C-terminal 120 residues show RNase H domain. The p51 of the subunit of HIV-RT corresponds to the polymerase domain of the p66 subunit. The crystal structure of RT has been resolved and identifies four individual subdomains: fingers, palm, thumb and connecting region. The overall folding of p66 and p51 is similar, but the spatial arrangements of the two subdomains are extremely different. Highly conserved regions located in the p66 region, together with two alpha helices of the thumb act as a clamp to position the template-primer relative to the polymerase active site, which is located in the palm region. The 3′ end of the primer terminus is close to residues Asp110, Asp185 and Asp186 in the catalytic site. A spatially distinct hydrophobic pocket comprising residues Tyr181 and Tyr188 in p66 form crucial components for binding non-nucleoside RT inhibitors. Inhibitors of RT can be divided into two categories. The first group include the nucleoside analogues (NRTI) such as zidovudine (ZDV, 3′-azido-2′3′-dideoxythymidine), didanosine (ddI, 2′3′-dideoxyinosine) and zalcitabine (ddC, 2′3′-dideoxycytidine), which compete with normal 2′-deoxynucleoside triphosphates (dNTP) precursors of DNA, whereas the second group comprise structurally diverse compounds referred to as non-nucleoside reverse transcriptase inhibitors (NNRTI). The chemical structures of some of the commonly used reverse transcriptase inhibitors are shown in **Fig. 1**.

Nucleoside inhibitors (NRTI)

Zidovudine (ZDV); didanosine (ddI); lamivudine (3TC, (−)-2′-deoxy-3′-thiacytidine), stavudine (d4T, 2′3′-dideoxythymidine) and zalcitabine (ddC) are currently licensed drugs but their efficacy has been limited owing to both pharmacological factors and emergence of resistant virus strains. Several newer compounds have greatly improved pharmacological properties. Some examples include: N^6-cyclopropylamino 2′3′-dideoxyguanosine (abacavir, BW1592U89), the acyclic phosphonate analogues 9-(2-phosphonylmethoxyethyl)adenine (adefovir, PMEA) and its propyl counterpart, PMPA (9-(2-phosphonylmethoxypropyl)adenine, and F-ddA (2′-β-fluoro-2′3′-dideoxyadenosine). All these com-

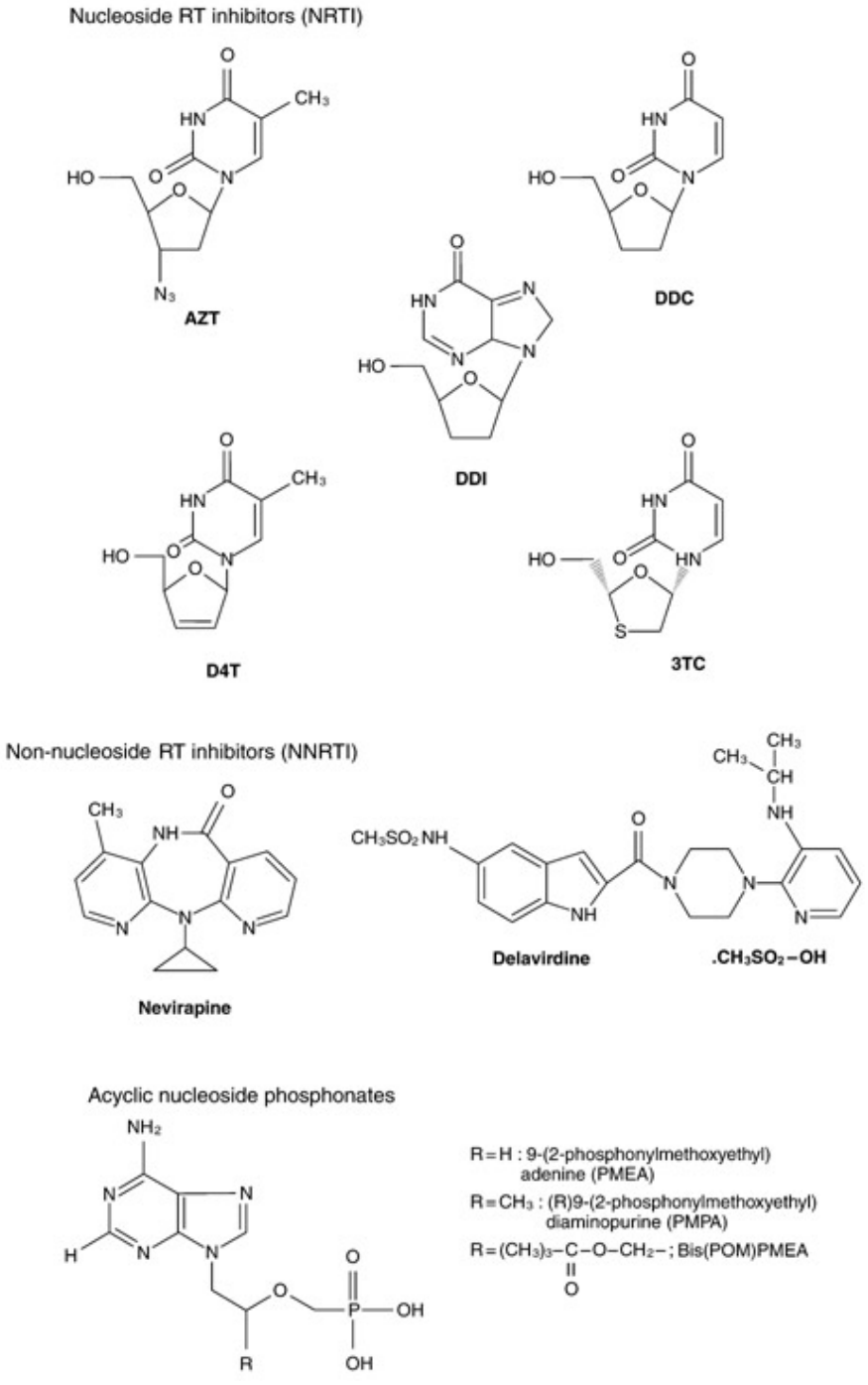


Figure 1 Structures of commonly used reverse transcriptase inhibitors.

pounds are currently undergoing clinical trials for the treatment of HIV infections in human subjects.

All nucleoside RT inhibitors are essentially prodrugs and require intracellular activation to the corresponding triphosphate (ddNTP) forms by cellular pathways. The initial kinase is a crucial step in the intracellular metabolism of the ddNs, and together with the intracellular half-life of the active triphosphate in the target cells can determine the antiviral efficacy of these agents. The dideoxynucleoside analogues such as ZDV and 3TC terminate the DNA chain growth due to lack of a 3′-hydroxyl group. The principal idea is that they replace the natural deoxynucleoside triphosphate as substrates during the viral

DNA synthesis, but terminate chain elongation because there is no 3′-OH group to continue chain growth. In both lymphocytes and monocyte/macrophages, the ddNs are first phosphorylated by either a nucleoside kinase or 5′-nucleotidase (acting as a phosphotransferase). The resulting monophosphate is further phosphorylated by nucleotide kinases to the active triphosphate. The nucleoside kinase activity of resting cells such as the monocytes/macrophages and dendritic cells may be insufficient to phosphorylate adequately certain ddNs, e.g. ZDV or d4T and limit their anti-HIV efficacy. One successful strategy to overcome this problem has utilized the acyclic nucleoside phosphonate analogues. Compounds such as adefovir and PMPA can be considered as monophosphate analogues, in which the first phosphate group has already been added as a phosphonate. These compounds enter cells by endocytosis and after addition of two phosphate groups by ubiquitous cellular nucleotide kinases inhibit the HIV RT. PMEA and PMPA also show a broad antiviral specificity and also inhibit other immunodeficiency viruses, such as simian immunodeficiency virus (SIV) as well as hepadnaviruses (e.g. hepatitis B virus, HBV). These compounds are currently being evaluated in the clinic for the treatment of both HIV and HBV infections in human subjects. A related phosphonate analogue (*S*)-1-[3-hydroxy-2-(phosphonylmethoxy)propyl] cytosine (HPMPC) is licensed for the treatment of cytomegalovirus infections in autoimmune deficiency virus (AIDS) patients.

Non-nucleoside RT inhibitors (NNRTI)

NNRTI are a chemically diverse group of compounds that function as highly selective, allosteric inhibitors of HIV-1 reverse transcriptase. Therefore, they do not compete with dNTP substrates to exert their antiviral effects, nor do they require intracellular activation. The NNRTIs are inactive against HIV-2, SIV and other lentiviruses. This specificity of the NNRTIs for the HIV-1 RT is due to the presence of a unique highly hydrophobic pocket in HIV-1 RT into which the NNRTI fit snugly. The NNRTI binding pocket of HIV-1 RT is functionally and spatially distinct from the substrate (dNTP) binding site on the palm domain of the p66 subunit. Most NNRTIs assume a butterfly-like (or Y-shaped) structure comprising two hydrophobic wings connected to a central polar body. The NNRTIs probably act as π electron donors to aromatic side chains of HIV RT residues around the NNRTI-binding pocket.

The first compounds to be described in that class are the tetrahydroimidazole[4,5,1-jk][1,4]benzodiazapin-2(1H)-one and thione (TIBO) derivatives. Other compounds soon followed such as 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT) derivatives, pyridinone, bis(heteroaryl)piperazine (BHAP), nevirapine, phenylethylthioureathiozole (PETT) derivatives, delaviridine and yet others. Although all these compounds may be assumed to bind to the same pocket site at the HIV RT, different classes of HIV-1 specific RT inhibitors may differ from each other with regard to the exact amino acids of the binding site with which they interact. This conclusion is drawn from the finding that different NNRTI do not exactly show crossresistance to each other, although they seem to overlap. Currently approved NNRTI include nevirapine (Boehringer-Ingelheim) and delaviridine (Rescriptor, Pharmacia-UpJohn). Other compounds, e.g. DMP-266, are in advanced clinical trials.

HIV drug resistance to reverse transcriptase inhibitors

Drug resistance is a major problem leading to treatment failure with antiretroviral agents. The patterns of crossresistance vary with different mutations, and a virus resistant to one agent is not automatically resistant to a related compound. A detailed list of various drug resistance associated mutations in HIV has been reported and a searchable database of drug resistance mutations in HIV is available on the web (*http://www.viral-resistance.com*). **Figure 2** shows an abbreviated list of RT mutations associated with NRTI resistance. Resistance to ZDV is associated with mutations of Met41 → Leu, Asp67 → Asn, Lys70 → Arg, Thr215 → Phe and Lys219 → Gln in the HIV RT gene. Single mutations appear early during treatments with ZDV but are not associated with high level of resistance until several mutations occur. In advanced HIV disease, phenotypic high levels of ZDV resistance and particularly the mutations at codon 41 and 215 are correlated with increased risk of clinical progression and death. Mutations at Leu74 → Val and Val75 → Thr in the RT gene confer resistance to both ddI and ddC. The Val75 → Thr mutation observed with d4T is also crossresistant to ddI, ddC, d4C and 2′-deoxy-5-fluoro-3′-thiacytidine (FTC). Met184 → Val is responsible for resistance to ddC and 3TC. A K65 → R mutation confers resistance to 3TC and PMEA. The genetic background of the virus also contributes to the phenotypic effect of resistance-determining mutations. For example, introduction of T215F/Y mutation in to wild-type RT leads to ZDV resistance, but does not confer ZDV resistance in viruses with mutant RT harboring M184V or L74V mutations.

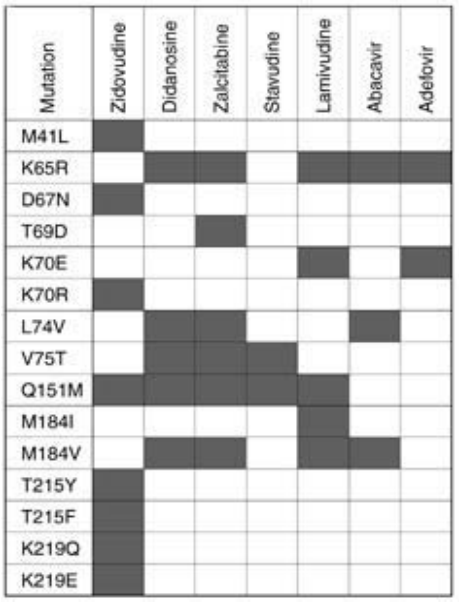


Figure 2 Reverse transcriptase mutations frequently associated with drug resistance to commonly used NRTI. The dark boxes show mutations verified to confer resistance.

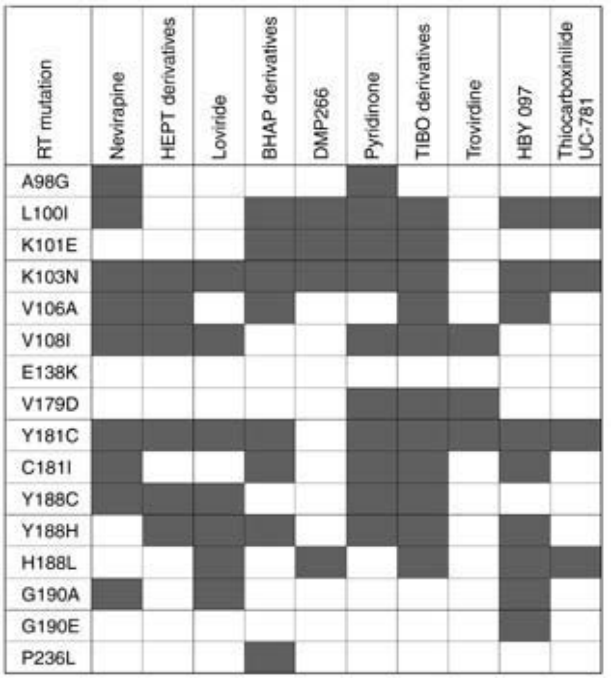


Figure 3 Reverse transcriptase mutations frequently associated with drug resistance to commonly used NNRTI. Dark boxes show mutations associated with resistance.

The observation that the development of resistance to one compound can reverse the effects of resistance mutation, and restore sensitivity to a second inhibitor has been clinically rewarding. The Met 184 → Val and Met 184 → Ile mutations emerge rapidly in patients treated with 3TC, and both mutations can suppress the effects of ZDV-resistant mutations. Other mutations that restore ZDV susceptibility in mutants harboring ZDV resistance mutations at codons 41, 67, 70, 215 and 219 include Leu74 → Val selected by ddI, Gln 161 → Leu and His 208 → Tyr mutations selected with the NNRTI Foscarnet, nevirapine or TIBO. The mechanism for these effects may be due to compensatory effects of certain combinations of mutations on template-primer binding.

The mutations observed with the ddNs do not lead to resistance to the NNRTI and vice versa. Resistance to the ddNs are located in the vicinity of either the substrate (dNTP) binding site or the template binding site. Resistance to the ddNs appear to develop more slowly than for the NNRTI, and as shown for ZDV resistance may develop in a stepwise manner, with each step leading to greater resistance.

Mutations leading to NNRTI resistance tend to cluster around the contact points between NNRTI and HIV-1 RT and often include RT residues L100, K101, K103, E138, V179, Y181, Y188, V189, G190 and P236. NNRTIs have been found to lead rapidly to emergence of drug resistant viruses both *in vitro* and *in vivo*. The mutation Tyr 181 → Cys is associated with resistance (or reduced sensitivity) to most of the NNRTIs (i.e. TIBO, HEPT, nevirapine, pyridinone, BHAP, 2′,5′,bis-O-(tert-butyldimethylsilyl)-3′-spiro-5′(4′-amino-1′,2′-oxathiole-2′,2′-dioxide) pyrimidine, alpha-anilinophenylacetamide (αAPA). The mutation Tyr 188 → His confers resistance to TIBO, pyridinone but not nevirapine, whereas the mutation Tyr 188 → Cys is associated with resistance to TIBO, pyridinone and nevirapine. Likewise, Val 106 → Asn determines resistance to TIBO, nevirapine, pyridinone and BHAP, whereas Val 106 → Ala accounts for resistance mainly to nevirapine and pyridinone. The mutation Leu 100 → Ile is associated mainly with resistance to TIBO and Glu 138 → Lys accounts for resistance to TSAO but none of the other compounds (**Fig. 3**).

Several observations have rekindled interest in the clinical development of NNRTI. Higher doses of NNRTI can be safely used to partially suppress the emergence of resistant variants. Mutations do not result in crossresistance to all NNRTI thus permitting a switch in therapy from one compound to another, or combination therapy with drugs that have different resistance patterns. Multiple sequential mutations are required to confer high level resistance to new NNRTIs, e.g., DMP-266. Some NNRTI mutations, e.g. Y181C selected by nevirapine, or L1001 selected by TIBO R82150 can suppress or reverse the effects

of ZDV resistance mutations. Likewise, mutations selected by delaviridine can sensitize RT over tenfold for nevirapine, TIBO R82913 and L-697,661. Some NNRTI mutations, e.g. G190E, reduce RT enzyme activity and reduce viral replication competence. Some NNRTI inhibit enzymes in the cytochrome P450 pathway which metabolize HIV protease inhibitors, and enhance their bioavailability.

Approaches to minimize emergence of resistant variants include use of combination therapy with multiple agents (NRTIs as well as other compounds, especially protease inhibitors) to ensure maximal virus suppression. A convergent therapy with multiple RT inhibitors was initially proposed with the idea that an enzyme that mutates to acquire resistance to multiple agents may be nonfunctional, but later studies have shown that the RT protein is highly flexible and can simultaneously show resistance to all the currently available compounds.

Integrase Inhibitors

The HIV integrase is an attractive target for selective anti-HIV therapy since there is no known functional counterpart in human cells. The only enzyme required for HIV-1 integration is the integrase (IN), a protein of 32 kDa encoded at the 3′ end of the *pol* gene. This enzyme is produced by protease-mediated cleavage of the gag-pol precursor during virion maturation. The integrase recognizes specific sequences in the long terminal repeat (LTR) of the viral DNA: 5′-ACTG ... CAGT-3′. The TG ... CA repeat has been conserved throughout evolution. In the first step of this reaction, termed 3′ processing, two nucleotides are removed from each 3′ end to produce new 3′ hydroxyl ends (CAOH-3′). This reaction takes place in the cytoplasm. After entering the nucleus, the processed viral DNA is joined to the host target DNA. The joining reaction includes a coupled 4–6 bp staggered cleavage of the target host DNA and the ligation of the processed CAOH-3′ viral DNA. Host enzymes probably accomplish repair of the remaining gaps. Oligonucleotide-based assays have been designed to mimic both the processing and joining reactions *in vitro*. Several DNA binding agents were found to inhibit the integrase. To date, only one integrase inhibitor, zintevir (AR-177), a synthetic 17mer oligonucleotide containing phosphorothioate linkage at the 3′ and 5′ terminal bonds is undergoing clinical trials as an intravenous formulation. AR-177 has an IC_{50} in the 30–50 nM range. AR-177 also inhibits virus binding to *CD4+* cells, and inhibits cell fusion at micromolar concentrations of AR-177.

Other inhibitors HIV IN include DNA-binding agents, topoisomerase inhibitors, aurintricarboxylic acid and cosalene analogues, caffeic acid phenylethyl ester (CAPE), and bis-catechols or other hydroxylated aromatic compounds. A new class, the anthraquinones (e.g. quinalizarin and purpurin) inhibits HIV preintegration complexes *in vitro*. Tyrphostins are protein kinase inhibitors that also exhibit activity against HIV IN. Hydroxycoumarin complexes, such as NSC 158393, have also been reported to have potent anti-HIV IN activity as well as activity against HIV protease. Recently, a novel class of IN inhibitors has been described – the dicaffeoylquinic acids (DCQAs) – which are isolated from medicinal plants used by the Kallawaya herbalists of Bolivia. Several reports have utilized computational analysis to search for potential inhibitors of IN, and identified a number of relatively low-potency inhibitors which could serve as lead compounds.

Protease Inhibitors (PI)

HIV protease is responsible for processing gag and gag-pol precursor polyproteins into essential structural proteins. Inactivation of protease gene by site-directed mutagenesis results in noninfectious virus, thus making HIV protease an attractive target for drug development. Currently four compounds – saquinavir, ritonavir, indinavir and nelfinavir – have been licensed for human use. The chemical structures of the currently licensed protease inhibitors are shown in **Fig. 4**. Amprenavir (Glaxo141) is in advanced multicenter clinical trials. Many other compounds, such as ABT378, PNU140690 and PD178393, are under various stages of development. HIV protease is an aspartic acid protease, and its crystal structure has been resolved. HIV protease is a dimer, and acts like a pair of molecular scissors to cleave target sites. Each 99-residue monomer contributes an aspartic acid residue to the site. The two aspartic acid residues deliver a water molecule to the cleavage site of the substrate to create a tetrahedral transition state intermediate that rapidly breaks apart to give a C-terminal acid and N-terminal amine. HIV protease inhibitors present some classic examples in drug design and development. Numerous inhibitors have been designed by incorporating a nonhydrolysable P1.P1′ cleavage site mimic into a substrate sequence, where P1 and P1′ refer to the first residue to the left and right, respectively, at the cleavage site. Saquinavir (invirase), the first protease inhibitor to be approved for human use, contains a hydroxyethylamine (HEA) at P1/P1′ transition state isostere. Ritonavir was synthesized to exploit the symmetric nature of HIV protease, and imposes an axis of symmetry on the hydroxyethylamine isostere at, or next to, the hydroxyl group of the transition state analogue. In other

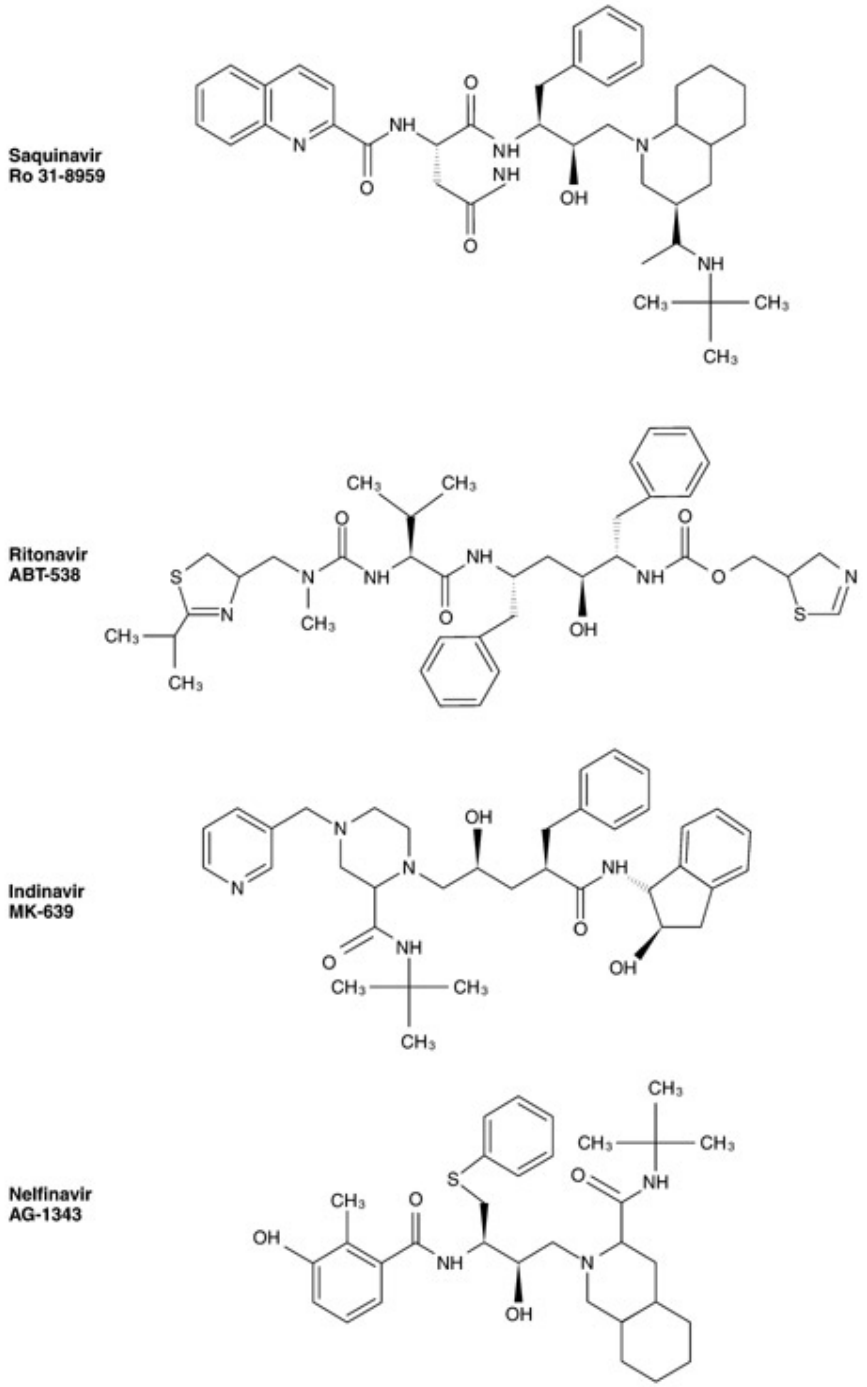


Figure 4 Structures of commonly used protease inhibitors.

studies Merck identified a heptapeptide containing hydroxyethylene isostere as a lead compound by screening compounds that were initially synthesized as an inhibitor of renin, also an aspartic protease. A series of compounds were synthesized to reduce the activity of this compound against renin, and increase activity against HIV protease finally leading to the development of indinavir. Nelfinavir was designed by refining saquinavir structure by replacing the enzymatically labile P3.P2 fragments with a simple hydroxytoluene amide group, and compensate the loss of potency resulting from this change by replacing the P1 phenyl group with a phenylthio group which can more efficiently fill the hydrophobic

pocket of HIV protease. Amprenavir (VX478 or BW141W94) was also designed by refining the saquinavir structure. Here the decahydroisoquinoline group was replaced with an isobutyl sulfonamide group, the P3-P2 groups were replaced with a tetrahydrofuran carbamate, and an amino group was introduced to the phenylsulfonamide group to increase water solubility and oral absorption.

All protease inhibitors show potent antiviral activity. Saquinavir has K_i of 1–8 nM against HIV-1 protease and its EC_{95} values <100 nM against various laboratory and clinical HIV isolates. The corresponding K_i and EC_{95} values are 0.015 nM and <150 nM for ritonavir, 0.4 nM and <100 nM for indinavir and 2 nM and <200 nM for nelfinavir. The HIV protease inhibitors are also highly effective *in vivo*, and accomplish >2-log (>99%) reduction in plasma viral RNA levels, compared ~0.5–1 log reduction by RT inhibitors. However, the major limitations of currently available HIV PIs is that they are all poorly soluble, high-molecular-weight compounds that show very high protein binding. These factors are associated with a poor oral bioavailability and increased hepatic clearance. Consequently, optimal dosing requires frequent administration of several large capsules or tablets, which promotes noncompliance, and also limits their use in younger children. Many PIs are metabolized by the cytochrome P_{450} pathway. The isozyme CYP3A4 is the major enzyme that metabolizes these compounds, and compounds that affect CYP3A metabolism can markedly influence the pharmacokinetics of HIV PI. For example, nevirapine stimulates CYP3A metabolism, and coadministration of nevirapine requires PI dose adjustments. Interestingly, ritonavir is also an inhibitor of CYP3A and can enhance serum concentrations of HIV PIs and other compounds metabolized by this pathway. This property is being exploited to enhance the bioavailability of saquinavir in combination therapies including ritonavir and saquinavir.

HIV resistance to protease inhibitors

Development of resistance is observed primarily when the plasma drug levels are low and insufficient to effectively block viral replication. This may occur either due to differences in individual variations in pharmacokinetics (e.g. poor absorption, increased clearance, food or drug-interactions etc.) or due to poor compliance in taking prescribed doses of medication. The different drugs select for different, although overlapping mutations (**Fig. 5**). All drug resistance mutations affect the substrate binding site. Commonly encountered mutations include substitutions at V82, I84 or L90 for indinavir and ritonavir, G48, I84 or L90 for saquinavir, and D30, I84 or L90 for nelfinavir. In additions to the mutations around the substrate-binding site, all PIs also select for overlapping sets of mutations elsewhere in the enzyme including L10, M46, L63, A71 and N88. Cyclic urea amide inhibitors currently under development (e.g. SD 146) show antiviral activity against HIV harboring protease gene mutations at 10I, 48V, 54T, 63Q, 71V, 77I and 82A and show resistance to currently available protease inhibitors.

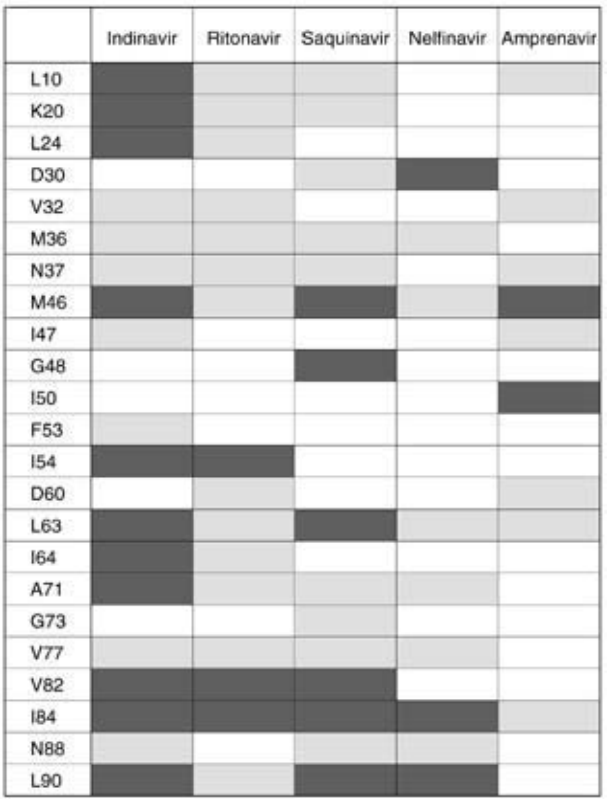


Figure 5 Protease mutations frequently associated with drug resistance. The dark boxes show phenotypically verified mutations. Lighter boxes identify mutations selected by the compound, but their role in determining resistance has not yet been confirmed.

Other Targets

Although potential antiviral targets are identified in other viral genes, no compound has progressed far enough to approach clinical development. Some of the potentially interesting targets and leads are indicated here.

The nucleocapsid protein NCP7 contains two C-X2-C-X4-H-X4-C motifs that are required for viral RNA encapsidation. The mature form NCP7 forms zinc-fingers in the presence of stoichiometric zinc. Certain oxidizing agents, including 3-nitroso-benz-

amides and cupric ions remove zinc complexed to NCp7 *in vitro*. Certain aromatic disulfide compounds also extract zinc from the NCP7. Compounds that extract zinc also inactivate HIV. Although currently tested zinc chelators are far too cytotoxic for therapeutic use, they provide a lead for development of a new class of antiviral agents.

Envelope gene of HIV plays a crucial role in virus entry into target cells. The CD4 molecule is the major cellular receptor for HIV. Although initial studies showed that soluble CD4 (sCD4) can inhibit laboratory-adapted HIV in cell lines, it was not very effective against primary isolates in PBMC. Small-molecular-weight compounds with therapeutic potential to inhibit CD4–gp120 interactions are still being sought.

Recently, various CC and CXC chemokine receptors have been shown to serve as co-receptors for HIV. The T-tropic, syncytium-inducing viruses, now called X-tropic viruses utilize fusin (a CXC chemokine receptor) molecule as coreceptor. AMD3100, a bicyclam, which was previously described as an inhibitor of HIV uncoating, has now been identified as an inhibitor of HIV gp120–fusin interactions. This compound has no antiviral effect against the macrophage-tropic, nonsyncytium inducing R5-tropic viruses that utilize CCR5 chemokine receptor. Peptide mimics of SDF ligands (amino acids 643–678) has also shown antiviral activity against X-tropic HIV *in vitro*. Likewise compounds like amino-oxypyranose and CC chemokine analogues can inhibit R5-tropic HIV *in vitro*.

More recently, a 36-amino acid synthetic peptide (T-20) derived from highly conserved sequences within the HIV-1 gp41 glycoprotein involved in membrane fusion has been evaluated as an antiviral agent. T-20 shows potent and selective antiviral activity *in vitro* against HIV-1 with $IC_{50} \sim 80$ ng ml^{-1} for the inhibition of infectivity and 1 ng ml^{-1} for the inhibition of syncytia formation. Biophysical evidence suggests that T-20 blocks membrane fusion by binding to a coiled-coil motif within the viral transmembrane protein and inhibiting critical virus-cell surface interactions that are necessary for virus entry. Studies performed *ex vivo* indicate that T-20 undergoes no detectable degradation when incubated at 37°C in human plasma preparations for up to 26 h. Pharmacokinetic analyses indicate that the plasma $t_{1/2}$ of T-20 is 2.3 and 3.1 h in rodents and primates, respectively. In primate models, T-20 exhibits relative bioavailabilities of approximately 75%, when given by either intramuscular or subcutaneous injection.

HIV Tat protein is an activator of viral transcription and it is essential for viral infectivity. The Tat inhibitor Ro 24-7429, a benzodiazepine compound is active *in vitro* and interacts with a host nuclear element to inhibit Tat function. However, clinical trials of this agent have failed to demonstrate antiviral activity, although plasma levels of the drug exceeded levels that inhibited HIV propagation *in vitro*. Ro24-7429 was highly protein-bound, which may account for its therapeutic failure. Recently, ALX40-4C a Tat protein mimic that interferes with the tat–TAR interactions has been shown to suppress HIV replication in both acutely and chronically infected cells. It is currently being evaluated.

Other potential antiviral targets include HIV Rev and Nef proteins. HIV Rev protein mediates nuclear export of unspliced or singly spliced viral RNAs and is essential for viral infectivity. The Nef protein of HIV, although indispensable for virus replication *in vitro*, is a major determinant of viral pathogenicity. Compounds that inhibit Rev-mediated RNA transport *in vitro* are either ineffective to prevent HIV replication in culture, or are cytotoxic and lack therapeutic potential. Likewise, macrolide antibiotics (e.g. concanamycin B and bafilomycin A1) that inhibit certain nef functions *in vitro* have been described, but they all lack therapeutic potential.

With increasing information available on the fine structure of various viral proteins, detailed molecular biology of their function, as well as recent advances in combinatorial chemistry and structure-aided drug design, we can be optimistic that new therapeutically effective inhibitors will be developed in the near future.

Chemotherapy of HIV Infections: Guidelines for the Use and Evaluation of Antiretroviral Agents

Currently five NRTI (ZDV, ddC, ddI, d4T, 3TC), two NNRTI (Nevirapine, Delaviridine) and four protease inhibitors (saquinavir, ritonavir, indinavir and nelfinavir) have been approved for human use, and this list is expanding rapidly. A large proportion of patients had earlier been treated by nucleoside monotherapy, primarily with ZDV and to a lesser extent with ddI. Subsequent studies soon showed that combination therapy with multiple agents is vastly superior to monotherapy. A guideline for the use of antiretroviral agents in HIV-infected adults and adolescents has recently been proposed and salient points are summarized below.

When to begin treatment

Treatment is recommended for all patients that exhibit symptomatic disease. Treatment should be initiated in asymptomatic individuals if there are plasma HIV RNA levels above 30 000–50 000 copies

ml^{-1} regardless of laboratory and/or clinical status; or there are plasma HIV RNA levels above 5000–10 000 copies ml^{-1} and a CD4+ cell count and/or clinical status suggestive of progression.

Recommended regimen

The treatment should include a protease inhibitor (either indinavir, nelfinavir, ritonavir or ritonavir plus saquinavir) and two nucleoside RT inhibitors. The suggested combinations of NRTI include ZDV + ddI, d4T + ddI, ZDV + ddC, ZDV + 3TC, and d4T + 3TC. Alternative regimens, in patients who cannot receive protease inhibitors, include one NNRTI (nevirapine or delaviridine) plus two NRTI, but are less likely to provide sustained virus suppression. Any monotherapy and some combinations (d4T + ZDV; ddC + ddI; ddC + d4T; ddC + 3TC) are not recommended since they are less effective, or show overlapping toxicities.

Changing antiretroviral regimen for suspected failure

Treatment changes may be warranted either due to drug intolerance or treatment failure. Single agents can be changed or the dose reduced in the event of drug intolerance. A suboptimal reduction in plasma viremia after initiation of therapy, reappearance of viremia after suppression to undetectable, significant increases in plasma viremia from the nadir of suppression, and declining CD4 T cell numbers all suggest a virological treatment failure and warrant treatment changes. It is important to use at least two new drugs and preferably to use an entirely new regimen with at least three new drugs when changing from a failing regimen. In some cases where patients have limited options for new regimens, it is rational to continue the prior regimen if partial viral suppression was achieved. For patients with no rational alternative options who have virologic failure with return of viral load to pretreatment levels and a declining CD4 T cell count, modification of therapy should be considered. The value of restarting a drug that the patient has previously received is not well understood. With ZDV, resistant strains are often replaced with 'wild-type' HIV when treatment is stopped, but resistance recurs rapidly if ZDV is restarted. Preliminary evidence suggests that this occurs with indinavir as well, but it is not known if similar problems apply to other nucleoside analogues, protease inhibitors, or NNRTIs.

Treatment effects on viral load correlate with clinical outcome including disease progression and survival. Initiation of highly active antiretroviral therapy (HAART) reduces plasma HIV-1 RNA levels below the limits of detection of currently available assays (<400–500 copies ml^{-1}), although this may not always be achievable, especially in HIV-infected children. A 3-fold (0.5-log) sustained reduction in viral load is a minimum indicator of effective therapy. Nucleoside monotherapy results in an average reduction in viral load of 0.5 log compared with 1- to 1.5-log reduction with nucleoside combination therapy and 2.0 to 2.5 log with combination including protease inhibitors. By using more drugs concomitantly, greater reduction in plasma viremia can be achieved, with a shorter time taken to reach the nadir. Changing therapy from a highly aggressive 'inductive' phase comprising three or more drugs, to a maintenance phase with less aggressive dual combination has been unsuccessful.

Can HIV infection be cured?

A small cohort of patients treated early, and aggressively with HAART have remained free of detectable plasma virus even with the most sensitive assay currently available (<40 copies ml^{-1}). It was hoped that these patients may actually have cleared HIV from the body. However, after treatment was stopped, HIV could be detected in the plasma of these patients. A very small fraction of latently infected CD4+ memory T cells was found to harbor the virus, which re-emerged after treatment was stopped. Current efforts are targeted at designing strategies to eradicate this subset of latently infected cells.

Use of Antiretroviral Agents for Chemoprophylaxis

Although postexposure prophylaxis is a highly attractive option, several factors restrict the use of current antiviral agents to certain circumstances. A combination of two NRTI and a protease inhibitor for a period of one month postexposure has been recommended for laboratory and health care workers accidentally exposed to HIV. ZDV administered to HIV-positive pregnant women during pregnancy and labor, with concomitant administration of ZDV to the infant for a period of six weeks has largely reduced perinatal transmission of HIV, and has become standard practice. Other antiviral regimens are also being evaluated for this purpose. Other approaches currently under development include the evaluation of regimens for their efficacy to reduce viral burden and virus-infected cells in mucosal and genital secretions and the development of virucidal agents for topical use with contraceptives.

Concluding Remarks

Antiviral agents have been highly effective in controlling the ravages of the AIDS epidemic, and have even raised hopes for a cure. Nevertheless, this very success poses even greater challenges for the future. There are problems in evaluating the efficacy of new compounds in the face of currently effective therapeutic regimens, as evidenced by the global debate on the ethics of performing placebo-controlled trials in developing countries. Monitoring treatment efficacy by studying viral dynamics is being challenged by current trends where patients on long-term PI therapy show continuing immunological improvement despite virological failure. Patients on combination therapy are running out of treatment options before new compounds are becoming available. Thus, better use of available antiviral compounds seems far more prudent than relying on new products. This approach includes the development of rational approaches for drug combinations, the development of optimal delivery systems to ensure better treatment compliance. Finally, we have to explore options for customizing treatment on an individual basis to select drugs and adjust dosage regimens based on pharmacological and pharmacokinetics parameters which take into account individual variables including past treatment history, and resistance profiles of viral isolates.

See also: **Antivirals; Human immunodeficiency viruses (*Retroviridae*): General features, Molecular biology.**

Further Reading

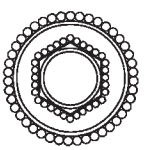
Centers for Disease Control (1998) Public Health Service Task Force Recommendations for the Use of Antiretroviral Drugs in Pregnant Women Infected with HIV-1 for Maternal Health and for Reducing Perinatal HIV-1 Transmission in the United States. *MMWR* RR-02.

De Clercq E (1995) Antiviral therapy for human immunodeficiency virus infections. *Clin. Microbiol. Rev.* 8: 200.

De Clercq E (1997) Development of resistance to human immunodeficiency virus (HIV) to anti-HIV agents: how to prevent the problem. *Int. J. Antimicrobial. Agents* 9: 21.

Flexner C (1998) HIV protease inhibitors. *N. Engl. J. Med.* 338: 1281.

HUMAN T-CELL LEUKEMIA VIRUSES (*RETROVIRIDAE*)



Contents

HTLV-1

Mitsuaki Yoshida, Department of Cellular and Molecular Biology, Institute of Medical Science, University of Tokyo, Tokyo, Japan

History

Human T-cell leukemia virus type 1 (HTLV-1) is the first exogenous retrovirus to have been established in humans. After a long and painstaking search for human retroviruses, HTLV-1 was isolated from adult T-cell leukemia (ATL) at the beginning of the 1980s. ATL was first described in 1976 in Japan as a unique malignancy of T cells affecting only adults. The disease is now known to be endemic in the southwest part of Japan, the Caribbean, South America and Africa. The human retrovirus HTLV was reported in the USA in 1980, by characterization of reverse transcriptase in a T-cell line established from peripheral blood T cells of a patient with T-cell lymphoma. This lymphoma was later diagnosed as ATL. Several T-cell lines were also established from Japanese patients with ATL by co-cultivation of normal cord-blood lymphocytes and leukemic cells from ATL patients, and retrovirus-like particles were detected in these established T cell lines. These cell lines expressed antigens that reacted with sera from ATL patients. These antigens were then identified as proteins encoded by a retrovirus (initially called ATLV), which was newly established by characterization of reverse transcriptase, specific core proteins and the proviral genome integrated in chromosomal

differ serologically. The molecular mechanism has not been resolved yet.

In eukaryotic viruses so far all host cell adaptation is explained by the appearance and selection of viral mutants. It has, however, not been investigated whether phenotypic M/R processes of genetically unaltered viruses do also occur in the kingdom of eukaryotes, possibly explaining the occurrence of reversible changes in the host cell tropism of viruses.

DNA Methylation in Eukaryotic Viruses

There is no unequivocal evidence of the existence of DNA M/R systems in eukaryotic cells. Enzymes resembling pairs of DNA methyltransferases and restriction endonucleases are, however, encoded by viruses infecting eukaryotic algae (*Chlorella* spp.); their biological functions remain still unclear. Other viruses, like Frog virus 3 and lymphocystis disease virus (members of the family *Iridoviridae*), encode their own DNA methyltransferases, and their genomes carry high numbers of 5-methylcytosine residues. Functional consequences of the extensive methylation have to be further elucidated.

In the DNA of eukaryotic cells 5-methylcytosine is usually generated by cellular methyltransferase activities and mainly but not exclusively occurs in CpG dinucleotides. DNA methylation is known to be involved in processes like transcription inhibition, genomic imprinting, cell differentiation and dedifferentiation (carcinogenesis), and hypermutability. Furthermore, DNA methylation is assumed to silence transcription from integrated retroviral genomes, whereas demethylation results in the reactivation of the provirus. For some human pathogenic viruses, such as herpes simplex viruses, cytomegalovirus, Epstein–Barr virus or papillomaviruses, a role of DNA methylation in gene regulation has been demonstrated.

See also: **Adenoviruses (*Adenoviridae*): Animal viruses, General features, Malignant transformation and oncology, Molecular biology; Algal viruses (*Phycodnaviridae*); Frog virus 3 (*Iridoviridae*); Herpes simplex viruses (*Herpesviridae*): General features, Molecular biology; Human immunodeficiency viruses (*Retroviridae*): Molecular biology, Anti-retroviral agents, General features; Coliphage lambda (*Siphoviridae*); P4 phage (*Satellites*); T7-like phages (*Podoviridae*); P2, 186 and related phages (*Myoviridae*).**

Further Reading

Arber W (1965) Host-controlled modification of bacteriophage. *Annu. Rev. Microbiol.* 19: 365.

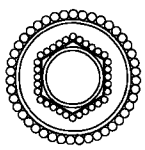
Bickle TA and Krüger DH (1993) The biology of DNA restriction. *Microbiol. Rev.* 57: 434.

Jost JP and Saluz HP (eds) (1993) *DNA Methylation: Molecular Biology and Biological Significance*. Basel: Birkhäuser.

Krüger DH, Kupper D, Meisel A, Reuter M and Schroeder C (1995) The significance of distance and orientation of restriction endonuclease recognition sites in viral DNA genomes. *FEMS Microbiol. Rev.* 17: 177.

McClelland M, Nelson M and Raschke E (1994) Effect of site-specific modification on restriction endonucleases and DNA modification methyltransferases. *Nucleic Acids Res.* 22: 3640.

HUMAN IMMUNODEFICIENCY VIRUSES (*RETROVIRIDAE*)



Contents

General Features

Luc Montagnier, Institut Pasteur, Paris, France and Queens College, NY, USA

History

The infectious origin of acquired immunodeficiency syndrome (AIDS) was recognized soon after the identification of the disease itself in 1981. The first AIDS cases diagnosed in 1982 in hemophiliacs

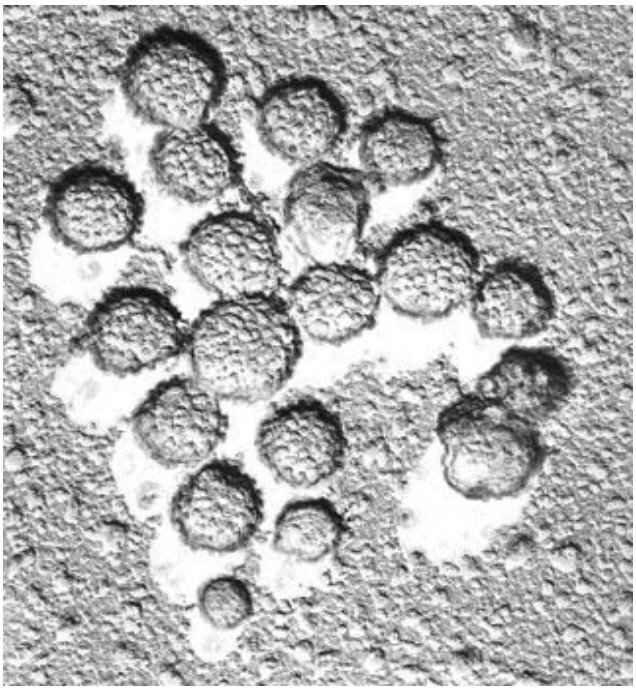


Figure 1 Replica surface of HIV-2 virions, showing the knobs formed by oligomers of the surface glycoprotein. (HIV-1 virions usually shed most of these knobs spontaneously.) (Courtesy of Drs H Gelderblom and M. Özel.)

indicated that filtered Factor VIII concentrates could transmit the disease, suggesting that a virus or a small bacterium which could pass through bacteriological filters was the etiological agent. Indeed a retrovirus was isolated in early 1983 by L. Montagnier, F. Barre-Sinoussi, J. C. Chermann and their colleagues from a culture of activated T-lymphocytes derived from a lymph node biopsy of a homosexual patient with lymphadenopathy. Other similar isolates from full-blown AIDS patients were made by the same group in 1983, who showed, along with D. Klatzmann and J. C. Gluckman, their tropism for CD4+ lymphocytes. The viruses were found not to be antigenically related to human T cell leukemia viruses (HTLVs) but to be more closely related to the animal lentiviruses. This was despite the fact that some other studies (R. C. Gallo) produced data in favor of an HTLV-related variant. In 1984, R. C. Gallo, M. Popovic and their co-workers described, under the name of HTLV-III, a virus which proved to be identical to the virus described by the Pasteur group. The essential role of the virus, now renamed human immunodeficiency virus (HIV), in AIDS was demonstrated by epidemiological studies, particularly in cases of transmission by blood, where HIV was the only detected common factor in the donor and the receiver. A second type of virus, named HIV-2, was isolated in 1986 from West-African patients with AIDS. The discovery of HIV as the etiological agent of AIDS has led to the development of rational therapeutic strategies such as reverse transcriptase inhibitors, protease inhibitors and vaccines.

Taxonomy and Classification

By its morphology, genetic structure, nucleotide sequence, the virus belongs to the second subfamily of retrovirus, the lentiviruses, which includes viruses causing slow pathologies in animals, such as Visna, equine infectious anemia (EIA) and feline immunodeficiency virus.

Retrolentiviruses of primates (SIVs, HIVs) are characterized by a tropism to CD4+ lymphocytes, a property not shared by lentiviruses of ungulates.

Virion Structure

Mature virions (diameter 100–120 nm) have a characteristic spherical morphology with a dense cone-shaped core surrounded by a bilayered phospholipid envelope in which knobs are inserted (**Fig. 1**). There are approximately 80 knobs covering the viral sphere. Each knob is made of several molecules of external glycoprotein, gp120, possibly as trimers or tetramers linked noncovalently to preformed oligomers of the integral membrane protein, gp41.

In the case of HIV-2, dimers or tetramers of the equivalent transmembrane proteins (gp36 or gp41) are so tightly bound that they appear as such (gp80 and gp160) in electrophoretic gels under classical denaturating conditions (heating at 100°C in 1% SDS).

Morphogenesis

Virus assembly takes place at the surface of the plasma membrane of lymphocytes or lymphoid cells and in the membrane of intracytoplasmic vacuoles in the case of macrophages. *Gag* polypeptides are necessary to induce budding, and the subsequent morphogenesis of the virion requires the presence of the precursor glycoprotein, gp 160.

Released immature particles with uncondensed cores are transformed into mature particles with condensed cores by proteolytic cleavage of the p17/p18 protein from the gag precursor, which binds to the inner layer of the viral envelope. As this occurs, the p24 protein wraps around the nucleocapsid, formed by the two RNA molecules and the basic proteins p6 and p9.

The correct morphogenesis and infectivity of the viral particles involves cleavage by a cellular protease

of the glycoprotein precursor, gp160 into gp120 and gp41.

Physical Properties

Virions are sensitive to acidic pH (total inactivation at pH 2), ethanol (20–70%), heat (6 log inactivation at 60°C 30 min, detergents (ionic and nonionic) and chlorine.

Virions are sensitive to radiation including UV light at 260 nM, and X and γ rays at a D10 value of over 500 000 rad (50 Gy).

Genome Structure

The genome is a single-stranded RNA molecule of 9400 base pairs; there are two molecules per virion linked by noncovalent bonds. Lysine tRNA is the primer of the reverse transcriptase, which is Mg^{2+} dependent (optimal concentrations of Mg 10 mM).

The structure of the human immunodeficiency virus genome is reflective of the complex nature of the limitations that are exerted during the replication of these viruses. Such limitations affect mainly the expression of viral genes, in an overall process that seems to optimize and synchronize expression of the viral proteins during acute replication, but could also in some instances control the expression of silent proviruses in chronically infected cells. The genome of HIV-1 and HIV-2 (**Fig. 2**), as well as of the different members of the simian immunodeficiency virus (SIV) group, contains several small genes in addition to the classical retroviral structural genes *gag*, *pol* and *env*. Some of these genes have a critical regulatory function, others appear not to be absolutely required for *in vitro* replication. In addition to these coding elements, the HIV genome is rich in *cis*-acting sequences active at different steps of replication, some of which are target sites for proteins regulating viral gene expression.

DNA Synthesis and Integration

Following entry into the cell, the viral genome, represented by a dimer of two identical genomic RNA molecules, is reverse transcribed into linear, double-stranded DNA. The HIV reverse transcription process does not fundamentally differ from what is known for other retroviruses. One interesting difference, however, is that the second (plus) strand of the viral DNA has two distinct origin sites for its synthesis instead of only one. The plus-strand origin is determined, in all retroviruses, by a polypurine tract (PPT) located at the 5′ boundary of the U3 region of the long terminal repeats (LTR). HIVs and other lentiviruses have a second PPT at the center of their genome, which defines a central discontinuity in the plus strand, reflecting its use as an additional origin site. This second origin appears to improve the efficiency of the reverse transcription process, as shown by a reduced replicative capacity of HIV mutants lacking the central PPT. This feature appears to be common to all lentiviruses and spumaviruses.

During the course of an acute infection, high copy numbers of unintegrated viral DNA molecules, either linear, circular with one LTR, or circular with two LTRs, can be observed in infected cells. Such accumulation of viral DNA appears to be the result of multiple events of infection in individual cells.

Following its synthesis in the cytoplasm, viral DNA is transported to the nucleus and integrated into the host cell genome. This transport is an active process, occurring through nuclear pores, mediated by the interactions of the preintegration complex with cellular proteins. It can occur in nondividing cells, unlike the case of oncoviruses. The integration reaction, which can be reproduced *in vitro*, is mediated by the viral integrase, encoded by the C-terminal region of the *pol* gene. The integrase reacts with the termini of the linear viral DNA molecule and with apparently random sites of the host genome. Integration is regarded as being required for expression of the viral genes.

Regulation of Gene Expression

Transcription

The 5′ LTR of the HIV genome contains the active viral promoter. The transcriptional start site defines the boundary between the U3 and R regions of the LTR. Transcription from the HIV promoter requires the presence of cellular transcriptional activators. Upstream of the transcription start site, surrounded by the usual transcriptional complex recognition signals, two important sets of sequences have been described: these are the three copies of the SP1 transcriptional activator binding site, and two copies of an enhancer element, which reacts with the transcriptional activator NFκB. Initially described as able to control transcription of the gene coding for the kappa chain of the immunoglobulins, NFκB is a ubiquitous transactivator whose components belong to a larger gene family that include the *rel* and *dorsal* proto-oncogenes. This factor can be activated in a number of cell types of the immune system, in particular following activation of the protein kinase C pathway. Further upstream in the U3 region of the LTR, is also found a variety of potential regulatory signals; a large segment of this region, termed NRE

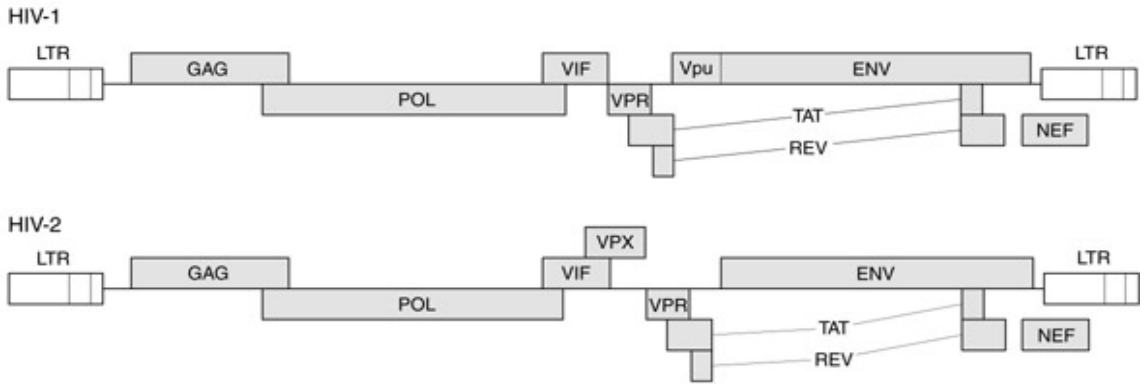


Figure 2 Genome structure of HIV-1 and HIV-2.

(negative regulatory element), has been described as exerting a negative regulatory action on transcription.

Viral regulation of transcription: the Tat system

Transcription of viral RNAs starts at the viral LTR and is mediated by cellular RNA polymerase II. But efficient synthesis of viral mRNAs only occurs in the presence of the viral protein Tat. This protein is produced from multiply spliced RNA, in which the two coding exons of the *tat* gene are linked together. The Tat protein acts by binding to a bulged stem–loop structure present at the 5′ end of all viral mRNAs, called TAR (Tat activation region). This binding is itself mediated by a cellular protein, cyclin T. Like other cyclins, cyclin T is associated with a protein kinase (CDK9) which will phosphorylate the C-terminus of RNA-polymerase II. This phosphorylation will allow efficient elongation of viral RNAs. These Tat cellular cofactors are only present in activated cells. Their absence will allow repression of transcription of proviral DNA which could thus enter a period of latency in T lymphocytes. This is another operative regulation that can allow either high virus production or quasi latency, in addition to the upstream control operated by transcription activators (NFκB) on the LTR promoter.

There is a good crosstransactivation of the HIV-2 LTR by HIV-1 Tat, but HIV-2 Tat is a poor HIV-1 transactivator: this could be consecutive to the presence of a second stem–loop structure on the HIV-2 TAR, required for efficient HIV-2 Tat binding.

Regulation of mRNA processing: the Rev system

In eukaryotic cells, only fully spliced mRNAs are exported to the cytoplasm and are translated. Unspliced RNAs are retained in the nucleus and eventually degraded.

Since HIV contains a single LTR promoter element, it encodes only a single, genome-length primary transcript. Selective expression of different viral genes is determined by differential splicing of this transcript. Splice sites (recognized by cellular proteins) are relatively inefficient, so that a pool of incompletely spliced RNAs could accumulate in the nucleus.

During acute infection, three major classes of viral transcripts can be observed: the 9 kb genomic RNA molecules, producing Gag and Pol proteins, that can also be encapsidated into viral particles; the singly spliced 4.3 kb RNA molecules, coding for the env glycoproteins; and the 1.8–2 kb, multiply spliced RNA molecules, coding for the regulatory proteins. The cytoplasmic export and stability of the unspliced and singly spliced HIV transcripts, coding for the structural proteins, is controlled by the Rev protein.

In the absence of Rev, only multiply spliced messages are seen in the cytoplasm of infected cells. When subgenomic Env constructs are studied, the effect of Rev appears to involve mostly cytoplasmic transport of unspliced RNAs. This restriction, however, seems to be dependent on the presence of splice sites. It is believed that Rev is able to oppose nuclear retention of RNA molecules by elements of the splicing machinery. It has also been suggested that Rev could favor cytoplasmic stability of RNA, association to polysomes, and even translation of unspliced or singly spliced transcripts. The target site for Rev is a complex multi stem-loop structure termed RRE (Rev responsive element), located in the intron separating the *tat* and *rev* coding exon, in the *env* coding region. Rev binds to the RRE but needs to multimerize to be active, which results in a threshold effect in Rev activity. Rev binds to the RRE through its N-terminal domain, whereas its C-terminal domain behaves as a nuclear export signal (NES) which binds to cellular proteins involved in transport from nucleus to cytoplasm.

Table 1 Proteins of HIVs

ORF	*Function*	*Location*	*HIV-1*	*HIV-2*
gag	*precursor*		p55	
	matrix	myristylated, outer virion shell	p17/18	p16
	capsid	virion: core	p24	p26
	nucleocapsid	virion: RNA associated	p7/p9	
	unknown	virion	p6	
pol	protease	virion	p10	
	RT (with RNase II domain)		p66	
	RT		p51	
	integrase		p34	p34
vif	enhances virus infectivity	infected cell	p23	
vpr	arrests cells in G2	virion	p15	
vpx	unresolved	virion (HIV-2, siv)	not found	p16
tat	transcriptional transactivator	infected cell: nucleus	p14	p20
rev	control of RNA splicing and transport	infected cell: nucleus and nucleolus	p19	p19
vpu	controls env-CD4 interaction	infected cell: membrane	p16	not found
env	*precursor*		gp160	gp140
	surface envelope glycoprotein, receptor binding	virion: surface	gp120	gp125
	transmembrane glycoprotein, membrane fusion		gp41	gp36
nef	modulates CD4 surface expression	infected cell: membrane	p27–30	p31

This effect has important consequences: during the early phase of a synchronous acute infection, the first transcripts to accumulate are the short multiply spliced species, followed by singly spliced and unspliced species. This shift from an early to a late phase of HIV expression appears to be under the control of accumulation of sufficient amounts of Rev. Similarly, some cells containing integrated HIV genomes bearing low basal expression levels do not produce structural RNAs; these can accumulate, however, after cell stimulation, in a process that is believed to involve accumulation of Rev. Therefore, the Rev system seems to promote synchronization of particle production during acute infection, and to delay structural protein expression in chronically infected cells, a phenomenon that could favor persistence of the infection in the context of a vigilant immune system.

Structural Proteins

Table 1 summarizes virion proteins, their functions, cellular location and compares HIV-1 with HIV-2 proteins.

Gag and Pol proteins

The Gag and Pol proteins are expressed from the full length, unspliced RNA molecules. In 1:100 of translation events, approximately, translation of *gag* is followed by a frameshift into the *pol* ORF. The Gag or Gag-Pol polyprotein precursors are then packaged into particles where the protease domain of the Gag-Pol precursor will cleave the final Gag and Pol products.

The Gag precursor is cleaved into four proteins. The first Gag protein, from the N-terminus of the precursor, is termed p17/18 for HIV-1 and p16 for HIV-2 (matrix protein); it carries a N-terminal myristic acid, which allows association of both the Gag and Gag-Pol precursors to the cell membrane. The largest Gag protein, p24 (p26 in HIV-2) is the principal constituent of the cone-shaped core characteristic of viruses of the HIV group (capsid protein). The next Gag protein, p7 or p9, is termed the nucleocapsid protein, and is tightly associated with the RNA genome: it has been found to promote both RNA dimerization and encapsidation. The most C-terminal of the Gag proteins is p6; although its function is not known, mutants lacking this protein exhibit a defect in particle budding.

The Pol region can be divided into three main functional elements. The most N-terminal is the protease, which acts as a dimer and cleaves Gag and Pol products during particle maturation. The central element is the reverse transcriptase, which acts as a heterodimer: the smaller component has polymerase activity, and the larger component has both polymerase and RNaseH activities. The action of RNaseH

is required to eliminate the RNA template, with the exception of selected sequences used as primers, and to allow strand transfer events.

env

The envelope glycoproteins of HIV-1 and HIV-2 are translated from a singly spliced RNA species. The glycoprotein is synthesized as a large glycosylated precursor which is then subjected to a series of biochemical rearrangements of its polysaccharide moiety and to a single cleavage event at a specific site carried out by a cellular convertase. This cleaveage separates the larger, outer portion of the envelope glycoprotein (gp 120) from the smaller, transmembrane protein (gp 41).

The gp 120 carries on a specific domain, in the form of a pocket, that binds with high affinity D1–D2 domain of the CD4 molecule present on susceptible cells. The gp 120 binding domain is conformational, involving several regions, which are conserved within HIV variants. Upon binding to CD4, the gp 120 molecule undergoes conformational changes, which exposes some other sites to cellular molecules favoring the tight association of gp 41 transmembrane protein with the plasma membrane. Unlike the CD4 binding site, these sites are located in variable regions or loops of gp 120, particularly the V3 loop. This explains why the virus can choose a variety of cell surface molecules for its entry generally in association but not always, with the CD4 main receptor. Among these surface molecules (called entry cofactors or coreceptors), two main species have been recognized, which are physiological receptors for chemokines: the CCR5 receptor for β chemokines (RANTES, MIP-1α, MIP-1β) and the CXC4 receptor for an α-chemokine (SDF-1).

Very interestingly, the use of each coreceptor corresponds to viruses with different biological properties and pathogenicity. Viruses isolated at the beginning of infection use in most cases the CCR5 coreceptor (R5 viruses). They are not cytopathic *in vitro* (nonsyncytium-inducing viruses) and replicate in macrophages and activated primary T lymphocytes expressing CCR5 and CD4. In full-blown AIDS cases, new viral species appear with high power of replication *in vitro*, cytopathic effect (syncytia and single cell lysis), poor ability to replicate in macrophages and ability to grow in tumor T cell lines. They use the coreceptor CXC4 (X4 viruses). With the characterization of more viral isolates, the situation appears more complex; there are dual-tropic viruses, using both CXC4 and CCR5 coreceptors, or using alternative chemokine coreceptors (CCR3, CCR2, CCR8). There is also evidence of binding of the V3 loop to nucleolin, a nuclear protein whose one isoform is expressed at the cell surface, and with a dipeptidase present on activated T cells, CD26.

The fusion between the viral membrane and the cellular membrane involves a change in the conformation of gp 41, itself induced by the conformational changes of gp 120. It involves exposure of a hydrophobic fusion domain present at the N-terminus of gp41, which enables it to insert itself in the cellular phospholipid bilayer. The transmembrane protein also displays an unusually long intracytoplasmic domain. This domain is particularly interesting in HIV-2 and SIV; indeed, tissue culture propagation of these viruses appears to favor truncation of the intracytoplasmic tail. The correction of this truncation leads to reduced infectivity *in vitro*.

An Important Regulatory Protein: Nef

This third early protein is expressed in far higher levels than Tat and Rev. Most of the protein is myristilated at its N-terminus and associated with the inner side of the plasma membrane. However, there is partial exposure of the C-terminus of the molecule at the outer cell surface and in experimental infections with Nef-expressing vectors, Nef is also in part excreted into the medium.

Expression of the intact Nef protein is not required for *in vitro* infection of peripheral blood leukocytes (PBLs) or of T cell lines. However Nef-deleted mutants of HIV and SIV are much less pathogenic *in vivo*, leading to persistent infection without disease, at least for a long period of time. Studies in the SIV/macaque model have shown that the initial virus load in lymph nodes is reduced by a factor of 10–50, and that formation of germinal centers in response to infection occurs more quickly than with the pathogenic Nef+ strains.

In the productive cycle of viral infection, several functions of Nef have been identified, which contribute to a higher or longer virus production. Nef has been shown to strongly downregulate the expression of cell surface CD4, by connecting CD4 with the cellular endocytic machinery. This prevents reinfection of the infected cell with newly produced virions, which could lead to its premature death.

Nef also downregulates to a lesser extent surface expression of MHG-1, resulting in some inhibition of recognition of the infected cells by specific cytotoxic T lymphocytes (CTL). Nef seems also, by independent, yet unclear, mechanisms, to enhance virion infectivity, perhaps by increasing the stability of the nucleocapsid at its entrance in the cell.

Perhaps the most relevant property of Nef for its role in AIDS pathogenesis is its capacity to activate

CD4+ T lymphocytes by modulating signaling pathways. An extreme case is that of the PBJ14 mutant of SIV which can induce within a few weeks an acute disease in pigtail macaques, and can activate both resting CD8+ and CD4+ lymphocytes *in vitro*. These unusual properties are linked to the creation in Nef by directed mutagenesis (or selection by passage in animals) of a new SH2 (Sarc homologous region 2) binding domain (YXXL motif).

The fact that CD8 cells which are not infectable by SIV are also stimulated, suggests some paracrine effect of Nef expressed or released at the surface of infected CD4+ cells. Nef from 'normally' pathogenic strains of HIV or SIV contains a proline-rich sequence which could bind to the SH3 domain of kinases involved in the activation pathway of T lymphocytes. When mutations are introduced in this region, SIV is no longer pathogenic, unless reversion to the wild-type occurs.

In addition, recombinant Nef protein, in the 10–50 ng/ml range, can act as a costimulatory factor in T lymphocytes in which the T cell receptor was subliminally activated and permits HIV replication in such cells. Thus, Nef produced by HIV-infected cells could recruit for infection neighboring T cells which were on the verge of activation and could not otherwise be susceptible to HIV infection.

This recruitment may be important at the beginning of infection when the number of activated T cells in lymphatic tissues is low and is a limiting factor for HIV expansion.

Other HIV Accessory Proteins: Vif, Vpr, Vpu, Vpx

All these proteins are encoded by single spliced mRNAs and therefore are produced late after sufficient Rev expression.

Vif The Vif protein is expressed at high levels in the cytoplasm of infected cells. Deletion of this gene reduces infectivity of HIV-1 virions in primary T4 cells and some tumor cell lines CH9. Some Vif proteins are also present in mature virions. It is believed that Vif can stabilize the virions upon entry in the cells and favor the formation of the reverse transcription complex.

Vpr This small basic protein is packaged in the virion nucleocapsid in amounts equivalent to the Gag proteins and therefore can be considered as a virion structural protein. Packaging depends on its association with the p6 protein released by proteolytic cleavage from the C-terminus of the p55 Gag precursor.

Two effects of Vpr have been recognized. One, which is controversial, is to contribute to the nuclear import of the preintegration complex, by interacting with nucleoporins, and other cellular proteins, especially in nondividing differentiated cells (macrophages).

The other function is to induce in cycling cells an arrest in G2. This effect is similar to that induced by DNA alkylating agents, and could result in the induction of the DNA repair machinery, which could be useful for integration of the proviral DNA. Rhesus macaques inoculated with a mutant SIV having a deletion in Vpr can still evolve towards AIDS-like disease, although some individuals show more resistance to disease progression.

Vpu The *Vpu* gene is unique to HIV-1 and the related virus isolated from chimpanzees. The product, Vpu, translated from the same mRNA that produces *env*, is an integral membrane protein with a C-terminal intracytoplasmic tail.

Vpu has two functions. The first is to allow selective degradation of CD4 when the latter associates with the viral envelop precursor in the endoplasmic reticulum. This will release the Env protein from this complex and allow its subsequent incorporation into new virions.

The second function is to facilitate virion release from the cell plasma membrane. Vpu-defective mutants show accumulation of intracellular viral particles.

Vpx The *vpx* gene is found only in HIV-2 and SIVs. Like Vpr, the protein is present in mature virions, in amounts roughly equal to that of the major Gag protein p24.

Inside the cell, Vpx is located at the inner side of the cell plasma membrane. Vpx deletion mutants have a reduced capacity to replicate in primary T lymphocytes and macrophages, although they grow normally in T cell lines.

Genetics

One striking feature of the HIVs arose as soon as the nucleotide sequence of several HIV isolates was known: HIVs display a high level of genetic variability. The most variable region of their genome is the outer region of the envelope glycoprotein, where divergence can reach 30%. The most conserved are the *gag* and *pol* genes. The leading cause of such a diversity is reverse transcriptase: this enzyme does not have any proof-correction activity, and appears to introduce errors every 10^4 nucleotides. Another cause is selection: having to replicate in the context of a vigilant immune

system, HIV needs to escape the antiviral immune response. This could explain why the envelope glycoprotein is the most variable region of the genome. It has also been shown that a single infected individual does not carry a single isolate, but a heterogenous population of subisolates, sometimes referred to as 'quasispecies', and that changing the conditions of viral replication (by growing the virus *in vitro*, for example) will modify the balance of that viral population. In addition the biological properties of the virus – as determined in the *in vitro* isolate – change during the course of the disease: in general, isolates made from lymphocytes of asymptomatic patients grow slower and are less cytopathic than isolates made from patients with full blown AIDS. These changes seem to be related to mutations in the envelope protein gp120, but no particular mutation can be assigned to the changes in biological properties.

Within the HIV-1 type, genetic diversity appears to be more striking when African isolates are compared with one another or with Western isolates. This is an indirect indication that African isolates may have evolved longer than their western counterparts.

Based on nucleotide sequence of the variable regions of the envelope protein gp120 (particularly V3), a cladistic classification of HIV-1 isolates is currently being used, using capital alphabetic letters, A to J. They all belong to the group M of HIV-1s (M for major). Since the early 1990s another group of HIV-1 has been described in Central Africa (Cameroun) with some sporadic cases in Europe. This group, which differs from the **M** group by around 50% of nucleotides sequences in the *env* gene, has been termed **O** (for outlayer). All HIV-1s are highly pathogenic but display various geographic locations (see below).

More radically divergent is the HIV-2 group, which was prominent in West Africa in the 1980s, but now tends to be less prevalent than HIV-1. By serology and nucleotide sequences, HIV-2, which also displays several subtypes, is closer to SIVs than to HIV-1. In fact, it probably originates from a SIV naturally infecting sooty mangabeys living in West Africa.

HIV-2 was initially found in some West African patients with AIDS, with symptoms comparable to HIV-1 disease. However, its pathogenic potential seems to be lower than HIV-1, displaying a lower viral load in chronically infected patients and a lower capacity of transmission by sexual contacts.

Origin and Geographic Distribution

HIV-1 seems to have originated in Central Africa. This is suggested by the larger extent of variability found in strains isolated from African patients.

Retrospective serological studies also indicate that the virus was present in the early 1970s in 0.25% of a population of young women living in Kinshasa (Zaïre). However, only retrospective sporadic cases could be detected in Africa before 1980 as well as in Europe and the United States. The HIV-1 directed epidemic started at about the same time in larger cities of Central Africa and of the United States.

Molecular analysis of the virus present in a serum taken from a Zaïrian patient in 1959 indicates that it could be an ancestor of several identified HIV-1 clades: B, which is now prominent in North America and Western Europe, D and F present in Central Africa.

In the phylogenetic trees, two isolates made from chimpanzees are clearly closer to the M group than from the O group, the origin of which cannot be traced to any simian lentivirus. This group may have been circulating in some human populations for a longer time.

In the 1990s HIV-1 infection has become pandemic, rapidly developing in new regions such as India, South-East Asia and South America. In Africa, the infection has spread to the East (Uganda, Kenya, Tanzania, Rwanda, Burundi) and to the North (Ivory Coast). Concerning HIV-2, its spread has been more limited from the areas where it was first detected: Guinea-Bissau, Cap Verde Islands, Senegal. HIV-2 is also present in other countries of the Western part of Africa (Ivory Coast, Burkina Faso, Cameroon, Liberia etc.) and Portuguese-speaking countries (Angola, Mozambique). An outbreak of HIV-2 infection has been detected in prostitutes of the Bombay area, India, suggesting that the virus has also reached Asia.

New rapidly spreading foci of HIV-1 infection have occurred in South Africa, Zimbabwe, Cambodia and China. Some subtypes are becoming prominent, according to the geographic location, such as A in Ivory Coast, C and D in Central and South Africa, E in Thaïland, F in Brazil. As the epidemic progresses, infection of the same individual by two variants becomes frequent, leading to the emergence of recombinants (particularly in the *env* gene) between subtypes.

As of 1998, the number of HIV-infected persons is estimated to be 30–40 million worldwide.

Serologic Relationships

Despite the large spectrum of genetic variability, the prototype strain of HIV-1, isolated at the Pasteur Institute, LAV LAI (otherwise named HTLV-IIIB) can still serve as source of antigen for the detection of HIV-1 antibodies in all geographic regions of the world, including antibodies against the envelope. By

contrast, little crossreactivity exists between SIV/HIV-2 group and HIV-1 glycoproteins, whereas crossreactivity remains important in Gag and Pol proteins of the two viruses.

The lack of crossreactivity in a major epitope of the transmembrane protein (gp41, gp36) has been used to differentiate between HIV-1 and HIV-2 infection, or to detect double infection. So far a few patients have shown, by western blot using HIV-1 and HIV-2 prototypes as antigen, atypical reactivity. Such cases, in which, for instance, no antibodies against the glycoprotein of HIV-1 or HIV-2 could be detected in serum, are probably due to variants distantly related to HIV-1 or HIV-2.

With regards to animal retroviruses, SIV-infected primates do react well with all HIV-2 proteins, confirming the close relationship between SIV (particularly SIV from sooty mangabey) and HIV-2. By contrast, other animal lentiviruses (Visna, CAEV, BIV, FIV) are too distantly related to show antibody crossreactivity, with the exception of equine infectious anemia virus which shows crossreactivity with HIV-1, at the level of the Gag p24 protein.

Host Range and Virus Transmission

HIV-1 has a very limited host range; human and chimpanzee are the only species known so far which can be chronically infected with the virus. However, no disease or deep immune depression have been observed in the hundreds of chimpanzees inoculated with human isolates of HIV-1, with one exception, whereas lack of symptoms is the exception in humans.

HIV-2 can chronically infect some macaque species (rhesus, cynomolgus); an AIDS-like syndrome has been observed in some experiments, but not in a reproducible way.

The two main routes of transmission of HIVs are blood and blood products and sexual contact. The efficiency of blood transmission (transfusion, needles, i.v. drug abuse) depends on several factors: number of virus particles, volume of blood, immune status of the receiver. Infection is particularly efficient in i.v. drug abusers.

Sexual transmission, homosexual and heterosexual, is the major mode of transmission today. All sexual practices are dangerous, but the risk is higher for anogenital intercourse, and is increased by some intercurrent genital infections (herpes, chlamydia etc.).

Transmission from mother to child is also a major mode. In the absence of treatment, 20–30% of seropositive women give birth to an infected child. Infection can occur in the second half of pregnancy, at delivery and also by breast feeding. Severe infection of children results in death in the first year of life. Otherwise the evolution follows that seen in adults.

Role of the Virus in AIDS Pathogenesis

Acquired Immune Deficiency Syndrome (AIDS, SIDA in French and Spanish-speaking countries) was defined in the early 1980s, biologically by a profound defect of cellular immunity associated with a deep shortage of CD4+ T lymphocytes and clinically by the occurrence of opportunistic infections and cancers. In Western countries, the most frequent infections are those of the lungs by *Pneumocystis carinii* and of the brain by toxoplasmas, followed by visceral and retinal infections by cytomegalovirus (CMV). Tuberculosis is frequent in tropical areas (Africa, Asia, South America).

Among cancers, the most frequent are disseminated and aggressive Kaposi's sarcoma caused by human herpesvirus 8 (HHV-8) and B-lymphomas, often caused by Epstein–Barr virus (EBV).

The causal relationship between HIV and the immune depression has been assessed as follows:

1. On the basis of epidemiological studies, particularly in blood donors and recipients;
2. From the tropism and cytopathic effect of HIV and viral glycoproteins on CD4+ cells;
3. On the reproduction of the disease in macaques by virus derived from molecular clones of SIV (close to HIV-2).

The natural history of HIV-1 infection has been thoroughy studied by histological and molecular techniques. Three phases can be distinguished: primary infection, a clinical latency phase, the clinical phase (full blown AIDS).

In the primary infection, entry of the virus through mucosa involves its association with denditric cells which then transport the virus to lymphatic tissues. The virus can rapidly multiply there in activated CD4+ lymphocytes and macrophages. Since the number of activated CD4 lymphocytes is small, it becomes a limiting factor which depends on intercurring microbial infections. Biologically, this phase is characterized by a peak of viral antigens (not always present) and a large number of viral RNA copies in blood, and a peak of γ and α interferons. Specific cytotoxic lymphocytes (CTL) then appear followed by the appearance of antibodies against viral glycoproteins and internal proteins. Clinically, when the level of viral replication is high, symptoms can be detected (fever, adenopathy, headaches). However,

the infection can be sometimes completely inapparent.

This episode is followed by a phase of clinical latency, with no symptoms. However, in a majority of cases, there is a slow and progressive degradation of the immune system, leading finally to clinical AIDS which will last on average ten years, in the absence of treatment. In a minority of individuals (5–10%) (long-term nonprogressors) this degradation does not take place or is very slow. In some others, on the contrary, immune depression can occur very rapidly within 1–2 years.

These variations reflect the complex interaction of HIV with the immune system of the host. In fact, the virus continuously replicates in lymphatic tissues (lymph nodes, spleen), with a rapid clearance from the blood. A relatively small number of infected cells is involved at the beginning and their destruction (either by the virus or by cytotoxic cells) cannot solely account for the large depletion of CD4+ cells. It is likely that indirect mechanisms of cell death are involved, bearing on cells which are not infected, but are in contact with noninfectious viral particles, or gp120 shed from cells or virions. Indeed, interaction of gp120 with the CD4 receptor or coreceptors could induce a wrong signaling leading to apoptotic cell death or anergy, when the T cell receptor is stimulated.

Evidence for the preapoptotic state of a large fractional circulating CD4 cells has been obtained. Defects in antigen-presenting cells, in bone marrow renewal of precursor cells, of faster thymic involution, of high oxidative stress, have also been involved to explain the specific CD4+ cell depletion. It is also clear that a state of chronic activation bearing not only on CD4+ cells, but also on CD8, NK and B cells exists all through this phase, associated with the production of inflammatory cytokines (interferons, interleukin (IL) 6, tumor necrosis factor).

In addition, a small pool of latently infected cells exists and is probably continuously renewed. This pool will escape any kind of treatment.

In the clinical phase, with the occurrence of more pathogenic variants (CXC4 tropic) and the decrease in cell-mediated immune response, the infection from local or regional becomes systemic and precipitates the fatal evolution.

Neurological signs are also frequent, particularly a subacute encephalopathy which can develop into a dementia syndrome and brain atrophy, and seems to be due to the virus itself. Some foci of brain macrophages infected with HIV can be detected in white matter, but neither neurons nor glial cells seem to be productibly infected in the same situation. Some astrocytes express large amounts of Nef protein. Therefore indirect mechanisms for the action of HIV (cytokines, nitric oxide?) have also to be postulated in order to explain the neuronal effects.

Immune Response

An immune response against HIV is mediated by T-helper cells. Antibody response against major HIV structural protein (Gag, Env) and also against Pol proteins and Nef appear within 2–3 weeks after HIV exposure, exceptionally after six months or more. Specific CD8+ cytotoxic T lymphocytes (CTLs) also appear, often earlier than the antibody response, directed against the same proteins. Antibodies against the viral envelope are poorly neutralizing, and are directed against variable regions of gp120, especially the V3 loop.

There is also induction of antiviral cytokines (interferons γ, α) but for unclear reasons, even large amounts of α interferon are not effective to control viral expansion. There is also *in vitro* evidence for viral inhibition by other soluble factors, particularly secreted by CD8+ T lymphocytes; the increase of β chemokines may inhibit by competition the entry of C5 viruses, but some other inhibitory factors produced by CD8+ cells have not yet been identified.

As the infection progresses, the specific CTL response tends to decrease and to lose its efficacy against mutant viruses, although a strong natural killer response (NK cells) can still also play a protective role.

The number and function of CD4+ T lymphocytes decreases progressively with some oscillations during the clinically latent phase. T-helper functions to recalled antigens are precociously affected, in line with a drop of IL-2 and an increase of Th2 cytokines (IL-4). The antibody response against viral proteins decreases at the clinical phase, reaching first the internal proteins. Owing to their higher titers, antibodies against gp120 and gp41 remain detectable even at late stages. These antibodies can also be detected in urine in lower titer.

Treatment

Until 1994, there was no effective treatment of HIV infection and AIDS. Monotherapy using nucleosidic or nonnucleosidic inhibitors of HIV reverse transcriptase gave disappointing results in clinical trials. It was realized that the inhibitory effect on virus replication was not strong enough to prevent the rapid occurrence of resistant mutants. Dramatic improvements appeared in 1995–1996 due to the design of a new class of potent antiviral drugs, the inhibitors of the viral protease, which prevent the

maturation of infectious virions by inhibiting the cleavage of the gag-pol precursor. Another important development was the general acceptance of the concept of combination therapy, in which two, three or even four inhibitors act synergistically and decrease the emergence of resistants. Finally the application of sensitive molecular techniques (polymerase chain reaction, branched DNA) to quantitate the viral load in plasma was an important tool to monitor the antiviral effect of the treatment.

The combined use of two inhibitors (such as azidothymidine (AZT) and 3-thiodeoxycitidine (3-TC)) of reverse transcriptase with a protease inhibitor (such as Ritonavir, Indinavir, Nelfinavir) results generally, in a strong decrease of viral load within weeks (2–4 logs), a slow but consistent increase of CD4+ cells and an improvement of the patient's condition.

Opportunistic infections are much less frequent and there is biological evidence of at least partial restoration of T helper-dependent immune functions (response to recall antigens, drop of apoptosis and cell activation markers). However, this treatment called HAART (highly active retroviral therapy) has some constraints and limitations.

1. It has to be taken daily and indefinitely.
2. Active virus multiplication is not completely suppressed, and multiresistant variants may appear, more rapidly if the patient does not adhere to strict compliance to the treatment.
3. There is a continuously renewed reservoir of cells which are latently infected and therefore have unexpressed proviral DNA which escapes the reverse transcriptase and protease inhibitors.
4. Important side effects, such as mobilization of lipids resulting in hypertriglyceridemia, insulin-resistant diabetes, can occur after long-term treatment.
5. The high cost precludes any generalization of its application for patients in developing countries.

Therefore there is a need for intensive research to develop new types of retroviral inhibitors, to find practical tests to evaluate the spectrum of resistance and sensitivity to available drugs of the patient's virus, to find complementary treatments aimed at reducing the dosage and duration of HAART and at achieving better restoration of the immune system.

Among possible promising developments are the use of: very low doses of IL-2 (a daily subcutaneous inoculation for 6 months); hydroxyurea; and inhibitors of cell activation and a specific immunotherapy against viral proteins.

Prevention and Vaccine

No vaccine is yet available and the only effective prevention at present is education about the ways of transmission, systematic testing of blood donors and the use of condoms. However, an important reduction of transmission from mother to child has been achieved by treatment with AZT of the mother at the end of pregnancy and at the time of delivery and of the newborn in the first weeks of life. A lighter regimen also seems to be effective and applicable to populations of developing countries.

A complete control of the AIDS epidemic cannot be achieved without the availability of a protective vaccine. Although the use of whole virus or whole surface glycoproteins has been disappointing, there are new promising approaches based on the use of DNA, mucosal adjuvants and live vectors, internal and regulatory proteins, and conserved parts of the surface glycoproteins.

Studies of naturally resistant HIV-exposed individuals have shown that cell-mediated immunity and the secretion of soluble inhibitory factors are more important for protection than antibodies, together with a limited role of genetic factors. Individuals homozygous for mutations impairing the expression of CCR5 coreceptors are totally resistant to infections by 'R5 tropic viruses'. However, these are rare (<2%) in the caucasian population. Individuals heterozygous for these mutations can still be infected, but seem to evolve more slowly to the clinical phase of AIDS.

In fact, a large majority of exposed noninfected individuals have acquired a specific immune resistance to infection, based on CTL and specific IgAs, although the role of other genetic factors (HLA) cannot be excluded.

The efficacy trials of candidate vaccine in large populations will raise important logistic and ethical issues difficult to solve, unless an important international mobilization greater than that achieved for vaccinal eradication of poliomyelitis, is achieved.

See also: **Bovine immunodeficiency virus (*Retroviridae*); Feline immunodeficiency virus (*Retroviridae*); Human T-cell leukemia viruses (*Retroviridae*): HTLV-1; HTLV-2; Immune response: Cell mediated immune response; General features; Interferons: General features; Latency; Persistent viral infection; Simian immunodeficiency viruses (*Retroviridae*); Visna-Maedi viruses (*Retroviridae*).**

Further Reading

Coffin JM, Hughes SH and Varmus HE (1998) *Retro-*

viruses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Cohen OJ, Kinter A, Fauci AS (1997) Host factors in the pathogenesis of HIV disease. *Immunological Reviews*, 159: p. 31–48.
Encyclopedia of AIDS (1998) Smith RA (ed) Fitzray Dearborn Publishers.
Hirsch MS and Curran J (1996) Human Immunodeficiency Viruses. In: Fields BN *et al.* (eds) *Virology*, 3rd edn, p. 1953–1975. Philadelphia: Lippincott-Raven Publishers.
Littman DR (1998) Chemokine Receptors: Key to AIDS Pathogenesis? *Cell*, vol. 93: p. 677–680.

Molecular Biology

Leon F García-Martínez, Southwestern Medical Center, Dallas, Texas, USA

J Victor García-Martínez, St Jude Children's Research Hospital, Memphis, Tennessee, USA

Human immunodeficiency viruses are members of the family *Retroviridae*, genus *Lentivirus*. The human immunodeficiency viruses (HIV) type 1 and 2 are the causative agents of acquired immunodeficiency syndrome (AIDS). Humans are the hosts for both viruses and they infect mainly CD4+ lymphocytes and monocyte/macrophages also expressing a coreceptor molecule. Characteristic of infection with HIV is an early high level of serum viremia that subsides in response to an aggressive immune response. However, HIV withstands this initial attack, is able to persist, and slowly erodes the immune system of the infected individual by depleting CD4+ lymphocytes at a rate of 2 billion per day. This article describes the molecular determinants responsible for virulence.

Properties of the Virion

HIV particles have a diameter of 100–120 nm and a spherical morphology classical of several other retroviruses. They consist of a dense truncated cone-shaped core surrounded by a membrane bilayer (envelope) decorated with spikes corresponding to the surface glycoprotein (SU). HIV particles are assembled beneath the cell surface. The internal structure of the virus particle rearranges after budding, a result of the proteolytic cleavage of the major structural components by a viral-encoded protease. This process is called maturation. Virions are sensitive to radiation, acidic pH, diluted ethanol, detergents, chorine and temperature (60°C). The genome consists of a homodimer of linear, positive-sense, single-stranded RNA of approx. 9.2 kb in size. The two strands of RNA are capped, polyadenylated, and noncovalently joined through the dimerization domain. Lysine tRNA is found bound to the viral RNA in the particle and serves as a primer for reverse transcription.

Genome Structure

The HIV genome is significantly more complex than the genome of murine retroviruses (**Fig. 1**). This complexity is likely to reflect the dynamic interplay between this virus and its host. Like all other retroviruses, the HIV genome encodes three structural genes *gag*, *pol* and *env*. In addition, it encodes several small genes (*vif*, *vpr*/*vpx*, *tat*, *rev*, *vpu* and *nef*) that regulate gene expression, protein expression, and to a great extent are determinants of virulence. In order to maximize its coding capacity, HIV uses several different means to pack information into a relatively small genome. Differential splicing of the viral genome is used to control in a temporal and advantageous manner the expression of structural and regulatory genes. In addition, the virus genome encodes several *cis*-acting elements involved in virion packaging (Ψ or psi), reverse transcription (primer binding site or PBS, polypurine tracts or PPT), RNA retention in the nucleus (negative regulatory elements or CRS), RNA export and processing (Rev-responsive element or RRE and polyadenylation signal), transcription (TAR), etc.

Virus Replication

The replication cycle of HIV is very similar to that of other retroviruses. However, it is under significantly tighter control. The replication cycle of HIV can be divided into three steps: (1) entry and integration; (2) gene expression; and (3) assembly and budding. Each of these steps involves several specific interactions between virus and cellular proteins. Like all highly capable parasites, HIV skillfully takes advantage of cellular processes to replicate very efficiently and evade destruction by the immune system.

Entry and Integration

The first specific interaction between HIV and its target cell is the binding of the surface viral glycoprotein (SU) to the CD4 molecule, its receptor on the surface of the target cell. For the most part, the interaction between SU and CD4 is required but not sufficient for virus entry. Entry is dependent on a second molecule or co-receptor that binds to a region of the SU protein that becomes accessible after it

viruses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Cohen OJ, Kinter A, Fauci AS (1997) Host factors in the pathogenesis of HIV disease. *Immunological Reviews*, 159: p. 31–48.
Encyclopedia of AIDS (1998) Smith RA (ed) Fitzray Dearborn Publishers.
Hirsch MS and Curran J (1996) Human Immunodeficiency Viruses. In: Fields BN *et al.* (eds) *Virology*, 3rd edn, p. 1953–1975. Philadelphia: Lippincott-Raven Publishers.
Littman DR (1998) Chemokine Receptors: Key to AIDS Pathogenesis? *Cell*, vol. 93: p. 677–680.

Molecular Biology

Leon F García-Martínez, Southwestern Medical Center, Dallas, Texas, USA

J Victor García-Martínez, St Jude Children's Research Hospital, Memphis, Tennessee, USA

Human immunodeficiency viruses are members of the family *Retroviridae,* genus *Lentivirus*. The human immunodeficiency viruses (HIV) type 1 and 2 are the causative agents of acquired immunodeficiency syndrome (AIDS). Humans are the hosts for both viruses and they infect mainly CD4+ lymphocytes and monocyte/macrophages also expressing a coreceptor molecule. Characteristic of infection with HIV is an early high level of serum viremia that subsides in response to an aggressive immune response. However, HIV withstands this initial attack, is able to persist, and slowly erodes the immune system of the infected individual by depleting CD4+ lymphocytes at a rate of 2 billion per day. This article describes the molecular determinants responsible for virulence.

Properties of the Virion

HIV particles have a diameter of 100–120 nm and a spherical morphology classical of several other retroviruses. They consist of a dense truncated cone-shaped core surrounded by a membrane bilayer (envelope) decorated with spikes corresponding to the surface glycoprotein (SU). HIV particles are assembled beneath the cell surface. The internal structure of the virus particle rearranges after budding, a result of the proteolytic cleavage of the major structural components by a viral-encoded protease. This process is called maturation. Virions are sensitive to radiation, acidic pH, diluted ethanol, detergents, chorine and temperature (60°C). The genome consists of a homodimer of linear, positive-sense, single-stranded RNA of approx. 9.2 kb in size. The two strands of RNA are capped, polyadenylated, and noncovalently joined through the dimerization domain. Lysine tRNA is found bound to the viral RNA in the particle and serves as a primer for reverse transcription.

Genome Structure

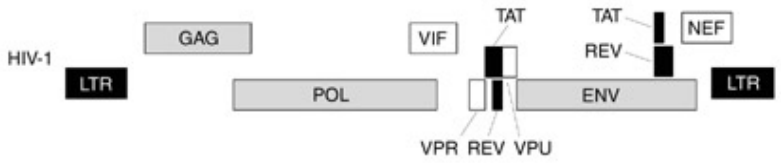
The HIV genome is significantly more complex than the genome of murine retroviruses (**Fig. 1**). This complexity is likely to reflect the dynamic interplay between this virus and its host. Like all other retroviruses, the HIV genome encodes three structural genes *gag*, *pol* and *env*. In addition, it encodes several small genes (*vif*, *vpr*/*vpx*, *tat*, *rev*, *vpu* and *nef*) that regulate gene expression, protein expression, and to a great extent are determinants of virulence. In order to maximize its coding capacity, HIV uses several different means to pack information into a relatively small genome. Differential splicing of the viral genome is used to control in a temporal and advantageous manner the expression of structural and regulatory genes. In addition, the virus genome encodes several *cis*-acting elements involved in virion packaging (Ψ or psi), reverse transcription (primer binding site or PBS, polypurine tracts or PPT), RNA retention in the nucleus (negative regulatory elements or CRS), RNA export and processing (Rev-responsive element or RRE and polyadenylation signal), transcription (TAR), etc.

Virus Replication

The replication cycle of HIV is very similar to that of other retroviruses. However, it is under significantly tighter control. The replication cycle of HIV can be divided into three steps: (1) entry and integration; (2) gene expression; and (3) assembly and budding. Each of these steps involves several specific interactions between virus and cellular proteins. Like all highly capable parasites, HIV skillfully takes advantage of cellular processes to replicate very efficiently and evade destruction by the immune system.

Entry and Integration

The first specific interaction between HIV and its target cell is the binding of the surface viral glycoprotein (SU) to the CD4 molecule, its receptor on the surface of the target cell. For the most part, the interaction between SU and CD4 is required but not sufficient for virus entry. Entry is dependent on a second molecule or co-receptor that binds to a region of the SU protein that becomes accessible after it



Structural genes	Encoded proteins	
gag	**Gag polyprotein**	**Pr 55^{gag}**
	Matrix	MA p17
	Capsid	CA p24
	Nucleocapsid	NC p7
pol	**Gag-pol polyprotein**	**Pr 160$^{gag\text{-}pol}$**
	Protease	PR p10
	Reverse transcriptase	RT → p51/66
	RNAse-H	RH
	Integrase	IN p32
env	**Envelope precursor**	**gp160**
	SU	gp120
	TM	gp41
Regulatory genes		
tat	**TAT**	**p14**
	Transcriptional transactivator	
rev	**REV**	**p19**
	Postranscriptional transactivator	
Accessory genes		
vif	**Viral infectivity factor (Vif)**	**p23**
	Enhances infectivity; cell type specific	
vpr	**Virus protein r (Vpr)**	**p15**
	Nuclear transport of preintegration complex; arrest in G2	
vpu	**Virus protein u (Vpu)**	**p16**
	Virion release; degradation of CD4 in ER	
nef	**Negative factor (Nef)**	**p27**
	Downregulation of CD4 and MHC class I; enhances infectivity; associates with kinases	

Figure 1 Diagram of the HIV-1 genome.

binds to CD4. Whereas all HIV isolates bind CD4, different HIV strains are specific for different co-receptors. These HIV co-receptors also function as receptors for chemokines and are members of the family of receptor proteins characterized by containing seven membrane-spanning segments. The discovery of the role of these co-receptors in HIV entry explained some aspects of HIV tropism that had puzzled investigators for many years and that are highly significant to virus replication and pathogenesis *in vivo*. The 'T' tropic viruses (also known as syncytium inducing or SI) are able to replicate in primary T cells and in cell lines expressing human CD4 but not in primary macrophages. These viruses use the CXCR4 molecule as co-receptor and are inhibited by the natural ligand for this receptor, stromal-derived factor (SDF-1). The 'M' tropic viruses (nonsyncytium inducing or NSI) infect human primary T cells and human monocyte/macrophages but fail to infect almost all human CD4+ cell lines. M-tropic viruses use the CCR5 molecule as co-receptor and are inhibited by its natural ligands the RANTES, MIP-1α, and MIP-1β chemokines.

The co-receptors for HIV are believed to play an important role in the fusion between the membranes of the virus and the cell. After fusion, the virus deposits its genetic information in the cytoplasm in the form of a preintegration complex. The preintegration complex consists of partially reversed transcribed RNA complex with matrix, polymerase, integrase and Vpr proteins. Of particular importance to the transport of the preintegration complex to the

nucleus are the matrix, Vpr and integrase proteins. All three have nucleophilic characteristics that are important for the active transport of the preintegration complex into the nucleus. This active transport mechanism is responsible for the ability of HIV to infect certain nondividing cells such as macrophages. Reverse transcription is completed in the cytoplasm and the finished double-stranded DNA product (provirus) is modified at both ends before integration into the host chromosome. For the most part, the process of reverse transcription for HIV is the same as for other retroviruses. One important exception is the use of a second central polypurine tract to complete the synthesis of the plus-strand. This unique feature of HIV seems to be important as mutations in this region have negative effects on virus replication.

Reverse transcriptases do not have a proof reading activity and therefore introduce nucleotide changes in the provirus with relatively high frequency (10^{-4}–10^{-5} per base incorporated). The error rate of HIV reverse transcriptase is not higher than that of other retroviruses but compounded with the high levels of HIV replication, it plays an important role in the viral diversity seen in patients. The endproduct of reverse transcription is a blunt-ended linear molecule. This molecule is modified by the viral integrase which cleaves the 3′ termini eliminating the terminal two bases from each 3′ end resulting in recessed 3′-hydroxyl groups. In the nucleus, the provirus preferentially integrates in highly bent DNA sites such as nucleosomes. After binding to the cellular DNA, the integrase–provirus complex positions the 3′-OH groups at the ends of the viral DNA to attack phosphodiester bonds on opposite strands of the cellular DNA. Then a *trans*-esterification reaction results in the joining of the proviral and cellular DNA ends. A 4–6 bp gap of mismatched viral 5′ ends are then trimmed, filled in and ligated. These last reactions are believed to be carried out by integrase but a cellular activity can not be ruled out. The net result of the integration process is the permanent incorporation of the provirus into the genome of the host.

Gene Expression

As for all other retroviruses, the HIV primary transcript initiates at the beginning of the R region of the 5′ long terminal repeat (LTR) and ends at the polyadenylation signal at the end of the R region of the 3′ LTR.Unlike most murine retroviruses which have a single splice donor (SD) and acceptor (SA) for the production of two mRNAs, the HIV RNA contains several SD and SA sites that allow for the production of numerous single and multiply spliced mRNAs. However, unlike murine retroviruses which depend solely on the cellular machinery to control the levels and types of viral transcripts, HIV encodes specific proteins that control the level and type of viral RNA expressed (full length, singly spliced and multiply spliced). The HIV LTR contains the typical components of a polymerase II enhancer–promoter which are responsible for the low basal level of transcription observed in the absence of the regulatory protein Tat (*trans*-activator of transcription). Unlike most promoters, the HIV LTR has a downstream activating *cis*-element called TAR (*trans*-acting response element) which is located in the first 80 bp of the transcript. Transactivation by Tat is mediated by the direct interaction between Tat and TAR and involves many different cellular proteins. Tat *trans*-activation results in a change in polarity of the transcripts from mainly short transcripts to fully elongated transcripts. Tat expression initiates a positive feedback loop that maintains high levels of transcription. Tat is encoded by two different exons from a multiply spliced mRNA that express a protein of 102 amino acids. All the known functional domains of Tat (TAR binding and nuclear localization) are found in the first exon. There is no definitive function for the second exon.

Early after infection there are only fully spliced viral RNAs in the cytoplasm of infected cells. These multiply spliced messages encode the Tat, Rev and Nef proteins. This differential distribution of viral RNAs is due to *cis*-acting nuclear retention sequences (CRS) present in the *gag*, *pol* and *env* coding regions of the viral RNA. These sequences are spliced out of the messages encoding Tat, Rev and Nef. As the amount of Rev in the cell reaches a threshold level, there is a significant increase in the levels of singly and multiply spliced messages in the cytoplasm. The transport of these messages into the cytoplasm is mediated by the Rev protein. Rev is a 117 amino acid phosphoprotein that binds to a *cis*-acting element, the Rev *r*esponsive *e*lement or RRE (234 bp) located within the *env* gene and found in all unspliced or singly spliced viral mRNAs. Whereas the exact stoichiometry of the Rev/RRE complex *in vivo* is not known, it is clear that many Rev molecules bind to a single RRE, hence the need for a high threshold level of Rev before export of the viral RNAs occurs. Based on the Rev-dependent export of viral RNA, investigators have divided the life cycle of the HIV into an early and a late phase. In the early phase only Rev-independent messages (*tat*, *rev* and *nef*) are exported and translated. In the late phase both Rev-independent and Rev-dependent (*gag-pol*, *env*, *vif*, *vpr*/*vpx* and *vpu*) messages are exported and translated. Unlike most cellular mRNAs, which are

monocistronic, several HIV mRNAs are multicistronic with open reading frames (ORFs) that partially overlap or are arranged in tandem. Ribosomal frameshift (−1) is involved in the expression of the Gag and Pol proteins from the same mRNA. In most translation events of the gag-pol mRNA, only the Gag protein is translated. At a much lower frequency (approx. 5%) the ribosome falls back one nucleotide at a specific site near the end of the *gag* coding sequence. The ribosome then continues translation of the *pol* ORF. This results in a Gag-Pol fusion protein that is subsequently processed by the virus protease during virion maturation. Other multicistronic mRNAs encode more than one protein and their respective expression levels are determined by the context of the first ATG. For example, all *env* messages also contain the *vpu* ORF. Because the context for the *vpu* ATG is suboptimal, the ribosome can skip over it and find the strong ATG for *env*. This level of control is important to maintain the correct equilibrium between the levels of the different viral proteins.

Assembly and budding

For the most part, assembly of the virus particle takes place on the inner surface of the cell membrane. In certain cell types like macrophages it might also take place in vacuoles. The major virion component is the Gag precursor $Pr55^{gag}$. In the virus particle, the $Pr55^{gag}$ precursor is cleaved into four major proteins: matrix (MA or $p17^{MA}$), capsid (CA or $p24^{CA}$), nucleocapsid (NC or $p7^{NC}$) and $p6^{gag}$. Gag is targeted to the cell membrane by virtue of being postranslationally modified by the addition of a myristyl group. $Pr55^{gag}$ is capable of associating into virion-like particles spontaneously without other viral or cellular proteins. $Pr55^{gag}$ directs the incorporation of the Gag-Pol polyprotein precursor ($Pr160^{gag\text{-}pol}$), the envelope protein, and Vpr. In addition, $Pr55^{gag}$ binds cyclophilin A. The significance of this observation is highlighted by the fact that cyclosporin and some of its analogues disrupt the association of $Pr55^{gag}$ with cyclophilin and inhibit HIV replication. The site of interaction of the Env protein with the forming virion is likely to be the matrix protein. The HIV-1 Env precursor (gp160) is cleaved by a cellular protease into the surface glycoprotein (SU or gp120) and a transmembrane glycoprotein (TM or gp41). Two nonstructural viral proteins Vpr and Nef have also been found in the virus particle. The nucleocapsid domain of Gag interacts directly with the encapsidation sequence (psi) of the HIV genomic RNA via its zinc finger domains. At this point the lipid bilayer begins to surround the viral core and budding occurs. Following assembly and release, proteolytic processing mediated by the viral protease separates the domains of the different polyproteins and sets the appropriate conditions for reverse transcription. Only the mature particles are competent for infection.

Viral Ancillary Genes

As indicated above, in addition to the two essential regulatory genes *tat* and *rev*, the HIV genome encodes several genes that although not essential for *in vitro* replication represent important determinants of virulence. Because these genes do not have an overt *in vitro* phenotype their study has been difficult and progress in these areas is slow. However, significant observations have been made that have partially unraveled their significance.

vif

The *v*iral *i*nfectivity *f*actor ORF overlaps with the 3′ end of *pol* and encodes a 23 kDa protein (Vif) found both in the cytoplasm and the inner portion of the cell membrane. Vif is required for infection of human primary lymphocytes and some cell lines. Whereas the mechanism of Vif action is not well understood, there is a consensus that it affects late events in the viral life cycle such as virus particle maturation. Because certain cells can complement the absence of Vif, the presence of a cellular homologue has been proposed. The infectivity of Vif defective virions produced in non-permissive cells can be 25–100 times lower than wild type. Vif has been found in virus particles at levels similar to Pol. However, since it can also be found in murine leukemia virus particles its incorporation appears not specific. Therefore, any possible significance of Vif incorporation into virions is yet to be determined.

vpr/vpx

The *v*irus *p*rotein *R* (and/or X) ORF encodes a protein (Vpr and/or Vpx) of about 120 amino acids (14 kDa) that is incorporated into the virion via $p6^{gag}$ at levels similar to those of Gag. Vpr is expressed from a singly spliced message which is dependent on Rev for transport into the cytoplasm. In HIV-infected cells, Vpr is found both in the cytoplasm and in the interior of the nuclear envelope. Vpr has two genetically separable functions: facilitating HIV infection of terminally differentiated macrophages and arrest of cells in G2 of the cell cycle. The role of Vpr in macrophage infection is to transport the proviral DNA into the intact nucleus of nondividing cells. This function of Vpr is partially restored by $p17^{MA}$ or integrase. The significance of G2 arrest is not clearly understood, but may result in increased levels of virus

production or perhaps in blocking apoptosis. An amino-terminus domain of Vpr is important for virion targeting and nuclear localization. A carboxyl-terminal domain is important for G2 arrest. Vpx is an additional ORF present in HIV-2 and related simian immunodeficiency virus strains. Vpx is also a virion protein but it does not induce G2 arrest.

vpu

The *v*irus *p*rotein *U* ORF encodes an 81 amino acids protein (Vpu). Vpu is expressed as part of a bicistronic message that also encodes the Env protein and is regulated by Rev. Vpu is a type I membrane protein with two well-described functions: degradation of CD4 in the endoplasmic reticulum and enhancement of virus release from the plasma membrane. The coordinated expression of Vpu and Env is important because CD4 degradation results in the release of Env from a complex with CD4 which otherwise would be retained in the endoplasmic reticulum. Therefore, Vpu expression in CD4+ cells would result in increased levels of Env at the site of particle assembly. A third function has also been described for Vpu, the ability to form a cation-selective ion channel. The role of this ion channel in Vpu activity is presently unknown.

nef

The protein product of the *nef* gene (Nef) was originally described as having a negative effect on virus replication and thus being a *ne*gative *f*actor hence the acronym. Subsequent *in vivo* and *in vitro* studies have shown that Nef has a positive effect on virus replication. The *nef* ORF encodes a 27–29 kDa protein. Nef is expressed from multiply spliced messages that are Rev independent and expressed at high levels early and throughout infection. Nef is myristoylated and phosphorylated in HIV-infected cells. Both post-translational modifications are important for function. Analysis by electron microscopy or immunofluorescence analysis indicates that Nef is found predominantly in the perinuclear region with a small fraction of Nef associated with the inner portion of the cell membrane. Biochemical analysis shows that a large proportion of Nef is associated with membranes through its N-terminus myristyl group. *In vitro* analysis indicates that Nef is a multifunctional protein. There are at least four reproducible functions for Nef: cell surface CD4 and major histocompatability complex (MHC) I downregulation, enhancement of particle infectivity, association with cellular serine/threonine kinases, and cellular activation. These functions of Nef contribute to the high level and efficient replication of HIV *in vivo*, but it is clear that the mechanisms of Nef action remain unknown.

See also: **Antivirals; Human immunodeficiency viruses (*Retroviridae*): Anti-retroviral agents, General features; Human T-cell leukemia viruses (*Retroviridae*): HTLV-1, HTLV-2; Retroviruses – type D (*Retroviridae*); Retroviruses of drosophila: The gypsy paradigm; Simian immunodeficiency viruses (*Retroviridae*).**

Further Reading

Coffin JM, Hughes SH and Varmus HE (1997) *Retroviruses*. Plainview, NY: Cold Spring Harbor Laboratory Press.

Pavlakis GN (1997) In: The molecular biology of Human Immunodeficiency Virus Type 1. De Vita VT, Hellman S and Rosenberg SA (eds) *AIDS*, 4th edn, p. 45. Philadelphia: Lippincott–Raven.

Antiretroviral Agents

Ranga V Srinivas and **Arnold Fridland**, Department of Infectious Diseases, St Jude Children's Research Hospital, Memphis, Tennessee, USA

Introduction

This review attempts to give a brief overview of antiretroviral agents currently used in the control of HIV-1 infections. Compounds in development, as well as other viral targets for the development of potential new drugs are considered briefly. An overview of compounds targeted against various viral gene products is presented and issues that affect the pharmacological response of the various compounds are raised.

Human Immunodeficiency Virus (HIV) Genome and Viral Targets for Drug Development

The HIV genome encodes both structural genes, *gag*, *pol* and *env*, that are common to all retroviruses, as well as additional regulatory/accessory genes unique to lentiviruses. In HIV, these genes include *tat*, *rev*, *nef*, *vif*, *vpu* and *vpr*. *Nef*, *vif*, *vpu* and *vpr* are not essential for virus replication *in vitro* although they play a role in viral pathogenesis. The HIV *gag* gene encodes a precursor polyprotein, which is processed to form the viral capsid which encases the viral

Concluding Remarks

Antiviral agents have been highly effective in controlling the ravages of the AIDS epidemic, and have even raised hopes for a cure. Nevertheless, this very success poses even greater challenges for the future. There are problems in evaluating the efficacy of new compounds in the face of currently effective therapeutic regimens, as evidenced by the global debate on the ethics of performing placebo-controlled trials in developing countries. Monitoring treatment efficacy by studying viral dynamics is being challenged by current trends where patients on long-term PI therapy show continuing immunological improvement despite virological failure. Patients on combination therapy are running out of treatment options before new compounds are becoming available. Thus, better use of available antiviral compounds seems far more prudent than relying on new products. This approach includes the development of rational approaches for drug combinations, the development of optimal delivery systems to ensure better treatment compliance. Finally, we have to explore options for customizing treatment on an individual basis to select drugs and adjust dosage regimens based on pharmacological and pharmacokinetics parameters which take into account individual variables including past treatment history, and resistance profiles of viral isolates.

See also: **Antivirals; Human immunodeficiency viruses (*Retroviridae*): General features, Molecular biology.**

Further Reading

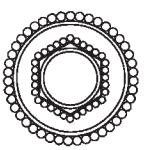
Centers for Disease Control (1998) Public Health Service Task Force Recommendations for the Use of Antiretroviral Drugs in Pregnant Women Infected with HIV-1 for Maternal Health and for Reducing Perinatal HIV-1 Transmission in the United States. *MMWR* RR-02.

De Clercq E (1995) Antiviral therapy for human immunodeficiency virus infections. *Clin. Microbiol. Rev.* 8: 200.

De Clercq E (1997) Development of resistance to human immunodeficiency virus (HIV) to anti-HIV agents: how to prevent the problem. *Int. J. Antimicrobial. Agents* 9: 21.

Flexner C (1998) HIV protease inhibitors. *N. Engl. J. Med.* 338: 1281.

HUMAN T-CELL LEUKEMIA VIRUSES (*RETROVIRIDAE*)



Contents

HTLV-1

Mitsuaki Yoshida, Department of Cellular and Molecular Biology, Institute of Medical Science, University of Tokyo, Tokyo, Japan

History

Human T-cell leukemia virus type 1 (HTLV-1) is the first exogenous retrovirus to have been established in humans. After a long and painstaking search for human retroviruses, HTLV-1 was isolated from adult T-cell leukemia (ATL) at the beginning of the 1980s. ATL was first described in 1976 in Japan as a unique malignancy of T cells affecting only adults. The disease is now known to be endemic in the southwest part of Japan, the Caribbean, South America and Africa. The human retrovirus HTLV was reported in the USA in 1980, by characterization of reverse transcriptase in a T-cell line established from peripheral blood T cells of a patient with T-cell lymphoma. This lymphoma was later diagnosed as ATL. Several T-cell lines were also established from Japanese patients with ATL by co-cultivation of normal cord-blood lymphocytes and leukemic cells from ATL patients, and retrovirus-like particles were detected in these established T cell lines. These cell lines expressed antigens that reacted with sera from ATL patients. These antigens were then identified as proteins encoded by a retrovirus (initially called ATLV), which was newly established by characterization of reverse transcriptase, specific core proteins and the proviral genome integrated in chromosomal

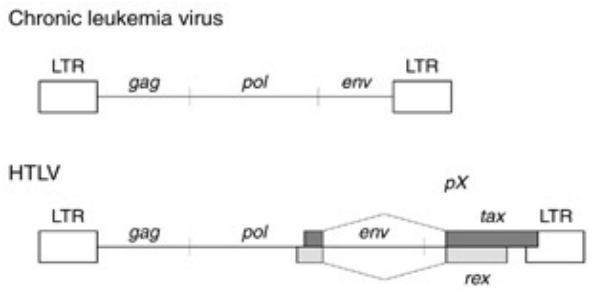


Figure 1 Genomic structures of HTLV and chronic leukemia virus. The pX region of HTLV-1 has overlapping open reading frames for Tax and Rex.

DNA of leukemic cells and established T cell lines. The background of the viral isolation provided strong evidence for the association of HTLV-1 with ATL. The isolation of HTLV-1 has disclosed new aspects of virally induced human cancers, prevention of specific types of leukemia and discovery of new diseases.

Taxonomy and Classification

HTLV-1 is a retrovirus in the genus *Lentivirus* of the *Retroviridae* family (*Gammaretrovirus* genus), with distinct properties from the general C-type retroviruses. The prototype of the retroviruses, chronic leukemia viruses, has the *gag*, *pol* and *env* genes on their genome encoding viral core proteins, reverse transcriptase and surface glycoprotein, respectively. Acute leukemia viruses have an oncogene of cellular origin substituting a part of *gag*, *pol* and/or *env* sequences. The HTLV-1 genome has *gag*, *pol* and *env* genes as general chronic leukemia viruses, but has an extra sequence, pX, in the 3′ terminal region of the genome (**Fig. 1**).

The HTLV-1 genome is very stable and is well conserved among remote endemic areas; however, it is classified into several types according to the restriction maps of the long terminal repeat (LTR). These are Cosmopolitan (C), Japanese (J) and African (A) and Melanesian (M) subtypes. No correlation of these subtypes with their pathogenesis has been observed.

Viruses similar to HTLV-1, simian T-cell leukemia viruses (STLVs), have been isolated from various species of nonhuman primates, including the Japanese macaque, African green monkey, pig-tailed macaque, gorilla and chimpanzee. The STLVs share 90–95% homology of the genomic sequence with HTLV-1 and are also very similar to each other. Although STLVs are widely distributed among monkeys, no leukemia similar to ATL has been commonly observed. Another member of the genus is bovine immunodeficiency virus.

After characterization of HTLV-1, another virus, which is immunologically similar to HTLV-1, was isolated from a patient with hairy T-cell leukemia and named HTLV-2. Similarity of the HTLV-2 genome to that of HTLV-1 is about 60%. HTLV-2 is frequently isolated from intravenous drug abusers, but only three cases have been isolated in patients with hairy T-cell leukemia; thus, its association with a specific disease has not been established.

Molecular Biology

Genome

The proviral genome is 9032 bp long and has a constitution of LTR-*gag*-*pol*-*env*-pX-LTR (**Fig. 1**). The presence of the pX sequence is unique among retroviruses and is essential for efficient replication of the virus. The pX sequence has no significant homology to human chromosomal DNA, and therefore, does not contain any typical oncogene.

The pX sequence contains three open reading frames (ORFs) that overlap each other. Two of these were identified as functional genes, *tax* and *rex*, coding for $p40^{tax}$ and $p27^{rex}$, and the other encodes for $p21^{x}$ whose function is not yet identified. Tax protein, $p40^{tax}$, is a *trans*-activator of transcription of the HTLV-1 genome, and is thus essential to viral gene expression. $p27^{rex}$ is a *trans*-acting modulator of RNA processing and enhances cytoplasmic expression of the unspliced and partially spliced mRNA for Gag and Env. Rex is therefore essential for HTLV-1 gene expression and replication. Regulation with these two viral regulators at the transcriptional and post-transcriptional levels is essential for efficient viral replication and escape from host immune responses. The protein of $p21^{x}$ is composed of the same amino acid sequence as $p27^{rex}$ except deletion at the N-terminal region by initiating translation at the inside of the coding frame for $p27^{rex}$, but its function is unknown.

Gene expression

The HTLV-1 genes are expressed through transcription and splicing of the transcript. The genomic RNA of 8.5 kb, a primary transcript, is the mRNA for Gag and Gag-Pol fusion proteins. A frameshift between *gag* and *pol* sequences suppresses the translational termination for Gag. The primary transcript is spliced into two forms to encode the Env and Tax, Rex and $p21^{x}$, respectively. The first splicing removes the *gag* and *pol* sequences, forming a 4.2 kb mRNA which is able to express Env protein. The second splicing

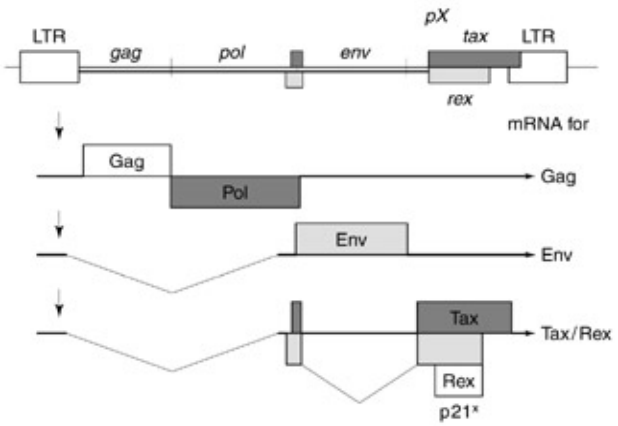


Figure 2 Transcription and splicings for the expression of HTLV-1 genes. Translation for $p21^x$ is initiated at the internal site of the frame for Rex.

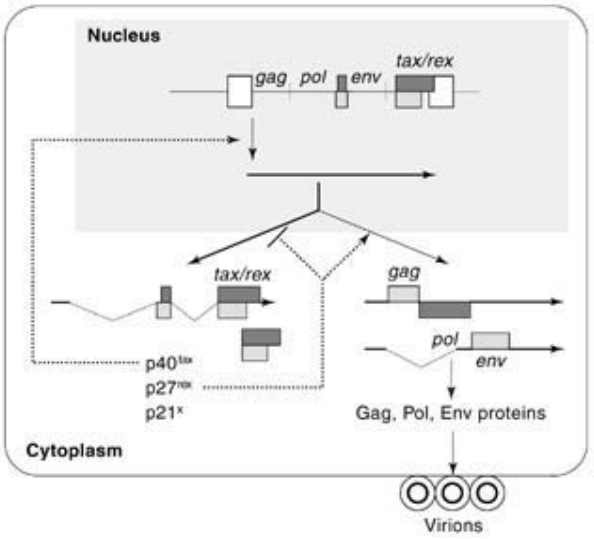


Figure 3 Regulation of HTLV-1 expression at the transcription level and RNA processing by two regulatory proteins, Tax and Rex. Dotted lines with arrow heads and bars represent enhancement and suppression, respectively.

further removes the *env* sequence, forming a 2.1 kb mRNA for the expression of Tax, Rex and $p21^x$ proteins (**Fig. 2**). Therefore, the expression of both unspliced and spliced mRNA in the cytoplasm is essential for viral replication.Transcription of the proviral genome is directed by the LTR. The LTR of HTLV-1 is transcriptionally active; furthermore, its activity is *trans*-activated over 100-fold by the presence of Tax protein. Enhancer sequences, which consist of three direct repeats of the 21 bp sequence containing an imperfect cAMP-responsive element (CRE), are required for *trans*-activation. Although Tax is an enhancer-activating protein, Tax does not bind directly to the 21 bp enhancer. Tax protein binds to CRE-binding protein (CREB) or CRE-modulating protein (CREM), which specifically bind to the 21 bp enhancer. On the other hand, Tax also binds to CREB binding protein (CBP) which is able to bind to CREB only when CREB is phosphorylated, responding to the signal through cAMP and protein kinase A. Therefore, the presence of Tax enables the complex of CREB-Tax-CBP to form even in the absence of cellular stimulation, to form CREB-P-CBP. Complex formation of this sort strongly activates transcription from the LTR.

Modulation of viral RNA processing is *trans*-activated by Rex protein. In the absence of Rex protein, all viral RNA is completely spliced and only the 2.1 kb mRNA for Tax and Rex is expressed in the cytoplasm. Then Rex protein thus expressed suppresses splicing of the viral RNA and enhances the expression of unspliced mRNA in the cytoplasm. Therefore, Rex is essential for the expression of Gag, Pol and Env proteins. This regulation by Rex, in return, reduces the level of spliced mRNA that encodes regulatory proteins, Tax and Rex, consequently resulting in a lower level of viral transcription (**Fig. 3**). In other words, Rex is a positive signal for the expression of viral structural proteins on one hand, but a negative signal for viral gene transcription on the other hand. The combination of these two regulators, Tax and Rex, makes the viral expression transient. This mechanism explains why HTLV-1 is so frequently repressed and how the infected cells are able to escape from the host immune response.

Rex binds to a *cis*-acting element (RxRE) in the target RNA, which is transcribed from the 3′ LTR. The mechanism by which Rex suppresses splicing and enhances the cytoplasmic expression is not clearly understood. The function of Rex is strikingly similar to that of Rev protein of human immunodeficiency virus (HIV), and in fact is able to respond to RvRE to which Rev binds.

Epidemiology

Geographic clustering

Nearly all ATL patients and HTLV-1-infected people have serum antibodies to HTLV-1 proteins, and virus carriers are defined by the presence of antibodies. HTLV-1 antibodies are recognized in 5–15% of adults in southwestern Japan, the Caribbean islands, South America, Central Africa, Papua New Guinea and the Solomon Islands. However, the prevalence of healthy, seropositive adults varies significantly from district to district and even from village to village within these

endemic areas. Why this virus is clustered in such remote regions is not clear; however, migration of races susceptible to HTLV-1 infection has been proposed. This proposal is based on the high conservation of the HTLV-1 genome among all endemic areas, suggesting a single origin of HTLV-1, viral transmission from mother to child, and unique haplotypes of HLA in patients, suggesting different responses of these individuals to HTLV-1 infection. Namely, populations susceptible to HTLV-1 infection moved from Central Asia to East Asia, to South Asia, and to South America through the Bering Strait and North America, and these populations were followed by those relatively resistant to HTLV-1 infection. Thus, the localization of HTLV-1 in such remote areas has been established. ATL and HAM/TSP (see below), which are induced by HTLV-1 infection, are similarly clustered, overlapping HTLV-1. ATL patients and healthy carriers are found sporadically all over the world, but most of them have moved from the endemic areas.

Age-dependent prevalence

The viral prevalence increases with age after 30, reaching a maximum in people between 40 and 50 years of age in Japan. The prevalence is 1.6 times higher in females than in males. This higher prevalence in females over 20 years old is attributed to sexual transmission of HTLV-1 from husbands to wives. Conversely, the findings indicate that HTLV-1 transmission from wives to husbands is infrequent. The age-dependent increase in both sexes was somewhat mysterious, but now is believed to reflect reduced risk of infection at the early stage of life, that is, 40–50 years ago. Artificial milk started to be popular around 40–50 years ago in these areas in Japan, and thus reduced the incidence of breast milk-borne infection of HTLV-1.

Familial aggregation

Familial aggregation of HTLV-1 infection is very apparent. Especially, if a mother is positive, her children are frequently positive. This aggregation is understood to be the result of viral transmission from husband to wife and from mother to child.

Genomic stability

Molecular analyses have demonstrated that the viral genome is well conserved in Japan, the Caribbean and Africa. The viral isolates from Papua New Guinea vary somewhat more but are still 90–95% homologous. The stability of the genome stands in sharp contrast to the highly labile genome of another human retrovirus, HIV.

Infection and Host Range

In vitro infection and transformation

Cell-free viral particles of HTLV-1 show extremely low infectivity *in vitro* and, usually, cannot establish an infection. Co-cultivation with virus-producing cells is able to transmit HTLV-1 to a variety of human and animal cells, human T and B lymphocytes, fibroblasts, and epithelial cells, as well as cells from monkeys, rats, rabbits, and hamsters, but curiously not mice. In these infected cells, the provirus is integrated into random sites in the chromosomal DNA. Only T cells with the CD4+ marker are frequently immortalized on infection with HTLV-1 *in vitro*. The immortalized cells express high levels of the α chain of IL-2R and proliferate in an IL-2R-dependent fashion and mimic leukemic cells *in vivo*.

Natural transmission and *in vivo* infection

Despite the broad range of host cell types *in vitro*, the cells infected *in vivo* are almost exclusively T cells with the CD4+ phenotype. Surprisingly, the vast majority of these infected cells, irrespective of whether or not they are leukemic, do not express the viral information *in vivo*. According to reverse polymerase chain reaction (PCR) analysis of the viral mRNA, over 99% of infected cells are negative for viral gene expression *in vivo*. These *in vivo* cells, however, can express the viral genes soon after cultivation *in vitro*; therefore, the viral latency is not due to structural defects of the proviruses.

HTLV-1 is transmitted through (1) blood transfusion, (2) nursing of infants by infected mothers and (3) sexual relations. Retrospective studies of blood transfusions showed that 60–70% of recipients of fresh, seropositive blood were infected with HTLV-1, but no recipients of seropositive plasma were infected. Thus, the transfer of infected cells from donor to recipient is required for viral transmission. HTLV-1 infection through transfusion is suggested not to induce ATL, but can induce HAM/TSP.

Viral transmission from mother to child was originally suggested by epidemiological studies: most mothers of seropositive children were seropositive and about 30% of the children of seropositive mothers were themselves seropositive. Breast milk is a source of transmissible virus. Milk taken from seropositive mothers could induce antibodies in adult marmosets. More direct evidence was obtained from a practical trial demonstrating that cessation of breast-feeding by seropositive mothers drastically reduced the seropositivity rate of their children.

Wives of seropositive husbands are frequently seropositive. Conversely, the husbands of seropositive wives show the same frequency of seropositivity as

those in the region under study. Thus, the virus appears to be transmitted from husband to wife but not *vice versa*. Infected T cells have been detected in semen and are thought to mediate the viral transmission from male to female.

Pathogenicity

Adult T-cell leukemia (ATL)

The onset of ATL is observed only in adults, most frequently being seen in people in their 40s and 50s. The male/female ratio of incidence of ATL is 1.4/1. Symptoms of ATL are variable and frequently complicated by skin lesions, enlargement of lymph nodes, liver and/or spleen, and infiltration of leukemic cells into various organs. Patients usually have antibodies to HTLV-1 proteins, show an increased level of serum LDH and suffer from hypercalcemia. In addition to the typical, acute form of ATL, smoldering, chronic and lymphoma types have been recognized. In smoldering ATL, patients commonly have a few or several percent of morphologically abnormal T cells in the peripheral blood, but do not show particular signs of severe illness for a long period. Patients with chronic ATL have rather high levels of HTLV-1-infected leukemic cells, but can maintain stable phenotypes for a certain period. In the acute phase, ATL is aggressive and resistant to any treatment; consequently, most patients die within six months of its onset.

Leukemic cells are T cells with the CD4+ phenotype. Frequently, the nucleus of leukemic cells is highly lobulated and this phenotype is unique to ATL. These cells are always integrated with HTLV-1 proviruses, and the site of integration is clonal in a given ATL patient. In most cases of ATL, one complete copy of the provirus is integrated into each leukemic cell. Some cases contain defective proviruses or more than two copies. The leukemic cells frequently express a high level of IL-2Rα on their surface. In almost all cases, leukemic cells carry chromosomal abnormalities, but no abnormality is common among ATL patients.

A causative role of HTLV-1 in ATL development has been proposed based on the clonal integration of the proviral DNA, since it implies that leukemic cells originated from a single HTLV-1-infected T cell. ATL patients are always infected with HTLV-1; therefore, viral infection is prerequisite for ATL development. Furthermore, HTLV-1 can immortalize human T cells *in vitro*, and the phenotypes of the immortalized T cells are similar to those of leukemic cells from ATL patients. The incidence of ATL among HTLV-1 carriers was estimated to be 2–5% over their lifetime.

Although the vast majority of ATL cases are associated with HTLV-1 infection, a form of ATL unrelated to HTLV-1 infection has been described. Patients with this disease are phenotypically indistinguishable both clinically and hematologically from patients with typical ATL, but they have no HTLV-1 antibodies and their leukemic cells carry no HTLV-1 proviral DNA. The etiologic factor in this disease has not been identified.

HAM/TSP and other diseases

HTLV-1 was found to be associated with a slowly progressive myelopathy known in tropical zones as tropical spastic paraparesis (TSP) and, in endemic areas of Japan, as HTLV-1-associated myelopathy (HAM). The unique phenotypes of HAM/TSP are chronic, symmetrical, bilateral involvement of the pyramidal tracts, at mainly the thoracic level of the spinal cord, and include progressive spastic paresis with spastic bladder and minimal sensory deficits. Patients with HAM/TSP are mostly adults; thus, the disease has a long latency after HTLV-1 infection. However, transfusion of seropositive blood can lead to HAM/TSP after an average interval of two years. This feature is different from ATL. However, screening for seropositive blood has greatly reduced the risk of acquiring HAM/TSP through transfusion.

Most patients with HAM/TSP have much higher (frequently one order higher) titers of antibodies against HTLV-1 antigens than asymptomatic carriers and ATL patients. These patients and their family members were reported to have particular types of human leukocyte antigens (HLA). The greater immunological response to HTLV-1 infection may be thus explained.

Despite their strong immunological responses to HTLV-1 infection, most HAM/TSP patients have much larger populations of infected T cells than do HTLV-1 carriers. Some of these infected T cells infiltrate the spinal cord particularly at the site of lesions and also the cerebrospinal fluid. Some of these virus-positive cells produce cytokines including cytotoxic tumor necrosis factor (TNF)-α in the spinal cord. The frequent or infrequent expression of cytotoxic cytokines is proposed to cause accumulation of damage in neuronal tissues as the mechanism of HAM/TSP.

HTLV-1 infection is also proposed to be associated with other diseases such as uveitis, chronic lung diseases, monoclonal gammopathy, and rheumatoid arthritis. The association of uveitis with HTLV-1 infection has been established; however, further

studies are required to elucidate the exact relationship of these diseases with HTLV-1 infection.

Molecular Biology of Leukemogenesis

Viral involvement

ATL cells have clonally integrated HTLV-1 proviruses. However, no common site of integration was observed among ATL patients. Therefore, the role of HTLV-1 in leukemogenesis is independent of its site of integration. In this feature, HTLV-1 differs from other chronic retroviruses of animals, in which integration was commonly adjacent to a proto-oncogene (*cis*-acting effect). As a consequence, a *trans*-acting function of HTLV-1 is postulated in leukemogenesis, and unique genes in the pX sequence have been the focus of interest.

The pX region is able to code for three proteins, $p40^{tax}$, $p27^{rex}$ and $p21^{x}$. Among these protein products, Tax was shown to immortalize human CD4-positive T cells, transform rodent fibroblasts in cooperation with *ras* oncogene, and induce mesenchymal tumors in its transgenic mice. These properties, together with the *trans*-acting functions of Tax protein, suggest that Tax may play critical roles in the development of ATL.

Tax

Tax was originally identified as a *trans*-activator of viral gene transcription as described above, and then also identified as a *trans*-activator of specific cellular genes. It was then identified as a *trans*-repressor of transcription of another set of cellular genes, and also as an inhibitor of tumor suppressor proteins and cell cycle regulators (**Fig. 4**).

Trans-activation of transcription by Tax protein requires a specific enhancer in the target gene; however, Tax itself does not bind to the enhancer directly. The mechanism includes Tax binding to enhancer binding proteins, CREB and CREM, that bind to the 21 bp enhancer of HTLV-1, NF-κB family proteins which bind to NF-κB binding site, and SRF which binds to serum responsive element (SRE). Through the binding to these enhancer binding proteins, Tax *trans*-activates transcription of the viral genome, interleukin (IL)-2Rα, IL-6, GM-CSF, TNF-α, PTHrP, major histocompatability complex (MHC) class I, c-Fos, c-Egr, and others. Abnormal expression of some of these genes obviously enhances abnormal proliferation of infected cells. Activation of IL-2Rα and PTHrP genes by Tax is a reasonable explanation for the abnormal expression of IL-2Rα chain on ATL cells and the high incidence of hypercalcemia among ATL patients, respectively. *Trans*-

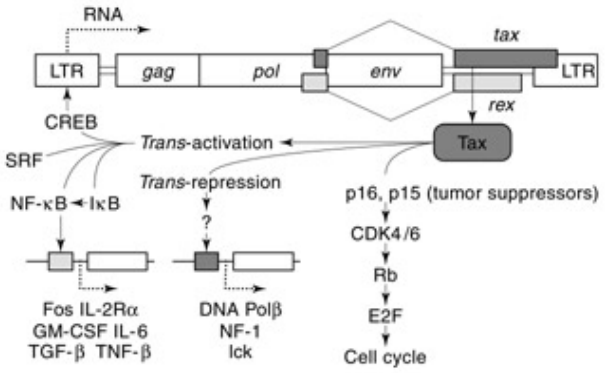


Figure 4 Schematic illustration of Tax action on cellular gene expression and cell cycle regulation.

activation of NF-κB-directed transcription is also achieved by suppression of the inhibitors, IκBα, β and γ, in the cytoplasm. Tax binds to IκBα and induces its rapid degradation, which is normally induced by stimuli for cell growth or differentiation. Consequently, NF-κB regulation is activated by Tax at two distinct steps; one is inactivation of the inhibitor IκB in the cytoplasm and the other is activation of NF-κB protein in the nucleus.

Trans-repression of transcription has been described for DNA polymerase β, NF-1 and lck. More cases will be found on other genes. The mechanism for *trans*-repression is not well characterized; however, the *trans*-repression of DNA polymerase β suggests inefficient DNA repair resulting in a high frequency of mutation. NF-1 gene is a tumor suppressor gene; thus, its suppression might contribute to induction of abnormal cell proliferation.

Inactivation of cell cycle inhibitors is also achieved by Tax; Tax binds to $p16^{ink4A}$ and $p15^{ink4B}$, which are also known to be tumor suppressor proteins, and inhibits their function. These INK family proteins inhibit CDK4 and CDK6, which phosphorylate and inactivate the negative function of Rb protein, and thus, arrest cells at the G1 phase of the cell cycle. Inactivation of these INK family proteins by binding with Tax releases cells from G1 arrest, promoting abnormal cell proliferation. Inactivation by Tax of cell cycle checkpoint protein is also reported at the G2/M phase.

Prevention and Control of Infection

Transfusion of seropositive blood transmits HTLV-1 to the recipients with a risk of two-thirds. With the introduction of HTLV-1 screening systems in blood banks, seropositive blood is now rejected in Japan,

and viral transmission through transfusion has been greatly reduced.

The major natural route of viral transmission is from mother to child via infected T cells in breast milk. Therefore, stopping breast-feeding should prevent infection of children. This possibility is being tested in Nagasaki City, Japan. With consent, pregnant women are surveyed for HTLV-1 antibodies; those who are seropositive are advised to avoid breast-feeding. The trial is still in progress, but the results so far observed indicate a drastic reduction in the incidence of seropositive children. Breast-feeding transmits the virus to the child with a risk of one-third, but bottle feeding reduced the risk to just a few percent. The success of this trial provides direct evidence for viral transmission through milk in humans, but unfortunately, not all children who were bottle-fed remained seronegative. The effect of breast-feeding over a shorter period such as 6 months is under investigation, since breast-feeding would be of great benefit to children.

Future

A regulatory gene, *tax*, of HTLV-1, whose primary role is activation of viral replication, is responsible for induction of abnormal growth of HTLV-1-infected cells. This seems to be facilitated by *trans*-activation or *trans*-repression of transcription of specific cellular genes and also by inhibition of some cell cycle inhibitors and tumor suppressor proteins. However, the long latency in the pathogenesis after infection clearly indicates that Tax alone is not sufficient for the malignant transformation of infected T cells. Abnormal promotion of cell proliferation by Tax basically results in a random population of infected T cells; however, final leukemic cells are always of clonal origin. It is generally accepted that tumorigenesis proceeds through multistep processes. Therefore, it is likely that HTLV-1 infection enhances proliferation of progenitor cells for tumor cells in a random fashion. One of the most critical questions is what kind of molecular events trigger a clonal expansion of infected cell into leukemic cells? Furthermore, there exist smoldering, chronic and acute ATL, and also lymphoma type of ATL. The leukemic cells in these different types of ATL are all clonal; therefore, it is an interesting question as to what kind of genes are involved in these different subtypes of ATL.

Another unique finding of HTLV-1 is its expression *in vivo*. The viral genes are expressed at extremely low levels and are detected only by sensitive PCR. The vast majority of infected T cells do not express any of the viral mRNA, irrespective of whether they are transformed or not. Therefore, most leukemic cells *in vivo* do not express Tax protein at all, suggesting that Tax may not be required for the maintenance of the transformed phenotypes of T cells *in vivo*. What kind of molecular events can mimic the function of Tax in these cells? The solution of these questions would provide clues for understanding ATL as well as cancers in general in humans.

Studies on HTLV-1 and modern technologies have now made it possible to prevent viral transmission, although only in part; however, cessation of breast-feeding might result in more serious problems in children in certain environments. Therefore, the development of an efficient vaccine is an urgent and essential issue for complete irradication of HTLV-1 and its associated diseases.

See also: **Bovine immunodeficiency virus (*Retroviridae*); Human immunodeficiency viruses (*Retroviridae*): Anti-retroviral agents, General features, Molecular biology; Human T-cell leukemia viruses (*Retroviridae*): HTLV-2; Simian immunodeficiency viruses (*Retroviridae*).**

Further Reading

Weiss R (1985) Human T cell retroviruses. In: Weiss R, Teich T, Varmus H and Coffin J (eds) *RNA Tumor Viruses*, Suppl., 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Cann AJ and Chen ISY (1990) Human T-cell leukemia virus types I and II. In: Fields BN, Knipe DM, Howley PM (eds) *Fields Virology*, 2nd edn, p. 1501. New York: Raven Press.

Takatsuki HY and Yoshida M (eds) (1992) *Advances in Adult T-cell Leukemia and HTLV-I Research*, Monograph on Cancer Research No. 39. Japanese Cancer Association, Tokyo: Japan Scientific Society Press, and London: CRC Press.

HTLV-2

Bobbie J Rimel, Joseph D Rosenblatt and **Vicente Planelles**, Departments of Medicine, Hematology-Oncology Unit and Microbiology and Immunology, University of Rochester, Rochester, New York, USA

History

Human T cell leukemia virus type 1 (HTLV-1), the first recognized human retrovirus, was discovered in 1980. HTLV-1 has been linked epidemiologically and molecularly to the pathogenesis of two distinct illnesses, a leukemia called adult T cell leukemia/

and viral transmission through transfusion has been greatly reduced.

The major natural route of viral transmission is from mother to child via infected T cells in breast milk. Therefore, stopping breast-feeding should prevent infection of children. This possibility is being tested in Nagasaki City, Japan. With consent, pregnant women are surveyed for HTLV-1 antibodies; those who are seropositive are advised to avoid breast-feeding. The trial is still in progress, but the results so far observed indicate a drastic reduction in the incidence of seropositive children. Breast-feeding transmits the virus to the child with a risk of one-third, but bottle feeding reduced the risk to just a few percent. The success of this trial provides direct evidence for viral transmission through milk in humans, but unfortunately, not all children who were bottle-fed remained seronegative. The effect of breast-feeding over a shorter period such as 6 months is under investigation, since breast-feeding would be of great benefit to children.

Future

A regulatory gene, *tax*, of HTLV-1, whose primary role is activation of viral replication, is responsible for induction of abnormal growth of HTLV-1-infected cells. This seems to be facilitated by *trans*-activation or *trans*-repression of transcription of specific cellular genes and also by inhibition of some cell cycle inhibitors and tumor suppressor proteins. However, the long latency in the pathogenesis after infection clearly indicates that Tax alone is not sufficient for the malignant transformation of infected T cells. Abnormal promotion of cell proliferation by Tax basically results in a random population of infected T cells; however, final leukemic cells are always of clonal origin. It is generally accepted that tumorigenesis proceeds through multistep processes. Therefore, it is likely that HTLV-1 infection enhances proliferation of progenitor cells for tumor cells in a random fashion. One of the most critical questions is what kind of molecular events trigger a clonal expansion of infected cell into leukemic cells? Furthermore, there exist smoldering, chronic and acute ATL, and also lymphoma type of ATL. The leukemic cells in these different types of ATL are all clonal; therefore, it is an interesting question as to what kind of genes are involved in these different subtypes of ATL.

Another unique finding of HTLV-1 is its expression *in vivo*. The viral genes are expressed at extremely low levels and are detected only by sensitive PCR. The vast majority of infected T cells do not express any of the viral mRNA, irrespective of whether they are transformed or not. Therefore, most leukemic cells *in vivo* do not express Tax protein at all, suggesting that Tax may not be required for the maintenance of the transformed phenotypes of T cells *in vivo*. What kind of molecular events can mimic the function of Tax in these cells? The solution of these questions would provide clues for understanding ATL as well as cancers in general in humans.

Studies on HTLV-1 and modern technologies have now made it possible to prevent viral transmission, although only in part; however, cessation of breast-feeding might result in more serious problems in children in certain environments. Therefore, the development of an efficient vaccine is an urgent and essential issue for complete irradication of HTLV-1 and its associated diseases.

See also: **Bovine immunodeficiency virus (*Retroviridae*); Human immunodeficiency viruses (*Retroviridae*): Anti-retroviral agents, General features, Molecular biology; Human T-cell leukemia viruses (*Retroviridae*): HTLV-2; Simian immunodeficiency viruses (*Retroviridae*).**

Further Reading

Weiss R (1985) Human T cell retroviruses. In: Weiss R, Teich T, Varmus H and Coffin J (eds) *RNA Tumor Viruses*, Suppl., 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Cann AJ and Chen ISY (1990) Human T-cell leukemia virus types I and II. In: Fields BN, Knipe DM, Howley PM (eds) *Fields Virology*, 2nd edn, p. 1501. New York: Raven Press.

Takatsuki HY and Yoshida M (eds) (1992) *Advances in Adult T-cell Leukemia and HTLV-I Research*, Monograph on Cancer Research No. 39. Japanese Cancer Association, Tokyo: Japan Scientific Society Press, and London: CRC Press.

HTLV-2

Bobbie J Rimel, Joseph D Rosenblatt and **Vicente Planelles**, Departments of Medicine, Hematology-Oncology Unit and Microbiology and Immunology, University of Rochester, Rochester, New York, USA

History

Human T cell leukemia virus type 1 (HTLV-1), the first recognized human retrovirus, was discovered in 1980. HTLV-1 has been linked epidemiologically and molecularly to the pathogenesis of two distinct illnesses, a leukemia called adult T cell leukemia/

lymphoma (ATLL), and a chronic neurologic disease, HTLV-1-associated myelopathy (HAM). Identification of HTLV-1 set the stage for the search for other human retroviruses.

A permanently immortalized T cell line, known as the Mo T cell line, was established in 1976 from the spleen of a patient with an unusual T cell variant of hairy-cell leukemia (HCL). This cell line was shown to contain a second highly related retrovirus distinct from any previous isolates of HTLV-1 by Kalyanaraman and Gallo. Although there was antigenic similarity between the structural gag-encoded proteins, p19 and p24 of HTLV-1 and the virus in the Mo T cell line, a competition radioimmunoassay using HTLV-1 virus lysate demonstrated only partial cross-reactivity. The Mo T cell virus was subsequently cloned and was found to have a similar genomic organization but less than 60% overall nucleotide homology to HTLV-1. Therefore, the virus in the Mo T cell line represented the first isolate of a new and different type of human retrovirus, now known as human T cell leukemia virus type 2 (HTLV-2).

To date, only sporadic cases of leukemia or human disease linked to HTLV-2 have been identified. The patient from whom the Mo T cell line was originally established had a disease characterized as a T cell variant of HCL. Whether the patient in fact had a true T cell HCL variant is still open to question, as direct analysis of leukemia cells was not performed and the T cell phenotype was established from an immortalized T cell line obtained from the patient's spleen. HCL is generally of the B cell lineage, with characteristic cytoplasmic protrusions from atypical lymphoid cells in the peripheral blood and bone marrow. HTLV-2 was again isolated from a second patient thought to have an unusual variant of HCL; however, detailed analysis of this patient demonstrated that he had a biphenotypic malignancy with a monoclonal B cell population with morphological features of HCL and an atypical clonal CD8+ T cell population predominant in the patient's bone marrow and peripheral blood that was infected with HTLV-2. As opposed to HTLV-1, where the virus has been repeatedly isolated from patients with a unique form of leukemia known as ATLL, HTLV-2 has yet to be convincingly associated with any human malignancy, except in anecdotal cases. Nevertheless, owing to its biologic, antigenic and molecular similarity to HTLV-1, HTLV-2 has afforded an excellent model for the study of gene regulation and transformation of infected T cells by both viruses.

Current epidemiologic data suggest that North and South American Indians have a significant prevalence of HTLV-2 seropositivity. High rates of infection have also been documented in populations of intravenous drug abusers (IVDA) in the USA and Europe. Detailed epidemiological studies of these HTLV-2-infected populations may eventually reveal a clearer relationship between HTLV-2 and human disease.

Taxonomy, Classification and General Properties

Retroviruses (*Retroviridae* family) were traditionally divided into three subfamilies, based primarily on pathogenesis rather than molecular data. Using nucleotide sequence relationships and genomic structure, the International Committee on the Taxonomy of Viruses (ICTV) has recently redefined the taxonomy of the *Retroviridae* to recognize seven distinct genera. According to this classification scheme, HTLV-2 is a member of the Deltaretrovirus (bovine leukemia virus (BLV) and HTLV) genus. The biological properties of these viruses and their molecular genetic structure distinguish them from human immunodeficiency virus type 1 (HIV-1) and 2 (HIV-2), which are members of the *Lentivirus* genus of retroviruses. Whereas HIV-1 and HIV-2 have cytopathic effects on human T cells and monocytes in culture, both HTLV-1 and -2 are capable of transforming T cells *in vitro*, resulting in immortalized T cell lines. However, both deltaretroviruses and lentiviruses appear to be capable of prolonged asymptomatic infection *in vivo*.

The molecular organization of HTLV-1, HTLV-2 and BLV is unique, in that all three viruses contain overlapping *trans*-acting regulatory genes located in the 3′ end of the genome. These genes, *tax* and *rex*, are analogous in function to the corresponding transcriptional and post-transcriptional regulators, *tat* and *rev* respectively, in HIV-1 and HIV-2. The *tax*- and *rex*-encoded proteins bear little amino acid homology to analogous lentivirus proteins, and appear to be unique to HTLV and BLV viruses. Additional viral proteins have been recognized by Franchine and co-workers in HTLV-1, and are thought to be encoded by HTLV-2 as well.

As in other retroviruses, the HTLV virion is believed to contain two copies of single-stranded HTLV genomic RNA encased within structural Gag proteins and covered by envelope glycoproteins. The virion also contains reverse transcriptase, integrase and RNase H (*pol* gene products), but not the *trans*-regulatory proteins, Tax or Rex, which are synthesized intracellularly, and, although not a functional part of the virion, are nevertheless crucial for viral replication.

Initial studies comparing genomic sequences of HTLV-2 isolates have clearly demonstrated the existence of two HTLV-2 subtypes: HTLV-2a (for-

merly known as HTLV-2 Mo) and HTLV-2b (formerly known as HTLV-2 NRA). This subtype discrimination is based on relative divergence of nucleotide sequences of the *envelope* (4.3%), *gag* (3.8%), and long terminal repeat (LTR) (5.7%) regions. The predicted amino acid sequence of the HTLV-2a and HTLV-2b Tax protein suggested a possible functional difference between the Tax proteins of these virus subtypes. A two-nucleotide substitution near the 3′ end of the HTLV-2b Tax coding sequence results in an Arg residue instead of the stop codon present in the HTLV-2a Tax protein. In transient transfection assays, HTLV-2b Tax is a fivefold more potent *trans*-activator of the HTLV-2 LTR than the HTLV-2a Tax protein. *In vivo* consequences of this functional difference remain to be established.

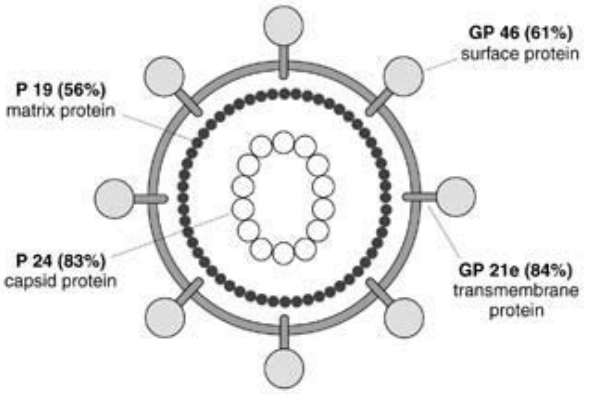


Figure 1 Amino acid homology between viral capsid and envelope proteins of HTLV-1 and HTLV-2. Reproduced with permission from Cann AJ and Chen ISY (1990) HTLV-I and -II. In: Fields BN, Knipe DM, Howley PM *et al.* (eds) *Fields Virology*, 2nd edn, p. 1501, New York: Raven.

Properties of the Genome and Proteins

The genetic organization of HTLV-1 and -2 is distinct from that of other retroviruses. Although the major virion protein components, the *gag*, *pol* and *env* genes common to all replication-competent retroviruses, are preserved, the 3′ end of the genome conserved between HTLV-1 and -2 is not common to replication-competent oncoviruses. This region was originally described in HTLV-1 by Seiki and Yoshida in Japan, and was designated the X region.

Although the epidemiology of HTLV-1 has been extensively studied worldwide and linked to the geographic distribution of HTLV-1-associated diseases, the epidemiology of HTLV-2 is still poorly characterized. Initially found in several patients with T cell leukemia, HTLV-2 was subsequently identified in acquired immune deficiency syndrome (AIDS) patients without associated malignancy, and in IVDA in the USA, UK and, more recently, Italy. A detailed study of IVDA in several locations in the USA by discriminatory PCR analysis of DNA within infected peripheral blood mononuclear cells (PBMC) demonstrated that many IVDAs, found to be seropositive by HTLV-1-based assays, are in fact infected with HTLV-2. A study in 1989 showed that of 23 seropositive IVDAs in New Orleans identified using an HTLV-1-based enzyme-linked immunosorbent assay (ELISA), 21 were found by discriminatory PCR to be infected with HTLV-2, and only two were found to be infected with HTLV-1. These studies highlight the serological crossreactivity of patient sera with HTLV-1 and -2 Gag antigens (see **Fig. 1**), and the need for more discriminatory analyses of seropositive individuals. Routine use of HTLV-1-based ELISA for screening of blood donor samples in the US has demonstrated an overall HTLV seroprevalence of approximately 0.04%.

Random screening of the blood supply with the HTLV-1-based ELISA has identified a number of otherwise healthy seropositive individuals. The use of discriminatory DNA amplification by PCR of mononuclear cell DNA to distinguish between HTLV-1 and -2 has demonstrated that over 50% of HTLV-seropositive random blood donors are in fact infected with HTLV-2. These studies also found that, whereas HTLV-1-infected blood donors tend to have lived in endemic areas or have had sexual contact with individuals from HTLV-1-endemic areas, most HTLV-2-infected donors appear to have come into intimate contact with IVDAs. Several populations that appear to be endemically infected at low rates with the HTLV-2 virus have been identified, including New Mexican Indians, Indians in Florida and, more recently, in Panama, Brazil and Venezuela. Rates of infection have varied from approximately 1% for the New Mexican Indian population to as high as 8% for the Guaymai Indians of Panama. The preliminary epidemiologic evidence would suggest that the indigenous native American population within the US and Central America may represent an endemic reservoir of HTLV-2 infection, and has led to speculation that HTLV-2 is a 'New World' virus. However, the degree of homology between HTLV-1 and HTLV-2 would suggest a common origin, making this speculation highly unlikely.

Since 1991, sporadic cases of HTLV-2 infection have been detected in West and Central Africa, and the presence of such infection in rural isolated populations, including pygmies, suggests a prior presence of HTLV-2 in this area. Recently, HTLV-2

has been isolated from a healthy male from Gabon, a pygmy from Cameroon and a group of Nigerians with sexually transmitted diseases. To date, HTLV-2 has not been detected in Japan or, with any regularity, in the Caribbean basin, both areas of endemic HTLV-1 infection.

A recent survey among Spanish IVDUs indicates that HTLV-2 has been introduced into Spain very recently. Interestingly, IVDUs in prisons showed higher HTLV-2 rates of infection in comparison with unincarcerated IVDUs, perhaps due to increased transmission through needle sharing. Hypothetically, the lack of opportunity to use clean needles in prison offers a unique occasion for the virus to be rapidly transmitted through needle sharing.

Host Range

HTLV-1 and -2 apparently cause disease only in humans, although some other mammalian species can be infected. Rabbits have been successfully infected with both HTLV-1 and -2. HTLV-1-infected rabbits reportedly may develop behavioral changes, but not leukemia. No pathology has been described in HTLV-2-infected rabbits to date.

Few conclusive data are available about cell types infected in humans. A wide variety of cells will support transcription from the HTLV-2 viral LTR, including cell lines of T cell, B cell and nonlymphoid origin *in vitro*. Physical separation of PBMC of carriers into T cell, B cell and adherent-cell populations and PCR analysis for the presence of integrated viral sequences suggested that, in the blood, the primary HTLV-2-infected cells are T cells.

Direct cell-to-cell contact may be required for efficient HTLV infection *in vitro*. In the laboratory, infection of cells with HTLV-1 or -2 is generally performed by cocultivating target cells with irradiated or mitomycin C-treated virus-producing donor cells. In contrast to HIV-1, infection by HTLV-1 and -2 of susceptible T cells appears to be inefficient, with a slow increment in the percentage of productively infected cells *in vitro*, occurring over several weeks to months. Infection of human PBMC by HTLV-1 or -2 results in productively infected T cell lines that are immortalized and can proliferate indefinitely in the absence of IL-2. Only T cells appear to be transformed by HTLV; nevertheless, viral replication can occur in other cell types. Epstein–Barr virus (EBV)-transformed B cell lines have been successfully infected with both HTLV-1 and -2, and HTLV-2 infection results in syncytia formation in the human B cell BJAB lymphoblastoid cell line.

The cellular receptor for HTLV-1 and -2 has not been identified, but it is thought to exist on a variety of human and animal cells, including lymphoid and nonlymphoid cells of primate, feline, rabbit and rat origin. The presence of the HTLV-1 and -2 receptor on these cells has been inferred by the ability to infect these cells with vesicular stomatitis virus (VSV) pseudotypes, which incorporate HTLV-1 or -2 Env proteins into VSV virions. Infection with HTLV-1 VSV pseudotypes can block infection by HTLV-2 VSV pseudotypes and vice versa, suggesting that HTLV-1 and -2 share a similar cellular receptor, which has been localized to human chromosome 17. Productive infection is also observed in several of the cell types identified by VSV studies, including cells of lymphoid and nonlymphoid origin such as human osteogenic sarcoma cells, human fibrosarcoma cells, feline kidney epithelial cells, transformed monkey kidney fibroblasts (COS) and human endothelial cells. Despite the variety of cell types that can be infected by HTLV-1 and -2, both HTLV-1 and -2 appear to preferentially transform T cell lines *in vitro*. These T cell lines have been of both CD4+ and CD8+ antigenic phenotype when transformed by HTLV-2, and will grow indefinitely in the absence of exogenous IL-2. The reasons for cell type-specific T cell transformation are not known. Cell lines doubly infected by HTLV-2 and HIV-1, as well as HTLV-1 and HIV-1, have been established.

Molecular Genetics

Unlike HIV-1, HTLV-1 exhibits an unusual degree of fidelity between viral isolates. Studies of HTLV-1 samples from several African countries, the Caribbean basin and South America showed little or no genetic drift within individuals followed serially over several months and mild intrastrain variation between various HTLV-1 isolates. A high degree of similarity was noted between West African, West Indian, Iranian and French Guyanan HTLV-1 isolates.

Initial sequencing of the LTR from the HTLV-2a and HTLV-2b strains obtained from two different leukemic patients showed approximately 95% homology. Mapping by restriction enzyme analysis demonstrated disparity in approximately 10% of restriction sites sampled between the two isolates. Recently, multiple complete sequences and partial sequences of HTLV-2 isolates have become available. Letourneur *et al.* performed multiple sequence alignments using the transmembrane portion of the glycoprotein gp21 sequence. Phylogenetic analysis of these alignments showed clustering of isolates into three subtypes; a, b, and c (**Fig. 2**).

The lack of variability between isolates of HTLV-1 as compared with the variability of isolates of HIV-1

may be explained by differences between the HTLV and HIV life cycles, kinetics or underlying molecular events. One hypothesis is that HTLV-1 reverse transcriptase (Pol) functions with increased fidelity relative to the HIV-1 reverse transcriptase. Increased fidelity of reverse transcriptase would result in fewer sequence changes in the DNA provirus, and thus, in the progeny virion with each cycle of replication. Alternatively, the reduced variability may be due to the significantly lower levels of HTLV-1/-2 viral replication in infected individuals as compared with HIV-1. Fewer errors with each replication cycle and/or a reduced number of cycles would decrease the mutation frequency of HTLV-1/-2 versus HIV-1. The low rate of replication for HTLV-1 may also reflect the tendency to remain latent in infected individuals. Instead of cycles of reinfection, proviral replication may depend to a degree on T cell division. Since the majority of T cells are resting and nondividing, viral replication *in vivo* would be reduced, and would account for a decreased rate of mutation.

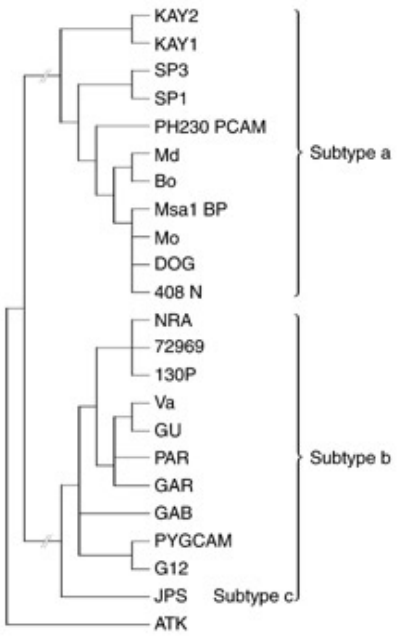


Figure 2 Phylogenetic analysis of 589 nucleotides of the *env* gene region encoding transmembrane glycoprotein gp21 of HTLV-2 isolates, showing distinction between subtypes. Adapted with permission from Letourneur F, d'Auriol L, Dazza MC (1998) Complete nucleotide sequence of an African human T-lymphotropic virus type II subtype b isolate (HTLV-II-Gab) molecular and phylogenetic analysis. *J. Gen. Virol.* 79: 274.

Serologic Crossreactivity of HTLV-1 and HTLV-2

Although they are distinct viruses, HTLV-1 and -2 are antigenically related. There is a 65% overall nucleic and sequence homology between sequenced HTLV-1 and -2 isolates. In the structural virion proteins, the homology is approximately 56% for p19 (Gag), 83% for p24 (Gag), 61% for gp46 (Env-SU) and 84% for gp21 (Env-TM) transmembrane proteins (**Fig. 1**). The homology is lowest (about 30%) within the viral LTR and the untranslated region in the 3′ end of the genome, increases in *gag* and *env* genes, and is highest within the 3′ overlapping *tax/rex* regulatory genes (about 75–80%). This high degree of homology between HTLV-1 and -2 is responsible for the antigenic crossreactivity between the viruses. The degree of virion structural and coat protein crossreactivity is illustrated in **Fig. 1.** In the past, most known carriers of HTLV-II were serologically detected using HTLV-1-based screening assays, due to cross-reactivity with Gag and Env proteins. Discrimination between HTLV-1 and -2 was performed by PCR assays. An ELISA, which discriminates between HTLV-1 and -2 based on the use of synthetic env peptides, is now commercially available. However, this assay is neither as specific nor as sensitive as PCR-based methods.

Clinical Features of HTLV-2 Infection

HTLV-2 has been found infrequently in the setting of human malignancy. The original isolate of HTLV-2 was obtained from a patient with an atypical T cell variant of HCL, but frank integration of HTLV-2 within leukemic cells *in vivo* was not demonstrated. Other HTLV-2 isolates were obtained from a patient with AIDS and a hemophiliac without coexistent malignancies. Evidence of HTLV-2 infection was seen in two patients with unusual T cell lymphoproliferative disorders and in asymptomatic IVDA in the US and UK.

Three viral mRNA species have been convincingly identified for HTLV-1 and -2. These include full-length genomic RNA encoding the *gag, protease* and *pol* genes, singly spliced *env* mRNA encoding four envelope proteins, and doubly spliced *tax/rex* mRNA encoding the sequences from the 3′ X region. Recently, investigators have suggested the existence of additional viral mRNA subspecies by using polymerase chain reaction (PCR) amplification techniques to detect subtle variations of viral mRNAs.

Two unique regulatory genes have been identified within the X region of HTLV-1 and -2. One gene, referred to as *tax,* encodes a transcriptional regulator of the HTLV LTR. It encodes a 40 kDa nuclear phosphoprotein, p40tax in HTLV-1 and a 37 kDa protein in HTLV-2, p37tax. A second gene, *rex,*

encodes two proteins in HTLV-1, p27XIII and p21XIII, and two protein species in HTLV-2, $p26^{x-b}$ and $p24^{x-b}$, in an alternate, partially overlapping reading frame.

The *gag* gene is translated as a polyprotein precursor with an apparent molecular mass of 70 kDa, which is cleaved to form mature Gag polypeptides. Mature Gag protein species, p19, p24 and p15, have been recognized. These represent the matrix, capsid and nucleocapsid proteins, respectively, of HTLV-1 and HTLV-2. The p24 protein, as noted, has high amino acid homology between HTLV-1 and -2, whereas the p19 protein shows a lesser degree of homology. Nevertheless, both p19 and p24 show considerable crossreactivity to intact proteins between HTLV-1- and -2-infected individuals. The p19 protein is thought to be myristoylated at its N-terminus, and this modification may facilitate association of p19 with the cell membrane.

An intact reading frame for a protease gene was first detected in the HTLV-2 sequence and subsequently described in some HTLV-1 clones. Initial noninfectious clones of HTLV-1 that were sequenced demonstrated stop codons within potential protease open reading frames (ORFs). The function of the protease has been examined for HTLV-2, and it has been demonstrated that, as in other retroviruses, the viral protease is necessary for appropriate cleavage and processing of Gag polyprecursor proteins into mature Gag structural proteins. Apparently the protease undergoes self-cleavage to generate a mature protease molecule. As in some other retroviruses, the protease ORF overlaps both Gag- and Pol-coding sequences. Therefore, in HTLV-1 and -2, the translation of the protease precursor likely occurs following ribosomal frameshifting.

The largest ORF in the HTLV genome is encoded in the polymerase region. In the case of HTLV-2, these sequences could encode a 982-amino acid Pol precursor. As in other retroviruses, the *pol* gene encodes the reverse transcriptase protein, as well as integrase and RNase H functions necessary for completion of the retroviral life cycle. HTLV-1 and -2 share approximately 56% amino acid homology within coding sequences of the *pol* gene. As previously noted, HTLV-1 and -2 show a lower rate of mutation than HIV-1, suggesting a higher fidelity of HTLV reverse transcriptase than that of HIV-1.

In both HTLV-1 and -2, the *env* gene begins upstream of the 3′ end of the *pol* gene and partially overlaps it. In HTLV-2, a high molecular mass glycosylated precursor of approximately 68 kDa is thought to be cleaved into a 46 kDa surface glycoprotein (gp46) and a 21 kDa transmembrane protein (p21). The Env proteins of HTLV-1 and -2 probably interact with similar as yet unidentified cellular receptors to mediate viral entry. The requirement for cocultivation of HTLV-infected cells to obtain efficient infection is unclear. However, the need for cocultivation suggests that HTLV-1 and -2 virions may be unstable in the extracellular milieu, or alternatively, that binding of Env alone to cellular receptors may be insufficient to infect cells. Experiments with both live and heat-inactivated HTLV-1 virions by Duc Dudon and Gazzollo and with HTLV-2 virions by Zack and Chen indicate that the virion coat may act to induce T cell proliferation nonspecifically in culture. This function has been speculatively attributed to Env proteins, although no direct mitogenic effects of Env have been demonstrated.

The *tax* and *rex Trans*-regulatory Genes

Unique genes are found in HTLV-1 and -2 encoded principally by sequences at the 3′ end of the genome, as noted previously. The protein products of these genes do not appear to be packaged within the mature virion. These genes are encoded by a 2.1 kb doubly spliced mRNA containing three exons, which is seen within infected cells. This multiply spliced RNA appears to encode two overlapping genes, designated *tax* and *rex*, respectively. The HTLV-1 *tax* gene encodes a protein of 40 kDa (p40tax), and the HTLV-2 *tax* gene has been demonstrated to encode a protein of 37 kDa (p37tax). These proteins localize primarily to the nucleus of infected cells, although small amounts of Tax have been found in the cytoplasm.

The Tax proteins of HTLV-1 and -2 are crucial to the viral life cycle, and mutations within the *tax* gene of HTLV-1 or -2 abolish viral mRNA transcription. The Tax protein acts to increase transcription from the viral promoter located in the 5′ LTR. A *trans*-acting transcriptional regulator in HTLV-infected cells was first demonstrated by experiments in which the HTLV-2 viral LTR was found not to transcribe efficiently in uninfected cells. Subsequently, several groups studied HTLV transcription by linking chloramphenicol acetyltransferase (CAT), a bacterial indicator gene, to the viral LTR. Cotransfections of the viral LTR linked to the CAT gene with expression plasmids containing Tax- and/or Rex-coding sequences into mammalian cells have demonstrated that expression of *tax* is necessary for efficient transcription from the viral LTR (**Fig. 3**). Similarly, transfection of whole HTLV-2 proviral mutants deficient in Tax and/or Rex have demonstrated that levels of transcription depend primarily on the presence of the Tax protein.

The Tax protein is a 37 kDa nuclear phosphoprotein that acts as a transcriptional activator via three

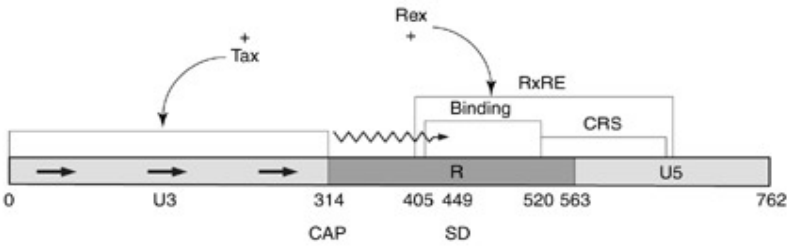


Figure 3 Interactions of *trans*-acting viral regulatory proteins with the HTLV-2 LTR. (Details in text.)

repeated 21 bp enhancer elements upstream of the transcriptional start site. Tax is thought to interact with transcriptional regulatory proteins without directly binding to DNA (**Fig. 3**). The HTLV-2 Rex protein is a 26 kDa phosphoprotein that binds to viral RNA within the Rex-responsive element (RxRE), and is thought to affect splicing and/or nuclear/cytoplasmic transport of viral mRNA. Transport appears to be inhibited in the absence of Rex by an adjacent *cis*-acting repressive element (designated the CRS). Rex function is required for efficient expression of unspliced *gag/pol* mRNA and partially spliced *env* transcripts (**Fig. 3**).

The mechanism by which the Tax protein elicits enhanced transcription from the viral LTR is the subject of extensive investigation. Studies by several investigators have established that Tax does not bind DNA, but rather, acts indirectly via three repeated enhancer elements located within the viral LTR. Tax can apparently interact with a variety of different cellular transcription factors. Investigators have identified transcription factors from the nuclear factor, kb (NF-kappaB), and the cyclic AMP responsive element binding protein (CREB) families, through which Tax appears to act in different promoters. In the HTLV-1 and HTLV-2 LTR, the Tax effect is mediated via three imperfect 21 bp repeat enhancer elements thought to bind CREB-like transcription factors (**Fig. 3**). The mechanism by which Tax interacts with various cellular transcription factors is not known.

CREB binding protein (CBP), thought to be a coactivator of CREB-mediated transcription, has been shown to bind both phosphorylated and unphosphorylated HTLV-1 CREB in the presence of Tax. Recent studies have shown that the CREB binding domain (KIX) domain of CBP can bind with Tax, and that Tax can promote binding of the KIX domain to DNA-bound CREB complexes.

Several heterologous promoters other than the HTLV-1/-2 LTRs, such as the adenovirus E3 promoter, the SV40 enhancer/promoter, the cellular interleukin 2 (IL-2) and IL-2α receptor promoters, the promoter for the c-*fos* oncogene, and the promoter for granulocyte–macrophage colony-stimulating factor (GM-CSF), have all demonstrated *trans*-activation by Tax. The pleomorphic effects of Tax on cellular promoters suggest that Tax may play a role in T cell transformation, and may contribute to the leukemic phenotype. Incorporation of the HTLV-1 *tax* gene into a herpes saimiri-derived expression vector and introduction into T cells resulted in proliferating T cell lines that expressed high levels of the IL-2 receptor but remained dependent on IL-2 for continued proliferation. It should be noted that fully transformed HTLV-1/-2-infected cell lines do not require IL-2 for continuous proliferation.",4> Introduction of HTLV-1 Tax expression vectors into transgenic mice resulted in thymic atrophy in some mice, and development of unusual tumors resembling neurofibromas in others. Thus, several lines of investigation have suggested a potential role for Tax in cellular transformation.

Recent investigations have demonstrated that Tax from HTLV-1 and -2 transactivates the intercellular adhesion molecule 1 (ICAM-1). ICAM-1 is the counter receptor for lymphocyte function-associated antigen (LFA-1). Since these adhesion molecules play important roles in cell-to-cell interactions and signal transduction, upregulation of ICAM-1 could have profound effects on the biology of infected T cells. First, it is possible that upregulation of ICAM-1 plays an important role in cell-to-cell-mediated transmission of HTLV-1. In addition, upregulation of ICAM-1 on HTLV-1 infected cells may facilitate transmission not only by promoting adhesion between infected T cells and target T cells but also by inducing the proliferation of the latter.

The *rex* gene of HTLV-1 and -2 has been demonstrated to encode two protein species in both viruses. In HTLV-1, the Rex 27 kDa and 21 kDa proteins appear to result from the use of alternative initiator methionine codons. However, in HTLV-2, the 26 kDa Rex protein has been shown to be a phosphorylated form of the 24 kDa Rex protein in which phosphorylation occurs on serine residue(s). The *rex* gene

products of HTLV-1 localize to nuclei of infected cells, and are found in the highest concentration within the nucleoli. A short conserved stretch of highly basic arginine and lysine amino acids at the N-terminus of Rex in HTLV-1 and -2 appears to be required for nucleolar localization and the RNA-binding function of the proteins. Studies of both HTLV-1 and -2 Rex suggest that the larger molecular weight species acts post-transcriptionally through *cis*-acting RNA Rex-responsive elements (RxRE) within the viral LTR to regulate viral mRNA transport. Cytoplasmic levels of *gag/pol* mRNA, and in HTLV-1, *env* mRNA, are increased in the presence of Rex.

The Rex protein of HTLV-2 has been purified using a baculovirus expression system, and purified Rex has been found to bind directly to HTLV-2 mRNA transcripts initiated from the 5′ LTR that contain the RxRE. Other investigators have noted that HTLV-1 Rex expressed in bacteria also binds to the corresponding HTLV-1 RxRE. The HTLV-2 Rex-binding domain may form a prominent stem–bulge–loop secondary RNA structure, and is entirely located within the R region of the LTR (**Fig. 3**). This RxRE secondary structure as well as critical sequences located within the 5′ splice site are required for binding, and are consequently necessary for Rex function. Rex may act by binding to the RxRE to increase export and/or prevent splicing of RxRE-containing mRNAs from the nucleus and cytoplasmic accumulation of unspliced *gag/pol* message. The mechanism by which Rex may affect the cellular fate of singly spliced *env* mRNA remains unclear. Additional RxRE and/or binding domains may be present elsewhere in the genome and allow differential processing of *env* mRNA. The net effect of Rex is thought to be accumulation of full-length *gag/pol* and possibly *env* mRNAs. Synthesis of Gag and Env proteins can then predominate in order to facilitate virion assembly.

Recently, the nuclear proteins hnRNP I and hnRNP AI have been shown to bind to cis acting repressive sequence (CRS) and may be involved in Rex regulation of RNA processing. HTLV-2 Rex has also been shown to inhibit early spliceosome assembly. Recent experiments indicate that Rex may also play a role in inhibiting transcription from the HTLV-2 LTR and, possibly, by this mechanism lead to the establishment of latent infection.

The *rev* gene in HIV-1 performs an analogous function. Both HTLV-1 and -2 *rex* can functionally substitute for the *rev* gene of HIV-1 and 'rescue' Rev-deficient HIV-1 clones, but reciprocal substitution of Rev for Rex has not been observed. Although Rex can substitute for Rev, binding of HTLV-2 Rex to the Rev-responsive element (RRE) in HIV-1 is of much lower affinity than to the homologous HTLV-2 RxRE. This indicates that like Tax, Rex may, somewhat nonspecifically, affect processing of other viral and/or cellular mRNAs, although direct evidence has not been obtained, except in the case of HIV-1.

Other proteins encoded by this pX region are not well characterized with regard to their function in HTLV infection or effects on host cell gene expression. HTLV-1 p12 protein shares structural similarity with the bovine papillomavirus E5 protein. Both proteins localize to the cellular endomembranes and are thought to interact with the H+ vaculolar ATPase complex that is involved in proton transport into cellular organelles. The p12 protein has also been shown to bind to subunits of the IL-2 receptor and decrease its surface expression. It has been hypothesized that p12 may play a role in altering cellular responses to IL-2 and other cytokines and thus may be involved in T cell transformation. A similar protein exists in HTLV-2.

Epidemiology

Molecular epidemiological studies have shown that HTLV-2a is the predominant infection in IVDAs in urban North America. HTLV-2b seems to be the predominant subtype in Indian groups in Panama, Colombia and Argentina. Recently, it has been suggested the existence of a third molecular subtype of HTLV-2 – HTLV-2c – in urban Brazilian and Indian populations. HTLV-2c has LTR and *env* nucleotide sequences more closely related to HTLV-2a, but a nucleotide and a predicted amino acid sequence for *tax* similar to that of HTLV-2b.

A second well-characterized isolate of HTLV-2 (HTLV-2b) was obtained from an elderly Caucasian male with HCL whose illness was marked by splenomegaly, inaspirable bone marrow and the presence of circulating tartrate-resistant acid phosphatase-positive (TRAP+) lymphoid cells with hairy cell morphology. The peripheral blood in this patient also contained a large number of atypical lymphoid cells that were TRAP-negative and of T cell origin. The patient's illness was observed over a 2-year period, and he was ultimately found to have two coexisting lymphoproliferative disorders of distinct T and B cell origin. Using cell-fractionation techniques, it was determined that oligoclonally integrated HTLV-2 DNA was detected in a CD8+ T cell-enriched fraction, but not in a CD4+ T cell-enriched fraction, a non-T cell fraction, or in B cells of the affected patient. It was concluded that the patient harbored a TRAP+ B cell malignancy without integrated HTLV-2, and a CD8+ T cell lymphoproliferative syndrome containing oligoclonally inte-

grated HTLV-2, which had arisen from a single virally infected precursor cell, similar to what is observed for HTLV-1 and adult T all leukemia (ATL). This case still represents the most convincing evidence for potential involvement of HTLV-2 in a human malignancy. Other anecdotal cases of T cell malignancy in the setting of HTLV-2 infection, including a case of T cell prolymphocytic leukemia, have been documented. Nevertheless, an etiologic role for HTLV-2 in leukemia/lymphoma remains to be established.

HTLV-2 has also been observed in only two cases of a progressive spastic myelopathy in patients co-infected with HIV-1. Additional cases of myelopathy among HTLV-2-infected New Mexican Indians and African Americans in the US have also been reported. It would therefore appear that like HTLV-1, infection with HTLV-2 might predispose to a spastic myelopathy affecting sphincter control and motor function in the lower extremities. In several patients with neurologic syndromes related to HTLV-2, severe cerebellar dysfunction leading to ataxia was also noted. In a study of HTLV-2-infected IVDA, most carriers were noted to be asymptomatic and have normal absolute lymphocyte counts and immunologic T and B lymphocytic subsets. A modestly elevated absolute lymphocyte count was noted in 4 of 21 HTLV-2-infected IVDA, and several patients had a CD8+ T cell lymphocytosis, as well as an overall increase in the percentage of CD8+ HLA-DR-positive lymphocytes. No overt pathology was demonstrated in this cohort, although several IVDA were also noted to have moderate elevations in serum creatine phosphokinase levels, suggesting possible myositis. This finding was intriguing in view of reports of polymyositis found in association with HTLV-1 infection. Thus, most HTLV-2-infected IVDA have been asymptomatic, and have had no overt hematologic, neurologic or immunologic abnormalities other than occasional lymphocytosis.

Infectious dermatitis has been described in children infected with HTLV-1 and similar cases of prolonged dermatitis and frequent staphylococcal skin infections have been described in IVDA found to harbor HTLV-2. Whether this represents a prototypic illness associated with the virus is unknown. Epidemiologic studies of small populations of New Mexican Indians, identified as an HTLV-2-infected endemic population, have not disclosed an increase in T cell malignancies, B cell chronic lymphocytic leukemia or HCL. Thus, a conclusive link between HTLV-2 and a specific malignant or neurological disease has not yet been established.

Recent studies have shown that HTLV-2-infected individuals had an increased risk of pneumonia, minor fungal infection, abscess and lymphadenopathy and bladder or kidney infections. This new finding of increased prevalence of a variety of infections in HTLV-2-positive donors suggests immunologic impairment. However, whether HTLV-2 has an etiological role in predisposing infected individuals to such infections will require further investigation.

Transformation by HTLV-2

T cell transformation is defined as continuous cellular proliferation *in vitro* in the absence of exogenous IL-2. Both HTLV-1 and HTLV-2 will transform normal human T cells *in vitro*. Infection by HTLV *in vitro* is successful only by cocultivation of irradiated virus-producing cells with PBMC; cell-free HTLV is generally noninfectious. HTLV does not encode a prototypical oncogene, and therefore cannot be classified as an acute transforming retrovirus. HTLV transformation also does not utilize oncogene/promotor-insertion mechanism of transformation, as seen for Moloney murine leukemia virus (MoMuLV) or other animal retroviruses.

The first noticeable change during coculture infection with HTLV is a polyclonal expansion of infected T cell subpopulations approximately two to four weeks after coculture. HTLV-transformed T cells also have increased endogenous expression of IL-2 and cell surface expression of the IL-2R molecule. Increased expression of IL-2 and IL-2R results in autocrine stimulation and is regarded as an important event in T cell transformation by HTLV. Further passage of infected cell cultures results in selective outgrowth of one or a few dominant clones. Although *in vitro* transformation by HTLV provides a model system to examine leukemogenesis by HTLV, there are distinct limitations and differences inherent in the system. Clonal populations of cells that predominate in culture are generally distinct from clonal populations that predominate in the patient. In addition, virus expression is relatively abundant in cells transformed *in vitro*, as well as tumor cells that have been adapted to grow in culture. This is in contrast with the extremely low level of viral RNA expression detected in circulating leukemic cells of patients, with adult T-cell leukemia (ATL).

The mechanism of transformation *in vitro* by HTLV-1 and -2 is unknown. Several hypotheses have emerged from molecular genetic studies of both HTLV-1 and -2. Much attention has focused on the potential role of the HTLV-2 *trans*-regulatory genes, *tax* and *rex*. Since Tax has been demonstrated to enhance transcription of a number of cellular regulatory genes, aberrant cellular gene expression may relate to immortalization of T cells. Tax *trans-*

activates the expression of a particular set of cellular genes, including IL-2, IL-2Rα, GM-CSF, as zinc-finger putative transcription factor, IL-3, IL-4, the proto-oncogenes, c-*sis* and c-*fos*, and the major histocompatability complex (MHC) I antigen. Activation of some of these genes involves induction of NFkappaB activity by Tax. Tax has been shown to repress transcription of the DNA polymerase gene, which encodes a protein involved in DNA repair, suggesting another mechanism that may be involved in transformation. How Rex may relate to transformation is unclear.

Both HTLV-1 and -2 virions are capable of mitogenically stimulating quiescent T cells to divide in the absence of actual viral infection and integration. The specific virion components responsible for the observed proliferative effects on T cells have not been identified, although the most likely mechanism would involve interaction of viral envelope proteins with a cellular receptor. Quiescent T cells infected with HIV-1 can be induced to produce HIV-1 following mitogenic stimulation with HTLV-1 or -2 virions, suggesting possible pathogenic consequences in individuals co-infected with HIV-1.

Detection and Control of HTLV-2

The vast majority of HTLV-2 carriers have been detected during routine screening using ELISA employing HTLV-1 virion lysate. Because of the serological crossreactivity of the viruses, HTLV-2-infected individuals are also detected (**Fig. 2**). Specific serological assays for HTLV-2, based on antigenic epitopes that discriminate between HTLV-1 and -2, have been developed, and their sensitivity and specificity are much improved. At the present time, the most reliable method of differentiating HTLV-1 from -2 infection is by amplification of PBMC DNA using PCR, which will reliably detect and differentiate between HTLV-1 and -2. Southern blotting for HTLV-2 DNA can be performed on cells that have been cultured for several weeks *in vitro*, but it is not practical on a routine basis. Without culture, PCR must be used because the levels of virus in the PBMC DNA are too low for detection by routine Southern blotting.

Discrimination between HTLV-1 and -2 is not a trivial issue, since the potential consequences of infection differ between the two viruses. In the case of HTLV-1, there is a low (4–5%) but documented risk of developing ATLL, and a lower risk of contracting HTLV-1-associated myelopathy. For HTLV-2, the risk of developing pathology is not yet known. If a seropositive individual is identified, counseling should be given with the aim of preventing further transmission of the virus through sexual transmission or breast-feeding. If possible, the individual should be referred to a facility that will perform additional testing to discriminate between HTLV-1 and -2.

The discovery and characterization of the human T cell leukemia viruses types 1 and 2 have led to unique insights into human oncogenesis and retroviral pathophysiology. HTLV-2, although closely related to HTLV-1, is of unknown pathogenic potential. Further identification of infected individuals and longitudinal follow-up of infected cohorts will likely yield clues as to the true pathogenic nature of this virus. The anecdotal reports of leukemia and myelopathy in association with the virus suggest that infection with HTLV-2 may have occasional neurologic or hematologic sequelae. Serological assays to discriminate between HTLV-1 and -2 infection are being developed, and these will undoubtedly provide useful epidemiologic information on a routine basis in the future.

The molecular events underlying HTLV-1/-2 replication and transformation of infected T cells will likely be better understood in the future. Insights gleaned from the study of the *trans*-acting genes of HTLV-2, *tax* and *rex*, have been applicable to the understanding of the regulatory mechanisms of other pathogenic human retroviruses such as HTLV-1, HIV-1 and HIV-2. New animal models for studies of HTLV-1/-2-induced malignancy such as transgenic and SCID-hu mice are being developed and tested.

See also: **Bovine leukemia virus (*Retroviridae*); Human immunodeficiency viruses (*Retroviridae*): Anti-retroviral agents, General features, Molecular biology; Human T-cell leukemia viruses (*Retroviridae*): HTLV-1.**

Further Reading

Feuer G and Chen ISY (1992) Mechanisms of human T-cell leukemia virus-induced leukemogenesis. *Biochim. Biophys. Acta* 1114: 223.

Franchini G (1995) Molecular mechanisms of human T-cell leukemia/lymphotropic virus type 1 infection. *Blood* 86: 3619.

Hollsberg P and Hafler DA (1993) Seminars in medicine of the Beth Israel Hospital, Boston. Pathogenesis of diseases induced by human lymphotropic virus type 1 infection. *N. Engl. J. Med.* 328: 1173.

Lee HH, Swanson P, Rosenblatt JD (1991) Relative prevalence of HTLV-I and HTLV-II infection in association with different risk factors. *Lancet* 337: 1435.

Newman C and Rosenblatt JD (1998) Human T-cell lymphotropic viruses. In: McCance D (ed.) *Human Tumor Viruses*, p. 329. Washington: American Society for Microbiology.

Murphy EL, Glynn SA, Fridey J *et al* (1997) Increased prevalence of infectious diseases and other adverse outcomes in human T-lymphotropic virus types I- and II-infected blood donors. Retrovirus Epidemiology Donor Study (REDS) Study Group. *J. Infect. Dis.* 176: 1468.

HYPOVIRUSES (*HYPOVIRIDAE*)

Donald L Nuss, Center for Agricultural Biotechnology, University of Maryland Biotechnology Institute, University of Maryland, College Park, USA

History

Interest in hypoviruses stems chiefly from the observation that they reduce virulence of their pathogenic fungal host, i.e. cause hypovirulence. Although future studies may reveal hypovirus-related genetic elements replicating in a range of fungi, their known natural host range is currently limited to the chestnut blight fungus *Cryphonectria parasitica*. A classic example of the havoc that can result from the introduction of an exotic organism, the North American chestnut blight epidemic, first reported in 1905, caused the destruction of an estimated 4 billion mature chestnut trees by 1950. *C. parasitica* was subsequently introduced into Italy during the 1930s, threatening European chestnut forests and orchards. However, for reasons still to be completely understood, the European chestnut blight epidemic was much less severe than that witnessed in North America. One clear contribution to this reduced severity was the prevalence of *C. parasitica* strains exhibiting a reduced virulence phenotype (hypovirulence), first described by an Italian forest pathologist in the 1950s. French investigators subsequently showed that the hypovirulence phenotype was transmissible following anastomosis (fusion of hyphae) between vegetatively compatible *C. parasitica* strains, implicating a cytoplasmic genetic element as the causative agent of the phenotype. The observation that a hypovirulent strain could produce a curative effect when inoculated on to existing cankers on diseased trees stimulated a successful government-sponsored biological control program using hypovirulent *C. parasitica* strains for management of chestnut blight in French chestnut plantations. Recent studies in Switzerland also support the view that natural hypovirulent strains retard disease progression in European forest ecosystems.

In 1977, Day and coworkers at the Connecticut Agricultural Experiment Station reported that hypovirulent *C. parasitica* strains harbor double-stranded (ds) RNAs, providing the first indication of the nature of cytoplasmic elements responsible for the phenotype. Subsequent surveys of dsRNAs associated with different North American and European hypovirulent strains revealed considerable variations in concentration, number and size of dsRNA components. By the late 1980s, it was clear that a detailed molecular analysis of the dsRNAs associated with a single hypovirulent strain was required to bring some measure of order to the mounting confusion generated by such surveys. This resulted in the cloning and complete sequence determination of the prototypic hypovirus, now designated CHV1-EP713, in 1991. This milestone was followed in 1992 by the construction of an infectious full-length cDNA clone of CHV1-EP713 RNA by Choi and Nuss. This development furnished direct evidence that hypoviruses are indeed the causative agents responsible for transmissible hypovirulence and provided the means for facile manipulation of the hypovirus genome. The subsequent development of a robust hypovirus transfection system using synthetic coding strand transcripts of the CHV1-EP713 dsRNA stimulated efforts to introduce the virus into pathogenic fungal species phylogenetically related to *C. parasitica*, thus expanding host range and virus-mediated virulence attenuation. Current hypovirus research focuses primarily on virus–host interactions, the effective use of natural and transgenic hypovirulent fungal strains for biocontrol and systematic comparative virology.

Taxonomy

A discussion of hypovirus taxonomy must be prefaced by a description of several distinguishing features relative to classic virus families. Hypovirus infection is nonlytic and persistent, resulting in very consistent phenotypic changes of the host. In a sense, these viruses have achieved the goal of contemporary gene

Murphy EL, Glynn SA, Fridey J *et al* (1997) Increased prevalence of infectious diseases and other adverse outcomes in human T-lymphotropic virus types I- and II-infected blood donors. Retrovirus Epidemiology Donor Study (REDS) Study Group. *J. Infect. Dis.* 176: 1468.

HYPOVIRUSES (*HYPOVIRIDAE*)

Donald L Nuss, Center for Agricultural Biotechnology, University of Maryland Biotechnology Institute, University of Maryland, College Park, USA

History

Interest in hypoviruses stems chiefly from the observation that they reduce virulence of their pathogenic fungal host, i.e. cause hypovirulence. Although future studies may reveal hypovirus-related genetic elements replicating in a range of fungi, their known natural host range is currently limited to the chestnut blight fungus *Cryphonectria parasitica*. A classic example of the havoc that can result from the introduction of an exotic organism, the North American chestnut blight epidemic, first reported in 1905, caused the destruction of an estimated 4 billion mature chestnut trees by 1950. *C. parasitica* was subsequently introduced into Italy during the 1930s, threatening European chestnut forests and orchards. However, for reasons still to be completely understood, the European chestnut blight epidemic was much less severe than that witnessed in North America. One clear contribution to this reduced severity was the prevalence of *C. parasitica* strains exhibiting a reduced virulence phenotype (hypovirulence), first described by an Italian forest pathologist in the 1950s. French investigators subsequently showed that the hypovirulence phenotype was transmissible following anastomosis (fusion of hyphae) between vegetatively compatible *C. parasitica* strains, implicating a cytoplasmic genetic element as the causative agent of the phenotype. The observation that a hypovirulent strain could produce a curative effect when inoculated on to existing cankers on diseased trees stimulated a successful government-sponsored biological control program using hypovirulent *C. parasitica* strains for management of chestnut blight in French chestnut plantations. Recent studies in Switzerland also support the view that natural hypovirulent strains retard disease progression in European forest ecosystems.

In 1977, Day and coworkers at the Connecticut Agricultural Experiment Station reported that hypovirulent *C. parasitica* strains harbor double-stranded (ds) RNAs, providing the first indication of the nature of cytoplasmic elements responsible for the phenotype. Subsequent surveys of dsRNAs associated with different North American and European hypovirulent strains revealed considerable variations in concentration, number and size of dsRNA components. By the late 1980s, it was clear that a detailed molecular analysis of the dsRNAs associated with a single hypovirulent strain was required to bring some measure of order to the mounting confusion generated by such surveys. This resulted in the cloning and complete sequence determination of the prototypic hypovirus, now designated CHV1-EP713, in 1991. This milestone was followed in 1992 by the construction of an infectious full-length cDNA clone of CHV1-EP713 RNA by Choi and Nuss. This development furnished direct evidence that hypoviruses are indeed the causative agents responsible for transmissible hypovirulence and provided the means for facile manipulation of the hypovirus genome. The subsequent development of a robust hypovirus transfection system using synthetic coding strand transcripts of the CHV1-EP713 dsRNA stimulated efforts to introduce the virus into pathogenic fungal species phylogenetically related to *C. parasitica*, thus expanding host range and virus-mediated virulence attenuation. Current hypovirus research focuses primarily on virus–host interactions, the effective use of natural and transgenic hypovirulent fungal strains for biocontrol and systematic comparative virology.

Taxonomy

A discussion of hypovirus taxonomy must be prefaced by a description of several distinguishing features relative to classic virus families. Hypovirus infection is nonlytic and persistent, resulting in very consistent phenotypic changes of the host. In a sense, these viruses have achieved the goal of contemporary gene

therapy, in that they stably alter host phenotype in a predictable and efficacious (hypovirulence) manner. As is observed for fungal viruses generally, hypoviruses exhibit no extracellular phase in their life cycle. Infections cannot be initiated by inoculation with an infected cell extract or enriched fractions. Instead, these viruses are transmitted via cytoplasmic mixing as a result of fusion (anastomosis) between vegetatively compatible strains or to a variable degree in asexual spores (conidia). Unlike most fungal viruses, hypoviruses encode no coat protein and form no discrete particle. Rather, hypovirus genetic information is found predominantly as dsRNA associated with membrane vesicles ranging in diameter from 50 to 80 mm. Based on amino acid comparisons, hypoviruses appear to be related ancestrally to the *Potyviridae* family of plant viruses.

The genus *Hypovirus* within the family *Hypoviridae* (*hypo* from *hypo*virulence) currently consists of three confirmed members. Designations used in the nomenclature include CHV for *Cryphonectria* hypovirus, a number indicating species relatedness and, following a hyphen, the fungal host strain from which the virus was isolated, e.g. EP713. As will be discussed in the next section, the primary nucleotide sequence for the coding strand of the prototypic member of the genus *Hypovirus*, CHV1-EP713, specifies two large open reading frames (ORFs) designated ORF A and ORF B. A second, closely related member, CHV1-EP747, has the same organization and shares approximately 90% identity at the nucleotide level with CHV1-EP713. The third confirmed member of the genus, designated CHV2-NB58, shares only about 60% nucleotide sequence identity with CHV1-EP713 and lacks a portion of ORF A that, for the CHV1 members, encodes a functional *cis*-acting cysteine protease. Preliminary characterizations of several additional hypovirus field isolates have indicated that designations CHV3 and CHV4 will be required in the very near future.

Genetic Organization and Expression Strategy

Although hypovirus genetic information is readily recovered from infected cultures as linear dsRNA, the absence of a discrete virus particle and an extracellular infection phase presents some difficulties in precisely defining the hypovirus genome. Synthetic copies of the coding strand are infectious by electrophoration into fungal spheroplasts, and phylogenetic analyses suggest a common ancestry with a group of positive-strand RNA plant viruses. Thus, one could consider hypoviruses as having a positive-strand RNA genome and the dsRNA as representing

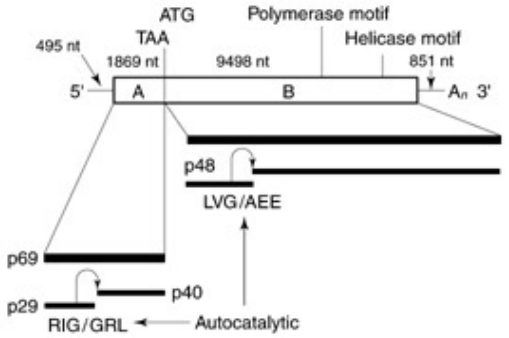


Figure 1 Hypovirus genetic organization and basic expression strategy. Details are discussed in the text. (Adapted from Shapira R, Choi GH, Hillman BI and Nuss DL (1991) *EMBO J.* 10: 741–746 by permission of Oxford University Press.)

accumulated replicative form RNA. Irrespective of this complication, direct analysis of hypovirus dsRNAs, cDNA cloning studies and *in vitro* translational analyses have provided the following view of hypovirus genetic organization and expression strategies. Depending on the species, hypovirus dsRNA ranges in size from 12 to 13 kb. One strand contains a 3′ poly(A) tail, while the complementary strand contains a 5′ poly(U) tract. All of the hypovirus coding information appears to reside within two contiguous ORFs on the polyadenylated strand, which in the case of CHV1-EP713 is 12 712 nucleotides in length, excluding the poly(A) tail (**Fig. 1**). ORF A encodes two polypeptides, p29 and p40, that are released from a polyprotein precursor, p69, by an autoproteolytic event between Gly248 and Gly249, mediated by a cysteine-like protease catalytic domain located within p29. ORF B has the capacity to encode a polyprotein of 3165 amino acids and contains unmistakable RNA-dependent RNA polymerase and helicase motifs. Proteolytic processing of only a portion of the ORF B polyprotein has been elucidated in the form of the autoproteolytic release of a 48 kDa protein, p48, from the N-terminus. This cleavage event occurs between Gly418 and Ala419 and is catalyzed by essential residues Cys341 and His388 within p48. The junction between ORF A and ORF B is defined by the sequence 5′-UAAUG-3′, in which the UAA portion clearly serves as the termination codon for ORF A and the AUG portion is thought to serve as the initiation codon for ORF B. While the mechanism involved in ribosome transition through the junction is not known, this unusual pentanucleotide sequence is found at the ORF A–ORF B junction for all confirmed species within the genus. There is clearly a need for additional fine detailed mapping of the processing cascades for hypovirus-encoded polyproteins.

In most hypovirus-infected C. *parasitica* isolates, the full-length viral dsRNA is accompanied by a constellation of shorter dsRNA species. These ancillary dsRNAs appear to be generated by internal deletion events, are replicated only in the presence of the full-length viral RNA and are not associated with any function or phenotypic effect.

Hypovirus-Mediated Alteration of Host Phenotype

In addition to reduced virulence, hypovirus infection also causes a number of associated phenotypic changes in its host. These can include: convenient experimental markers, e.g. reduced orange pigmentation; vital host functions, e.g. reduced asexual reproduction (conidiation); or a highly complex process, e.g. reduced virulence. Several lines of evidence, including results obtained with the differential mRNA display technique, have clearly indicated a significant and stable alteration in the pattern of host gene transcript accumulation as a result of hypovirus infection. These combined observations led to speculations that hypovirus infection results in alterations in host signal transduction pathways that regulate the constellation of phenotypic traits that are modified in hypovirulent strains. Indeed, results of very recent studies do indicate that a major way in which hypoviruses alter fungal phenotype, including virulence attenuation, is by disrupting G-protein signal transduction.

Following the cloning of the gene for a C. *parasitica* G-protein α subunit of the Gi family, designated CPG-1, Western analysis revealed a reduced accumulation of this subunit in CHV1-EP713 infected cultures. Like hypovirus infection, transgenic cosuppression of CPC-1 accumulation, in the absence of virus infection, was also shown to result in attenuation of fungal virulence. In related studies, hypovirus infection and CPG-1 cosuppression were both shown to prevent the induction of C. *parasitica*-encoded cellulases, enzymes expected to have some role in degrading plant host cell walls during the infection process. Consistent with the prediction that CPG-1, like mammalian Giα subunits, may function to negatively regulate adenylyl cyclase, hypovirus infection and CPG-1 cosuppression were shown to independently result in constitutively elevated cAMP levels. Moreover, effects of hypovirus infection on the accumulation of specific host transcripts could be mimicked by artificial elevation of cAMP levels. Taken together, these results led to the proposal that CHV1-EP713 alters host phenotype primarily through disruption of CPG-1-regulated, cAMP-linked signal transduction. Results of targeted disruption of *cpg-1* were entirely consistent with this view, causing both elevated cAMP levels and a set of phenotypic changes similar to, but more severe than, those observed for CHV1-EP713-infected C. *parasitica* cultures. Mechanisms involved in the apparent hypovirus-mediated reduction in CPG-1 accumulation remain to be elucidated.

Information concerning the role of hypovirus-encoded proteins in host phenotypic alterations is limited. In this regard, the development of a full-length infectious CHV1-EP713 cDNA clone provides powerful approaches with which to identify virus-encoded symptom determinants. For example, deletion of the p29-coding domain within the context of the infectious CHV1-EP713 cDNA clone resulted in a replication-competent viral RNA that caused little reduction in host pigment production and only a moderate reduction in conidiation, relative to the wild-type virus, while retaining the ability to confer wild-type levels of hypovirulence. Thus, while not essential for either viral replication or virulence attenuation, p29 clearly contributes to a number of hypovirulence-associated traits. One can easily imagine an extension of these studies to determine roles for other hypovirus-encoded protein products. The ability to uncouple hypovirulence from associated traits, such as reduced levels of conidiation or reduced pigment production, also established the feasibility of engineering infectious viral cDNA for construction of hypovirulent fungal strains with specific phenotypic traits.

Biological Control

A practical prediction associated with the development of an infectious CHV1-EP713 cDNA clone was that transgenic hypovirulent C. *parasitica* strains would exhibit expanded capacity for hypovirus transmission. Transmission of hypovirus RNA by natural hypovirulent strains appears to be restricted by a vegetative compatibility (v-c) system that regulates the ability of C. *parasitica* strains to anastamose, and is complicated in North American forest ecosystems by a high level of v-c diversity in native fungal populations. C. *parasitica* strains transformed with the CHV1-EP713 cDNA contain a chromosomally integrated copy of the viral cDNA in addition to cDNA-derived cytoplasmically replicating RNA. As a result, these transgenic hypovirulent strains have the capacity to transmit hypoviruses via nuclear inheritance to ascospore progeny. Meiotic transmission to ascospore progeny represents an entirely novel mode of hypovirus dissemination that is expected to circumvent barriers imposed by the vegetative compatibility system. Additionally, since

the ascospore progeny represent a spectrum of different vegetative compatibility groups, launching of the cytoplasmic viral cDNA into these new vegetative compatibility groups should result in the potential for expanded vegetative dissemination of hypovirus genetic information. Stable transmission of virus at a high frequency to asexual spores of transgenic strains has also been demonstrated in the laboratory. These combined transmission properties are, in turn, predicted to confer substantially enhanced biological control potential. In this regard, studies designed to test dissemination and persistence properties of transgenic hypovirulent *C. parasitica* strains under actual field conditions are currently in progress in Connecticut and West Virginia.

Future Perspectives

As more field isolates are characterized it is becoming apparent that there is considerable diversity in the spectrum and severity of symptoms associated with hypovirus infections, even for isolates that are closely related at the nucleotide level. It is anticipated that comparative molecular studies of different hypovirus isolates, now progressing in several laboratories, will reveal fine details of hypovirus genome structure/function relationships and protein function. By incorporating hypovirus infectious cDNA transformation and transfection technologies, such studies may also provide the most effective approach for identifying viral determinants responsible for alterations of host phenotype. The identification of virus-encoded determinants responsible for hypovirulence and the range of hypovirulence-associated traits will, in turn, facilitate engineering of more effective transgenic hypovirulent fungal strains for biomanagement and will drive mechanistic studies of hypovirus-mediated disruption of host signal transduction. Progress in these areas would further strengthen the potential application of hypoviruses for purposes of understanding and controlling fungal pathogenesis.

Further Reading

Anagnostakis SL (1982) Biological control of chestnut blight. *Science* 215: 466.

Heiniger U and Rigling D (1994) Biological control of chestnut blight in Europe. *Annu. Rev. Phytopathol.* 32: 581.

Hillman BI, Fulbright DW, Nuss DL and Van Alfen NK (1995) Hypoviridae. In: Murphy FA, Fauquet CM, Bishop DHL *et al* (eds) *Virus Taxonomy*, p. 261. New York: Springer.

MacDonald WL and Fulbright DW (1991) Biological control of chestnut blight: use and limitations of transmissible hypovirulence. *Plant Dis.* 75: 656.

Nuss DL (1992) Biological control of chestnut blight: an example of virus-mediated attenuation of fungal pathogenesis. *Microbiol. Rev.* 56: 561.

Nuss DL (1996) Using hypoviruses to probe and perturb signal transduction processes underlying fungal pathogenesis. *Plant Cell* 8: 1845.

IDAEOVIRUS

MA Mayo and **AT Jones**, Scottish Crop Research Institute, Invergowrie, Dundee, UK

History

The genus *Idaeovirus* contains a single species, raspberry bushy dwarf virus (RBDV). Bushy dwarf disease became apparent in Scotland in the 1950s, causing widespread damage in the dominant raspberry cultivar 'Lloyd George'. RBDV was consistently associated with this disease, but later work showed that it did not induce the disease itself, but only when it was part of a complex of viruses. However, RBDV is the causal agent of yellows disease in raspberry, and a cause of 'crumbly fruit' in some *Rubus* species and cultivars. Some strains are associated with a lethal disease in some red raspberry cultivars.

Taxonomy and Classification

RBDV is the sole species in the genus *Idaeovirus*. The genus is not classified into a family. However, there are a number of similarities between RBDV and viruses classified in the family *Bromoviridae*. RBDV particles are readily deformed on electron microscope grids and the virus is pollen-transmitted, both features of viruses in the genus *Ilarvirus*, family *Bromoviridae*. The expression strategy for the coat protein gene of RBDV is very like that for the coat protein genes of viruses in all the genera in the family *Bromoviridae* (e.g. alfalfa mosaic virus) (**Fig. 1**). Also, there are some similarities in amino acid sequence between the gene products of RBDV and viruses in most of the *Bromoviridae* genera (**Fig. 2**). Nevertheless, the current official ICTV position is that the genus *Idaeovirus* is distinct by virtue of the bipartite genome of RBDV.

Geographic Distribution

RBDV occurs worldwide, wherever its *Rubus* hosts (raspberry, blackberry, hybrid berry) are grown. This includes Europe, North America, South America, the former USSR and Australasia.

Host Range and Virus Propagation

RBDV has a moderately wide experimental host range but induces symptoms in only a few species. Reported hosts are in 55 species in 12 families of dicotyledonous plants. RBDV is usually propagated in *Chenopodium quinoa* plants. Virus particles can be purified by a combination of clarification and/or precipitation at low pH, followed by polyethylene glycol precipitation and differential and sucrose density gradient centrifugation.

Properties of the Virion

RBDV particles are about 33 nm in diameter but, unless fixed by aldehyde treatment, they often appear distorted when examined by electron microscopy. Particles sediment as a single component of about 100 S. They are readily disrupted by treatment with high salt concentrations or relatively low concentrations of sodium dodecyl sulfate. Particles are assumed to have a $T = 3$ symmetry on the basis of estimates of molecular weights of virion RNA and protein subunits.

Properties of the Genome

Virus particles contain three single-stranded RNA species. The genome of the R15 isolate of RBDV comprises RNA1 of 5449 nucleotides and RNA2 of 2231 nucleotides (**Fig. 1**). Virions contain a third RNA molecule (RNA3) which is a copy of the 3′-terminal 946 nucleotides of RNA2. RBDV RNA molecules do not have 3′-terminal poly(A) and are thought to be capped at the 5′ end. The 3′-terminal sequences of RNA molecules can be folded into highly ordered structures. RNA extracted from infected plants using phenol-based methods contains substantial amounts of double-stranded RNA. This RNA corresponds in size to double-stranded forms of RNA1 and RNA2 (about 6 kbp and 2.5 kbp); no

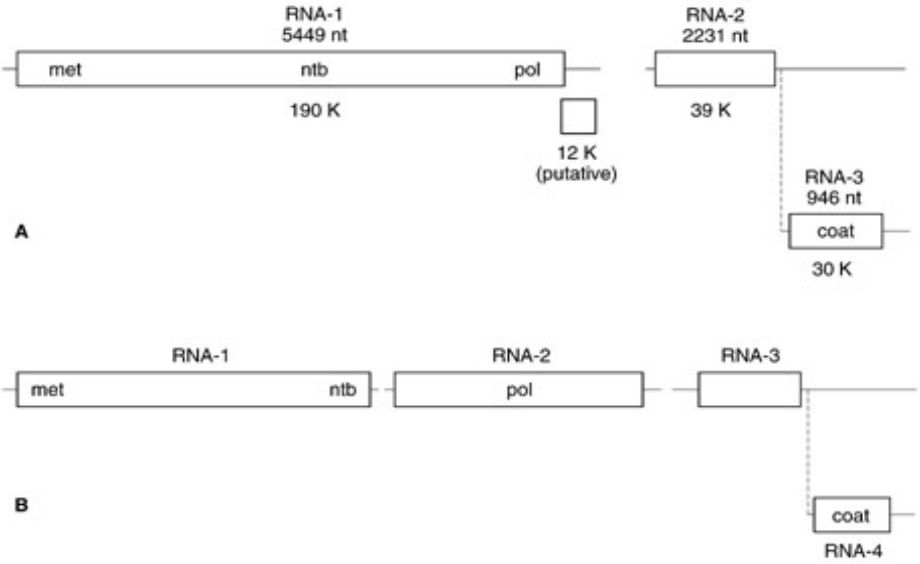


Figure 1 Organization of the bipartite genome of (**A**) raspberry bushy dwarf virus and comparison with the organization of the tripartite genome of (**B**) alfalfa mosaic virus. RNAs are represented as horizontal lines, open reading frames are represented as boxes and the dashed lines illustrate the production of a subgenomic mRNA for translation to yield coat protein. The approximate positions of conserved domains are shown as met (methyl transferase), ntb (NTP-binding) and pol (RNA polymerase).

double-stranded RNA is present that corresponds in size to double-stranded RNA3.

Properties of the Genome (Genes)

RNA1 encodes a large polypeptide with an M_r of *c.* 190 000 (190K), which accounts for most of its coding potential (**Fig. 1**). A small open reading frame is present near the 3′ end of R15 RNA1. It is also present in genomes of a number of other isolates and may therefore be functional. Its product would be a polypeptide with an M_r of about 12 000 (R15 isolate, **Fig. 1**). The 190K polypeptide contains regions with amino acid sequence domains that resemble those thought to be associated with methyl transferase, NTP-binding and RNA-dependent RNA polymerase activities in proteins of other viruses (**Fig. 1**).

RBDV RNA2 encodes two polypeptides. These are a 39K polypeptide at the 5′ end and a 30K polypeptide at the 3′ end (**Fig. 1**). The genes are separated by 118 nucleotides of noncoding sequence. The 39K polypeptide has limited sequence similarities with the

			AIMV	CiLRV	BMV	BBMV	CMV	TAV	OLV2	RBDV
Bromoviridae	*Alfamovirus*	AIMV		37	26	28	26	25	17	27
	Ilarvirus	CiLVR	43		26	28	23	23	14	25
	Bromovirus	BMV	29	25		65	45	46	18	29
		BBMV	25	23	50		43	44	18	28
	Cucumovirus	CMV	29	27	39	36		73	16	29
		TAV	27	27	37	35	58		17	27
	Oleavirus	OLV2	12	12	12	12	13	13		16
	Idaeovirus	RBDV	33	32	37	35	28	28	14	

Figure 2 Matrix showing the percentage identities between pairs of corresponding proteins of alfalfa mosaic virus (AlMV), citrus leaf rugose virus (CiLRV), brome mosaic virus (BMV), broad bean mottle virus (BBMV), cucumber mosaic virus (CMV), tomato aspermy virus (TAV), olive latent virus 2 (OLV2) and raspberry bushy dwarf virus (RBDV). Taxonomic relationships among the viruses are shown to the left. Values above and to the right of the diagonal line are comparisons between putative polymerases, values to the bottom and left are for comparisons between putative NTP-binding/methyl transferases.

putative movement proteins of viruses in the family *Bromoviridae*, and the genera *Dianthovirus* and *Furovirus*, which has led to the suggestion that the function of the 39K protein is, at least in part, to facilitate virus movement. The 30K polypeptide is the coat protein. The sequence of RNA3 encodes the coat protein and the gene has a leader sequence of 40 nucleotides. *In vitro* translation of RNA2 yields the 39K protein but not the coat protein. This is produced when RNA3 is translated.

Evolution

The RNA2 of RBDV resembles the RNA3 of viruses in the family *Bromoviridae* in size (*c.* 2 kb), organization (two open reading frames) and expression strategy (coat protein expression from a subgenomic RNA). Some properties of the virions, as well as features of virus transmission (see below), also suggest a link between RBDV and viruses in the family *Bromoviridae*. Pairwise comparisons of whole proteins (**Fig. 2**) or especially conserved domains support this suggestion. These show that the proteins of viruses in the genera *Alfamovirus, Bromovirus* and *Cucumovirus* resemble those of RBDV more than any of these resemble proteins of olive latent virus 2, the type member of the genus *Oleavirus*, which is also classified in the family *Bromoviridae*. These comparisons suggest that the genus *Idaeovirus* could be classified in the family *Bromoviridae*, and more tentatively point to a possible common origin of the viruses in these genera, or at least some of the proteins that they encode.

Serologic Relationships and Variability

Most isolates of RBDV are indistinguishable serologically but minor serological variants are reported from black raspberry. However, some isolates are known that differ in *Rubus* host range. Several of these are able to overcome the RBDV resistance gene *Bu* which is present in some red raspberry cultivars.

Transmission and Tissue Tropism

RBDV spreads from plant to plant in association with pollen. Plants can be infected this way, either as progeny from the pollination (vertical transmission) or as the pollinated mother plants (horizontal transmission). It is not clear how the mother plants become infected following inoculation with pollen from infected plants.

In infected *Rubus* plants, RBDV is often erratically distributed, which can complicate sampling for diagnostic purposes. However, RBDV will multiply in most, if not all, tissues of infected plants.

It is likely that RBDV has been distributed worldwide by the trade in *Rubus* planting material and seed.

Pathogenicity

RBDV can cause significant crop loss in susceptible cultivars of *Rubus*. Recently, in Europe, the emergence of RBDV strains capable of overcoming the principal RBDV resistance gene (*Bu*) has caused further concerns.

Prevention and Control

The only effective control of RBDV infection available currently is the use of certified healthy stock and the exclusion of virus sources near crops, or the cultivation of resistant cultivars. Durable resistance is being sought by both conventional selection breeding and by transgenic methods. Tests with *Nicotiana* plants transformed with cDNA to RBDV genes have shown that transgenes can confer resistance to RBDV multiplication.

See also: **Bromoviruses (*Bromoviridae*); Alfamovirus and Ilarviruses (*Bromoviridae*); Cucumoviruses (*Bromoviridae*): General features, Molecular biology.**

Further Reading

Jones AT and Mayo MA (1998) Raspberry bushy dwarf virus. AAB Descriptions of Plant Viruses No. 360.

Jones AT, Mayo MA and Murant AF (1996) Raspberry bushy dwarf idaeovirus. In: Harrison BD and Murant AF (eds) *The Plant Viruses*, vol. 5, p. 283. New York: Plenum Press.

Murant AF and Mayo MA (1995) Genus *Idaeovirus*. In: Murphy FA, Fauquet CM, Bishop DHL *et al* (eds) *Virus Taxonomy. Sixth Report of the International Committee on Taxonomy of Viruses*, p. 458. Vienna: Springer.

Ilarviruses *see* **Alfamoviruses and Ilarviruses**

associations. Studies in animal models suggest that Th1-type lymphokines are involved in the genesis of many organ-specific autoimmune diseases.

See also: **Adenoviruses (*Adenoviridae*): Animal viruses, General features, Malignant transformation and oncology, Molecular biology; Dengue viruses (*Flaviviridae*); Epstein-Barr virus (*Herpesviridae*): General features, Molecular biology; Human immunodeficiency viruses (*Retroviridae*): Molecular biology, Anti-retroviral agents, General features; Influenza viruses (*Orthomyxoviridae*): General features, Molecular biology, Structure of antigens; Measles virus (*Paramyxoviridae*); Persistent viral infection; Vaccines and immune response; Immune escape mechanisms; Immune response: Cell mediated immune response; Virus structure: Principles of virus structure.**

Further Reading

Ada G and Ramsay A (1997) *Vaccines, Vaccination and the Immune Response*, p. 1. Philadelphia: Lippincott-Raven.

Brown LE, Hampson AW, Webster RG (1996) *Options for the Control of Influenza, III*. Amsterdam: Elsevier.

Fields BN, Knipe DM, Howley PM *et al* (eds) (1997) *Fields Virology*, 3rd edn. Philadelphia: Lippincott-Raven.

Romagnani S (1997) The Thi/Th2 paradigm. *Immunol. Today* 18: 263.

Sprent J (1997) Immunological memory. *Curr. Opin. Immunol.* 9: 371.

Cell Mediated Immune Response

Janice Riberdy and **Peter Doherty**, St Jude Children's Research Hospital, Memphis, Tennessee, USA

General Features

Cell-mediated immunity (CMI) is concerned with the elimination of infected cells from organ sites of virus growth. This terminates the production of new virus, preventing the further spread of the infectious process. While virus-specific immunoglobulin (Ig) molecules are very important for neutralizing free virions, and thus play a central part in resistance to reinfection, antibody transfer experiments indicate that Ig-mediated mechanisms are less prominent in the clearance of virus from solid tissues. Removing the infected cell generally depends on the involvement of virus-specific thymus-derived lymphocytes, or T cells, the effectors of CMI. The immune system has evolved largely to deal with pathogens, optimizing the effective synergism between Ig-mediated and T cell-mediated functions.

There are two broad categories of T cells, each expressing a clonally distributed T cell receptor (TCR) heterodimer characterized as either $\alpha\beta$ or $\gamma\delta$. The $\alpha\beta$ or $\gamma\delta$ TCR molecules are associated with the multimeric CD3 complex, which is involved in mediating the signaling events that control T cell stimulation and differentiation. Most current understanding of virus-specific CMI is concerned with the $\alpha\beta$ T cell subsets, which express either the CD4 or CD8 coreceptor molecules (**Fig. 1**). The $\gamma\delta$ T cells are a minority in mice and humans, constituting less than 5–10% of the peripheral T cells in blood and secondary lymphoid tissue, while they are much more prevalent (20–30%) in pigs, ruminants and chickens. The role of the $\gamma\delta$ T cells in virus infections is not yet understood, though these lymphocytes are known to accumulate in some viral pneumonias and may play a part in regulating the inflammatory process.

The CMI effector mechanism is targeted to the surface of virus-infected cells because the antigen-specific $\alpha\beta$ TCR recognizes viral peptides presented in association with self major histocompatibility complex (MHC) glycoproteins (**Fig. 1**). The developing T cells are selected in the thymus to be tolerant (non-responsive) of self MHC molecules encountered in the thymic microenvironment but reactive, after leaving the thymus, to these same glycoproteins when they are modified by the binding of nonself peptides. This phenomenon is known as 'MHC restriction'. Tolerance of viral peptide–MHC glycoprotein complexes can also be induced if, as in mice infected *in utero* with murine lymphocytic choriomeningitis virus (LCMV), the antigen is encountered during thymocyte differentiation.

A critical lymphocyte in most virus-induced inflammatory processes is the CD8+ $\alpha\beta$ T cell. Lymphocytes of this T cell subset recognize viral peptides presented in the context of the class I MHC glycoproteins, the strong transplantation antigens that are expressed (or can be induced) on most cells throughout the body. The CD8 molecule binds to a constant region of the MHC protein, thereby increasing the avidity of the interaction between the T lymphocyte and the antigen-presenting stimulator or target cell. This may be particularly important at the initial stages of the induction of a primary T cell response. The pore-forming proteins (perforins) and enzymes (granzymes) characteristic of cytotoxic effector function are prominent in the cytoplasm of activated,

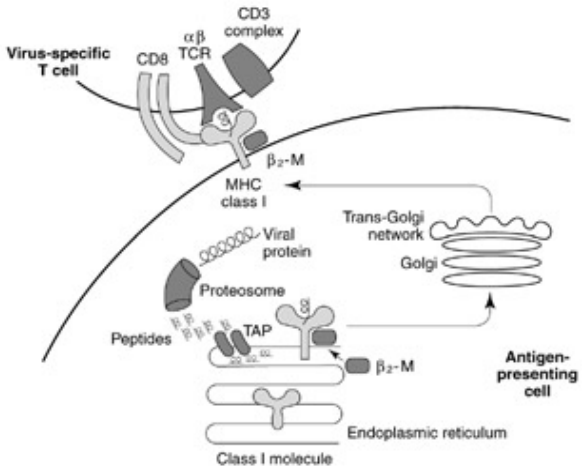


Figure 1 *T cell surveillance.* The virus-infected cell signals that it is abnormal by presenting a nonself peptide in the groove of the MHC class I protein complex. The MHC class I complex comprises an alleleic form of the variant heavy chain bound to the constant β_2-microglobulin (β_2-m) molecule. This is recognized by a clonotypic $\alpha\beta$ T cell receptor expressed on the surface of a recirculating, CD8+T cell. The lymphocyte–target cell interaction is thought to be further stabilized by the concurrent recognition of a conserved region of the MHC molecule by the CD8 dimer. The fact that the great majority of cells thoughout the body express, or can be induced to express, class I MHC glycoproteins makes this monitoring mechanism very effective. *Endogenous processing pathway.* A proportion of the viral proteins produced in the cytoplasm during the course of virus synthesis and assembly are degraded by the proteasome complex and transported into the endoplasmic reticulum, where they form a stable complex with the class I heavy chain and β_2-m. It is this trimeric complex that is ultimately transported to the plasma membrane for recognition by the CD8+T cell.

virus-specific, CD8+ T cells. These cytotoxic lymphocytes (CTLs) apparently make direct contact with the infected cells *in vivo* and lyse the target cells.

CD4+ T cells or helper T cells work in concert with the CD8+ lymphocytes and B cells to provide a complete immune response to viral infections. Secretion of lymphokines such as interferon-γ induces expression of both MHC class I and class II glycoproteins, which in turn enhances the specific recognition of MHC–viral peptide complexes by the T cells themselves (**Fig. 2**). CD4+ T lymphocytes recognize antigenic peptides in the context of MHC class II proteins. MHC class II molecules have a more limited range of expression that includes macrophages, dendritic cells and B cells. This is an important feature that focuses the CD4+ T cell response to cells that specifically require 'help', such as the B cell population. CD4+ 'helper' T cells secrete lymphokines, such as interleukin (IL)-2 and IL-4, which in turn promotes the clonal expansion and differentiation of B cells essential for the generation of the antibody-secreting plasma cells.

The MHC restriction of the $\alpha\beta$ T cells thus focuses the CD4+ and CD8+ subsets into appropriate anatomical niches. The CD4+ helpers encounter high concentrations of class II MHC molecules in lymphoid tissue, where they promote the development of the immune response. The CD8+ effectors recognize modified class I MHC glycoproteins expressed on any cell throughout the body, and act to eliminate these infected cells and thus limit further spread of the pathogen.

Antigen Processing, Presentation and Levels of Response

Whether or not a viral peptide is presented by a class I or class II MHC glycoprotein depends, in large part, whether the antigen is inside or outside the cell. During an infection, viral proteins are made in the cytoplasm of the cell. A portion of these proteins are proteolytically degraded by the proteasome complex and the resulting peptides are transported into the endoplasmic reticulum via a proteinaceous pore formed by the TAP molecules. MHC class I molecules are cotranslationally transported into the endoplasmic reticulum where they bind the nonpolymorphic molecule β_2-microglobulin (β_2-m). This process is mediated by a series of chaperone molecules such as calnexin and calreticulin and results in the binding of MHC class I–β_2-m complexes to the TAP molecules via the tapasin protein. Antigenic peptides transported through the TAP complex can then bind and stabilize the class I–β_2-m complex such that it is now competent for transport to the plasma membrane where it can interact with the TCR of a CD8+ T cell. Whether or not a given peptide has sufficient binding affinity for a class I complex is largely dictated by the structural constraints of the particular class I allele. The endogenous (class I) antigen processing/presentation pathway occurs in all virus-infected cells except the few, such as some categories of sensory neurons, that seem unable to express class I MHC molecules. Whether or not the viral protein that gives rise to the antigenic peptide is ever found on cell surface is irrelevant. Many of the peptides that are important for CMI are derived from early proteins that are internal to both the virion and to the virus-infected cell and thus serve to focus the CD8+ T cells to the surface of infected cells.

Even with very large and complex viruses, the CD8+ T cell response is often dominated by the recognition of a single viral peptide presented in the

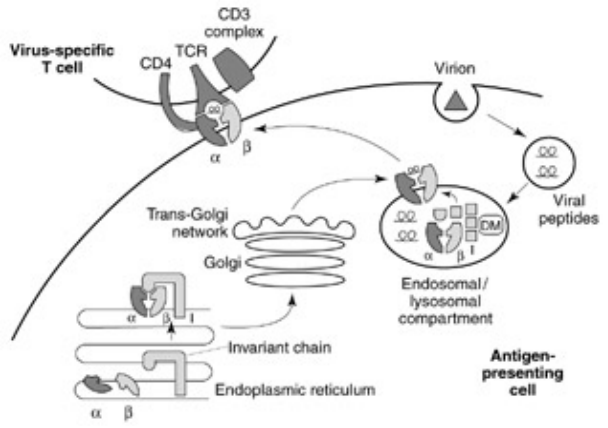


Figure 2 *Exogenous processing pathway.* Invariant chain associates with the α and β chains of MHC class II glycoproteins in the endoplasmic reticulum and blocks peptides from binding the complex. A sorting signal encoded in the invariant chain molecule itself targets the $\alpha\beta$ I complex to an endosomal/lysosomal compartment where the invariant chain is proteolytically degraded. Virions can be taken into cells such as macrophages or dendritic cells by phagocytosis. Alternatively, virions can be specifically endocytosed by clonotypic B cell receptors (Ig). These virions are degraded in lysosomal/endosomal compartments where the resultant peptide fragments associate with MHC class II $\alpha\beta$ heterodimers via interactions with H-2 M (murine) or HLA-DM (human) molecules. The MHC class II–peptide complex is transported to the plasma membrane where it serves as a target for CD4+ helper T cells which secrete lymphokines (IL-2, IL-4) and promote B cell responses and antibody production. Cells that normally express MHC class II molecules, such as dendritic cells, macrophages and B cells, tend to be located in lymphoid tissue with the lymph nodes and spleen providing the 'nurturing' environment necessary during the initial stages of the immune response.

context of one of the 4–6 allelic forms (**Fig. 1**) of the class I MHC glycoproteins (encoded at the HLA-A, B, C loci in humans; H-2K, D, L in the mouse) expressed on the surface of most cells in any individual. Absence of this 'immunodominant' allele may allow the emergence of a response associated with another peptide–MHC complex. The existence of three genetic loci with essentially similar functions, together with the extreme polymorphism of the class I MHC genes, ensures that a state of complete anergy during the course of a virus infection is comparatively rare for the CD8+ T cells. As a consequence, despite the fact that MHC-related effects on patterns of resistance can be measured in virus diseases, these are generally not the main host genes determining susceptibility.

In contrast, exogenous proteins such as virions are taken into the cell as a consequence of phagocytosis and are proteolytically degraded into peptide fragments in an endosomal/lysosomal compartment (**Fig. 2**), shared by the class II MHC molecules (HLA-DP, DQ, and DR in humans; I-A and I-E in mice). MHC class II molecules are comprised of two transmembrane proteins. The α- and β-chain proteins of the class II molecule are also cotranslationally translocated into the endoplasmic reticulum where they associate with a third molecule, the invariant chain. This protein serves two significant functions. First the association with invariant chain prevents the $\alpha\beta$ dimer from binding peptides in the endoplasmic reticulum and also provides a sorting signal to target newly synthesized MHC class II molecules to the endosomal/lysosomal compartment where antigenic peptides from exogenous proteins reside. The invariant chain is proteolytically degraded in the endosomal/lysosomal compartment and antigenic peptide binding is finally mediated by a complex similar in structure to classical class II molecules (termed HLA-DM in humans and H-2 M in mice). At this point, the MHC class II peptide complex is transported to the plasma membrane for recognition by CD4+ T cells. Phagocytosis of exogenous antigens is the typical antigen-presenting route of macrophages and dendritic cells. These cells are critical in initiating the immune response, as they express key costimulatory signals necessary to activate naive T cells.

The B lymphocyte is also a key antigen-presenting cell in the virus-specific immune response. Precursor B cells expressing surface Ig molecules (the B cell receptor) specific for epitopes exposed on a viral coat protein will be crosslinked as a consequence of multipoint binding to these repeated antigenic determinants (**Fig. 2**). The virus–Ig complex is internalized, degraded in lysosomes, and peptides derived from the various viral proteins presented in the context of class II MHC glycoproteins. This process focuses CD4+ T cells to the surface of B cells that have surface Ig molecules specific for viral epitopes. This is critical for the developing B cells to get the appropriate cytokine help needed for progression to Ig secreting plasma cells. In brief, antigen processing and presentation pathways serve to direct cytotoxic T cells to infected targets and helper T cells to B cells that require cytokine signals.

Stimulation of Cell-mediated Immunity

The generation of T cell effectors during the primary phases of the host response to an invading virus usually occurs in organized lymphoid tissue (**Fig. 3**). The architecture of the lymph node constitutes a circumscribed anatomical niche to facilitate contact between the relatively rare, naive B cell and T cell precursors and the antigen-presenting macrophages

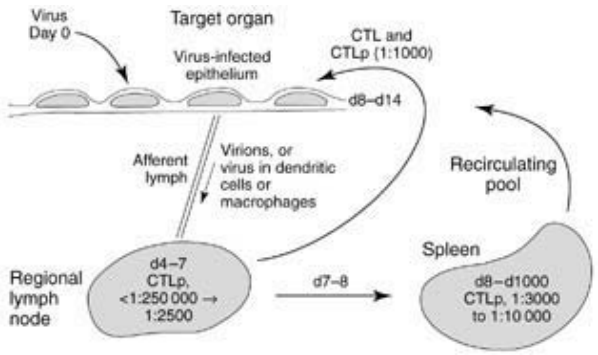


Figure 3 Primary T cell responses are normally generated in organized lymphoid tissue, particularly regional lymph nodes. Viral antigens may reach the lymph node as either free virions, or as processed peptides presented on the surface of dendritic cells or monocytes. The prevalence of virus-specific cytotoxic T lymphocyte precursors (CTLp) greatly increases over 6–8 days, with many of the CD8+T cell effectors then exiting from the lymph node and traveling via the blood to the organ(s) supporting virus growth. Thereafter, at least for the life of a laboratory mouse, there is an expanded pool of virus-specific, memory CTLp.

and dendritic cells. In particular, the microarchitecture of lymph nodes includes delineated T cell and B cell zones, as well as clusters of follicular dendritic cells and germinal centers. All of the factors that contribute to the generation and maintenance of these microstructures are not known, but molecules such as lymphotoxin α are key players in these processes. The functional consequences of this unique microenvironment are particularly important for lymphokines secreted at short range by the CD4+ helper T cells which promote the proliferation and differentiation of antigen-specific B cells and ultimately result in the generation of high-affinity antibodies.

Viral antigens reach the lymph nodes or spleen via blood or lymph, or encounter lymphoid tissue aggregates (the tonsils, Peyer's patches) associated directly with mucosal surfaces. The virus may be in the form of free virions, or processed peptides presented on the surface of monocytes/macrophages or dendritic cells that have traveled from the initial site of infection to the regional lymph node in afferent lymph. Early in the course of the disease process there is a shutdown of T cell recirculation (with resultant lymphopenia) due to the massive recruitment of all classes of lymphocytes into lymphoid tissue. This nonselective process is mediated, at least in part, by the virus-induced production of interferon $\alpha\beta$. The numbers of virus-specific CD8+ CTLp and CD4+ Thp then dramatically increase over the next 6 or 8 days.

Many of the virus-immune CD8+ T cells, and fewer of the CD4+ subset, then leave the lymph node and travel via the blood to the major target organ(s) supporting virus growth. The lymphocytes invade the infected tissue, where the CD8+ effectors generally clear the infectious process completely within 24–48 h. Large numbers of other T cells and monocytes/macrophages are also recruited into the site of pathology. Both CD4+ and CD8+ T cells can promote this delayed-type hypersensitivity response. The inflammatory process resolves after the virus is eliminated, though substantial numbers of lymphocytes and macrophages may persist through the time that tissue repair is continuing.

The immune system incorporates many levels of protection, which at first glance may appear redundant. However, as many viruses evolve to circumvent immune detection, the importance of overlapping features is revealed. For instance, mice that are first depleted of CD4+ T cells and then exposed to a cytomegalovirus (CMV) clear the infection from the lung, but not from the salivary gland. Perhaps an antibody-mediated process, operating via T cell help to responder B cells, is involved in eliminating infected cells that are for some reason inaccessible to (or not recognized by) the CD8+ effectors. Mechanisms dependent on the virus-specific Ig response seem to offer the only possibility for dealing specifically with virus-infected cells that are, for example, constitutively MHC negative.

T Cell-mediated Immunopathology

The almost synchronous destruction of large numbers of virus-infected cells by the emerging CMI response can lead to an acute physiological defect and even, in an extreme situation, to death. The classical model of CD8-T cell mediated immunopathology in a virus infection is murine LCM. The rather nonlytic LCMV grows in cells of the choroid plexus, ependyma and meninges following intracerebral injection of adult mice. Death results when CD8+ class I MHC-restricted CTLs damage the LCMV-infected cells and cause a massive breakdown of the blood–brain barrier. Suppression of the T cell response prevents this process from occurring; the mice survive and become asymptomatic carriers of the virus.

Immunopathology is thus the Janus face of normal CMI. Elimination of cells supporting the growth of a lytic virus is essential if the individual is to survive. Whether or not this process will bring on a crisis depends on the extent of the infection, and how central the infected cells are to maintaining the functional integrity of the organism. Perhaps this is the reason that evolution has favored a mechanism

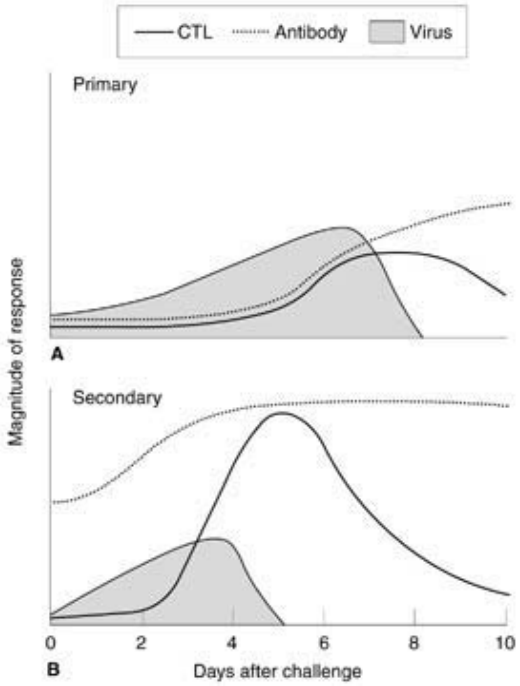


Figure 4 The characteristics of a primary cytotoxic T lymphocyte (CTL) and antibody response are illustrated in (**A**), the important point being that CTL effector function is not maintained for long after the virus is eliminated, though there is a greatly expanded pool of virus-specific, CD8+ memory T cells which are poised to respond should there be a subsequent infection (Fig. 3). Antibody persists, and a secondary response (**B**) to challenge with the same virus will generally result in the neutralization of the virus. However, some viruses (notably the influenza A viruses) share CTL epitopes on internal viral proteins, though their surface glycoproteins are different (e.g. H1N1 and H3N2). Cross-challenge with such viruses will give the secondary CTL response shown in (**B**). It is important to note that there is still a lag phase of 2–3 days before these CTL effectors are available to clear the virus.

that allows many categories of neurons, which cannot be replaced, to remain MHC negative.

T Cell Memory

Before an initial encounter with a virus, there are generally less than 1:250000 virus-specific CTLp in lymphoid tissue. The number increases to 1:2000–1:3000 by 6 or 7 days after infection (**Fig. 3**). Similarly, the frequency of virus specific Thp is greatly elevated during the course of an infection. Enhanced CTLp frequency is maintained for several years without further exogenous antigenic challenge in (for example) mice that have been infected with an influenza A virus, though there seems to be more variation in the magnitude of the Thp. The nature of the homeostatic mechanisms which regulate the size and function of these memory populations are key questions that remain as yet, unanswered.

Immunization to promote T cell memory may clearly be beneficial. However, though memory T cells are both present at higher frequency and more readily stimulated than their naive precursors, it is still the case that such lymphocytes are not usually activated to be fully functional effectors until several days after the secondary challenge (**Fig. 4**). This lag phase means that memory T cells, unlike circulating antibody, do not prevent the establishment of infection, though the process of virus clearance may be somewhat enhanced. For instance, there is evidence from a human study that the presence of such virus-specific CTLp in peripheral blood is correlated with less severe influenza virus infection. Also, there is encouraging preliminary evidence that people immunized with the gp160 of human immunodeficiency virus (HIV) in an alum adjuvant are maintaining a population of HIV-specific CTLp in peripheral blood for a considerable period. Thus, the maintenance of long-term memory populations may well enhance the overall well-being of individuals, but the specific regulation of these lymphocytes remains elusive.

HIV Cell-mediated Immunity

Infection with HIV poses a particular problem for immunity for two reasons. The first is that the viral coat glycoproteins vary greatly, which limits the effectiveness of vaccines depending on the presence of pre-existing neutralizing antibody. The second is that the cell surface receptor for HIV is the CD4 molecule expressed on the helper T cell and, in humans, on some macrophages. Chemokine receptors have recently been identified as coreceptors for HIV infection. The development of the acquired immune deficiency syndrome (AIDS) is associated with the progressive, inexorable loss of the CD4+T cells. At least part of this may reflect elimination of the infected CD4+T cells by the virus-specific CD8+T cells, which remain fully functional CTL effectors during the course of this persistent infection. Also, stimulation of the CD4+T cells activates the virus growth cycle and thereby increases viral load. A further complicating factor is that virus reservoirs persist in association with follicular dendritic cells in the lymph node. This provides a continuous supply of infectious virus as CD4+T cells pass through the lymph node. Relatively few virus-infected CD4+cells are found in peripheral blood, which could indicate that these lymphocytes are being effectively eliminated when they become antigenic in lymphoid tissue.

The lack of CD4+T cells in patients with AIDS results in much greater susceptibility to a variety of opportunistic infections, particularly those caused by fungi and protozoa. Secondary infections with other viruses are not as prominent, presumably because the CD8+T cell response is less compromised, though infection with herpesviruses may eventually become a problem. Herpesviruses, such as Epstein–Barr virus (EBV) and CMV, tend to persist in sites where they cannot be eliminated by CD8+T cells, though such infections are generally controlled in people who are not immunosuppressed. The severely impaired immune function of AIDS patients reflects the necessity of a complete immune system with many functioning branches.

History: or How We Got to Where We Are

Studies in laboratory mice with three different virus systems (LCMV, poxviruses and influenza) have contributed enormously both to our understanding of CMI and to immunobiology in the general sense. The persistent carrier state in mice infected *in utero* with LCMV, characterized by Traub (1939), was one of the factors that led Burnet and Fenner to formulate the concept of immunological tolerance (1949). Burnet (1970) went on to develop the immunological surveillance hypothesis, which he argued in the context of tumor immunity. This is undoubtedly applicable to the lymphoma and nasopharyngeal carcinoma associated with EBV infection, the basic mechanism being that illustrated in **Fig. 1.**

The operational basis of surveillance by class I MHC-restricted CTLs was discovered by Zinkernagel and Doherty (1974), while analysing the immunopathological disease caused by LCMV in previously unexposed adult mice. The 'single T cell receptor/altered self' model developed from these experiments provided an explanation (1975) for both variations in the level of T cell responsiveness associated with the expression of particular MHC alleles (the so-called immune response gene effects), and the extreme polymorphism of the class I MHC genes. Zinkernagel (1978) also used the LCM model to establish the canalizing role of the thymus in the establishment of the class I MHC-restricted T cell repertoire. They were awarded the Nobel Prize for this work in 1996.

The first vaccine, introduced for smallpox by Jenner (1798), depends on CMI effector mechanisms. Systematic analysis of the ectomelia (mousepox) model by Fenner (1947) set the stage for modern studies of CMI, with Blanden showing some years later (1970) that primed Thy-1+ cells promote virus clearance. Recombinant poxvirus (vaccinia) technology has also been used very effectively for the identification of the antigenic proteins/peptides involved in virus-specific T cell recognition. This approach (pioneered by Paoletti and Moss) has led to practical consequences, for example the recombinant rabies vaccine developed by Koprowski and his colleagues for use with feral populations.

An unexpected crossreactivity for virus-specific CD8+ CTLs was discovered independently (1977) in the laboratories of Askonas and Doherty, both of whom were analyzing CMI to the naturally occurring influenza A virus recombinants. These T cells were later found (1984–1985) by Townsend and McMichael, Bennink and Yewdell (using vaccinia virus recombinants) to be specific for conserved, internal proteins of the virus, such as the ribonucleoprotein (RNP). Townsend (1986) then had the major insight that the influenza-specific, CD8+ CTLs were recognizing peptides of the RNP. This made sense of MHC restriction when considered in the context of the emerging understanding of the TCR (Davis and Mak) and the crystallographic analysis of the HLA-A2 molecule by Bjorkman, Wylie, Strominger and colleagues. The new field of antigen processing in the cytoplasmic/endogenous (class I MHC) rather than the lysosomal/exogenous (class II MHC) compartment was opened as a consequence (**Figs 1** and **2**).

The experimental analysis of virus-specific CMI has thus led to several major (and many minor) original findings and pointed the way to some key concepts concerning the nature of immunity. The need to deal with virus infections has obviously been central to the evolution of the immune system. Rigorous analysis of the host response to viruses will continue to provide new insights into general mechanisms in immunity.

See also: **Autoimmunity; Cytomegaloviruses (*Herpesviridae*): Animal cytomegaloviruses, General features (human), Molecular biology (human), Murine cytomegaloviruses; Epstein-Barr virus (*Herpesviridae*): General features, Molecular biology; Human immunodeficiency viruses (*Retroviridae*): Molecular biology, Anti-retroviral agents, General features; Influenza viruses (*Orthomyxoviridae*): General features, Molecular biology, Structure of antigens; Lymphocytic choriomeningitis virus (*Arenaviridae*): General features, Molecular biology; Persistent viral infection; Vaccines and immune response; Vaccinia virus (*Poxviridae*), Vectors: Animal viruses, Plant viruses.**

Further Reading

Ada GL and Jones PD (1986) The immune response to

influenza infection. *Curr. Top. Microbiol. Immunol.* 128: 1.

Doherty PC, Allan W, Eichelberger M and Carding SR (1992) Roles of $\alpha\beta$ and $\gamma\delta$ T cell subsets in viral immunity. *Annu. Rev. Immunol.* 10: 123.

Yewdell JW and Bennink JR (1990) The binary logic of antigen presentation and processing. *Cell* 62: 203.

Zinkernagel RM and Doherty PC (1979) MHC-restricted cytotoxic T cells: studies on the biologic role of polymorphic major transplantation antigens determining T-cell restriction specificity, function and responsiveness. *Adv. Immunol.* 27: 51.

Infectious Pancreatic Necrosis Virus *see* **Birnaviruses – Animal**

INFLUENZA VIRUSES (*ORTHOMYXOVIRIDAE*)

Contents

General Features

Robert G Webster, St Jude Children's Research Hospital, Memphis, Tennessee, USA

History

The highly contagious acute respiratory illness known as influenza has afflicted humans since ancient times. The pattern of outbreaks of respiratory diseases that appear suddenly, persist for a few weeks and then suddenly disappear is sufficiently characteristic to permit the identification of numerous major epidemics in the distant past. One such epidemic was recorded by Hippocrates in 412 BC, and several were described in the Middle Ages. Historical data from 1500–1800 indicate that: (1) epidemics occurred relatively frequently but irregularly, with the disease seeming to disappear for periods of time; (2) epidemics varied in severity but usually caused mortality in the elderly; and (3) some epidemics, such as those in 1781 and 1830, spread across Russia and Asia. There are similarities with our current knowledge of the epidemiology of influenza, including age-related mortality and emergence of epidemics in China.

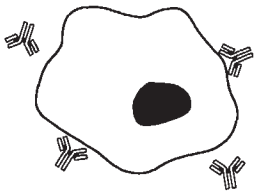
The 1918 'Spanish influenza' pandemic was particularly severe, killing between 20 and 40 million people worldwide, and partial analysis of the virus genome from a lung sample in a paraffin block of a soldier that died in 1918 supports the belief that the virus originated from swine. In the late 1920s, Richard Shope showed that swine influenza could be transmitted with filtered mucus, suggesting that the causative agent was a virus. Human influenza A virus was first isolated in ferrets in 1933 by Wilson Smith, Sir Christopher Andrewes and Sir Patrick Laidlaw, in the UK. Influenza B was first isolated by Thomas Francis in 1940.

Fowl plague, a lethal form of influenza in chickens, was first reported in Italy in 1878 but it was not until 1955 that Walter Shafer in Germany showed that this disease was actually caused by an influenza virus.

Taxonomy and Classification

Influenza viruses belong to the *Orthomyxoviridae* family of RNA viruses and is constituted of four genera; *Influenzavirus* A, *Influenzavirus* B, *Influenzavirus* C and *Thogovirus*. These viruses have segmented negative-strand RNA genomes enclosed in a lipid envelope derived from the host cell. The particles are covered in a layer of spikes, contained in the envelope, which are of three kinds: a hemagglutinin (HA) which agglutinates erythrocytes, an enzyme neuraminidase (NA) which releases the virus from cells and a small number of copies of the M2 protein that serves as an ion channel. Beneath the lipid envelope is a membrane or matrix (M) protein surrounding a helical complex containing the nucleoproteins (NP) and three poly-

IMMUNE RESPONSE



Contents

General Features

Gordon L Ada, Australian National University, Canberra, Australia

History

The germ of immunology grew out of the observation in ancient times that those who survived a clinical infection seldom suffered a second, similar sickness. Repeated exposure to a disease like smallpox, which leaves characteristic pock marks after the first attack, strengthened this belief. Some understanding of the mechanisms of protection from a second infection came much later and mainly from the study of bacteria and their products. The discovery in the 1890s that soluble toxins produced by some bacteria induced all the symptoms of the disease led to the finding that the body produced a 'substance' which 'neutralized' the activity of the toxin – the antitoxin or antibody. This in turn led to extensive studies of antigen–antibody reactions and to the use of immune serum therapy. Such studies dominated immunological work in the first half of the twentieth century.

It was also suggested in the late nineteenth century that leukocytes could play a role in the defense of the body, based on the observations of the activity of phagocytic cells. However, the declaration of the Clonal Selection Theory by Burnet in 1957 demolished the earlier ideas of the chemists about the mechanism of antibody formation and production, and ushered in an era of intense study of first B-cell- and later T-cell-mediated immune reactions.

The use of viruses as tools for elucidating immune responses came much later, and has substantially added to our understanding of immunological principles. Infections by viruses containing mosaics of identical protein molecules contributed to our understanding of the importance of antigen conformation and recognition by antibodies. Viruses with RNA genomes which show great antigenic variation illustrated the contribution of individual amino acid residues in influencing the specificity of antigen–antibody reactions. From the mid-1970s until the present day, the use of viruses as infectious immunogens provided the major approach for studying an important cellular response, cytotoxic T cell generation and activity.

Though it is likely that the immune response of vertebrates was largely fashioned by the need to overcome infections, immunologists have taken advantage of this only during the last quarter century.

Nature of Immune Responses

It is convenient to divide 'immune' reactions into two classes: adaptive responses, often called specific responses; and nonadaptive or innate responses. Adaptive responses are mediated by lymphocytes which express two unique characteristics: exquisite specificity and memory. This response is characteristic of vertebrates and reaches its highest development in primates.

Nonadaptive immune responses

In nonimmunized hosts, there is a lag period after infection of at least some days (and sometimes considerably longer, e.g. human immunodeficiency virus, HIV) before components of the adaptive response are detected. It is especially during this period that nonadaptive responses are important for limiting viral responses. In humans and ferrets, a mild influenza virus infection may be limited to the upper respiratory tract, where the mucus contains substances that can inhibit influenza virus infectivity. Infection induces an inflammatory response in which a variety of cells, including monocytes and polymorphonuclear leukocytes, are extruded on to the site of infection. There is a correlation between the extent of fever caused by pyrogens such as interleukin 1 (IL-1) and the rate of decline of viral titer. Activation of natural killer (NK) cells by interferons (IFN-α and IFN-β) results in lower viral titers. Mice bearing the *Mx* gene are resistant to influenza virus infection due to enhanced IFN production. Depletion of NK cells in some strains of mice makes them more susceptible to some viral infections (cytomegalovirus and vaccinia and mouse hepatitis viruses).

Activation of the complement cascade following infection may result in lysis of enveloped viruses such as influenza.

The innate system predates the adaptive system in evolution and it was first thought that the two

Table 1 Some properties of antigen-presenting cells

Molecules expressed	*Dendritic cells*	*Macrophages*	*B cells*
Class I MHC	Constitutive	Constitutive	Constitutive
Class II MHC	High, IFN-γ enhances	IFN-γ enhances	IL-4 enhances
Costimulator	High	Activation enhances	Activation enhances
T cell target	CD4+,CD8+	Mainly CD4+	Activated CD4+

systems are unrelated, but it has become increasingly clear that the activation and maturation of the former may facilitate the effectiveness of the latter. IFN-γ, originally called immune interferon because it was produced by T cells, is now known to be also produced by NK cells. The classical and alternate pathways of complement activation, which differ only slightly in the properties of the first of six components, serve the innate and adaptive systems respectively.

Adaptive immune responses

Role of antigen-presenting cells (APCs) APCs may interact with two classes of lymphocytes: the B or bone marrow-derived lymphocytes, but especially with the T or thymus-derived lymphocytes. APCs process antigen to a form which is recognized by T cells. Noninfectious antigenic material (e.g. inactivated viral particles) is endocytosed into the APC and degraded in lysosomes. Some of the peptides so produced associate with class II major histocompatibility complex (MHC) antigens; the complex is transported to and expressed at the plasma membrane, where it is specifically recognized by a T cell receptor. This response is termed a class II MHC-restricted response and the recognizing cell has the CD4 membrane marker.

In contrast, an infectious particle, e.g. a virus, may recognize and bind to a receptor on the APC, be endocytosed and replicate (either abortively or productively) within the cell. Some of the newly synthesized proteins within the cytoplasm are degraded to peptides, a few of which may associate with class I MHC antigens. Following expression at the cell surface, the complex may be recognized by the receptor on T cells, which also express the CD8 marker. The response is called a class I MHC-restricted response.

In order for T cell activation, differentiation and replication to occur, the APC must also express a costimulator molecule, CD 80, 86 (B7.1 or 7.2), which is recognized by specific receptors on the T cell (CD 28). The activities of APCs can be modified by certain cytokines. **Table 1** lists some properties of three different APCs. Dendritic cells (DCs) are particularly important for CD8+ T cell stimulation, whereas B cells are important antigen presenters to already activated CD4+ T cells (T–B cell interaction).

Activation of T cells There are now known to be subclasses of both CD4+ and CD8+ T cells, each characterized by a particular pattern of cytokine secretion after interaction with APCs and differentiation into helper/effector cells. The properties of these are outlined in **Table 2**. Because of the similarities in expression and secretion of different cytokines, the terms type 1 and type 2 T cell responses have come into general use. Th1 and Tc1 responses are referred to as type 1 responses and Th2 and Tc2 responses as type 2 responses. Two cytokines in particular not only have a dominant role in inducing these responses, IL-12 inducing a type 1 response and IL-4 a type 2 response, but also in suppressing other responses. For

Table 2 Some properties of subclasses of T lymphocytes

Class	*Subclass*	*Cytokine pattern*	*Effector activities*
CD4+	Th1	IL-2, 3; IFN-γ, TNFα, β	Provides help for IgG2a (mice) and IgG1 (humans); mediates DTH, but usually not cytotoxic activity
	Th2	IL-3, 4, 5, 6, 10, 13, TNFα	Provides help for IgG1, IgA and IgE production
	Th0	IL-2, 4, 5, 10, IFN-γ	Provides help to B cells
CD8+	Tc1 (CTL)	IL-2, IFN-γ, TNFβ	Mediates early lysis of virus-infected cells Usually, poor mediator of DTH reactions
	Tc2	IL-4, 5, 10.	Suppresses CTL activity

CTL, cytotoxic T lymphocyte; DTH, delayed-type hypersensitivity; TNF, tumor necrosis factor.

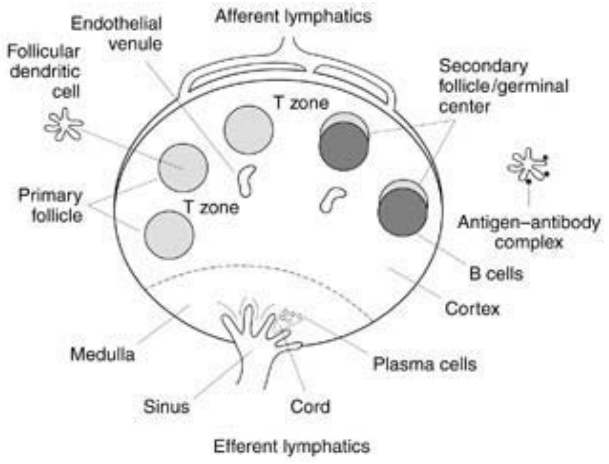


Figure 1 Cell:cell interactions within lymph nodes.

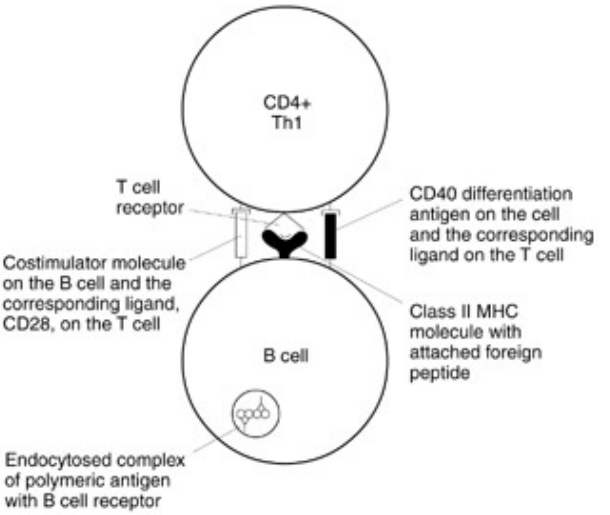


Figure 2 B cell activation by interaction with effector Th cells.

example, IL-4 and Tc2 can suppress the formation/activity of Tc1 cells.

T cells can also be distinguished because of the composition of their receptors for antigen recognition. Most have α,β-peptide chains, but some have γ,δ chains. The latter are a diverse group and are discussed elsewhere.

In vivo, afferent lymphatic vessels convey antigen, some perhaps already taken up by DCs, to the cortical and medullary areas of draining lymph nodes (or to the spleen) via the afferent lymphatics (**Fig. 1**). Particulate material may also be phagocytosed by macrophages in the medullary sinuses. Lymphocytes enter the node via endothelial (postcapillary) venules and T cells localize in the paracortex (T zone), where interaction with APCs occurs.

Activation of B cells An epitope of either a simple, a complex or aggregate of antigens may be specifically recognized by IgM receptors on B cells in the cortex of the lymph node. The complex so formed is endocytosed and degraded within the cell in a fashion thought to be similar to the process in macrophages or dendritic cells. Levels of costimulator molecules as well as class II MHC antigen expression are enhanced and the peptide–MHC antigen complex is transported to the cell membrane. The B cell now migrates to the T zone in the node where the MHC–peptide complex may be recognized by the T cell receptor of usually an already activated T cell. This interaction is illustrated in **Fig. 2**.

In the case of a T cell-independent antigen, e.g. a saccharide, the B cell may secrete IgM antibodies of low affinity, but because of their high avidity, they may be an important early defense mechanism against some pathogens, including viruses.

Early-formed antibody may interact with free antigen and the complex bind via complement receptors to special cells with a very high surface area, the follicular dendritic cells (FDCs) in primary lymphoid follicles (**Fig. 1**). T cell-activated B cells expressing different Ig isotypes (centrocytes) migrate to the follicles and undergo somatic hypermutation, leading to affinity maturation of the Ig receptors. Interaction with antigen held at the surface of FDCs preferentially selects those cells with receptors of higher affinity, and these differentiate into plasma cells. This activity causes morphological changes in the follicle so that they become secondary follicles or germinal centers. The plasma cells enter medullary cords and exit the node via efferent lymphatics. Many migrate to the bone marrow, but most have a relatively short lifespan. Cells not selected by antigen in this way die by apoptosis.

Though there are examples where protective antibodies may be formed to continuous (linear) sequences of a viral antigen, increasing evidence indicates that the most effective neutralizing antibodies recognize conformational epitopes formed by adjacent protein molecules. Examples are the hemagglutinin of influenza virus which exists as a trimer, the VP1 protein multimer of picornaviruses and, more recently, the env antigen of HIV which exists as a trimer. These oligomers/polymers are normally resistant to proteases. This is especially so for glycoproteins, and in the case of a retrovirus, enzymatic removal of the terminal neuraminic acid of the env antigen enhances the susceptibility to protease degradation. In fact, intact HIV particles have been found attached to FDCs. These findings imply that, *in vivo*,

Table 3 Sequence of immune responses following intranasal inoculation of a nonlethal dose of influenza virus to mice.

Type of response	*Events*	*Time period (days)*
Virus	The virus attaches to receptors on epithelial cells, replicates, infects other cells. Maximum titers are reached on days 3–6, thence they decrease and virus is eliminated	0–10
Th cells	CD4+ Th cells are first detected	2
CTLs	CD8+ CTLs are detected, reach peak levels at about 3 days after peak viral titers, then decrease and activity becomes undetectable	4–12
	Memory CTLs are formed and remain at high levels for more than 18 months	14–500+
ASCs	IgM SCs detected at about 7 days, reach peak levels at about 3 months, and are present for about 12 months	7–300+
	IgG SCs detected at about 10 days, reach peak levels at about 3 months and are present for more than 18 months	10–500+
	IgA SCs detected at about 10 days, reach peak levels at about 3 months, and are present for about 12 months; memory ASCs reach peak levels at about 3 months	7–300+

ASCs, antibody-secreting cells; CTLs, cytotoxic T lymphocytes; Th, T helper cells.

these antigens should retain their quaternary conformation while in the extracellular medium, i.e. for selection of B cells and persistence on FDCs. In contrast, once inside an APC such antigens should be more readily degradable to peptides. There is less need for internal antigens of a virus to be protease resistant; often, such antigens (e.g. the nucleoproteins of influenza and lymphocytic choriomeningitis viruses) are a rich source of peptides which bind to class I MHC antigens.

Formation of immunological memory

B cells Some centrocytes in the follicles convert to memory B lymphocytes which circulate around the body. When they later traverse a lymph node or the spleen which contains specific antigen attached to FDCs, the process of activation and selection for cells with high-affinity receptor or reconversion to memory cells begins again. In this way, provided antigen persists for a long period in an appropriate conformation, specific antibody of high affinity may be present for a very long period after an infection or immunization. For example, the H1N1 strain of influenza virus circulated before 1957. It was 'displaced' then by H2N2 and later by H3N2 strains. When the H1N1 strain later appeared in 1977–1978, severe infections mainly occurred in those born since 1957.

An alternative or additional explanation for continued antibody production is the establishment of an idiotypic–anti-idiotypic antibody network, but there is little direct evidence for a long-acting mechanism of this kind.

Compared to naive cells, memory B cells express IgG, IgA or IgE receptors so are readily distinguished from naive B cells. *In vivo*, it is difficult to estimate the number of memory B cells at any one time, but *in vitro* experiments suggest that after an infection the number may be 100-fold or higher, compared with the number of naive cells of that specificity.

T cells The mechanism of formation of T memory cells is shrouded in mystery. There is no indication that it occurs in any special site, affinity maturation of the T cell receptor does not occur, and it is now generally accepted that memory T cells have a long life in the absence of specific antigen. They have a higher level of some cell surface markers, including CD45RO, than naive cells. *In vitro*, limit dilution analyses indicate that after a specific infection or immunization, the numbers of specific Tc1 cells may increase by a factor of 20 or more, but there is some doubt about the validity of such findings *in vivo*, as it is now recognized that memory cells are more readily activated than naive cells. There is also discussion as to whether some memory cells exist in an 'activated' state, whereas others behave more like naive cells.

Sequence of responses in an acute viral infection

Primary response Influenza virus infection in the mouse has been studied in some detail. Following intranasal inoculation of a sublethal dose of influenza virus, some innate responses in the lung can be detected within hours. The subsequent sequence of adaptive responses is outlined in **Table 3**. Specific antibody is the only mechanism for entirely preventing infection (sterilizing immunity), though this probably occurs quite rarely if at all. Many viruses infect via a mucosal surface, such as the gut, and the

respiratory and genitourinary tracts. A special feature is the induction of secretory IgA (sIgA), which is secreted into the lumen from the mucosa. It is very stable, resisting degradation by most proteases, so is ideal as a first line of defense. In cases where viremia may also occur, such as polio virus and measles virus infections, serum Ig forms a second line of defense. Infection must occur for a cell-mediated immune response to develop.

Secondary response If subsequently exposed to virus of an identical or closely similar antigenic specificity, the pre-existing antibody should neutralize the infectivity of nearly all of the incoming virus. Escaping virus would initiate infection, but the progeny in turn would likely be neutralized by the antibody so that the infection would be subclinical. A primary cytotoxic T lymphocyte response should also contribute to clearing the residual infection. However, if the antigenic (B cell) specificity of the challenge virus is different, as occurs especially with influenza virus pandemics, little neutralization of virus will occur, but there will be a more rapid and enhanced cell-mediated immune response, especially cytotoxic T lymphocytes, to the infection because of the memory response to conserved T cell epitopes. Thus, the clinical infection is controlled more rapidly, which could mean the difference between life or death in some cases.

Evading the Immune Response

Viruses may use a number of strategies to evade the immune response.

Evasion of antibody

Antigenic variation Many DNA viruses occur in antigenically different forms, known as serotypes. The number varies from a few (e.g. hepatitis B) to many (e.g. adenoviruses, >40). Each serotype is antigenically stable, and the distribution may vary in different localities. Prior infection with one serotype often does not protect against infection by a different serotype. Owing to the lack of corrective mechanisms for RNA synthesis, RNA viruses sometimes show greater antigenic variation. Mutations leading to changes in the sequence of nucleic acid bases and hence of amino acids, which may occur during viral replication, may persist. This is called antigenic drift and the classical examples are influenza (orthomyxovirus) and HIV (retrovirus). There are also said to be more than 100 serotypes of rhinovirus (an RNA virus) but it seems possible that some are mutants which emerged because of immune pressure. It is not clear why some RNA viruses (e.g. measles, polio) show much less antigenic variation in the field compared to influenza virus. Usually, internal antigens (e.g. nucleoprotein) show much less drift than surface antigens (e.g. influenza), so that they have conserved T cell epitopes. Though HIV shows very high antigenic drift in many proteins, there are some conserved T cell epitopes in the internal antigens.

Viruses with a segmented genome can, in addition, undergo gene reassortment and this may result in major antigenic differences between parent and progeny virus. This process is called antigenic shift and has led to several pandemics of influenza virus.

Immunosuppression Sometimes, antibody formation in response to an infection is poor or delayed or both. For example, HIV possesses suppressor sequences, and synthetic peptides corresponding to these sequences have been shown to inhibit T cell proliferation *in vitro*. In HIV infection, there is often a marked delay before neutralizing antibody is detected.

Evasion of cell-mediated immune responses

Latency Latency is a stage after infection when infectious virus in a cell cannot be detected, but under certain conditions, such as stress, the virus may be reactivated and infectious progeny again produced. Classical examples are herpes viruses, Epstein–Barr virus (EBV) and HIV.

Sanctuary sites Some sites are not easily accessible to the immune system; for example, the brain and epididymis, which are separated from the blood by a barrier, and the kidney. HIV, for example, is present in the brain and semen. This may provide an explanation of why many HIV-infected individuals who generate strong cytotoxic T lymphocyte responses do not clear the virus. Herpes and measles viruses infect neurons and plasmodia infects red blood cells, neither of which express class I MHC. Even lymph nodes can act as a sanctuary. Infectious HIV has been found attached to FDCs in lymph nodes, even though neutralizing antibody was present.

Regulation of class I MHC expression Infection of cells by pox, cytomegalo, herpes and adeno viruses can result in downregulation of class I MHC antigen expression. A variety of mechanisms is used but a common one is the expression in the infected cell of an antigen which binds to the MHC molecule and prevents its expression at the plasma membrane. The net result is that the infected cell is not recognized by cytotoxic T lymphocytes.

Table 4 Putative immune diseases associated with viral infections

Disease	*Agent*
Systemic lupus erythematosus	C-type viruses
Rheumatoid arthritis	Epstein–Barr virus
Autoimmune disease syndrome	HIV
Idiopathic thrombocytopenic purpura	Various viruses
Multiple sclerosis	Parainfluenza, measles viruses, HTLV-1, etc.
Diabetes mellitus	Coxsackie virus B
Thyroiditis	Mumps virus
Postinfectious encephalomyelitis	Measles, rubella viruses

Information courtesy Dr N. R. Rose.

Antigenic drift in cytotoxic T lymphocyte epitopes Like B cell epitopes in some RNA viruses, antigenic drift can occur in T cell epitopes, notably during HIV infection. This may be particularly important if it occurs in a dominant cytotoxic T lymphocyte epitope, as other conserved epitopes may be less well recognized.

Immunological tolerance Infection of neonates by some viruses, such as hepatitis B and lymphocytic choriomeningitis virus (LCMV), results in the persistence of the virus and a carrier state.

Infection of effector cells A wide range of viruses can infect cells involved in immune processes. HIV infects Th cells, DCs and macrophages, and this leads to immune incompetence. The structure of lymph nodes and follicles can be destroyed, and opportunistic infections occur. EBV infection can flourish in immunosuppressed, organ-transplanted people, but transfer of syngeneic cytotoxic T lymphocytes has controlled the infection.

Adverse immune reactions to viral infections

Immune complex formation It is almost inevitable that the immune response to a viral infection will induce some immunopathology. Immune complexes may be deposited in places such as renal glomeruli and the synovium of joints which have filtering basement membranes. If viral antigen is continuously released, such as in a persistent hepatitis B infection, the complexes are deposited over a prolonged period, causing chronic inflammation.

Immune enhancement If a virus replicates in cells which express Fc receptors, then the formation of complexes of virus and nonneutralizing antibody may facilitate infection of the cell by the virus. The classical example of immune enhancement is a dengue virus (flavivirus) infection, leading to immunopathological effects *in vivo*, such as the dengue shock syndrome. Infection-enhancing antibodies have been found in HIV-infected individuals, especially during the later stages of the infection.

Immunopathology There is a variety of experimental models in which a viral infection can cause considerable pathology, sometimes leading to death. Mice injected intracerebrally with LCMV may die due to the formation and activity of cytotoxic T lymphocytes. This does not happen if infection occurs by other routes. Transfer of cloned specific cytotoxic T lymphocytes in small numbers to mice infected intranasally with respiratory syncytial virus enhances recovery; transfer in large numbers enhances disease. Transfer of virus-specific class II MHC-restricted immune cells expressing delayed-type hypersensitivity activity to mice infected with influenza virus can enhance disease.

Molecular mimicry, autoimmunity and autoimmune disease

The presence of similar amino acid sequences between some viral and host proteins is more common than originally supposed. Data-based searches have shown considerable sequence homology, the extent observed increasing with the length of the sequence. Some monoclonal antibodies to viral proteins bind to host proteins. Experimentally, immunization with a peptide from a viral antigen which shared 60% homology with a myelin basic protein induced pathological lesions in the brain. It is important that the homology be not too great.

Autoimmunity is common in human populations and it is very likely that most viral infections induce some autoimmunity. Infection by some viruses may initiate or be associated with a particular autoimmune disease. **Table 4** contains a list of possible

associations. Studies in animal models suggest that Th1-type lymphokines are involved in the genesis of many organ-specific autoimmune diseases.

See also: **Adenoviruses (*Adenoviridae*): Animal viruses, General features, Malignant transformation and oncology, Molecular biology; Dengue viruses (*Flaviviridae*); Epstein-Barr virus (*Herpesviridae*): General features, Molecular biology; Human immunodeficiency viruses (*Retroviridae*): Molecular biology, Anti-retroviral agents, General features; Influenza viruses (*Orthomyxoviridae*): General features, Molecular biology, Structure of antigens; Measles virus (*Paramyxoviridae*); Persistent viral infection; Vaccines and immune response; Immune escape mechanisms; Immune response: Cell mediated immune response; Virus structure: Principles of virus structure.**

Further Reading

Ada G and Ramsay A (1997) *Vaccines, Vaccination and the Immune Response*, p. 1. Philadelphia: Lippincott-Raven.

Brown LE, Hampson AW, Webster RG (1996) *Options for the Control of Influenza, III*. Amsterdam: Elsevier.

Fields BN, Knipe DM, Howley PM *et al* (eds) (1997) *Fields Virology*, 3rd edn. Philadelphia: Lippincott-Raven.

Romagnani S (1997) The Thi/Th2 paradigm. *Immunol. Today* 18: 263.

Sprent J (1997) Immunological memory. *Curr. Opin. Immunol.* 9: 371.

Cell Mediated Immune Response

Janice Riberdy and **Peter Doherty**, St Jude Children's Research Hospital, Memphis, Tennessee, USA

General Features

Cell-mediated immunity (CMI) is concerned with the elimination of infected cells from organ sites of virus growth. This terminates the production of new virus, preventing the further spread of the infectious process. While virus-specific immunoglobulin (Ig) molecules are very important for neutralizing free virions, and thus play a central part in resistance to reinfection, antibody transfer experiments indicate that Ig-mediated mechanisms are less prominent in the clearance of virus from solid tissues. Removing the infected cell generally depends on the involvement of virus-specific thymus-derived lymphocytes, or T cells, the effectors of CMI. The immune system has evolved largely to deal with pathogens, optimizing the effective synergism between Ig-mediated and T cell-mediated functions.

There are two broad categories of T cells, each expressing a clonally distributed T cell receptor (TCR) heterodimer characterized as either $\alpha\beta$ or $\gamma\delta$. The $\alpha\beta$ or $\gamma\delta$ TCR molecules are associated with the multimeric CD3 complex, which is involved in mediating the signaling events that control T cell stimulation and differentiation. Most current understanding of virus-specific CMI is concerned with the $\alpha\beta$ T cell subsets, which express either the CD4 or CD8 coreceptor molecules (**Fig. 1**). The $\gamma\delta$ T cells are a minority in mice and humans, constituting less than 5–10% of the peripheral T cells in blood and secondary lymphoid tissue, while they are much more prevalent (20–30%) in pigs, ruminants and chickens. The role of the $\gamma\delta$ T cells in virus infections is not yet understood, though these lymphocytes are known to accumulate in some viral pneumonias and may play a part in regulating the inflammatory process.

The CMI effector mechanism is targeted to the surface of virus-infected cells because the antigen-specific $\alpha\beta$ TCR recognizes viral peptides presented in association with self major histocompatibility complex (MHC) glycoproteins (**Fig. 1**). The developing T cells are selected in the thymus to be tolerant (non-responsive) of self MHC molecules encountered in the thymic microenvironment but reactive, after leaving the thymus, to these same glycoproteins when they are modified by the binding of nonself peptides. This phenomenon is known as 'MHC restriction'. Tolerance of viral peptide–MHC glycoprotein complexes can also be induced if, as in mice infected *in utero* with murine lymphocytic choriomeningitis virus (LCMV), the antigen is encountered during thymocyte differentiation.

A critical lymphocyte in most virus-induced inflammatory processes is the CD8+ $\alpha\beta$ T cell. Lymphocytes of this T cell subset recognize viral peptides presented in the context of the class I MHC glycoproteins, the strong transplantation antigens that are expressed (or can be induced) on most cells throughout the body. The CD8 molecule binds to a constant region of the MHC protein, thereby increasing the avidity of the interaction between the T lymphocyte and the antigen-presenting stimulator or target cell. This may be particularly important at the initial stages of the induction of a primary T cell response. The pore-forming proteins (perforins) and enzymes (granzymes) characteristic of cytotoxic effector function are prominent in the cytoplasm of activated,

influenza infection. *Curr. Top. Microbiol. Immunol.* 128: 1.

Doherty PC, Allan W, Eichelberger M and Carding SR (1992) Roles of $\alpha\beta$ and $\gamma\delta$ T cell subsets in viral immunity. *Annu. Rev. Immunol.* 10: 123.

Yewdell JW and Bennink JR (1990) The binary logic of antigen presentation and processing. *Cell* 62: 203.

Zinkernagel RM and Doherty PC (1979) MHC-restricted cytotoxic T cells: studies on the biologic role of polymorphic major transplantation antigens determining T-cell restriction specificity, function and responsiveness. *Adv. Immunol.* 27: 51.

Infectious Pancreatic Necrosis Virus *see* **Birnaviruses – Animal**

INFLUENZA VIRUSES (*ORTHOMYXOVIRIDAE*)

Contents

General Features

Robert G Webster, St Jude Children's Research Hospital, Memphis, Tennessee, USA

History

The highly contagious acute respiratory illness known as influenza has afflicted humans since ancient times. The pattern of outbreaks of respiratory diseases that appear suddenly, persist for a few weeks and then suddenly disappear is sufficiently characteristic to permit the identification of numerous major epidemics in the distant past. One such epidemic was recorded by Hippocrates in 412 BC, and several were described in the Middle Ages. Historical data from 1500–1800 indicate that: (1) epidemics occurred relatively frequently but irregularly, with the disease seeming to disappear for periods of time; (2) epidemics varied in severity but usually caused mortality in the elderly; and (3) some epidemics, such as those in 1781 and 1830, spread across Russia and Asia. There are similarities with our current knowledge of the epidemiology of influenza, including age-related mortality and emergence of epidemics in China.

The 1918 'Spanish influenza' pandemic was particularly severe, killing between 20 and 40 million people worldwide, and partial analysis of the virus genome from a lung sample in a paraffin block of a soldier that died in 1918 supports the belief that the virus originated from swine. In the late 1920s, Richard Shope showed that swine influenza could be transmitted with filtered mucus, suggesting that the causative agent was a virus. Human influenza A virus was first isolated in ferrets in 1933 by Wilson Smith, Sir Christopher Andrewes and Sir Patrick Laidlaw, in the UK. Influenza B was first isolated by Thomas Francis in 1940.

Fowl plague, a lethal form of influenza in chickens, was first reported in Italy in 1878 but it was not until 1955 that Walter Shafer in Germany showed that this disease was actually caused by an influenza virus.

Taxonomy and Classification

Influenza viruses belong to the *Orthomyxoviridae* family of RNA viruses and is constituted of four genera; *Influenzavirus* A, *Influenzavirus* B, *Influenzavirus* C and *Thogovirus*. These viruses have segmented negative-strand RNA genomes enclosed in a lipid envelope derived from the host cell. The particles are covered in a layer of spikes, contained in the envelope, which are of three kinds: a hemagglutinin (HA) which agglutinates erythrocytes, an enzyme neuraminidase (NA) which releases the virus from cells and a small number of copies of the M2 protein that serves as an ion channel. Beneath the lipid envelope is a membrane or matrix (M) protein surrounding a helical complex containing the nucleoproteins (NP) and three poly-

merase proteins. *Influenzavirus* A and B have eight RNA segments whereas *influenzavirus* C and *Thogovirus* have seven, each coding at least one protein. Influenza A viruses are divided into subtypes which are based on the host of origin (pigs, horses, birds), geographic origin, strain number and year of isolation, the subtype is given in parenthesis, e.g. A/swine/Iowa/15/30 (H1N1). By tradition, the host of origin of human strains is not included, e.g. A/Hong Kong/1/68/(H3N2).

Geographic and Seasonal Distribution

Human influenza occurs worldwide, is endemic in tropical regions and occurs year round. In the temperate and subarctic regions, influenza in humans and swine is a 'winter disease'. The occurrence of influenza in winter is not well understood and may be associated with crowding, low humidity, and increased virus survival. Avian influenza viruses are prevalent in the fall of the year in wild ducks when the young congregate prior to migration and in shorebirds in the US in spring.

Equine, swine and avian influenza viruses have a wide geographic distribution; the avian influenza viruses from Europe and Asia can be separated phylogenetically from those in the Americas.

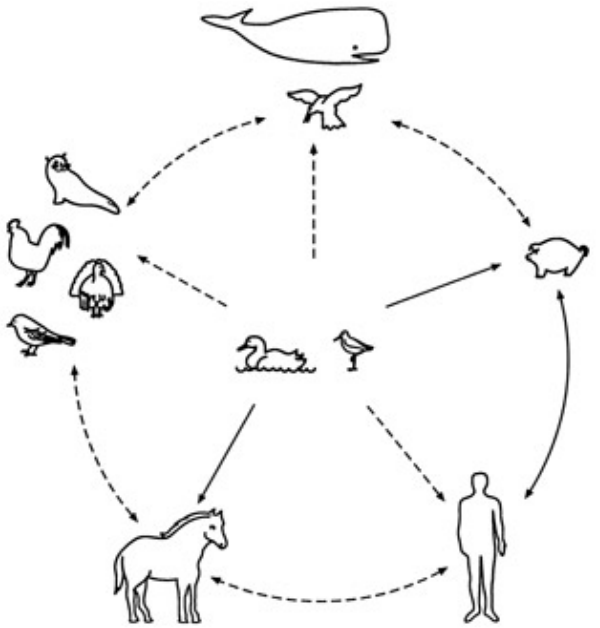


Figure 1 The reservoir of influenza A viruses. The working hypothesis is that wild aquatic birds are the primordial reservoir of all influenza viruses for avian and mammalian species. Transmission of influenza has been demonstrated between pigs and humans (solid lines). There is extensive evidence for transmission between aquatic birds and other species including pigs and horses and indirect transmission to humans through pigs and evidence for direct transmission to humans from chickens.

Host Range and Viral Propagation

Influenza A viruses commonly cause disease in humans, pigs, horses and occasionally in birds. Since 1933, three HA subtypes have been identified in humans, two in pigs, and two in horses. All of the 15 subtypes are found in aquatic birds, mainly in wild ducks and shorebirds; they usually cause no disease in these hosts but serve as reservoirs of influenza A genetic information (**Fig. 1**). Influenza viruses (of avian origin) are occasionally isolated from seals, mink and whales. Influenza B viruses occur only in humans, whereas influenza C has been found in humans and pigs.

Experimental hosts include ferrets, which develop febrile rhinitis after infection with influenza A and B viruses. Ferrets have been used extensively to study the biology of influenza viruses and as a source of reference antisera. Other experimental animals include mice, monkeys and New World primates. Mice are not naturally infected with influenza viruses, but both influenza A and B viruses can be adapted to mice. The virus replicates in the upper and lower respiratory tract and, after adaptation can cause pneumonia and death. Most strains of mice can support influenza virus after adaptation, and inbred strains have been valuable in elucidating cell-mediated immune responses to respiratory viral infection. The A2G mouse is resistant to influenza and carries a dominant allele (*Mx* gene) that specifies a protein which is induced by interferon and inhibits influenza virus.

Influenza viruses can be grown in embryonated hens' eggs or in a number of primary tissue culture systems. Cultivation of influenza viruses in eggs remains the system for production of vaccine and for large quantities of virus used in laboratory studies. The continuous Madin Darby canine kidney cell line and primary monkey, calf, hamster and chicken kidney cell cultures will support influenza viral replication, provided trypsin is added to cleave the HA. Influenza virus replication is detected by hemagglutination of erythrocytes.

Genetics

Influenza viruses show genetic changes by accumulation of point mutations in their RNA segments and by reassorting their segments after two or more influenza viruses infect a single cell. The mutation rate of influenza A is approximately 1.5×10^{-5} mutations per nucleotide per replication cycle. In human influenza A viruses, up to 50% of the nucleotide

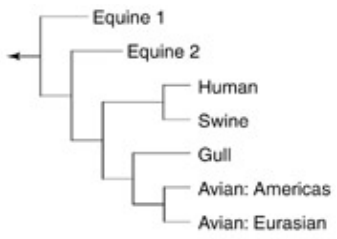


Figure 2 Generalized phylogeny of influenza A virus. Generalized phylogenetic tree of the nucleotide sequences of the nucleoprotein gene of influenza viruses. The most ancient influenza lineage (Equine 1) may have disappeared from horses. Human and swine viruses have a brother–sister relationship. Avian lineages are geographically separable into those from the Americas and those from Eurasia.

changes result in amino acid changes. Amino acid changes are less frequent in influenza B and C and in avian influenza A. Human influenza A viruses are evolving more rapidly than other influenza viruses and also reassort their genomes after mixed infection with another influenza A virus.

Evolution

Results of sequence analysis of each of the genes of influenza A viruses support the idea that there are five host specific lineages, that the avian lineages are in evolutionary stasis and can be separated in American and Eurasian varieties (**Fig. 2**). The influenza A viruses currently circulating in humans probably originated approximately 150 years ago from influenza viruses in avian species. The available information suggests that the catastrophic 1918 'Spanish' influenza originated from swine supporting the notion of the pig as the intermediate host. The highly virulent strains died out but gave rise to the current human influenza A lineage. The influenza A viruses circulating in swine and horses, and occasional isolates from seals and mink, also originate from avian sources.

Serologic Relationships and Variability

Influenza A and B viruses are distinguished antigenically on the basis of their NP and M proteins. The subtypes of influenza A are distinguished by serologic differences between their HA and NA proteins. Influenza A and B viruses of mammals are unique among the respiratory tract viruses in that they undergo continuous variation; thus, vaccines must be changed almost every year. The surface antigens of the influenza A viruses undergo variation by both antigenic drift and antigenic shift; antigenic drift occurs in types B and C virus but shift occurs only in influenza A.

Antigenic drift in the HA and NA occurs by point mutations in the gene, leading to an accumulation of amino acid sequence changes that alter the antigenic sites such that they are no longer recognized by the host's immune system. Antigenic drift can be mimicked in the laboratory by growing influenza viruses in the presence of monoclonal antibodies to the HA or NA.

Antigenic shift occurs only in influenza A viruses and is caused by a more radical change in the HA and/or NA. After antigenic shift, influenza viruses appear in the population with surface antigens unlike those of viruses immediately preceding them. Since the first human influenza virus was isolated in 1933, antigenic shifts have occurred in 1957, when H2N2 subtype (Asian Strain) replaced the H1N1 subtype, in 1968, when the Hong Kong (H3N2) virus appeared, and in 1977, when the H1N1 virus reappeared. All these major antigenic shifts in the virus occurred in China. Serologic evidence suggests that since 1890 there have been six antigenic shifts (**Fig. 3**). There has been a cyclic appearance of three HA subtypes: H2N8 viruses in 1890, H3N8 in 1900, H1N1 in 1918, H2N2 again in 1957, and H3N2 in 1968, and H1N1 in 1977.

Epidemiology

The available evidence indicates that new human influenza strains may result from genetic reassortment between influenza virus in humans and in lower animals and birds and that these viruses originate in China. There is ample evidence for genetic reassortment of the segmented RNA genome, and sequence analysis has established that three of the eight segments of the Asian 57 strain came from avian sources (HA, NA and one polymerase PB1). The 1968 Hong Kong virus obtained an HA and a PB1 gene from an avian source. Pigs are susceptible to infection with avian and human influenza viruses and the possibility has been raised that they may serve as the mixing vessel.

It is about 30 years since a novel influenza subtype emerged in humans and a new pandemic is considered imminent. The direct transmission of an avian H5N1 influenza virus to humans in Hong Kong in 1997 with 18 confirmed cases and six deaths might represent the early stages of development of a pandemic. The H5N1 virus was prevalent in the live bird markets and was highly pathogenic for chickens but nonpathogenic in ducks. This virus failed to transmit efficiently from human to human and transmission to humans from avian sources was interrupted by slaughter of the poultry in Hong Kong. The depopulation of poultry, cleaning of markets and the new policy of marketing

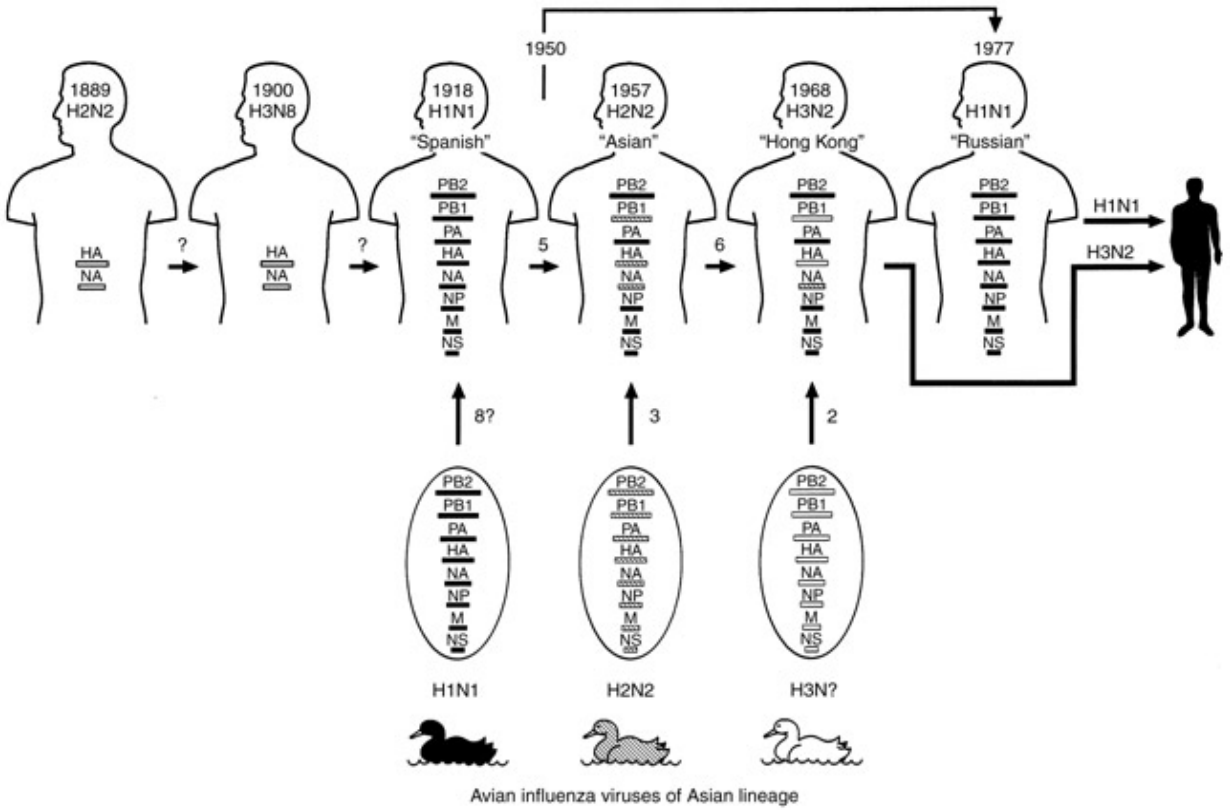


Figure 3 Emergence of influenza A pandemic viruses. Seroarcheology suggests that H2N2 and N3N8 influenza viruses circulated in humans in 1989 and 1990 respectively. Phylogenetic evidence suggests that an influenza virus possessing eight gene segments from avian influenza reservoirs was transmitted to pigs and before 1918 it spread and replaced the 1900 strain. This virus was reportedly carried from North America to Europe by American troops and caused the catastrophic Spanish influenza pandemic of 1918. In 1957 the Asian pandemic virus acquired three genes (*PBI, HA, NA*) from the avian influenza gene pool in wild ducks by genetic reassortment and kept five other genes from the circulating human strain. After the Asian strain appeared, the H1N1 strains disappeared from humans. In 1968 the Hong Kong pandemic virus acquired two genes (*PBI, HA*) from the duck reservior by reassortment and kept six genes from the virus circulating in humans. After the appearance of the Hong Kong strain, the H2N2 Asian strains were no longer detectable in humans. In 1977 the 'Russian' H1N1 influenza virus that had circulated in humans in 1950 reappeared and spread in children and young adults. This virus probably escaped from a laboratory and has continued to cocirculate with the H3N2 influenza viruses in the human population.

live chickens separate from aquatic birds may influence interspecies transmission.

It is unclear how all of the subtypes of influenza A viruses are maintained in aquatic birds from year to year. Influenza viruses are cleared from ducks in approximately 7 days, and there is no evidence of persistence of influenza virus genetic information in any species. The available data support the hypothesis that the viruses are maintained by circulation at low levels in the avian population during migration and overwintering.

Transmission and Tissue Tropism

Influenza viruses of humans and other mammals are spread by aerosols, including sneezing. The virus replicates in the cells of the upper and lower respiratory tract, reaching a peak at 2–3 days after infection, and is usually cleared in 7 days. Children experiencing their first infection can shed virus for up to 13 days.

Influenza viruses of birds are usually spread by fecal contamination of water but can also be spread by aerosols. Unlike mammalian influenza viruses, which combine with receptors in the respiratory tract, avian influenza viruses combine with receptors in both the respiratory and lower intestinal tracts. The HA molecule combines with cell surface sialic acid-bearing glycoproteins and glycolipids. In aquatic birds, viruses are shed in high concentrations in the feces, which probably serve as the main vector for transmission to domestic poultry.

Pathogenicity

Influenza virus strains differ in pathogenicity: some viruses cause substantial mortality in their hosts, whereas others do not. The immune status of the population and the extent of antigenic differences between strains are of key importance. Since 1957, the most serious outbreaks of disease in humans have corresponded to the introduction of 'new' pandemic strains, but interpandemic variants can also be associated with high mortality rates. The disease resulting from influenza viral infection is a complex event involving both the virus and the host. The viral genes responsible for high degrees of pathogenicity are better resolved for influenza viruses in chickens than in humans. The HA is of critical importance, but the correct combination of genes must also be present and usually include the NP, one of the polymerase genes and NA. In avian strains, a series of basic amino acids at the cleavage site between HA1 and HA2 is correlated with the potential for high pathogenicity (*See also:* **Influenza viruses – molecular biology** and **structure of antigens**); avirulent strains have a single arginine at this site. It is not known whether this finding extends to mammalian influenza strains. Equine H7 influenza virus, which possesses such a sequence, is not highly pathogenic in horses.

Clinical Features of Infection

In humans, influenza is characterized by the sudden onset of an acute respiratory illness with headaches, chills and nonproductive cough, followed by high fever, muscle aches, generalized weakness and loss of appetite. The fever declines by the third day and is usually gone by the sixth day, but cough and weakness can persist for an additional 2 weeks. More severe illness can develop if primary influenza pneumonia or secondary bacterial pneumonia occurs. People over 65 years of age and those with health problems, such as heart conditions, emphysema, asthma or acquired immune deficiency syndrome (AIDS), and children and adults receiving cancer chemotherapy are at increased risk for complications from influenza and should receive yearly vaccination. At least 10 000 excess deaths (over expected) have been documented in each of 19 different US epidemics in the period 1957–1986; in three epidemics there were >40 000 excess deaths. Approximately 80–90% of the excess deaths attributed to pneumonia and influenza were in people aged 65 years or older. Reye's syndrome, an often-fatal disorder, may occur in children after influenza infection most often with influenza B with brain and liver damage. Reye's syndrome is associated with the use of aspirin compounds and its incidence has declined with reduced use of these compounds.

Disease signs in pigs and horses are similar to those in humans. Influenza also occurs in birds, mainly aquatic species, with no detectable disease signs. In domestic species such as chickens and turkeys, the symptoms are usually mild with respiratory signs. However, two subtypes, H5 and H7, can cause generalized infection with central nervous system involvement and very high mortality in chickens and turkeys.

Pathology and Histopathology

Human influenza A virus induces pathologic changes throughout the respiratory tract, with the most significant pathology seen in the lower respiratory tract. Bronchoscopy of persons with uncomplicated influenza infections shows a diffuse involvement of the larynx, trachea, and bronchi, with acute mucosal inflammation and edema. Light microscopic studies reveal that within 1 day after the onset of symptoms, there is severe desquamation of the ciliated and mucus-producing epithelial cells, and thickened hyalinized basement membrane is exposed. Submucosal edema and hyperemia occur with an infiltration by neutrophils and mononuclear cells. Viral antigen is present predominantly in the epithelial cells and the mononuclear cells. Complete resolution of the epithelial necrosis probably takes up to a month. Viral pneumonia involves the spread of virus to the lung parenchyma. Combined viral and bacterial pneumonia often occurs in elderly and immunocompromised patients.

Immune Response

Recovery from influenza viral infection involves both humoral and cell-mediated responses. The surface glycoproteins are of the most importance in the humoral response, and internal proteins predominate in the cellular response. Mucosal [immunoglobulin (Ig)A] and serum (IgG) antibodies to the HA molecule neutralize viral infectivity and are primarily responsible for resistance to infection. This is the basis of vaccination against current epidemic strains with killed virus. The Ig response to the HA is subtype specific, but accumulation of point mutations (antigenic drift) permits infectious virus to escape antibody-mediated destruction. Five antigenic sites associated with protection have been identified on the globular head of the H3 molecule. Antibodies to the NA do not prevent infection, but reduce the spread of virus.

Influenza viruses are normally cleared by CD8+T virus-specific T lymphocytes. CD4+T cells do not clear influenza virus as effectively as CD8+T cells; however, their presence is required for antibody production although they can also contribute to the lung consolidation that is characteristic of influenza pneumonia. Infection with any influenza A virus primes for a secondary T cell response to any other influenza A virus because many of the CD8+T cells are specific for peptides of conserved internal viral proteins that are presented in a class I-restricted manner. The NP is particularly prominent in this regard, although the M protein, as well as NS and polymerase proteins may contribute to antigenicity. Macrophages, natural killer cells and immune T lymphocytes all play a role in virus clearance and release interferon, which inhibits influenza viral replication.

Prevention and Control of Influenza

Two options are available for the control of influenza A virus: vaccination or therapy with influenza specific antiviral agents. With increased global surveillance by the World Health Organization, vaccines and epidemic strains are usually well matched and effective at providing protection against influenza A and B.

The available vaccines include purified inactivated egg-grown whole virus or purified surface antigens. Only surface antigens (subvirion vaccines) are recommended for children under 12 years, because the whole-virus vaccine causes febrile responses in children. Persons with known allergies to eggs should not be given egg-grown vaccine. Cold-adapted attenuated live influenza vaccines have been shown to be safe and effective in experimental trials and are currently under review for licensing for general use.

The antiviral agents amantadine and rimantadine that target the M2 ion channel are only efficacious on influenza A viruses and resistant variants arise rapidly. Antiviral agents targeting the NA (Zanamivir, GS4071 and GS4104) are efficacious against influenza A and B viruses and clinical testing shows considerable promise.

Future Perspectives

Continuing antigenic drift and irregular antigenic shift are neither predictable nor preventable. The natural reservoirs of influenza viruses in aquatic birds throughout the world means that influenza is not eradicable. Thus the long-term perspective for influenza is the development of improved control strategies. It is considered inevitable that there will be another human influenza pandemic and as time passes the likelihood increases. Planning for the next human pandemic has been done in many countries and is promoted by the World Health Organization. Pandemic plans include improved ability to respond to antigenic drift on an annual basis with increased surveillance, rapidity of preparing vaccines, and the utilization of available antiviral drugs. Improvements in dealing with antigenic drift will ensure the ability to rapidly implement a pandemic plan.

It is recognized that we are ill equipped to deal with the emergence of a 1918-style pandemic that immediately causes high mortality and transmits rapidly. Increased human surveillance particularly in China and the possibility of an early warning system that involves the detection of novel influenza viruses in pigs may provide additional lead-time for vaccine preparation. There is an urgent need for highly efficacious anti-influenza drugs. The NA inhibitors are showing promise but additional targets need to be investigated. Stockpiling of amantandine and rimantadine must be considered. The preparation of high growth reassortant strains to each influenza A subtype and methods to broaden the immune response with crossreactive determinants including the use of new adjuvants are necessary. Other strategies including live attenuated vaccine strains to all subtypes and DNA vaccine strategies requires additional study and incorporation into a strategic plan that is updated on a regular basis – minimally every 3 years.

See also: **Antivirals; Epidemiology of viral diseases; Geminiviruses (*Geminiviridae*); Immune response: Cell mediated Immune response, General features; Influenza viruses (*Orthomyxoviridae*): Molecular biology, Structure of antigens; Respiratory viruses; Virus structure: Atomic structure, Principles of virus structure.**

Further Reading

Brown LE, Hampson AW and Webster RG (eds) (1996) *Options for the Control of Influenza III*. Amsterdam: Elsevier Press.

Monto AS (1997) Pandemic influenza: confronting a re-emergent threat. *J. Infect. Dis.* (Suppl.) 176: S1.

Murphy BR and Webster RG (1996) Orthomyxoviruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 1397. Philadelphia: Lippincott–Raven.

Molecular Biology

Peter Palese and **Adolfo García-Sastre**, Mount Sinai School of Medicine, New York, USA

Properties of the Virion

Influenza viruses are spherical enveloped viruses of approximately 120 nm in diameter, but large filamentous forms can also be observed in the electron microscope, especially when polarized cells are infected. The viral glycoproteins hemagglutinin (HA) and neuraminidase (NA) are inserted in the external viral envelope and form spike-like structures. The HA protein is the major surface glycoprotein in influenza A and B viruses, and appears as an elongated homotrimer, while the NA protein forms a mushroom-shaped homotetramer. In contrast to influenza A and B viruses, influenza C viruses appear to have only one type of glycoprotein, the hemagglutinin/esterase (HE or HEF), which is anchored in the lipid bilayer membrane. In addition, the envelope of influenza A, B and C viruses contains small amounts of another integral membrane protein, the M2, NB and CM2 proteins, respectively. Just beneath the viral envelope of all influenza viruses is a protein layer made up of the M1 protein. The viral ribonucleoprotein (RNP) core consists of the viral RNAs which are associated with the nucleoprotein (NP) and the polymerase proteins (PB1, PB2 and PA). A scheme of the organization of an influenza A viral particle is shown in **Fig. 1**.

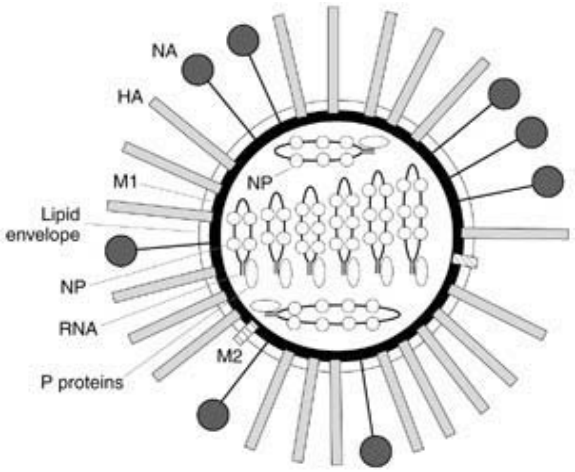


Figure 1 An influenza A viral particle. The virion contains a lipid envelope in which three different types of transmembrane proteins are anchored: the hemagglutinin (HA) and the neuraminidase (NA) form the characteristic viral spikes which can easily be seen by electron microscopy; the M2 protein is a nonabundant short transmembrane polypeptide. Inside the envelope there is a protein layer constituted by the M1 protein (filled ring), which surrounds the viral core or ribonucleoproteins (RNPs). Each RNP consists of one single-stranded RNA segment associated with the nucleoprotein (NP) and the three polymerase (P) proteins. In addition, the NEP(NS2) protein might also be a structural component present in the inside of the viral particle. RNA segments exist in a circular conformation stabilized by base-pairing between their 3′ and 5′ ends. Eight different RNA segments can be found per virion.

Properties of the Genome

The genomes of influenza A and B viruses consist of eight different single-stranded RNA segments of negative polarity; that of influenza C viruses has only seven segments. The coding capacity of these genomes is approximately 13 600, 14 600 and 12 900 nucleotides for A, B and C viruses, respectively, and the sequences of many different strains have been obtained. The RNA segments of influenza A viruses exist in the virion in a circular conformation due to a terminal panhandle/fork/corkscrew structure stabilized by base-pairing between the 3′ and 5′ ends of the RNA segments. Although it has not yet been proven, it is assumed that this is the case for B and C viruses as well.

When influenza viruses are grown at high multiplicity, defective interfering (DI) particles may be produced. These DI particles contain one or more extra RNA segments whose 3′ and 5′ ends are identical to those of normal influenza viral RNAs. The DI RNAs are caused by internal deletion of influenza viral RNA segments, and their presence makes the virus defective. It also appears that the DI RNAs may interfere with viral replication.

Properties of the Proteins

The major surface glycoprotein of the virus is the HA, a polypeptide of approximately 560 amino acids. The HA undergoes various post-translational modifications during its transport to the plasma membrane, including trimerization, glycosylation, disulfide bond formation, palmitoylation, proteolytic cleavage and conformational changes. A diagram of the three-dimensional structure of the mature HA is shown in **Fig. 2A**. After proteolytic cleavage, two polypeptides are produced, the HA1 and the HA2; they remain linked by a cysteine bridge. Two principal functions have been found for the HA protein: (1) it is responsible for attachment of the virus to host-cell receptors, which are believed to be glycoproteins or gangliosides containing sialic acids; and (2) it possesses fusion activity. After attachment, the virus is

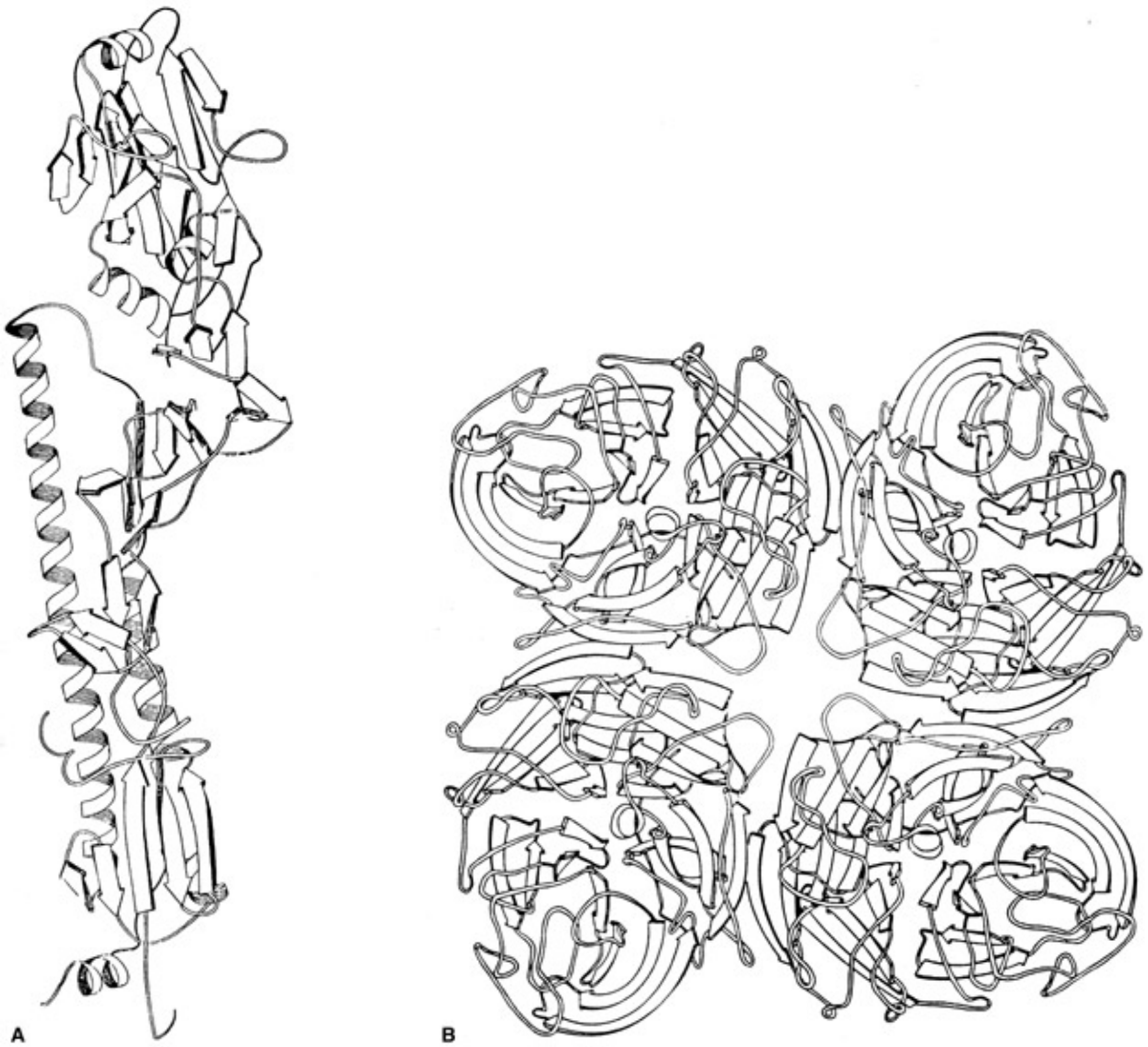


Figure 2 (**A**) Diagram of an H3 hemagglutinin kindly provided by Dr D. Wiley. The chain tracing shows a side view of a single monomer. The β chains (broad arrows) and the helices are indicated. (**B**) The four identical subunits of an NA spike viewed from above. The chain tracing has been kindly provided by Dr G. Air and Dr M. Luo and is based on the x-ray diffraction analysis of the crystallized molecule. Each of the four subunits has an unusual array of six β sheets. Each of these β sheets (resembling blades of a propeller) consists of four connected strands (wide arrows).

internalized into a cellular endosome. Acidification of this vesicle provokes a conformational change in the HA. The N-terminal region of the HA2, which is hidden inside the HA molecule, rearranges to form an extended coiled-coil structure, leading to the interaction of the endosome membrane with the hydrophobic N-terminus of the HA2. As a result, the viral envelope and the endosome membranes are fused, and the viral nucleocapsid is released into the cytoplasm of the host cell. The fusion activity of the HA is dependent on its being cleaved, and the susceptibility of cleavage of the HA has been correlated with virulence of virus in tissue culture and animals. In addition to the receptor binding and fusion activities, the HA protein contains the principal antigenic determinants, and it is assumed that antigenic variation of the HA is the major factor which allows the virus to escape the host immune response.

Like the HA, the NA is also a transmembrane glycoprotein. It contains approximately 460 amino acids, distributed along a short internal tail, a transmembrane domain, a stalk and a globular head. The three-dimensional structure of the head is represented in **Fig. 2B**, and consists of six β-sheet structures resembling a propeller with six blades. The NA protein is an integral membrane glycoprotein (of

type II) anchored in the lipid envelope by its N-terminus. It is found in the virion as a homotetramer. Because of its sialidase (neuraminidase) activity, this protein is responsible for the receptor-destroying activity of the virus. It appears that the NA facilitates the release of the new viruses from the host cell. In the absence of this enzyme, viruses remain bound to the cellular receptors by their HA proteins. The NA also removes the sialic acid present on the virus particles, preventing their aggregation to one another.

Influenza C viruses contain only one type of viral spike glycoprotein, the HEF protein, which is responsible for the receptor-binding, receptor-destroying and fusion activities of the virus. The cellular receptor for the influenza C virus is known to be the 9-*O*-acetyl-*N*-acetylneuraminic acid, and its receptor-destroying enzyme is not an NA, but a neuraminate-*O*-acetylesterase. Like the HA protein of A and B viruses, the HEF of influenza C viruses must be cleaved in order to exhibit membrane fusion activity.

The M2 protein of influenza A viruses is a small polypeptide ($M_r \approx 11\,000$) encoded by a spliced form of the mRNA derived from RNA segment 7. It contains a cytoplasmic C-terminus, a transmembrane domain and an external N-terminus and it is relatively abundant as a homotetramer in the plasma membrane of infected cells, but very rare in the virion (20–60 molecules per virus). In order to explain this discrepancy, an exclusion mechanism from the viral envelope could be proposed for the M2 protein. Mutations in the M2 protein have been associated with the resistance of the virus to amantadine and rimantadine, which are chemotherapeutic agents against influenza. It has been found that this polypeptide forms an ion channel, which is important during the uncoating as well as the viral maturation steps. Thus, the M2 protein pumps protons from the endosome to the inside of the virus during viral entry, leading to acidification of the viral cores and to dissociation of the M1 protein (uncoating) from the viral RNP complexes.

The RNA segment 6 of influenza B viruses codes for the NB protein and the NA protein; the latter is coded for via a second open reading frame with an alternative AUG initiation codon. The NB is a small glycosylated transmembrane polypeptide ($M_r \approx 18\,000$), structurally similar to the M2 protein of A viruses. It also serves as an ion channel and it may have functions similar to those of the M2 protein in influenza A viruses. Influenza C viruses also express a small integral homotetrameric membrane glycoprotein (the CM2 protein) from the same RNA segment which encodes the M1 protein. This glycoprotein, as the M2 and NB proteins, seems to be incorporated at low levels into the viral envelope and may also be an ion channel involved in viral uncoating.

The M1 protein is a polypeptide of $M_r \approx 28\,000$ and is encoded by RNA segment 7 of influenza A virus. It is the most abundant protein in the virion, and appears as a peripheral membrane protein surrounded by the viral lipid envelope. During uncoating, the M1 protein must dissociate from the RNPs, which then are transported into the nucleus. During the late period of viral replication, the newly synthesized M1 proteins appear to inhibit viral RNA transcription and they may have an indirect or direct role in promoting the exit of the RNPs from the nucleus into the cytoplasm. In any case, the M1 protein seems to be responsible for preventing the reimport of the RNPs into the nucleus. In addition, it has been postulated that the M1 protein associated with the RNPs interacts with the plasma membrane and possibly with the viral HA, NA and M2 proteins and/or with host membrane proteins, playing a key role in the budding of the virus from the host cell.

The polymerase proteins PB1, PB2 and PA are encoded by the three largest RNA segments. Their molecular weights are each around 80 000–90 000 and together they form a complex which possesses RNA-dependent RNA polymerase activity. Approximately 50 polymerase complexes are found per virion. The P proteins contain karyophilic signals in their sequences and they accumulate readily in the nucleus of infected cells. The PB2 protein seems to be involved in the recognition and cleavage of the type I cap structures of the cellular mRNAs, which then are used as primers for influenza viral RNA transcription. The PB1 protein is the catalytic polymerase subunit and is required for the initiation and elongation of the newly synthesized viral RNA. The PA protein may be involved in viral RNA replication and, in addition, the expression of the PA protein in infected cells has been associated with proteolytic activity. The functional significance of the latter activity is not yet understood.

The NP protein is the major structural protein of the RNPs and it has been found to be associated with the viral RNA segments. The NP is an arginine-rich protein ($M_r \approx 55\,000$) of approximately 500 amino acids. Each NP molecule covers approximately 20 nucleotides of the viral RNAs. The NP mediates the transport of the incoming viral RNPs from the cytoplasm into the nucleus by interacting with the cellular karyopherinα/importinα transport machinery. In addition, the NP plays an important role during viral RNA synthesis, and free NP molecules are required for full-length viral RNA synthesis, but not for viral mRNA transcription. The NP protein is the major antigenic target for cytotoxic T lympho-

cytes. It contains an immunodominant T cell epitope which is recognized in association with class I proteins of the major histocompatibility complex.

The NS1 (M_r between 26 000 and 18 000, depending on the virus strain) and NEP (NS2) ($M_r \approx 11\,000$) proteins are viral proteins derived from RNA segment 8 by alternative splicing of the mRNA. The nonstructural NS1 protein is an RNA binding protein which can be found in the cytoplasm and in the nucleus of infected cells. Several different functions have been proposed for this protein. In the nucleus, it seems to inhibit the processing of the cellular mRNAs at several steps, including polyadenylation, splicing and RNA transport mechanisms. The NS1 also appears to specifically enhance in the cytoplasm the translational rate of the viral mRNAs. Finally, the ability of the NS1 protein to bind double-stranded RNA, which could be formed during viral RNA replication and transcription, might prevent the activation of antiviral (interferon) pathways by these RNA species.

The NS2 or NEP (*n*uclear *e*xport *p*rotein) of influenza A viruses has recently been shown to have a nuclear export signal similar to the Rev protein of HIV. Since, in addition, the NEP is synthesized at late times during viral infection, it has been proposed that it mediates the nuclear export of the viral RNPs which is a prerequisite for viral budding. Interestingly, the NEP and M1 proteins interact in *in vitro* assays, and it is thought that the nuclear export of RNP molecules is facilitated through the association of the M1 protein with the NP (and the NEP). It will be interesting to investigate whether the NS2 proteins of influenza B and C viruses have similar nuclear export activities. In contrast to the previously believed nonstructural character of the NS2/NEP, this protein appears to be incorporated into virus particles via its interaction with the M1.

Another viral polypeptide (BM2), of $M_r \approx 12\,000$, has been identified in influenza B virus-infected cells. This protein has no known counterpart in influenza A and C viruses. It is encoded by a second open reading frame of RNA segment 7 of influenza B virus, and its function has not been determined.

Physical Properties

In general, influenza viruses show a sensitivity to denaturing agents comparable to that of other enveloped viruses. Differences in pH, ionic strength and ionic composition of the surrounding medium influence the viral resistance to physical and chemical agents. The infectivity can be preserved in saline-balanced fluid of neutral pH at low temperatures. Influenza viruses are relatively thermolabile, and are rapidly inactivated at temperatures higher than 50°C. Agents affecting the stability of membranes, proteins or nucleic acids, such as ionizing radiation, detergents, organic solvents, etc., reduce or completely destroy the infectivity of the virus.

Replication

Strategy of replication of nucleic acid

The replication and transcription of the genome of influenza virus takes place in the nuclei of infected cells (**Fig. 3**). In the nucleus, the viral RNP is used as template for the viral RNA polymerase complex (PB1, PB2, PA) to produce two different species of RNA: complementary RNA (cRNA), a full-length copy of viral RNA (vRNA); and mRNA, which is capped at the 5′ end and lacks the 3′ 15–16 nucleotides of the cRNA, having instead a poly(A) tail. The minimal promoter for the synthesis of mRNA and cRNA is formed by both the 5′ and 3′ noncoding regions of the vRNA. The cRNA is used as template to generate new vRNA and it also contains a promoter formed by its 5′ and 3′ ends. Promoter sequences of the cRNA and vRNA are similar but not identical. Both promoters consist of short sequences (12–13 nucleotides) which are highly conserved in all RNA segments of influenza A viruses, and they are localized at the precise 3′ and 5′ ends of the RNA. There is ample experimental evidence showing that these ends adopt a secondary structure (in form of a panhandle, fork or corkscrew) which appears to be required not only for replication, but also for transcription, polyadenylation, and packaging of vRNAs. However, other regions outside the conserved RNA ends also seem to influence these processes. Primers have not yet been identified for full-length replication of influenza virus RNAs *in vivo*, but short primers (such as ApG) are required for an optimal *in vitro* reaction.

Characterization of transcription

The vRNA is not only used as template for replication, but also for the synthesis of mRNA. This process is primed *in vivo* by m^7GpppXm-containing short capped RNAs derived from host-cell mRNAs. The primers are generated by endonuclease activity of the viral PB2 protein, which cleaves the 5′ ends of cellular mRNAs when vRNA specific sequences are also present. Binding of the viral polymerase complex to the 5′ ends of the viral mRNAs might prevent their cleavage by the PB2. Polyadenylation of the viral mRNA requires the panhandle structure of the vRNA and a juxtaposed stretch of five to seven uridines. It has been hypothesized that the panhandle structure and/or proteins bound to this panhandle, such as the

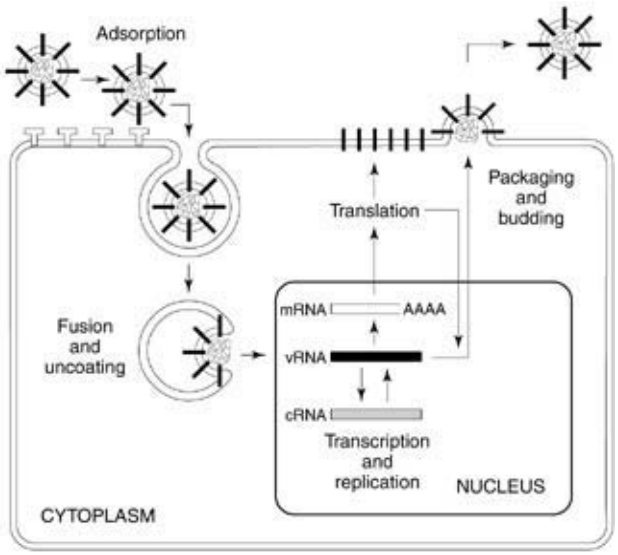


Figure 3 Replicative cycle of influenza virus. (1) Adsorption: the virus interacts with sialic acid-containing cell receptors via its HA protein, and is internalized by endosomes. (2) Fusion and uncoating: the HA undergoes a conformational change mediated by the acid environment of the endosome, which leads to the fusion of viral and cellular membranes. The inside of the virus also gets acidified due to proton trafficking through the M2 ion channel. This acidification is responsible for the separation of the M1 protein from the ribonucleoproteins (RNPs), which are then transported into the nucleus of the host cell thanks to a nuclear localization signal in the NP. (3) Transcription and replication: the viral RNA (vRNA) is transcribed and replicated in the nucleus by the viral polymerase. Two different species of RNA are synthesized from the vRNA template: (a) full-length copies (cRNA), which are used by the polymerase to produce more vRNA molecules; and (b) mRNA. (4) Translation: following export into the cytoplasm the mRNAs are translated to form viral proteins. The membrane proteins (HA, NA and M2) are transported via the rough endoplasmic reticulum and Golgi apparatus to the plasma membrane. The viral proteins possessing nuclear signals (PB1, PB2, PA, NP, M1, NS1 and NEP) are transported into the nucleus. (5) Packaging and budding: the newly synthesized NEP protein appears to facilitate the transport of the RNPs from the nucleus into the cytoplasm by bridging the RNPs with the nuclear export machinery. M1–RNP complexes are formed which interact with viral proteins in the plasma membrane. Newly made viruses bud from the host cell membrane.

viral polymerase complex, provides a physical barrier for transcription, and that the RNA polymerase stutters at the stretch of uridines, thus adding the poly(A) tail. The melting of the panhandle structure in the presence of free viral NP could itself be the *cis* signal for the switch from mRNA to cRNA synthesis.

Characterization of translation

The influenza viral mRNAs use the cellular translation machinery to synthesize the viral proteins. While translation of viral mRNAs is a very efficient process, translation of the host cell mRNAs is inhibited in influenza virus-infected cells. The precise mechanism for this inhibition is not well understood. Interestingly, the virus has evolved several mechanisms by which it can increase its coding capacity. They include: partial splicing of the mRNA, coupled stop–start translation of tandem cistrons and ribosomal choice between two possible initiation codons.

The RNA segment 7 of influenza A virus encodes two proteins, M1 and M2. The M2 protein is produced by translation of an mRNA derived from the M1 mRNA by splicing; it shares only its first eight amino acid residues with the M1 protein. Because of a frameshift resulting from the splicing, the remaining amino acids of the M2 are different from those of the M1. The mRNA coding for the M1 protein of influenza C virus also appears to be spliced. Surprisingly, the spliced mRNA actually encodes for the M1 protein. Recently, a protein product, the CM2 protein, which is translated from the unspliced mRNA has been identified. The CM2 protein is most likely generated by cleavage of the precursor protein.

RNA segment 8 of influenza A and B viruses and RNA 7 of C viruses also have coding capacities for two different proteins, the unspliced forms of their mRNAs code for the NS1 proteins, and the spliced forms for the NS2 (NEP) proteins. The ratio of spliced to unspliced mRNA is about 1:10. The viral splicing sequences share the consensus sequences of eukaryotic mRNAs, which in general undergo complete (efficient) splicing. It has been suggested that both *cis*- and *trans*-acting signals (i.e. the NS1 protein) are involved in the regulation of splicing in influenza viral mRNAs.

Two different polypeptides, the NA and NB proteins, can be translated from the influenza B virus segment 6. If initiation of the translation takes place at the first AUG of the mRNA, the NB protein is synthesized; the second AUG, separated from the first by four nucleotides, initiates the synthesis of the NA protein.

Segment 7 of influenza B viruses is used for the synthesis of the M1 protein. Cellular ribosomes, after the stop codon for the M1 protein, appear to reinitiate translation at an AUG codon overlapping the stop codon UAA in the pentanucleotide sequence UAAUG. This process, which is similar to that observed in multicistronic bacterial genes, leads to the synthesis of the BM2 protein.

Post-translational processing

The viral protein that incurs the largest number of post-translational modifications is the HA protein.

While it is being synthesized, a signal peptide in the N-terminal sequence of the nascent polypeptide mediates its attachment to the membrane of the rough endoplasmic reticulum (RER). The signal peptide is then cleaved and the HA molecule remains attached to the membrane of the RER by its C-terminal sequence. After synthesis is completed, the HA protein follows the exocytic cellular pathway, and in this process it is folded, assembled into trimers, glycosylated in its extracellular domain, and palmitoylated in the cysteine residues of its cytoplasmic tail. Finally, the molecule is cleaved into its two subunits, HA1 and HA2, which remain bound by a disulfide bridge. Cleavage of the HA is essential for its fusogenic activity.

The NA protein also follows the exocytic cellular pathway: it is post-translationally folded, assembled into tetramers and glycosylated. However, in contrast to the HA and the majority of cellular transmembrane proteins, its signal peptide is not cleaved, and the NA is attached to the plasma membrane by its N-terminal sequence. Other viral proteins that undergo post-translational modifications are the M2 protein, which is phosphorylated and palmitoylated at its cytoplasmic tail, the NB and CM2 proteins of influenza B and C viruses, which in addition are glycosylated, and the NP, M1, NS1 and NEP(NS2) proteins, which can be phosphorylated. The precise functional significance of all these modifications is not yet clear.

Packaging of vRNA, assembly and release of virus

At late times in infection, the newly synthesized RNPs move back from the nucleus to the cytoplasm. The precise regulation of this transport is not yet understood. It has been proposed that the late viral protein NEP plays a key role in this process (see above). In addition, the M1 protein and unknown phosphorylation events might be involved. Once in the cytoplasm, the RNPs (in association with the M1 protein) interact with the plasma membrane, where the viral transmembrane proteins HA, NA and M2 are inserted. The M1 protein is probably responsible for the virus budding, selecting the viral envelope proteins via specific interactions. Although the cytoplasmic tails of the HA, NA and M2 proteins are the most likely candidates for these specific interactions with the M1 protein, viable viruses in which the cytoplasmic domains of the HA and the NA were deleted have been generated. In any case, the new viral progeny buds from the infected cell, with the viral envelope derived from the plasma membrane of the infected host cell.

It has been demonstrated that the 3′ and 5′ noncoding regions of the vRNAs contain the *cis* signals required for packaging, as reporter genes containing only these noncoding sequences are packaged into virions. The question then arises as to whether the eight vRNA segments are selectively chosen one by one for packaging into a viral particle or whether they are randomly packaged. The difficulty of elaborating a mechanism of selective packaging that would involve complex and specific interactions among each RNA segment and possibly the nucleocapsid proteins and the M1 protein favors the random packaging hypothesis. In this model, it is assumed that influenza viruses package on average 11–12 RNA segments per particle; these segments are randomly taken from a pool of eight different vRNAs, all of which carry the same packaging signal. Only virus particles with a full complement of the eight different RNA segments (among the 11–12 randomly packaged ones) are infectious. Based on statistical analysis, this model is compatible with the finding that only a small percentage (less than 10%) of influenza virus particles are infectious. Supporting this theory, it was shown directly that influenza viruses can package additional segments. Using genetic engineering methods (see below), a virus was constructed which required nine different RNA segments in order to be infectious. However, observations that DI RNA segments derived from specific influenza virus vRNAs are able to compete for packaging into virus particles with their parental vRNA segment would favor a selective packaging model.

Genetic Manipulation of Influenza Viruses

Unlike the RNA of positive-strand RNA viruses, the RNA of negative-strand RNA viruses is not infectious. As a consequence, it is not possible to obtain recombinant influenza viruses containing site-specific mutations by solely transfecting cDNA-derived viral genomic RNA into cells. However, several systems have been developed which allow the *in vitro* or *in vivo* reconstitution of a biologically active RNP complex and subsequent rescue of infectious virus using plasmid-derived RNA. Briefly, synthetic viral RNA transcribed from plasmid cDNA *in vitro* is incubated with purified viral NP and polymerase proteins and the reconstituted RNPs are transfected into cells. Subsequent infection with a helper virus allows the introduced RNP to be replicated, transcribed and packaged (**Fig. 4**). Alternatively, RNPs can be reconstituted *in vivo* by transfecting into cells expression plasmids for vRNA and NP and polymerase proteins. These RNPs then can be packaged into new virions if the cells are

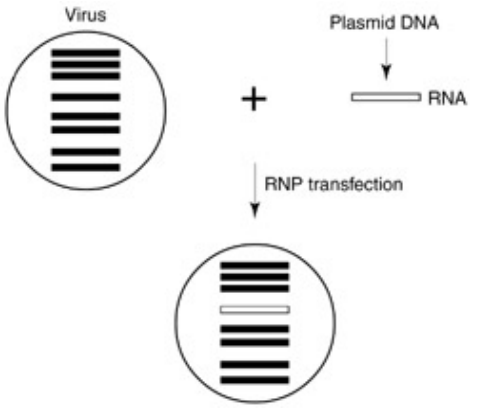


Figure 4 Genetic manipulation of the influenza virus genome via cDNA-derived RNA. Plasmid DNA is used to transcribe viral RNA *in vitro*. This RNA (or RNA derived from a genetically altered plasmid) is mixed *in vitro* with purified influenza viral NP and polymerase complex. The resulting ribonucleoproteins (RNP) are transfected into cells which have been infected with helper virus. Genetically altered transfectant viruses can then be rescued from the infected cell.

also infected with a helper virus. These powerful systems now permit mutations to be introduced into the viral genome so that the *cis*-acting sequences required for RNA transcription, replication and packaging may be studied. In addition, the function and properties of the individual viral proteins can be studied using this methodology. Finally, genetically altered influenza viruses are now being made in the laboratory to display specific biological features, such as attenuation properties or expression of foreign genes and antigens. Such an approach may ultimately lead to the development of greatly improved vaccines against influenza and/or other diseases.

See also: **Antivirals; Genetics of animal viruses; Influenza viruses (*Orthomyxoviridae*): General features, Structure of antigens; Vaccines and immune response.**

Further Reading

García-Sastre A and Palese P (1993) Genetic manipulation of negative-strand RNA virus genomes. *Annu. Rev. Microbiol.* 47: 765.

Lamb RA and Krug RM (1996) *Orthomyxoviridae:* the viruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 1353. Philadelphia: Lippincott-Raven.

Murphy BR and Webster RG (1996) Orthomyxoviruses. In: Fields BN, Knipe DM, Howley PM *et al.* (eds) *Fields Virology*, 3rd edn, p. 1397. Philadelphia: Lippincott-Raven.

Structure of Antigens

Jonathan W Yewdell and **Jack R Bennink**, Laboratory of Viral Diseases, NIAID-NIH, Bethesda, Maryland, USA

W Graeme Laver, Influenza Research Unit, John Curtin School of Medical Research, The Australian National University, Canberra, Australia

Introduction

Influenza viruses are classified into three types, A, B and C, on the basis of their type-specific nucleoprotein and matrix protein antigens. Type A influenza viruses are further classified into subtypes based on the antigenic characteristics of their surface antigens, hemagglutinin (HA) and neuraminidase (NA). Fourteen distinct HA subtypes and nine NA subtypes are now recognized in the nomenclature system of the influenza A viruses recommended by the World Health Organization. This section will describe those structures recognized by the antigen specific receptors of B lymphocytes (antibodies; Ab) and T lymphocytes (T cell receptor; TCR).

Structure of the Hemagglutinin (HA)

In type A influenza virus, the HA accounts for about 25% of the viral protein. It is responsible for the attachment of virus to cells and for the penetration of the virus into the cell during the initial stages of infection. Antibodies to the HA neutralize the infectivity of the virus. Variation in the HA glycoprotein is mainly responsible for the continually occurring outbreaks of influenza and for our inability to control these by vaccination.

The HA of the 1968 influenza virus strain A/Aichi/2/68 (H3N2) is synthesized as a single polypeptide chain of 550 amino acids (**Fig. 1**), which is shortened by removal of a single peptide and subsequently cleaved by removal of Arg329 into two chains, HA1 (36.3 kDa) and HA2 (25.7 kDa). These chains are covalently attached by a disulfide bond from HA1 position 14 to HA2 position 137, and the two-chain monomers are associated noncovalently to form trimers on the surface of membranes. Bromelain treatment of virus yields a soluble trimer, BHA of the extracellular regions, containing all of HA1 and the first 175 to 221 amino acids of HA2 but missing the hydrophobic membrane-anchoring peptide.

Three-dimensional structure of the HA

Bromelain-released HA from some influenza A viruses can be crystallized, and from X-ray diffraction

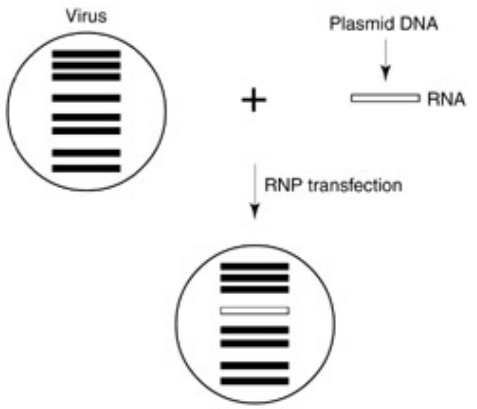


Figure 4 Genetic manipulation of the influenza virus genome via cDNA-derived RNA. Plasmid DNA is used to transcribe viral RNA *in vitro*. This RNA (or RNA derived from a genetically altered plasmid) is mixed *in vitro* with purified influenza viral NP and polymerase complex. The resulting ribonucleoproteins (RNP) are transfected into cells which have been infected with helper virus. Genetically altered transfectant viruses can then be rescued from the infected cell.

also infected with a helper virus. These powerful systems now permit mutations to be introduced into the viral genome so that the *cis*-acting sequences required for RNA transcription, replication and packaging may be studied. In addition, the function and properties of the individual viral proteins can be studied using this methodology. Finally, genetically altered influenza viruses are now being made in the laboratory to display specific biological features, such as attenuation properties or expression of foreign genes and antigens. Such an approach may ultimately lead to the development of greatly improved vaccines against influenza and/or other diseases.

See also: **Antivirals; Genetics of animal viruses; Influenza viruses (*Orthomyxoviridae*): General features, Structure of antigens; Vaccines and immune response.**

Further Reading

García-Sastre A and Palese P (1993) Genetic manipulation of negative-strand RNA virus genomes. *Annu. Rev. Microbiol.* 47: 765.

Lamb RA and Krug RM (1996) *Orthomyxoviridae:* the viruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 1353. Philadelphia: Lippincott-Raven.

Murphy BR and Webster RG (1996) Orthomyxoviruses. In: Fields BN, Knipe DM, Howley PM *et al.* (eds) *Fields Virology*, 3rd edn, p. 1397. Philadelphia: Lippincott-Raven.

Structure of Antigens

Jonathan W Yewdell and **Jack R Bennink**, Laboratory of Viral Diseases, NIAID-NIH, Bethesda, Maryland, USA

W Graeme Laver, Influenza Research Unit, John Curtin School of Medical Research, The Australian National University, Canberra, Australia

Introduction

Influenza viruses are classified into three types, A, B and C, on the basis of their type-specific nucleoprotein and matrix protein antigens. Type A influenza viruses are further classified into subtypes based on the antigenic characteristics of their surface antigens, hemagglutinin (HA) and neuraminidase (NA). Fourteen distinct HA subtypes and nine NA subtypes are now recognized in the nomenclature system of the influenza A viruses recommended by the World Health Organization. This section will describe those structures recognized by the antigen specific receptors of B lymphocytes (antibodies; Ab) and T lymphocytes (T cell receptor; TCR).

Structure of the Hemagglutinin (HA)

In type A influenza virus, the HA accounts for about 25% of the viral protein. It is responsible for the attachment of virus to cells and for the penetration of the virus into the cell during the initial stages of infection. Antibodies to the HA neutralize the infectivity of the virus. Variation in the HA glycoprotein is mainly responsible for the continually occurring outbreaks of influenza and for our inability to control these by vaccination.

The HA of the 1968 influenza virus strain A/Aichi/2/68 (H3N2) is synthesized as a single polypeptide chain of 550 amino acids (**Fig. 1**), which is shortened by removal of a single peptide and subsequently cleaved by removal of Arg329 into two chains, HA1 (36.3 kDa) and HA2 (25.7 kDa). These chains are covalently attached by a disulfide bond from HA1 position 14 to HA2 position 137, and the two-chain monomers are associated noncovalently to form trimers on the surface of membranes. Bromelain treatment of virus yields a soluble trimer, BHA of the extracellular regions, containing all of HA1 and the first 175 to 221 amino acids of HA2 but missing the hydrophobic membrane-anchoring peptide.

Three-dimensional structure of the HA

Bromelain-released HA from some influenza A viruses can be crystallized, and from X-ray diffraction

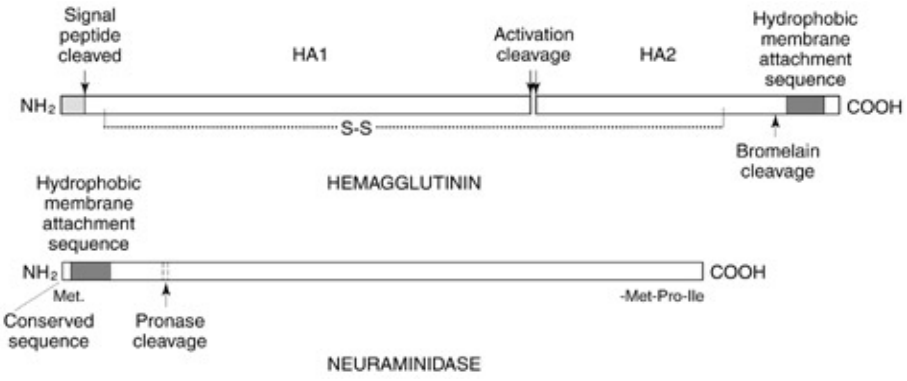


Figure 1 Some features of the HA and NA polypeptides. The HA is synthesized as a single polypeptide. Following its synthesis, an N-terminal signal peptide is cleaved off and the molecule is cleaved further into HA1 and HA2 with the removal of one or more intervening amino acids. This latter cleavage is necessary for the virus to be infectious. HA1 and HA2 remain linked by a single disulfide bond, and each HA spike contains three of these dimers. A sequence of hydrophobic amino acids near the C-terminus of HA2 serves to anchor the HA in the lipid of the viral membrane. Treatment with bromelain removes this hydrophobic region without damaging the rest of the molecule which, in some cases, can be crystallized. The NA is oriented in the viral membrane in the opposite way to the HA. No post-translational cleavage of the NA polypeptide occurs, no signal peptide is split off and even the initiating methionine is retained. No processing at the C-terminus takes place; the C-terminal sequence, Met-Pro-Ile, predicted from the gene sequence, is of N_2NA found in intact NA molecules isolated from virus and in the pronase-released NA heads. A sequence of six polar amino acids at the N-terminus of the NA polypeptide, which is totally conserved in at least eight different NA subtypes, is followed by a sequence of hydrophobic amino acids which probably represents the transmembrane region of the NA stalk. This sequence is not conserved at all between subtypes (apart from conservation of hydrophobicity). Pronase cleaves the polypeptide in the positions shown, removing the stalk and releasing the enzymatically and antigenically active head of the NA which, in some cases, can be crystallized.

data the three-dimensional structure of Hong Kong (H3N2) HA has been determined (**Fig. 2**). Conservation of the structural features such as disulfide bonds suggests that HA molecules from other subtypes will have highly similar structures. The HA glycoprotein of influenza virus is a trimer built of two structurally distinct regions: a triple-stranded coiled-coil of α helices that extends 7.6 nm from the membrane and a globular region of antiparallel β sheet which contains the receptor-binding site. The variable antigenic determinants are located on the globular domain. Each subunit has an unusual loop-like topology; it begins at the membrane, extends 13.5 nm distally, and folds back to enter the membrane (**Fig. 2**).

Structure of the Neuraminidase (NA)

The NA is the second subtype-specific glycoprotein on the influenza virion and is composed of a single polypeptide chain. It exists as a mushroom-shaped spike with a box-like head made out of four coplanar and roughly spherical subunits and a centrally attached stalk containing a hydrophobic region by which it is embedded in the viral membrane. The role of the NA in the life cycle of the virus is still unclear. Mutants of influenza virus lacking NA activity form aggregates at the surface of infected cells, suggesting that one function of the NA is the removal of receptors from the HA, from itself and from the

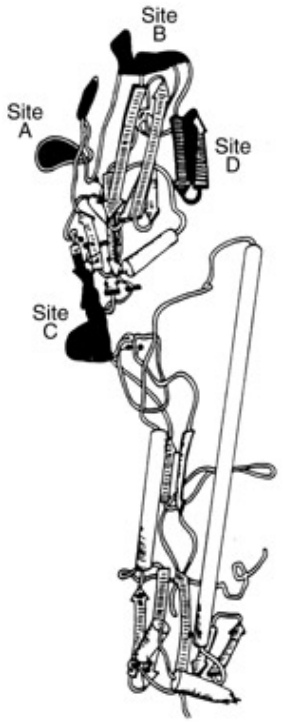


Figure 2 The Hong Kong HA monomer, showing folding of the HA1 and HA2 polypeptides. The four shaded areas show where four independent antigenic areas may be located. Note that in the HA spike, which is composed of three of the monomers, side D is buried and may not be involved in antibody binding.

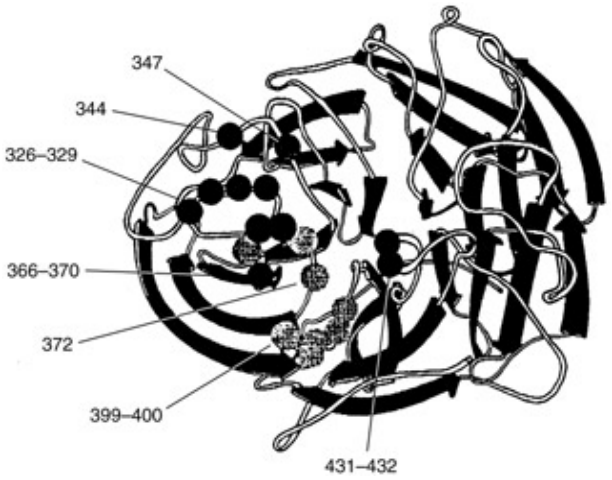


Figure 3 The NC41 epitope on N8 NA. The chain fold in the monomer is viewed down the fourfold axis. The 19 amino acid residues on the NA in contact with antibody are shown as circles: ●, contact to L chain; ◍, contact to H chain; ●, contact to H and L chains.

infected cell surface. This prevents self-aggregation, promotes virus release from the infected cell, and enables the virus to escape from nonproductive interactions, e.g. binding to noncellular sialic acid containing substances such as mucus.

The NA is oriented in the viral membrane in the opposite way to the HA, i.e. the transmembrane portion is at the N terminus (**Fig. 1**). No post-translational cleavage of the NA polypeptide occurs, a signal peptide is not split off and even the initiating methionine is retained. Processing at the C-terminus does not take place, i.e. the C-terminal sequence predicted from the gene sequence is found in intact NA molecules isolated from virus and in the pronase-released NA heads. A sequence of six polar amino acids at the N-terminus of the NA polypeptide, which is totally conserved in at least eight different NA subtypes, is followed by a sequence of hydrophobic amino acids that probably represents the transmembrane region of the NA stalk. Apart from conservation of hydrophobicity, this sequence is not conserved between subtypes. Pronase cleaves the polypeptide, removing the stalk and releasing the enzymatically and antigenically active head of the NA which, in some cases, can be crystallized. The influenza C virus does not have NA activity.

Three-dimensional structure of the NA

Pronase-released NAs have been crystallized, and from X-ray diffraction data the three-dimensional structure has been determined. Influenza viral NA provides the first known example of a tetrameric protein with circular fourfold symmetry. The polypeptide chain folds into six topologically identical four-stranded antiparallel β sheets which are themselves arranged like the blades of a propeller (**Fig. 3**).

The product of catalysis, sialic acid, is observed to bind in a large pocket on the distal surface, flanked and surrounded by nine acidic residues, six basic residues and three hydrophobic residues, all of which are strictly conserved in all known influenza viral NA sequences. The conservation of amino acids around the catalytic site suggests that antibodies cannot exert pressure on that site for its modification or that mutations in this site are lethal.

Structure of Antigenic Determinants (Epitopes) on Proteins

Of an almost infinite variety of B cell epitopes (or antigenic determinants) on protein molecules, the complete structures of very few are known. The single method that can determine the complete structure of an epitope is preparation of a complex of a monoclonal antibody Fab fragment with its antigen, crystallization of this complex and determination of its structure using X-ray diffraction methods.

The term epitope was coined by Niels Jerne in 1960, when he proposed that 'an antigen particle carries several epitopes' (= surface configurations, single determinants, structural themes, immunogenic elements, haptenic groups, antigenic patterns, specific areas). Hidden epitopes which become immunologically available only after breakage, decomposition or denaturation of the antigen are cryptotopes. Inherent in this definition is the concept that an epitope occurs on the surface of a native protein, whereas a linear peptide sequence seen after protein unfolding could occur on the inside or the outside of the folded polypeptide. Processed peptides that would be recognized by T cells in association with major histocompatibility complex (MHC) molecules would actually be included in the latter category and are more appropriately considered cryptotopes than T cell epitopes, as they are now known.

The term T cell epitope is in common usage to describe the peptide sequence on the original protein, although it is not in this native form that it is recognized by the T cell. In contrast, antibodies do recognize epitopes on native proteins, and the complementary paratope on the antibody is exquisitely specific for the native conformation.

The epitopes so far characterized by X-ray crystallography are of the discontinuous type, encompassing 2–5 surface loops. The highly complementary interface between antigen and antibody is absolutely dependent on the folding of the native protein. The

antibody-combining site is seen to be an irregular, rather flat surface with protrusions and valleys formed by amino acid side chains. The epitopes each contain between 15 and 22 residues on the antigen in contact with a similar number of residues on the antibody paratope. Although the interpretation of contacts is somewhat arbitrary (and may differ between laboratories) and the contribution of each to the binding energy (both positively and negatively) is unknown, these provide the best descriptions of epitopes on native proteins so far available.

Despite the consensus that X-ray crystallography has provided of the structure of protein epitopes, there still is a widely held misconception that epitopes on native proteins consist of segments of about seven amino acid residues that can be mimicked or mapped utilizing synthetic peptides of a similar length. In fact, numerous studies claimed to have localized epitopes on native proteins by studying synthetic peptides corresponding to short linear sequences within the protein. However, the success of such approaches with well-defined antibodies has been limited, and most of the crossreactivity seen in these experiments represents binding to denatured protein present in the antigen preparation. Conversely, those cases in which free peptides appear to give good crossreactivity with antiprotein antibodies in solution assays probably represent antibodies originally elicited by denatured protein.

Epitopes on Influenza Viral HA and NA and the Mechanism of Antigenic Drift

The location of epitopes on the HA of influenza viruses is known, but crystals of the complexes of HA and antibody Fab fragments have not yet been reported, so the three-dimensional structure of epitopes on the HA remains to be determined. Escape mutants, isolated by growing virus in the presence of neutralizing monoclonal antibodies to the HA, have single amino acid sequence changes in the HA1 polypeptide that cluster in four regions on the top of the HA globular domain (**Fig. 2**). The crystal structure of these escape mutants (which do not bind the antibody used for their selection) shows that the changes in structure of the HA are restricted to the side chains of the amino acid residues involved, indicating, first, that these residues form part of the epitope recognized by that particular monoclonal antibody, and, second, that conformational alterations in the HA are not required to greatly reduce antibody affinity.

Crystals of influenza viral NA complexed with monoclonal antibody Fab fragments have been obtained, and the three-dimensional structure of one epitope on the NA recognized by monoclonal antibody NC41 has been determined to high resolution, and structures of other antibody–NA complexes are underway. This antibody-binding site (epitope) on NA of the N9 subtype is discontinuous and comprises five chain segments and 19 residues in contact, while 33 NA residues in eight segments have 8.99 nm^2 of surface area buried by the interaction (to a 0.17 nm probe), including two hexose units. Seventeen residues in the NC41 Fab fragment lying in five of the six complementarity-determining regions (CDRs) make contact with the NA, and 36 antibody residues in seven segments have 9.16 nm^2 of buried surface area (**Fig. 3**).

Why B and T Cell Determinants Differ

As alluded to above, influenza virus-specific T cells, unlike most antibodies induced by influenza viral infection, recognize sequential/continuous types of determinants in influenza viral proteins. The different nature of B and T cell determinants reflects a fundamental difference in how antigens are presented to antigen receptors expressed on the surface of B and T cells. Antibody secretion by B cells is triggered by antigen crosslinking of surface antibody. Although there are probably peptides or larger fragments of viral proteins that are either released from virus-infected cells or created by the action of extracellular proteases, these fragments are poorly immunogenic, possibly because their monovalent nature prevents them from crosslinking B cell receptors. By contrast, native influenza viral glycoproteins are highly immunogenic, as they form repeating structures by virtue of their assembly into homo-oligomeric structures that themselves are incorporated in macromolecular assemblies in virions.

T cell activation is similar to B cell activation in requiring antigen–receptor crosslinking. Unlike B cells, however, T cells recognize antigens in a complex with MHC (in humans termed HLA) class I or class II glycoproteins. These proteins are encoded by highly polymorphic genes: indeed, these are the most polymorphic human genes known. For each HLA class I or II locus there are hundreds of alleles present in appreciable frequencies among various populations. Individuals usually express 4–6 different class I molecules and a similar number of distinct class II molecules. The sequential nature of T cell determinants reflects the properties of the antigen-binding sites on MHC molecules. Solution of the structure of class I molecules by X-ray crystallography reveals the antigen-binding site to be a groove comprised of two parallel α helices separated by 1.8 nm, laying atop a platform of eight antiparallel β sheets to form a

2.5 nm long cleft, 1 nm deep. This groove accommodates peptides of 8–9 residues in a fairly extended form. Although longer peptides are capable of binding to class I molecules, they do so with lower affinity, at least in part because the N- and C-termini of the optimally sized peptides form important contacts with residues near the ends of the binding site. Most peptides directly isolated from MHC class I molecules are 8–10 residues in length, although longer peptides are occasionally isolated. The binding of peptides to class I molecules is governed by the highly specific interaction of one or two critical residues in the peptide ('dominant anchors'), with several other residues interacting in a less critical manner with the binding site. The exact type and position of these critical residues differs for interaction with the various class I alleles. In this way, the host is able to sample 4–6 distinct sets of peptides. By pool sequencing peptides recovered from different class I molecules, the dominant anchor residues recognized by numerous human class I molecules have been determined. Using this information, it is often possible to identify peptides in viral proteins that bind the respective class I molecule. Mere binding of the peptide to the class I molecule, however, does not guarantee that the peptide will be immunogenic. Binding is just one factor in an equation that includes the efficiency of antigen processing (see below) and the presence of T cells with TCRs able to recognize the class I–peptide complex.

The three-dimensional structure of a TCR interacting with class I molecules complexed with peptide has been determined. The TCR interacts with the class I molecule over a broad surface, much like the interaction of antibodies with globular proteins. The TCR contacts both the side chains of the 3–4 residues in the peptide that point out of the binding site, as well as residues present in the α helices that form the side walls of the antigen-binding groove.

Despite a limited amount of amino acid homology, the structure of class II molecules is uncannily similar to that of class I molecules. Indeed, in side-by-side representations it is difficult to distinguish them at a glance. There are subtle differences, however, in the nature of the binding pocket that enables class II molecules to bind peptides that extend beyond the binding groove with little or no cost in binding energy compared to binding of smaller peptides. Consequently, there is much greater heterogeneity in the sizes of peptides bound by class II molecules. As a rule, peptides bound to class II molecules are generally around 15 residues in length, although peptides are represented in a continuum ranging from eight residues to full length proteins. As with class I molecules, peptide binding is due largely to the interactions of a few side chains with pockets in the binding groove. Due to the heterogeneity in length, however, rules governing the interactions of peptides with class II molecules are in more primitive state.

Two T Cells

The activation of T cells often requires more than the simple binding of the T cell antigen receptor to a foreign protein bound to MHC molecules. A number of other cell surface molecules on T cells and antigen-presenting cells are usually actively involved in T cell activation. Of particular importance functionally are the interactions of two cell surface glycoproteins, termed CD4 and CD8, the expression of which on almost all mature T cells is mutually exclusive. These molecules function to target the T cell antigen receptor to class II or class I molecules. This is achieved by the direct interaction of peptide-bearing class II and I molecules with CD4 and CD8 molecules, respectively.

The expression of CD4 or CD8 molecules on T cells is coordinately regulated with T cell function. The primary function of T_{CD8+} is to limit the replication of influenza virus. This is achieved by destroying virus-infected cells, and, possibly, by releasing lymphokines with antiviral activity. The primary function of CD4-expressing cells (T_{CD4+}) is to enhance antibody responses and specific and nonspecific cellular immunity. Consistent with these functions, class I molecules are expressed by virtually all cell types, while class II molecules are expressed largely by B cells and inflammatory cells (monocytes, macrophages). Upon exposure to any of several cytokines (most importantly interferon γ and tumor necrosis factor α), class II molecules are induced in many cell types, enabling T_{CD4+} to participate more directly in exerting antiviral effector functions.

General Characteristics of T cell Determinants Derived from Influenza Viral Proteins

Obviously, the first criterion for a given sequence of influenza viral protein to be recognized by T cells is that it bind class I or II molecules with sufficient affinity. Although strict rules have not been established, it appears that the lower end of the dissociation constant required is in the micromolar range (the highest affinity peptides exhibit dissociation constants in the one-tenth nanomolar range). The second criterion is that the determinant be liberated from the viral protein and find its way to the appropriate intracellular compartment where binding to class I or II molecules occurs. The general term for this series of

events is antigen processing. For most peptides destined to bind class I molecules, antigen processing begins in the cytosol. There are two sources of viral proteins in the cytosol. First, during the process of viral penetration, small amounts of viral proteins are delivered to the cytosol from the incoming virions. Second, viral proteins are synthesized in the cytosol by ribosomes from viral mRNAs produced by the viral polymerases from the negative-stranded genome. The important practical consequence of this feature of class I-associated processing is that infectious virus is required to stimulate T_{CD8+} responses.

Viral proteins in the cytosol are cleaved by cellular proteases, including, but probably not limited to, proteasomes. Proteasomes are extremely abundant macromolecular structures that play the essential role of disposing of damaged or unwanted cellular proteins. They are highly conserved in nature, and even proteasomes from yeast are able to produce peptides cabable of binding to class I molecules. Indeed, T cells may have evolved to take advantage of the ability of proteasomes to produce peptides.

Cleavage products are then transported into the endoplasmic reticulum apparatus by TAP, the transporter associated with antigen processing. TAP is a resident endoplasmic reticulum protein encoded by two genes located in the class II region of the MHC. It transports peptides of between 8 and $\sim$16 residues and demonstrates a preference for C-terminal residues bound by HLA class I molecules (hydrophobic or positively charged residues). Peptides with N-terminal extensions are probably trimmed in the endoplasmic reticulum to the optimal length for binding to class I molecules, possibly as the peptides are tethered to class I molecules by their C-termini. TAP also functions to bind newly synthesized class I molecules, presumably to increase the efficiency of peptide loading. This binding is mediated by a newly discovered protein termed tapasin. Peptide association with class I molecules induces their release from TAP, and the peptide–class I complex is then rapidly transported to the cell surface through the secretory pathway.

At this point, the final criterion for T cell recognition must be met: the immune system must have the capacity to encode a T cell antigen receptor that is able to distinguish the viral peptide–class I complex from class I complexes containing peptides derived from host proteins.

The same three criteria apply to T_{CD4+} recognition of antigens in association with class II molecules. In this case, however, antigen processing occurs not in the cytosol but in an endosomal compartment. Viral proteins or particles that have entered cells by either absorptive endocytosis or, far less efficiently, by pinocytosis, are unfolded and degraded by proteases located in an early endosomal compartment. Peptides generated in this manner then bind to newly synthesized class II molecules, which are specifically targeted to endosomes during their transport to the cell surface. Due to the ability of class II molecules to bind long peptides, class II molecules are also able to bind peptides in unfolded proteins, and for some antigens this step probably occurs prior to liberation of the peptide from the proteins.

As a consequence of the endosomal origin of class II-associated viral peptides, vigorous class II restricted responses are elicited by immunization with viral proteins or noninfectious intact viral preparations. Such responses are not, however, identical to those elicited by infectious virus. There are two general types of determinants that arise only during a viral infection. First, there are determinants derived from viral proteins not present in virions that are synthesized during the infectious cycle ('nonstructural proteins'). Second, there are some determinants within viral structural proteins that, for reasons that are not completely understood, are preferentially derived from proteins that have been synthesized by the antigen-processing cell.

Specific Characteristics of T Cell Determinants Derived from Influenza Viral Proteins

In theory, the special characteristics of a protein could limit the ability of the antigen processing machinery to liberate antigenic determinants contained within it. In practice, the antigen processing machinery does not appear to distinguish between the various influenza viral proteins. All of the viral proteins have been found to be processed for recognition by T_{CD8+}. Although not as extensively examined, it also appears likely that all viral proteins are presented to T_{CD4+}. Importantly, the recognition of proteins from different influenza A virus isolates by a single T cell clone generally reflects the overall degree of amino acid homology exhibited by the proteins. For example, recognition of nucleoproteins derived from any influenza A virus isolate by either class I- or II-restricted T cells is highly crossreactive, in keeping with the greater than 90% amino acid homology exhibited between these proteins. By contrast, the viral glycoproteins, which exhibit antigenic drift and shift, are often recognized in a strain-specific manner. As a practical manner, this means that T cells capable of reacting with an influenza A isolate can be easily elicited by immunization with the highly conserved internal viral structural and nonconstructural proteins.

With the type of criteria that must be met for a given sequence in a protein to act as an antigenic determinant, it is hardly surprising that the number of determinants contained within each protein capable of eliciting a T cell response restricted to a given MHC molecule is limited. Influenza proteins generally have 3–5 or more nonoverlapping sequences capable of eliciting T cells restricted by a given class II allele. By contrast, the same proteins contain much fewer determinants capable of eliciting T cells restricted by a given class I allele, ranging from 2 to 1, or, fairly often, even 0. Thus, while immunization with an isolated protein elicits T_{CD4+} responses among individuals of various HLA types, the same protein, even when delivered in the appropriate manner to elicit class I-restricted responses (i.e. in a way that allows for cytosolic access), can fail to elicit T_{CD8+} responses in an appreciable number of the same individuals. A given peptide determinant is usually recognized in association with a single class I or II allele, but there are some examples in which overlapping sequences have been shown to be recognized by both class II- and class I-restricted T cells.

DNA Vaccination

Great excitement has greeted the possibility of using purified DNA encoding viral proteins to vaccinate against viral infections. The mechanism of presentation of DNA-encoded antigens to the immune system remains to be established but, in principle, DNA vaccination is similar to vaccination with infectious virus. Thus, the synthesis of viral proteins by antigen-presenting cells transfected by the vaccinating DNA will provide peptides to class I molecules, and viral proteins released from transfected cells should be processed and presented with class II molecules by professional antigen-presenting cells. Viral proteins released from transfected cells also stimulate B cells. Indeed, it has been found that neutralizing antibodies, T_{CD4+} and T_{CD8+} responses are induced following immunization in animals with DNA encoding influenza virus proteins. The immune response is, however, less potent than that obtained following immunization with live attenuated influenza virus, and it remains to be seen whether DNA immunization for influenza virus offers any real advantages in efficacy, safety or cost over existing vaccines or new vaccines.

See also: **Influenza viruses (*Orthomyxoviridae*): General features, Molecular biology; Immune response: Cell mediated immune response, General features.**

Further Reading

Air GM and Laver WG (1989) The neuraminidase of influenza virus. *Proteins Struct. Funct. Genet.* 6: 341.

Braciale TJ and Braciale VL (1991) Antigen presentation: structural themes and functional variations. *Immunol. Today* 12: 124.

Laver WG *et al.* (1990) Epitopes on protein antigens: misconceptions and realities. *Cell* 61: 553.

Wiley DC and Skehel JJ (1987) The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Annu. Rev. Biochem.* 56: 365.

Yewdell JW and Bennink JR (1992) Cell biology of antigen processing and presentation to major histocompatibility complex class I-molecule restricted T lymphocytes. *Adv. Immunol.* 52: 1.

Yewdell JW and Hackett CJ (1988) The specificity and function of T lymphocytes induced by influenza A viruses. In: Krug R (ed.) *The Influenza Viruses*. New York: Plenum.

INSECT PEST CONTROL BY VIRUSES

Davin Miller and **David O'Reilly**, Department of Biology, Imperial College of Science, Technology and Medicine, London, UK

David Dall, Division of Entomology, CSIRO, Canberra, Australia

Introduction

Around one-third of agricultural losses recorded annually are caused by insects. Attempts to minimize these losses through insect control measures are currently based largely on the use of chemical pesticides; however, there are serious problems with pests developing resistance to these chemical control measures in many parts of the world, and in addition there is mounting concern about the impact of these

With the type of criteria that must be met for a given sequence in a protein to act as an antigenic determinant, it is hardly surprising that the number of determinants contained within each protein capable of eliciting a T cell response restricted to a given MHC molecule is limited. Influenza proteins generally have 3–5 or more nonoverlapping sequences capable of eliciting T cells restricted by a given class II allele. By contrast, the same proteins contain much fewer determinants capable of eliciting T cells restricted by a given class I allele, ranging from 2 to 1, or, fairly often, even 0. Thus, while immunization with an isolated protein elicits T_{CD4+} responses among individuals of various HLA types, the same protein, even when delivered in the appropriate manner to elicit class I-restricted responses (i.e. in a way that allows for cytosolic access), can fail to elicit T_{CD8+} responses in an appreciable number of the same individuals. A given peptide determinant is usually recognized in association with a single class I or II allele, but there are some examples in which overlapping sequences have been shown to be recognized by both class II- and class I-restricted T cells.

DNA Vaccination

Great excitement has greeted the possibility of using purified DNA encoding viral proteins to vaccinate against viral infections. The mechanism of presentation of DNA-encoded antigens to the immune system remains to be established but, in principle, DNA vaccination is similar to vaccination with infectious virus. Thus, the synthesis of viral proteins by antigen-presenting cells transfected by the vaccinating DNA will provide peptides to class I molecules, and viral proteins released from transfected cells should be processed and presented with class II molecules by professional antigen-presenting cells. Viral proteins released from transfected cells also stimulate B cells. Indeed, it has been found that neutralizing antibodies, T_{CD4+} and T_{CD8+} responses are induced following immunization in animals with DNA encoding influenza virus proteins. The immune response is, however, less potent than that obtained following immunization with live attenuated influenza virus, and it remains to be seen whether DNA immunization for influenza virus offers any real advantages in efficacy, safety or cost over existing vaccines or new vaccines.

See also: **Influenza viruses (*Orthomyxoviridae*): General features, Molecular biology; Immune response: Cell mediated immune response, General features.**

Further Reading

Air GM and Laver WG (1989) The neuraminidase of influenza virus. *Proteins Struct. Funct. Genet.* 6: 341.

Braciale TJ and Braciale VL (1991) Antigen presentation: structural themes and functional variations. *Immunol. Today* 12: 124.

Laver WG *et al.* (1990) Epitopes on protein antigens: misconceptions and realities. *Cell* 61: 553.

Wiley DC and Skehel JJ (1987) The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Annu. Rev. Biochem.* 56: 365.

Yewdell JW and Bennink JR (1992) Cell biology of antigen processing and presentation to major histocompatibility complex class I-molecule restricted T lymphocytes. *Adv. Immunol.* 52: 1.

Yewdell JW and Hackett CJ (1988) The specificity and function of T lymphocytes induced by influenza A viruses. In: Krug R (ed.) *The Influenza Viruses*. New York: Plenum.

INSECT PEST CONTROL BY VIRUSES

Davin Miller and **David O'Reilly**, Department of Biology, Imperial College of Science, Technology and Medicine, London, UK

David Dall, Division of Entomology, CSIRO, Canberra, Australia

Introduction

Around one-third of agricultural losses recorded annually are caused by insects. Attempts to minimize these losses through insect control measures are currently based largely on the use of chemical pesticides; however, there are serious problems with pests developing resistance to these chemical control measures in many parts of the world, and in addition there is mounting concern about the impact of these

Table 1 Insect virus classification

Virus family (Subfamily/genus)	*Genome*	*Known insect host range*
Nodaviridae	ssRNA	Coleoptera, Diptera, Hymenoptera, Lepidoptera
Picorna-like insect viruses	ssRNA	Diptera, Lepidoptera, Orthoptera
Tetraviridae	ssRNA	Lepidoptera
Reoviridae (Cypovirus)	dsRNA	Diptera, Coleoptera, Hymenoptera, Lepidoptera
Parvoviridae (Densovirinae)	ssDNA	Diptera, Lepidoptera, Orthoptera
Ascoviridae	dsDNA	Lepidoptera
Iridoviridae (Iridovirus, Chloriridovirus)	dsDNA	Coleoptera, Diptera, Lepidoptera, Orthoptera
Poxviridae (Entomopoxvirinae)	dsDNA	Coleoptera, Diptera, Lepidoptera, Orthoptera
Nonoccluded viruses	dsDNA	Coleoptera, Lepidoptera
Baculoviridae (Nucleopolyhedrovirus, Granulovirus)	dsDNA	Coleoptera, Diptera, Hymenoptera, Lepidoptera, Trichoptera

chemicals on the environment. These problems have led to an upsurge of interest in biological control agents, including the use of insect-specific microorganisms, for pest control. Among these microorganisms are the viral pathogens of insects.

The major virus groups with members that infect insects are shown in **Table 1**; as discussed below, the suitability of members of these groups for use as pest control agents varies considerably.

Desirable attributes of any ideal viral insecticide include an appropriate host range, an ability to cause virulent infections in the field, and a rapid speed of pest kill; in addition, any ideal agent should be easy to produce and apply, and should show stability both in storage and once released into the environment. Finally, such an agent should also have a well-understood biology and ecology. In this entry we discuss each of the virus groups listed in **Table 1** in turn, examining how they compare against these various criteria. It is apparent that many of the groups have little potential as control agents, and these are dealt with briefly. In contrast, most of the entry deals with members of the *Baculoviridae*, on which the majority of development work has been done to date.

RNA Viruses

Nodaviridae

These small RNA viruses have received little consideration as control agents, in part because they seem unable to produce frequent infections with high levels of mortality in the wild, leading to concerns over their ease of transmission to the target species. The other main concern is the documented ability of some of these viruses to replicate in both vertebrate and plant tissue cultures, leading to worries over the safety of nontarget organisms within the environment.

Tetraviridae

Members of the *Tetraviridae* are small nonenveloped RNA viruses that are specific for lepidopteran hosts. Symptoms of infection vary from mild weight loss to severe stunting and death. The viruses can be transmitted horizontally, and it is suspected that vertical transmission between generations is possible; in addition, tetraviruses also demonstrate substantial levels of persistence in the environment. One of the better studied members of this family is the *Heliothis armigera* (cotton bollworm) stunt virus (HaSV) whose relatively simple genome consists of a total of three genes on two RNA segments. Unfortunately, the current lack of cell culture systems for any tetravirus, and, as a consequence, the high costs associated with *in vivo* production have thus far hindered the development of these viruses as pest control agents. Also, the widespread distribution of crossreactivity in vertebrate sera to members of this group is a factor whose basis needs to be resolved before uncontained application could proceed in the field. Nevertheless, the simplicity of the genome of these viruses has been recognized as lending them to genetic manipulation. Thus, in recent work HaSV has been produced from chromasomally-integrated genes in transgenic plants, a route that suggests two possible approaches to circumvent the cost of production problems. In one, it could be envisaged that transgenic plants expressing infectious particles of HaSV could be protected against *Heliothis* larvae; alternatively, the ability to produce the virus in, for example a plant cell culture system might provide an economically feasible alternative to *in vivo* production.

Insect picorna-like viruses

This is a broadly-based group of small RNA viruses that are found in invertebrates and that show physical similarities to picornaviruses. Ultimately, however, many of these viruses are likely to be assigned to

families other than the *Picornaviridae*. Current members include two viruses that have been reasonably well characterized, namely cricket paralysis virus (CrPV) and *Drosophila* C virus (DCV). Although found initially in crickets, CrPV has also been found to infect several species of lepidoptera (moths and butterflies), thus demonstrating a broad host range. This virus has been used on several occasions as a pest control agent, in one instance in olive plantations to suppress populations of *Parasa viridissima* (West African slug caterpillar). In this case a dose-dependent mortality rate of 11–61% was recorded 7 days postapplication, and after 14 days this rose to up to 92%. Another 'picorna-like' virus, *Gonometa* virus, has been used to control *G. podocarpi,* a pest of pine trees in Uganda. Both these examples have, however, been noncommercial in nature, and the commercialization of any picorna-like insect viruses for pest control purposes currently appears unlikely. However, the increased use of molecular biology techniques, together with the possibility of engineering transgenic plants to produce infectious virions, as discussed above, could facilitate the exploitation of specific members of this group, such as aphid viruses, as pest control agents in the future.

Reoviridae

The best-characterized insect-specific members of the *Reoviridae* are assigned to the genus *Cypovirus,* and are more commonly known as cytoplasmic polyhedroviruses (CPVs). Infection with these viruses results in formation of polyhedral occlusion bodies in the cytoplasm of infected cells; these occlusion bodies provide protection for the virions during the process of transmission between hosts. CPVs have been identified in a wide range of insect orders, including dipterans (flies), lepidopterans and coleopterans (beetles). Their ability to produce occlusion bodies, their highly infectious nature, and their collectively wide host range are all viewed as positive attributes with respect to their development as pest control agents. Their speed of kill, however, is slow in comparison to chemical pesticides and some other viruses, so their use is likely to be limited to situations where some crop damage can be tolerated. Only one commercial CPV product (Matsukemin), has been produced to date, for use against *Dendrolimus spectabilis,* a pest of pine trees in Japan. A number of trials have also been carried out in China to assess potential for control of *D. punctatus* (pine caterpillar), a serious defoliating pest of Massonian pine plantations in Southern China. Trials are also being conducted, mainly in Japan and China, using a mixture of the insecticidal bacterium *Bacillus thuringiensis* and *Dendrolimus* CPV.

DNA Viruses

Densovirinae

Comprising a subfamily of the *Parvoviridae*, the *Densovirinae* contains members (DNVs) that are invertebrate-specific viruses, and that have a single-stranded linear DNA genome. Densonucleosis viruses (DNVs) have been shown to cause lethal infections in hosts from the orders Lepidoptera, Diptera and Orthoptera (locusts and crickets). The virions are relatively stable in the environment, but are apparently highly susceptible to ultraviolet (UV) light. Horizontal transmission is either via excretion of infected cells, as documented in, for example, a virus from *Sibine fusca* (SfDNV), or by cannibalistic ingestion of infected insects as is the case for, for example, *Galleria mellonella* (greater wax moth) densovirus (GmDNV).

Although not an extensively studied virus group, several examples of successful pest control using DNV have been documented. For example, SfDNV has been successfully used to control *S. fusca* on Colombian palm oil trees. In one trial, a dose of 1–5 infected larval equivalents per hectare was sprayed from the air, resulting in a dose dependent mortality rate of 73–97% at 15 days postapplication. A similar result was achieved in the control of *Casphalia extranea* in Ivory Coast palm oil and coconut plantations, using between 50 and 100 infected *C. extranea* larval equivalents per hectare. To date, the reported broad host range of DNVs for insects, together with their extreme sensitivity to UV light, has limited the development of these viruses as control agents.

Ascoviridae

Ascoviruses contain a linear double-stranded DNA genome, and to date have been identified only as pathogens of the Noctuid family of Lepidoptera. The viruses cause a chronic infection, taking between 2 and 6 weeks to kill the host. This slow speed of kill, together with apparently poor levels of transmission between hosts, and the absence of examples of natural epizootics, suggest that they have little to offer as biological control agents. At this time there is no record of attempts to use these viruses for insect control. However, it has been noted that ascovirus infection is associated with impairment of larval development and feeding. This fact, together with the specificity of their host range, which includes several

economically important pests, suggests that they may merit more detailed study.

Iridoviridae

This family contains viruses that infect both vertebrates and invertebrates, but members of two genera, *Iridovirus* and *Chloriridovirus,* are insect specific. The prefix 'irido' is derived from the characteristic iridescent appearance that can occur in infected insects; recently, however, it has been shown that the incidence of the virus within natural populations is far greater than suggested by the number of iridescent insects. This group of viruses has been found in coleopteran, lepidopteran and dipteran hosts, including important crop pests such as *Heliothis* spp., and blackflies (*Simulium* spp.), which are vectors of human disease.

Unfortunately, little is known about the transmission route or life cycle of these viruses and this, together with their apparent lack of virulence, has discouraged their serious consideration as pest control agents. However, their ability to infect dipteran hosts, a group from which relatively few other insect viruses are recorded, as well as their potential amenability to genetic engineering, is likely to encourage the further investigation of these viruses as potential pesticides.

Entomopoxvirinae

The *Entomopoxvirinae* (EPV) are a subfamily of the *Poxviridae* that are specific to insect hosts. EPV infections have been documented in four major orders of insect pests: the Lepidoptera, Coleoptera, Orthoptera and Diptera. The EPVs resemble the baculoviruses in a number of ways, including their production of occlusion bodies and their amenability to genetic modification. In addition, however, EPVs are capable of infecting a number of economically important pest species not affected by the *Baculoviridae,* including the desert locust (*Schistocerca gregaria*), possibly the most destructive pest species worldwide, and a variety of scarab beetle pests. To date the EPVs have received little attention as potential pest control agents, due largely to their slow speed of kill. However, several EPVs are capable of replicating in insect cells in culture, and this has enabled researchers to investigate the possibility of genetically modifying EPVs to enhance their speed of kill. One EPV showing particular promise in this respect is the *H. armigera* EPV (HaEPV). This virus infects several different economically important lepidopteran pests, grows readily in tissue culture, and can be genetically modified. Work is currently underway to increase the speed of kill of this virus in the same manner as for baculoviruses (see below).

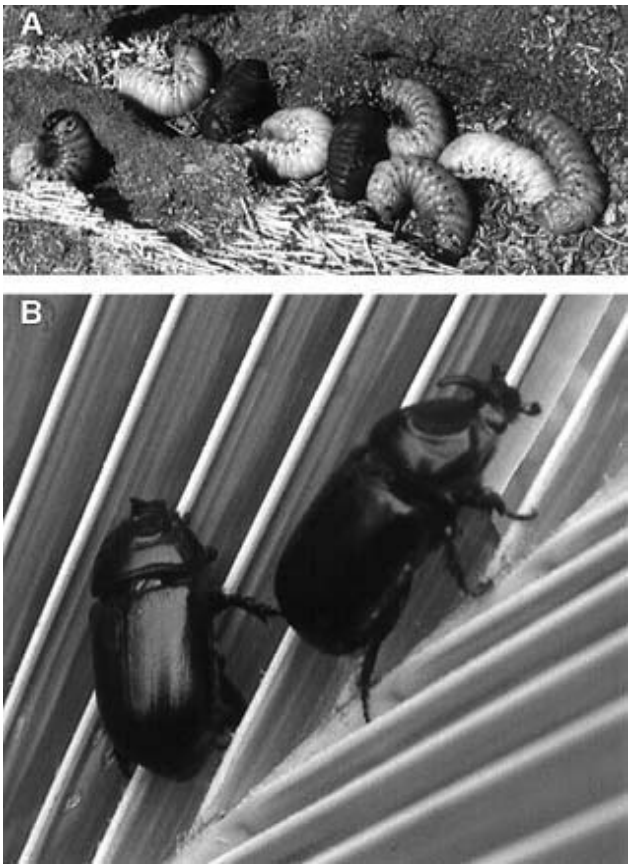


Figure 1 (**A**) *Oryctes rhinoceros* (coconut rhinocerus beetle) larvae and pupae at a breeding site. (**B**) *Oryctes rhinocerous* adults. (Courtesy of Dr D F Waterhouse, CSIRO, Australia.)

Non-occluded DNA viruses

This now unclassified group of viruses was formerly considered to be a subfamily of baculoviruses, with which they share many physical characteristics. Unlike members of that group, however, they do not produce occlusion bodies, and can infect both adult and larval insect hosts. Three viruses with large double-stranded DNA genomes have been assigned to this group, and all are apparently specific to insects. The first of these to be discovered was the *Oryctes* virus (Or-1V) which infects *O. rhinoceros* (the coconut rhinoceros beetle). The other two members of the group (Hz-1V and Hz-2V) have both been found in *Helicoverpa zea* (corn ear worm). Both Ov-1V and Hz-1V are able to replicate in tissue culture and so have been relatively well characterized. Hz-2V infects the reproductive tissue of adult *H. zea,* causing atrophy of the reproductive organs. Symptoms of

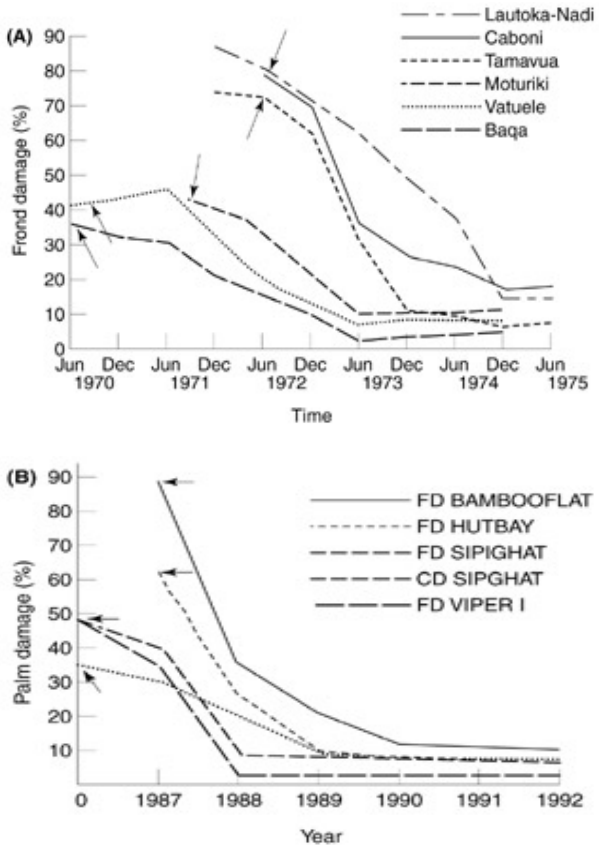


Figure 2 (**A**) Effect of *Oryctes rhinocerous* virus on palm damage by *O. rhinoceros* in the Fiji Islands. Arrows indicate time of virus introduction; virus spread naturally into Lautoka-Nadi area by mid-1973. (Modified from Bedford GO (1980) with permission from the *Annual Review of Entomology*, Volume 25 1980 by Annual Reviews.) (**B**) Decline in palm damage in the Indian Andaman Islands 1987–1992. Arrows indicate date of *O. rhinocerous* virus release; FD, frond damage; CD, crown damage. (Modified with permission from Jakob TK (1996) *Bull. Entomol. Res.* 86: 257–262.)

infection include shrunken testes in males and possible absence of ovaries in females. Infected adults that are not sterilized by the disease have been shown to produce apparently healthy larvae, but adults that develop in turn from these larvae can also show the characteristic atrophy of the reproductive organs. This effect of host sterilization, together with the capacity for vertical transmission and the importance of the host as a pest species, indicate a possible future for Hz-2V as a pest control agent.

Or-1V is currently the most successful example of the use of a viral biological control agent for insect pest control. This virus was isolated from *O. rhinoceros* (**Fig. 1**) in Malaysia in 1963 and was subsequently found throughout southeast Asia. However, it was found to be absent from the South Pacific countries where *O. rhinoceros* was a serious pest of palm trees. As a consequence, a program to introduce the virus into Western Samoa was started in 1967. The virus was initially introduced into the natural breeding sites for the beetle using logs or sawdust infected with Or-1V. Later, artificial breeding sites contaminated with the virus were set up, and, in addition, adult males were infected in the laboratory and returned to the breeding areas. Adult beetles infected with the virus can transmit it to other adults during mating, by accidental contact or via infected excreta at the breeding site; larvae that ingest this infected excreta may also become infected, and subsequently fail to reach adulthood. The initial trial on Western Samoa was so successful that a control program was introduced throughout the South Pacific. Results throughout this region indicated dramatic reductions in palm damage (**Fig. 2A**). In Fiji, damage was reduced from 75–90% in the worst affected areas in 1970 to around 10% by late 1974. Similar results were seen on Mauritius, where in 1978 palm damage was reduced by 60–95% from previrus release levels in 1970. The success of this program led to the use of Or-1V in the Indian Andaman Islands, with similar results (**Fig. 2B**). Both these programs have now resulted in long term economic control of this important pest, and the only maintenance required for continued control has been the sporadic reintroduction of the virus on some islands.

Baculoviridae

General biology The baculoviruses are a well-characterized group of invertebrate-specific viruses that are divided into the genera *Nucleopolyhedrovirus* (containing the NPVs) and *Granulovirus* (containing the GVs). NPVs are found mainly in insect hosts, and have been widely recorded from larvae of lepidopteran species, as well as in members of the Hymenoptera, Diptera, Coleoptera and Trichoptera. There are also reports of NPV infections in crustaceans. In contrast, the GVs are apparently restricted to lepidopteran hosts. Like CPVs and EPVs (see above), baculoviruses produce large proteinaceous occlusion bodies which serve to protect the virions during periods of transmission between hosts. NPV occlusion bodies always contain multiple virus particles, whereas in GVs only one virus particle is occluded in each occlusion body.

Most baculovirus isolates have a narrow host range, and many are highly virulent (**Fig. 3**), causing widespread epizootics in the natural environment, and playing a significant role in natural regulation of insect populations. This combination of characters has led to baculoviruses being perceived as the best

Figure 3 *Heliothis armigera* (cotton bollworm) cadaver after infection by HaNPV.

suited of the insect viruses for development as commercial pest control agents.

Past and current uses of baculoviruses Baculoviruses have been used for many years as pest control agents, being exploited both commercially (**Table 2**) and noncommercially. Early reports of use include the Chinese army using mortars to fire infected larvae into the canopy of forests infested with caterpillars. Nevertheless, until recently the relatively slow speed of action of baculoviruses has meant that most commercial use of the viruses has been directed towards forestry, or other production systems where commodities can tolerate levels of damage typically suffered in the time between infection and death of a pest. Examples of semicommercial use include the US Forestry Service products Gypcheck (*Lymantria dispar* NPV) used against *L. dispar* (gypsy moth) and Neocheck-S (*Neodiprion sertifer* NPV) used to control *N. sertifer* (pine sawfly). There are relatively few examples of natural baculoviruses being used to protect field crops. The most successful example is the use of *Anticarsia gemmatalis* NPV (AgNPV) to control *A. gemmatalis*, the velvetbean caterpillar, in soybean crops in Brazil. A long-term program there has now led to establishment of a large *in vivo* production industry, and the annual spraying of over two million hectares with the virus. Other notable examples of baculovirus use include GemStar™, a wild-type *H. zea* NPV being evaluated for use in a US Department of Agriculture bollworm/budworm suppression program and control of *H. armigera* in grain crops in Australia, and SPOD-X LC™, a commercial formulation of the wild-type baculovirus *Spodoptera exigua* (beet armyworm) NPV (SeNPV). Since 1994 SPOD-X LC™ has been marketed in the USA, Thailand and Holland for the control of *S. exigua* on ornamental, vegetable and field crops. Results of use reported so far have indicated that it can achieve a level of control equivalent to that of commercial chemical products, demonstrating that commercial use of natural baculoviruses can be a successful strategy on high-value crops.

Advantages and disadvantages of baculoviruses as pest control agents In natural settings, baculoviruses are known to cause large-scale epizootic events that are responsible, in part, for the regulation of populations of many insect species. Under artificial circumstances (e.g. when used as a pest control agent) baculoviruses also have a significant number of advantageous characteristics. Thus, when administered *per os* they are extremely virulent, and usually cause lethal infections. Their invertebrate specificity and limited host range means that a baculovirus-based pesticide is unlikely to cause problems to beneficial insects and other nontarget organisms. Despite their individually narrow host range, baculoviruses are collectively capable of infecting a wide range of

Table 2 Examples of commercial baculovirus pesticides

Product name	*Virus*	*Pest*	*Crop*
Elcar	*Helicoverpa zea* NPV	Multiple pests	Cotton
Gypcheck	*Lymantria dispar* NPV	*L. dispar*	Forestry
TM Biocontrol-1	*Orgyia pseudotsugata* NPV	*O. pseudotsugata*	Forestry
Neocheck-S	*Neodiprion sertifer* NPV	*N. sertifer*	Forestry
SPOD-X LC	*Spodoptera exigua* NPV	*S. exigua*	Vegetables
GemStar	*Heliocoverpa zea* NPV	Multiple pests	Cotton
Anticarsia virus	*A. gemmatalis* NPV	*A. gemmatalis*	Soybeans
Cydia pomonella	*C. pomonella* GV	*C. pomonella*	Apples

economically important pests, so that it is likely that technology developed for one virus–pest combination will also be applicable to the development of other viral pesticide products. Finally, development of pest resistance to these complex agents seems less likely to occur – at least in the short term – than has frequently been the case with simple chemical compounds.

Baculoviruses do, however, also have a number of limitations that have prevented their widespread use as biological insect control agents. Thus they are currently rather expensive to produce, either *in vitro* (in cell culture systems) or *in vivo* (in insect larvae). Similarly, while their narrow host range is an advantage from a safety perspective (see above), it can also be a disadvantage when more than one pest species occurs on a crop. Further, these viruses are also highly susceptible to inactivation by UV light in sunlight, so that complex formulation strategies must be used. However, perhaps their principal disadvantage is their relatively slow speed of action. Most baculoviruses take 7–10 days to kill an infected host, and substantial damage can thus be caused to a crop between the time of virus application and the eventual death of the target pest.

In recent years considerable efforts have been made on several fronts to decrease these limitations. One strategy being pursued to reduce production costs is the development of efficient cell culture systems. Continual progress is being made in increasing the size and capacity of fermentation vessels for growth of large and high-density cell cultures, as well as in optimizing cheap culture media and the highly productive cell lines necessary for commercial-scale virus production. Work is also being done with respect to characterization of the genetic basis of baculovirus host range/specificity, and a number of genes have now been demonstrated to play a role in this phenomenon. A detailed understanding of the mechanism of host range determination at a molecular level should provide an ability to modify virus host range, thus making these engineered baculoviruses more attractive as pest control agents.

It seems likely that the problem of UV sensitivity will be solved by the development of appropriate formulations containing effective UV protectants that can be applied to the crop with the viral pesticide. Finally, substantial progress has been made in increasing the speed of action of baculoviruses by genetic manipulation, as reviewed in the following section.

Genetic manipulation of baculoviruses Two broad and complementary approaches have been taken to improve the insecticidal properties of NPV baculoviruses by genetic manipulation; namely, the insertion of genes from other sources into the viral genome, and the manipulation/deletion of normally occurring viral genes. The types of foreign genes inserted into the virus genome can be subdivided into those that encode insect-specific toxins, and those that encode hormones or enzymes designed to disrupt host homeostasis (**Table 3**). Genes encoding insect-specific toxins have been isolated from sources ranging from scorpions and spiders to mites, bacteria and sea anemones. The effects of these toxins, when expressed by a recombinant virus, on the insecticidal properties of that virus have been mixed. Of those for which published data are available, the AaHIT toxin of the North African scorpion (*Androctonus australis*), and a toxin from *Pyemotes tritici* (straw itch mite) have proved most effective, resulting in demonstrated pest kill times of 48–72 hours.

Table 3 Genes used in attempts to improve insecticidal activity of baculoviruses

Foreign gene inserted	*Target*
Lesser Asian scorpion toxin	Sodium channels
North African scorpion toxin	Sodium channels
LQH scorpion toxin	Sodium channels
Straw itch mite toxin	Nervous system
Funnel web spider toxin	Sodium channels
Sea anemone toxin	Sodium channels
Bacillus thuringiensis toxin	Cell membranes
Wasp antigen 5	?
Maize mitochondrial toxin (URF13)	?
Insect diuretic hormone	Water balance
Prothoracicotropic hormone	Ecdysteroid levels
Juvenile hormone esterase (JHE)	JH levels
Eclosion hormone	Insect development

The insertion of genes designed to disrupt insect homeostasis has proved less effective, although overexpression of a mutant form of the *H. virescens* juvenile hormone esterase has been reported to improve the insecticidal properties of NPVs under some circumstances.

The other approach taken to enhancing the insecticidal activity of baculoviruses is to delete genes from the viral genome. One viral protein which baculoviruses use to control host development is ecdysteroid UDP-glucosyl transferase (EGT); this protein inactivates the host hormones that trigger molting, initiating a virally-induced developmental arrest. This molting arrest is thought to be advantageous to the virus in prolonging the duration of the feeding stage of host larvae, and ultimately leading to a higher level of virus production. Deletion of the *egt* gene has produced viruses that kill their hosts up to 30% more quickly than the wild-type parental virus,

in the process reducing overall host feeding levels by 20–30%.

Field trials of genetically modified baculoviruses A number of field trials of recombinant baculoviruses have now been done. Trials in Oxford (UK) involved the release, under controlled conditions, of a recombinant *Autographa californica* NPV (rAcMNPV) containing the AaHIT gene on cabbage infested with *Tricoplusia ni* larvae. These trials demonstrated that the enhanced speed of kill of the virus led to a 25% reduction in crop damage. Larger scale trials have been carried out in the USA by the agrochemical company American Cyanamid. These involved the use of both an *egt*-minus AcMNPV and an *egt*-minus AcMNPV containing the AaHIT gene. Trials in 1993–1995 demonstrated that under field conditions the *egt*-minus virus killed target species up to 20% faster than wild-type virus, and trials using *egt*-minus rAcMNPV containing the AaHIT gene showed still further improvement in insecticidal activity. A trial on cotton in Louisiana demonstrated that control of *H. virescens* by this *egt*-minus AaHIT-expressing virus was comparable with that of a commercial insecticide and a *B. thuringiensis* bacterial biopesticide.

Conclusions

The advent of powerful techniques of molecular biology together with increasing environmental concerns over chemical pesticides have led to a resurgence of interest in the use of insect viruses in pest control regimens. The possibility of using EPVs, CPVs and tetraviruses has been greatly enhanced by advances in genetic engineering. However, it is the baculoviruses, and in particular the NPVs, that have been the subject of most dramatic progress. Recombinant baculoviruses with significantly enhanced speed of action are now available, and field trials using these have produced encouraging results.

None the less, some issues remain to be resolved before such viruses are taken through to full commercial development. One area of concern is the possible effects of toxins produced in infected insects on nontarget organisms. Work done so far has shown that the amount of AaHIT produced by rAcMNPV is not sufficient to harm either mammals or predatory/scavenging insects feeding on infected caterpillars or their cadavers. Thus it seems that this issue will not present any problem. Another area of concern is the possibility of genetic exchange between the virus and other organisms. It is conceivable that a toxin gene for instance, could become inserted in the genome of another organism, possibly upsetting the natural balance between that organism and those others it interacts with in the environment. Present research indicates that such an event is unlikely, but further research of the risk associated with that phenomenon is required. Currently, it seems likely that the first recombinant baculovirus pesticides will be available on a commercial basis within the next few years.

See also: **Ascoviruses (*Ascoviridae*); Baculoviruses (*Baculoviridae*): Granuloviruses, Nucleopolyhedrovirus; Nonoccluded baculoviruses; Densonucleosis viruses (*Parvoviridae*); Entomopoxviruses (*Poxviridae*); Nodaviruses (*Nodaviridae*); Parvoviruses (*Parvoviridae*): Cats, dogs and mink, Molecular biology, General features; Picornaviruses – insect (*Picornaviridae*); Reoviruses (*Reoviridae*): General features, Molecular biology; Tetraviruses (*Tetraviridae*); *Iridoviridae* – invertebrate.**

Further Reading

Arif BM (1995) Recent advances in the molecular biology of entomopoxviruses. *J. Gen. Virol.* 76: 1.

Beadle DJ, Bishop DHL, Copping LG, Dixon GK and Hollomon DW (eds) (1993) *Opportunities for Molecular Biology in Crop Production*, monograph 55. Farnham: British Crop Protection Council.

Bedford GO (1980) Biology, ecology and control of palm rhinoceros beetles. *Annu. Rev. Entomol.* 25: 309.

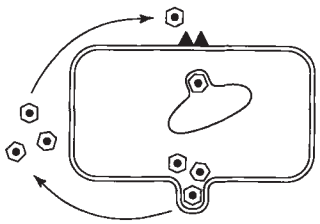
Black BC, Brennan LA, Dierks PM and Gard IE (1997) Commercialization of baculoviral insecticides. In: Miller LK (ed). *The Baculoviruses*, p. 341. New York: Plenum Press.

Hanzlik TN and Gordon KHL (1997) The *Tetraviridae*. *Adv. Virus Res.* 48: 101.

King LK, Possee RD, Hughes DS *et al* (1994) Advances in insect virology. In: Evans PD (ed). *Advances in Insect Physiology*, vol. 25. London: Academic Press.

INTERFERENCE

Julius S Youngner and **Patricia Whitaker-Dowling**, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA



History

From the earliest days of quantitative virology it was recognized that when two different viruses infect the same cell one virus frequently affects the growth of the other. When interference between the two viruses occurs it is traditionally subdivided into two categories: homologous and heterologous interference, depending on the closeness of the relation of the two viruses. For many years the mechanisms involved in these phenomena remained obscure and subject to much speculation. The discovery of interferon in 1957 offered a potential mechanism for virus interference and many of the examples that had been observed in the past were explained by the action of this antiviral protein. The revival of interest in virus interference led to increasing recognition that in many cases the interferon mechanism is not involved and that other factors play a role in the inhibitory interactions between viruses.

Types of Virus Interference

There are several mechanisms that can be invoked to explain the interference that is observed between related and unrelated viruses. It should be noted that two of the types of interference referred to below have been extensively investigated and are covered in more depth in other entries in this volume.

Interferons

As mentioned above, the discovery of interferon by Isaacs and Lindenmann led to the clarification of the mechanism involved in a number of the interference models that had been studied. The interferon system is composed of a family of proteins (designated α-, β-, and γ-interferons) which are induced in cells infected by a variety of RNA and DNA viruses. The ability to induce interferon can vary widely with different viruses and has even been shown to vary with different mutants of the same virus. Furthermore, the antiviral effects of interferon are not virus-specific; the inhibitor will act against the virus that induced it as well as against heterologous viruses. Co-infection of cells with two viruses with different interferon-inducing abilities can result in a situation in which the growth of one of the viruses is hindered by the interferon induced by the other virus. A detailed summary of the characteristics of interferon and the mechanisms of its antiviral action are described elsewhere in this volume.

Incompatibility of heterologous viruses

Interference between heterologous viruses usually is produced by one virus inactivating or inhibiting a host cell function required by the other virus. A prime example of this type of incompatibility is seen in cells infected with poliovirus and an unrelated RNA virus, vesicular stomatitis virus (VSV). Infection with poliovirus results in the inactivation of the cap-binding protein complex of the infected cell, an action that blocks the translation of capped mRNAs. Since VSV produces capped mRNAs, VSV protein synthesis is inhibited in poliovirus-infected cells.

Another example of heterologous interference is the inhibition of the growth of influenza A virus in cells doubly infected with this virus and VSV. Influenza virus requires continuous host mRNA synthesis during its replicative cycle because it 'steals' the 5′ end of host mRNAs and uses these oligonucleotides as primers for the synthesis of its own mRNAs. Since VSV infection produces an efficient and early shutdown of host RNA transcription, in doubly infected cells there are no newly synthesized host mRNAs from which influenza virus can obtain the cap sequences necessary for its own mRNA; its replication is thus blocked.

Heterologous interference also can occur when one virus has a faster replication cycle and rapidly shuts down a host function that is needed by a second virus that is replicating with a slower growth cycle. For example, VSV inhibits the growth of a herpesvirus, pseudorabies, in doubly infected cells by shutting off the host protein synthesis machinery before the herpesvirus has the opportunity to express all of its proteins.

An alternative mechanism which can cause incompatibility of heterologous viruses is based on the interaction of their gene products. This type of interference occurs when two different viruses encode proteins that are similar enough to form heterologous multimeric complexes. If these multimeric complexes are nonfunctional, interference results. Such a mech-

anism may explain the interference observed between two VSV serotypes (Indiana and New Jersey).

Superinfection exclusion

Several families of viruses have evolved strategies to block the invasion of a cell that has been previously infected. This block can occur at the level of adsorption, penetration, or later in the replicative cycle. For example, certain retroviruses inhibit the expression of their cell surface receptor following infection, thus blocking the adsorption of superinfecting virus. Members of the orthomyxovirus and paramyxovirus families encode a transmembrane glycoprotein with receptor-destroying activity which blocks virus binding. This activity not only allows the efficient escape of newly formed progeny virus but also prevents superinfection by related viruses.

VSV is also capable of preventing superinfection at the level of penetration; this block is caused by a VSV-induced inhibition of endocytosis, a pathway used by many viruses to enter cells.

The term 'intrinsic interference' is used to describe a phenomenon in which cells infected with a noncytopathic RNA virus, such as rubella, are resistant to superinfection with an unrelated RNA virus, Newcastle disease virus. This resistance is not due to interferon induction since the only cells in the culture that are resistant are those that are actually infected with rubella. Indirect studies suggest this interference is due to the synthesis of protein(s) of the rubella virus rather than some host protein, but the mechanism is not understood.

Defective interfering (DI) particles

Serial high-multiplicity passages of most viruses produce mutant viruses that have part of their genome deleted or rearranged. Because these mutants are defective for replication, they require a helper function that is provided in *trans* by the parental virus. However, in the course of this co-infection the replication of the defective virus is favored and the production of the parental virus is reduced significantly. A detailed description of the generation of DI particles and their inhibiting effect on the replication of parental virus is given elsewhere in this volume.

Dominant-negative mutants

A sizable body of information has accumulated reporting instances in which viruses with mutations that produce *ts*, host range, small plaque, or nonsense mutants behave in a dominant manner and can inhibit the growth of the standard wild-type virus in mixed infections. These mutants differ from DI particles in that there is no absolute requirement for a helper virus in order for them to grow, and they interfere by mechanisms that differ from those used by DI particles.

The documentation of interference by dominant-negative mutants covers a wide range of RNA and DNA animal viruses, as well as plant viruses and bacteriophage. In the case of plant viruses, this phenomenon has been a subject of interest for decades. It was originally investigated in connection with its effect on viral pathogenesis in plants. The term 'cross-protection' is used to describe the resistance afforded against a virulent strain of virus by prior infection with an attenuated strain of the same virus. Since plants do not mount an antiviral response analogous to the interferon system or have any known counterpart to the immune system, it is thought that cross-protection arises from virus interference.

Detailed Consideration of Dominant-negative Mutants

Dominant-negative mutants have been derived spontaneously or by mutagenesis from nearly every family of animal viruses. In fact, viral mutants with the ability to interfere have proved relatively easy to obtain and for some viruses, such as VSV, have been identified in every complementation group. The ease with which this phenotype arises may be due to the fact that the majority of virus gene products function as components of multimeric complexes; only a small proportion of viral gene products function as single-subunit, soluble proteins. Dominant mutants may also have a selective advantage and may prevent the successful growth of revertants by virtue of their dominant phenotype. Mutant viruses that evolve in long-term persistent infections in cell culture model systems are of particular interest.

Such viruses frequently express two phenotypes that are crucial to the maintenance of the persistent state. First, the viruses derived from persistent infections have a reduced capacity to kill cells; second, these mutant viruses are also dominant over the parental wild-type virus in mixed infections. Both phenotypes would be expected in viruses that are selected in long-term persistence. In fact, establishing persistent infections may provide an effective selection technique for obtaining viruses that are attenuated, dominant-negative mutants.

It is important to distinguish the ways in which dominance can be manifested. In the case of some dominant-negative mutants, only their phenotypes are dominant in mixed infection with wild-type virus.

Although co-infection with these mutants can significantly affect the ability of the wild-type virus to grow, the reduced yield from these limited replication cycles reflects the same ratio of the two viruses as in the input inoculum. This can be termed 'phenotypic dominance'. For example, double infection at the nonpermissive temperature with equal amounts of a dominant-negative, temperature-sensitive mutant and a wild-type virus may reduce the yield of the wild-type virus by several orders of magnitude, but the composition of the total yield will be 50% mutant and 50% wild-type virus. This type of dominance prevents the overgrowth of revertants which may arise within the population, but since it provides no reproductive advantage to the dominant mutant, this virus will be unable to establish predominance within a mixed virus population. An alternate manifestation of a dominant-negative phenotype occurs when the mutant dominates at the level of phenotypic expression and also suppresses the growth of wild-type virus relative to its own. In this instance the yield from the mixed infection is predominantly mutant virus. This variety of dominance can be termed 'genotypic dominance'. Any mutants with this trait will have a strong selective advantage in a mixed virus population and thus be able to both establish and maintain predominance within a mixed virus population.

Possible mechanisms of interference by dominant-negative viruses

Despite the large number of instances in which dominant-negative viruses have been described, there is a paucity of direct biochemical data dealing with the mechanism of interference. There has been a good deal of speculation based on observations of reductions in virus yield, genome replication, or viral gene expression. Out of this speculation some recurrent themes have emerged concerning mechanisms that may be involved in this type of interference but the authors do not intend to imply that this is an exhaustive list. Of the five suggested mechanisms described next, the first three would be expected to show phenotypic dominance whilst the last two would be predicted to result in genotypic dominance. It should be noted that some of these mechanisms also may apply to cases of heterologous interference or interference by superinfection exclusion.

The 'rotten apple' hypothesis Perhaps the most common hypothesis to explain the mechanism of interference by a mutant virus is that a defective polypeptide enters into a multimeric complex and thereby inactivates or inhibits the activity of all the components of that complex. The formation of such hybrid structures has been suggested in the case of certain bacterial and animal viruses. A possible example of this mechanism is seen with a *ts* mutant of VSV that encodes a lesion in the nucleocapsid protein (N). In mixed infections of cell cultures with wild-type and mutant viruses, the pool of N protein in the cell would consist of a mixture of both normal and defective N polypeptides. Should the defective product participate in the polymerization of N protein on the viral RNA, a premature termination of assembly or defective nucleocapsids might result. Either of these outcomes would effectively incapacitate both the viral RNA molecule and all normal N protein in the complex. If this mechanism were operating, relatively small amounts of the defective polypeptide would cause significant levels of interference.

The 'road block' hypothesis In this model the defective function of the mutant virus irreversibly sequesters the pool of a factor or site required for the replication of both viruses. A possible example of this mechanism is the ability of a mutant of VSV that contains a lesion in the RNA polymerase protein (L) to interfere with wild-type virus in mixed infections. This defective polymerase may bind preferentially to RNA synthesis initiation sites on the wild-type genome and thus prevent access by the functional wild-type enzyme. Such a mechanism is particularly applicable in the case of mutated *trans*-activating elements. Viral proteins which function to activate enhancer regions on viral genomes could mutate in a manner that retains their binding activity but loses their activating function. In some instances involving DNA viruses, it is proposed that the dominant-negative phenotype may be mediated by binding of the mutant protein to the replication origin, thus inhibiting the initiation of viral DNA synthesis.

The 'all things in moderation' hypothesis It has been demonstrated in several bacteriophage and plant virus systems that gene dosage is another mechanism that may explain interference. In these instances, an imbalance in the relative amounts of certain structural proteins leads to the improper or inhibited assembly of virus particles or affects gene expression. In this case a termination mutation at any site on a particular gene may be sufficient to cause the dominant phenotype.

The 'direct competition' hypothesis In double infections with two viruses, competition for some limiting factor or site of either viral or host origin may play a role in interference. One of the interacting viruses may have the advantage of a higher binding

affinity for such a specific site or factor, and thus outcompete the other virus in a mixed infection. From this model it would be expected that as the degree of interference with the wild-type virus increases, the amount of the mutant progeny would also increase since the total yield of virus is dependent on the limiting factor in the growth cycle. Such an outcome has been reported in a double infection involving wild-type and mutant Sendai virus. In this case, despite significant reduction in wild-type virus progeny, net yield of virus is unchanged.

The 'attractive genome' hypothesis A mechanism for interference may operate that is similar to what has been proposed for the action of defective-interfering particles. Extensive studies with VSV have suggested that DI particles interfere by successfully competing with the parental genome for the available RNA polymerase complex. In a similar manner, the genomes of dominant-negative viruses may possess binding sites for the viral replication machinery, or its components, that are more attractive or in greater abundance than the binding sites of the wild-type virus.

Complementation and reassortment inhibition

That a detrimental mutation results in the phenotypic absence of that particular function and that phenotypic rescue can be accomplished by supplying that function in *trans* are commonly held tenets of genetics. Complementation analysis is based on these assumptions. Interference by dominant-negative mutants may explain some of the difficulties encountered in demonstrating significant complementation. Examples are cited in the literature for several families of DNA and RNA viruses, e.g. herpesvirus HSV-1 and VSV, where co-infection of cells with complementation incompetent mutants interferes with the successful growth of wild-type virus. This test can be used to differentiate dominant-negative mutants from mutants that are complementation incompetent due to alteration in *cis*-acting functions. In addition to an inhibition of complementation, reassortment of viral genes of segmented RNA viruses can also be affected when one of the two parental viruses is able to dominate the other. Such an inhibition of reassortment has been shown to occur with both influenza and reoviruses.

Dominant-negative mutants as antiviral agents

The potential for using dominant-negative mutants to prevent or treat disease in humans or animals is being increasingly recognized. Two approaches are being pursued. One approach involves the performance of what is essentially gene therapy, i.e. the use of expression vectors carrying naturally occurring or genetically engineered dominant-negative viral genes. Akin to antisense messenger RNAs, which are genetically engineered products whose design is directed at blocking the expression of a specific gene, dominant-negative mutants function by blocking the activity of a specific gene product. When introduced into cells susceptible to, or infected with, a virulent wild-type virus, expression of the mutant gene would produce a protein that would act as a dominant-negative inhibitor.

There are several examples of the effective use of dominant-negative mutant proteins derived from viral genes and delivered by expression vectors in cell culture model systems. The growth of wild-type virus is suppressed in these transfected cells. It has been reported that in the case of human immunodeficiency virus type 1 (HIV-1), expression of mutant *tat*, *gag* or *rev* proteins in transfected cells results in dominant-negative inhibition of the replication of wild-type HIV-1. The replication of a herpesvirus, HSV-1, is inhibited in transfected cells expressing a mutated form of a structural protein, glycoprotein B, or expressing a mutated viral *trans*-activator necessary for early gene expression. The expression of dominant-negative mutant genes in cells has been termed 'intracellular immunization'. The transfection of dominant-negative genes also offers the possibility for the creation of transgenic animals carrying genes which confer on the animals a genetic resistance to infection with specific viral pathogens. The application of these cell culture models to disease states in humans is fraught with the problems associated with gene therapy in general, foremost among them the delivery to and appropriate expression of the dominant-negative genes in the correct target cells in the body.

An alternate approach, and one which might be achievable in the near future, is the use of attenuated, dominant-negative mutant viruses to modify disease processes prophylactically or therapeutically by interfering with their virulent counterpart. The use of attentuated intact viruses carrying dominant-negative mutations circumvents the problem of delivery since the mutant virus is already targeted to the appropriate cell population. Furthermore, the proper expression of the mutant protein is assured since the gene appears in its natural context. The feasibility of this approach has been demonstrated in experimental animals in the case of an attenuated influenza A strain that is a dominant-negative mutant. Simultaneous co-infection of animals with this attentuated virus and wild-type influenza virus results in the complete suppression of the disease symptoms seen in animals infected

with the same amount of wild-type virus alone. The advantages of the use of attenuated viruses are that the effective dominant gene(s) are delivered in the intact animal directly to the appropriate target cells and their expression is transient and does not cause the permanent alteration of the host genome.

Live virus vaccines should contain dominant negative mutations

In this connection it should be noted that the ability to interfere with the growth of wild-type virus would be a particularly desirable trait in the case of live virus vaccines. The use of vaccine viruses with attenuating mutations that also confer a dominant-negative phenotype would add a significant degree of protection against virulent revertants that may arise during the immunizing infection or against epidemic viruses that may be circulating concurrently in the population. In addition to their immunizing potential, dominant-negative vaccine viruses could offer short-term antiviral protection until the immune response to the virus is firmly established.

See also: **Antivirals; Defective interfering viruses; Interferons: Therapy of aids and cancer; Vesicular stomatitus viruses (*Rhabdoviridae*).**

Further Reading

D'Aloja P, Olivetta E *et al.* (1998) gag, vif, and nef genes contribute to the homologous viral interference induced by a nonproducer human immunodeficiency virus type 1 (HIV-1) variant: identification of novel HIV-1-inhibiting viral protein mutants. *J. Virol.* 72(5): 4308–4319.

Friedman AD, Triezenberg SJ and McKnight SL (1988) Expression of a truncated viral *trans*-activator selectively impedes lytic infection by its cognate virus. *Nature* 335: 452.

Herskowitz I (1987) Functional inactivation of genes by dominant negative mutations. *Nature* 329: 219.

Whitaker-Dowling P and Youngner JS (1987) Viral interference-dominance of mutant viruses over wild-type virus in mixed infections. *Microb. Rev.* 51: 179.

Whitaker-Dowling P, Maassab HF and Youngner JS (1991) Dominant-negative mutants as antiviral agents: simultaneous infection with the cold-adapted live-virus vaccine for influenza A protects ferrets from disease produced by wild-type influenza A. *J. Infect. Dis.* 164: 1200.

[This article is reproduced from the 1st edn (1994).]

INTERFERONS

IF

Philip I Marcus, University of Connecticut, Storrs, Connecticut, USA

History

Interferons (IFNs) represent the prototype of a family of cytokines with an extraordinary wide range of biological effects including their action as antiviral agents – the basis of their discovery. Interferons have emerged as some of the most active biological substances known. Their study reveals myriad effects on cells, contributing to our understanding of basic cellular, immuno- and developmental biology. The IFN system, both its induction and action, represents the first line of defense against viral diseases, and possibly other infections agents, and has attained official sanction as an acceptable therapeutic agent, fulfilling the dreams of its early investigators.

Virologists long recognized that exposure of cells to one kind of virus often rendered the cells refractory to infection by another virus, even of heterologous origin. The simultaneous occurrence of two different childhood virus diseases is unprecedented. The shroud of mystery surrounding these examples of 'viral interference' was lifted with a seminal discovery by Isaacs and Lindenmann in 1957. They found that chicken cells exposed to heat-inactivated influenza virus released a factor which, when added to fresh cells, reduced their capacity to produce new virus. Based on this ability to interfere with virus replication the factor aptly was termed 'interferon'. During the first decade after its discovery, IFN was viewed as a curiosity, the research object of a small number of virologists; yet some had visions of its use therapeutically as an antiviral agent. Initial attempts to purify interferon, a prerequisite for clinical use, were unsuccessful because cells produced only a few molecules following its induction. However, these molecules were extremely potent biological response

with the same amount of wild-type virus alone. The advantages of the use of attenuated viruses are that the effective dominant gene(s) are delivered in the intact animal directly to the appropriate target cells and their expression is transient and does not cause the permanent alteration of the host genome.

Live virus vaccines should contain dominant negative mutations

In this connection it should be noted that the ability to interfere with the growth of wild-type virus would be a particularly desirable trait in the case of live virus vaccines. The use of vaccine viruses with attenuating mutations that also confer a dominant-negative phenotype would add a significant degree of protection against virulent revertants that may arise during the immunizing infection or against epidemic viruses that may be circulating concurrently in the population. In addition to their immunizing potential, dominant-negative vaccine viruses could offer short-term antiviral protection until the immune response to the virus is firmly established.

See also: **Antivirals; Defective interfering viruses; Interferons: Therapy of aids and cancer; Vesicular stomatitus viruses (*Rhabdoviridae*).**

Further Reading

D'Aloja P, Olivetta E *et al.* (1998) gag, vif, and nef genes contribute to the homologous viral interference induced by a nonproducer human immunodeficiency virus type 1 (HIV-1) variant: identification of novel HIV-1-inhibiting viral protein mutants. *J. Virol.* 72(5): 4308–4319.

Friedman AD, Triezenberg SJ and McKnight SL (1988) Expression of a truncated viral *trans*-activator selectively impedes lytic infection by its cognate virus. *Nature* 335: 452.

Herskowitz I (1987) Functional inactivation of genes by dominant negative mutations. *Nature* 329: 219.

Whitaker-Dowling P and Youngner JS (1987) Viral interference-dominance of mutant viruses over wild-type virus in mixed infections. *Microb. Rev.* 51: 179.

Whitaker-Dowling P, Maassab HF and Youngner JS (1991) Dominant-negative mutants as antiviral agents: simultaneous infection with the cold-adapted live-virus vaccine for influenza A protects ferrets from disease produced by wild-type influenza A. *J. Infect. Dis.* 164: 1200.

[This article is reproduced from the 1st edn (1994).]

INTERFERONS

IF

Philip I Marcus, University of Connecticut, Storrs, Connecticut, USA

History

Interferons (IFNs) represent the prototype of a family of cytokines with an extraordinary wide range of biological effects including their action as antiviral agents – the basis of their discovery. Interferons have emerged as some of the most active biological substances known. Their study reveals myriad effects on cells, contributing to our understanding of basic cellular, immuno- and developmental biology. The IFN system, both its induction and action, represents the first line of defense against viral diseases, and possibly other infections agents, and has attained official sanction as an acceptable therapeutic agent, fulfilling the dreams of its early investigators.

Virologists long recognized that exposure of cells to one kind of virus often rendered the cells refractory to infection by another virus, even of heterologous origin. The simultaneous occurrence of two different childhood virus diseases is unprecedented. The shroud of mystery surrounding these examples of 'viral interference' was lifted with a seminal discovery by Isaacs and Lindenmann in 1957. They found that chicken cells exposed to heat-inactivated influenza virus released a factor which, when added to fresh cells, reduced their capacity to produce new virus. Based on this ability to interfere with virus replication the factor aptly was termed 'interferon'. During the first decade after its discovery, IFN was viewed as a curiosity, the research object of a small number of virologists; yet some had visions of its use therapeutically as an antiviral agent. Initial attempts to purify interferon, a prerequisite for clinical use, were unsuccessful because cells produced only a few molecules following its induction. However, these molecules were extremely potent biological response

modifiers as measured during the action phase; detectable at about 10 molecules per cell, or 10^{-15} M.

Recognition of the full spectrum of the actions of IFNs came slowly, hampered by difficulties inherent in purifying a protein produced so sparingly by cells. As IFNs became available through recombinant DNA technology, the scope and magnitude of research broadened and the pace quickened. As part of a complex network of cytokines, IFNs display attributes beyond those of an antiviral agent. They function as negative cell growth regulators, immunomodulators, regulators of differentiation and development, inhibitors of some nonviral pathogens, and a reproductive hormone in ruminant ungulate species.

The small number of IFN molecules produced per cell initially limited human IFN for clinical use. For many years the Finnish Red Cross was the sole source of IFN, obtaining it from white blood cells infected with Sendai virus as the inducer. Its production limited clinical studies. In contrast, *Escherichia coli* that express human IFN can produce more than 10^9 units l^{-1}, representing over 10% of the bacterial mass, whereas clinical doses are on the order of 10^6 units per patient per day. Although IFN made by bacteria is not glycosylated, it is biologically active. All of the human IFN genes and many of animal origin, along with avian IFN genes, have been cloned and their respective IFNs produced in large quantity. Cloning and expression of chicken IFN completed the cycle of research started by the discovery of IFN in that species some 40 years ago. IFN molecules altered and produced by genetic engineering have enhanced specific activity, broadened species specificity and increased stability. Ingenuity, not amount, now limits the use of IFN.

Interferon Genes and Molecules

Cells produce two distinct types of IFNs, types I and II. These are distinguished by several biological and molecular attributes: the types of cells that express them, antigenicity, the 5′ regulatory regions of their respective genes, structural coding sequences, three-dimensional structure, the specific cellular receptors to which they bind and initiate signaling, and the signal transduction pathways (shared in part) that lead to activation of IFN action genes. IFNs collectively termed type I consist of four major family members, IFN-α, -β, -ω, -τ, and a new member, IFN-δ. Type II IFNs contain a single member, IFN-γ.

Type I interferons (α, β, ω, τ, δ) While antigenically distinct, these share a common ancestry and biological and biochemical properties, including their hallmark stability to heat ($\approx$ 70°C, 30 min) and acid (pH 2). Type I IFNs compete to varying degrees for common receptors on the cell surface ubiquitously distributed among virtually all cell types. Although many attributes are shared, each family displays distinct characteristics.

IFNs-α are produced primarily from leukocytes upon stimulation by double-stranded RNA (dsRNA) or viruses because of dsRNA formed during their replication. The human IFN-α family consists of 14 intronless genes and four pseudogenes located on chromosome 9. The 5′-flanking regions of the IFN-α genes contain sequences responsible for the inducible transcription that characterizes all IFN genes. Sequences in the 3′-flanking region contain polyadenylation sites and a motif common to genes that are characteristically expressed transiently due to an instability conferred on that class of mRNA. The primary proteins of the IFN-α family contain signal and mature protein sequences of 23 and 166 amino acids, respectively, and produce a molecule of $\approx$19.5 kDa. The biologically active molecules of human (Hu)IFN-α contain two intramolecular disulfide bonds, of which only one is essential (Cys29–Cys138), contributing to the heat and pH 2 stability that characterize type I IFNs.

IFN-β molecules are produced primarily by fibroblasts and epithelial cells following induction by dsRNA or viruses, and, in contrast to the HuIFN-α family, constitute the product of a single intronless gene. This gene also is located on chromosome 9, and shares about 30% homology with HuIFN-α genes. The HuIFN-α/β genes appear to have diverged about 250 million years ago, and are expressed in mammals, birds, reptiles and fish. All members of these families possess the IFN system, but those of mammals are best characterized. The fully processed, glycosylated and secreted molecule of HuIFN-β contains 166 amino acids and has a molecular weight of $\approx$23 kDa. The nonglycosylated molecule produced in *E. coli* has a molecular weight of 20 kDa and is fully active. HuIFN-β contains three cysteine residues. When Cys17 is replaced by serine, dysfunctional intramolecular sulfide bonds are precluded and the specific biological activity of this IFN-β_{ser} is increased. Murine IFN-β consists of five α helices, a structure shared by other cytokines. There is extensive sequence homology between the 5′-flanking regions of HuIFN-α and -β genes upstream from the TATA box, often resulting in the coordinate induction of both. In avian cells a dsRNase is coordinately induced with type I IFN.

IFN-ω (omega) is an antigenically distinct type I IFN that shares several attributes with IFN-α/β, including common receptors. The mature proteins of IFN-ω contain 172 residues, six amino acids longer

than IFN-α/β. IFN-ω makes up to 30% of the IFN produced by virus-induced peripheral blood mononuclear cells. IFN-ω, like IFN-α/β, induces an antiviral state, downregulates cell growth and modulates the immune response. The expression of IFN-stimulated genes may be sustained longer in IFN-ω-treated cells and hence this cytokine may prove to be more effective in the clinic. There are at least six HuIFN-ω genes, five of which are pseudogenes. IFN-ω is widely distributed among mammals, dogs being the exception, and likely diverged from IFN-α some 200 million years ago.

IFNs-τ (trophoblast) constitute type I IFNs induced by an unknown means in massive amounts from embryonic trophectoderm during maternal recognition of the conceptus and are the major product of the preimplantation embryo of ruminant ungulates. Like IFN-ω, the mature protein of IFN-τ contains 172 amino acids. Trophoblast IFNs are thought to have evolved from IFN-ω some 50 million years ago. Though IFN-τ and -ω share up to 75% sequence identity they are immunologically different and are derived from genes with distinctive promoter sequences. The unique action of IFN-τ as a reproductive hormone suggests that other novel IFNs may exist.

IFN-δ (delta) is a novel truncated type I IFN that is transiently expressed in porcine trophoblast during implantation in the uterus. The mature protein contains 149 amino acids following loss of a 21 amino acid signal peptide. IFN-δ is produced from an intronless gene, contains distinct regulatory sequences, and is antigenically distinguishable from other porcine IFNs. IFN-δ, like IFN-τ, represents a class of IFNs induced under physiological conditions and not significantly by viruses.

Type II interferon (IFN-γ, or immune IFN) This is produced by T lymphocytes following their exposure to mitogens, specific antigens or various cytokines. HuIFN-γ is encoded by a single gene on chromosome 12 which transcribes RNA containing three introns. The mRNA from the four exons produces a primary and signal-processed mature protein of 166 and 143 amino acids, respectively. The 16.8 kDa mature protein is glycosylated at one or two sites, to produce molecules of 20 kDa or 25 kDa. The active molecule does not contain cysteine residues, but functions biologically as a homodimer. The acid and heat lability of IFN-γ distinguish it from type I IFNs. The 5′-flanking region of the HuIFN-γ gene functions as an inducible transcriptional enhancer and contains sequences in regions comparable with those of other cytokines expressed in lymphocytes.

Induction of Interferons

Probably all cell types are capable of producing IFN when appropriately induced by viruses, dsRNA, mitogens or other cytokines. Most RNA viruses are good inducers of IFN because they generate dsRNA during replication and complete the induction process before host cell macromolecular synthesis is compromised. RNA viruses that fail to induce IFN invariably suppress its induction, even in cells already programmed to produce it. Some DNA viruses induce IFN because they generate dsRNA through convergent transcription of their genome. Even organisms as diverse as mycoplasmas, rickettsia, bacteria and protozoa can induce IFN, albeit less efficiently.

Once dsRNA appears in the cytoplasm it is recognized by a cellular protein with high binding affinity for helical RNA, an endogenous protein kinase (PKR) that is activated by dsRNA. For HuIFN-β this activation leads to phosphorylation and subsequent proteolysis of IκB, the inhibitor of the ubiquitous transcription factor NFκB. NFκB freed of its inhibitor is translocated to the nucleus where it functions as one of several positive regulatory factors for transcription.

In the fully competent but uninduced cell, expression of the HuIFN-β gene is tightly repressed by several protein factors bound to the 5′-promoter region of the genes that act cooperatively as negative regulators (HMGI/Y, OCT, IRF2, PRDI-BFI, PRDII-BFI, NRE-BP). IFN induction leads to activation of transcription factors that act positively (ATF2/CREB, HMGI/Y, IRF1, NFκB/Rel). These displace and/or rearrange extant negative regulators on the DNA sequence which contains four positive regulatory domains (PRDI-IV) and one overlapping negative regulatory element (NRE) upstream from the TATA box that precedes the transcription start site. This chain of events begins within minutes after a cell is exposed to an IFN inducer. Most RNA viruses are excellent inducers of IFN because they form dsRNA during replication or contain it as genome. The cell is exquisitely responsive to dsRNA – a single molecule constitutes the threshold for IFN induction. Helical RNA with as few as 40 base pairs suffice to activate dsRNA-dependent enzymes, albeit not as efficiently as longer sequences. Some chemicals, like imiquimod, an imidazoquinolinamine, induce IFN following oral administration.

IFN mRNA appears in the cell within an hour after virus infection/induction, reaches peak rates of synthesis by 5–10 h, and declines rapidly thereafter. Downregulation of transcription of positive regulatory factors, coupled with the programmed short half-life of IFN mRNA, contribute to the character-

istic pulse-like yield of IFN observed over 24 h. This highly regulated process results in a quantum, or finite, yield of IFN, the absolute value of which varies with both inducer and cell.

IFN production can be enhanced by 'priming' cells, i.e. pretreating them with IFN, or by superinduction. Priming induces higher levels of transcription factors, like IRF-1 (*IFN regulatory factor*), thus shortening the period to new IFN production and enhancing its yield. Superinduction is achieved through the sequential use of inhibitors of translation and transcription, and can enhance the yield of IFN several hundred-fold, by blocking production of repressor molecules and thereby prolonging transcription of IFN mRNA, and in some cases by extending its half-life.

The acquisition of IFN inducibility is linked to development. Embryonic cells of murine or avian origin cannot be induced to make IFN until after 5–7 days of gestation. Thereafter, the cells' capacity to produce IFN increases with embryonic age. Undifferentiated embryonal teratoma cells do not acquire IFN inducibility until they differentiate. The absence of *trans*-acting factors or IFN-stimulated response elements (ISRE)-binding proteins in the undifferentiated cell indicates developmental events regulate the process, as does the failure of viruses to activate NFκB and IRF-1 in young chicken embryo cells.

The Antiviral State

Within hours after virus infection, newly produced and released IFN is circulating in the body. Cells most proximal to the initially infected cell, and hence most vulnerable to infection, receive IFN first and in the highest concentrations. The binding of as few as ten molecules of IFN to specific receptors on the cell surface starts the action phase of the IFN system, where a broad spectrum antiviral state develops within hours. IFN is comparable in its action against viruses with that of broad-spectrum antibiotics against bacteria.

Receptor–IFN binding This initiates a cascade of events that terminate in an antiviral state. Receptors on the cell surface constitute specific sites to which IFNs bind with high affinity ($K_D \approx 10^{-10}$ M). Receptors number in the thousands per cell, and are transmembrane proteins molecularly distinct for types I and II IFN. Cellular receptors are responsible for species specificity. Thus, chicken IFN does not act on human cells, and vice versa; there are some exceptions. Human type I IFN receptors (IFNAR) are encoded by genes on chromosome 21 that express three subunits: IFNAR-1, IFNAR-2.1 and IFNAR-2.2. Both IFN-α/β bind to these IFNAR as monomeric ligands. Cells trisomic for chromosome 21 contain more receptors than diploid cells and consequently display an enhanced sensitivity to the action of type I IFN. HuIFN-γ binds as a homodimer to a specific transmembrane receptor, IFNGR, that contains two subunits: IFNGR-1, a high binding affinity molecule encoded on chromosome 6, and IFNGR-2, an accessory protein required for signaling that is encoded on chromosome 21. IFN-γ functioning as a homodimeric ligand may in turn dimerize two sets of IFNGR subunits to initiate the signaling that culminates in the expression of IFN action genes.

Signal transduction pathways Development of the antiviral state starts with IFN binding to specific cell receptors and ends with the expression of a coterie of rapid response genes, some of whose products confer an antiviral state. Between these two events lies a complex signal transduction pathway. Within minutes after the ligand IFN binds to its receptors a series of signal-responsive events convert latent cytoplasmic transcription factors into active proteins, IFN stimulated gene factors (ISGF), including a family of IFN-regulatory factors (IRF) that when properly assembled bind to specific DNA sequences: the IFN stimulated response elements (ISRE) for type I IFNs, and the gamma activated sequences (GAS) for type II IFN. There follows transcription of the myriad IFN-stimulated genes (ISG) whose products result in the broad range of antiviral and other effects that characterize IFN action. IFNs share parts of the signaling pathways with other cytokines and growth factors. The pathway for type I IFN induced action includes TYK2 (tyrosine kinase), JAK1 (Janus kinase), STAT1/STAT2 (signal transducer and activator of transcription) and p48 (a DNA binding protein). Upon ligand binding, the two kinases, located on the cytoplasmic side of IFN receptors, become activated through phosphorylation, and in turn phosphorylate and thus activate STATs, which complex with p48 to form ISGF-3. ISGF-3 translocates to the nucleus where, along with other transcription factors, it binds to ISRE for activation of type I IFN genes. In the type II IFN signaling pathway ligand binding activates by phosphorylation, JAK1/JAK2 which in turn phosphorylate and thus activate STAT1. The homodimer STAT1/STAT1 translocates to the nucleus and along with other transcription factors bind to GAS to initiate type II IFN gene activation. This receptor–JAK/STAT pathway distinguishes between the two types of IFN through extracellular binding and by selective use and activation of JAK/STAT members intracellularly. This results in selective activation of action genes of the two IFN families, with some overlap. In this way

events at the outer cell membrane are converted into DNA transcription signals and the selective expression of IFN action genes.

Antiviral systems activated by IFN action Following binding of IFN to cellular receptors and the resultant transduction signaling cascade, there is enhanced or new expression of over 100 genes. Gene activation generally does not require *de novo* protein synthesis, enabling cells to respond rapidly to IFN. Some genes are induced by either type I or II IFNs, others exclusively by one or the other. Only a few of these newly expressed proteins thus far have been linked to the antiviral state. These are considered below.

2–5 Oligoadenylate synthetase/2–5 (A)/RNase L system

2–5 Oligoadenylate synthetase (2–5 (A) synthetase) is constitutively present in a latent state at low basal levels in cells, and increases 10- to 1000-fold in response to IFN.

The latent enzyme is activated in a novel manner, namely through the high affinity binding ($K_D \approx 10^{-10}$ M) of dsRNA – a signature molecule of virus infection. Once activated by dsRNA the 2–5 (A) synthetase polymerizes ATP into a series of 2′–5′ linked oligomers. The trimer [(2′–5′)pppA(pA)$_n$], called 2–5A, often the most abundant species, activates a latent cellular endoribonuclease (RNase L), itself inducible by IFN, which cleaves single-stranded (ss) RNA and hence blocks protein synthesis through degradation of mRNA. The highest concentration of 2–5 A accumulates near the site of 2–5 (A) synthetase activation by viral dsRNA and hence activates first RNase L molecules closest to viral RNA synthesis. This results in a localized and preferential degradation of viral ssRNA or mRNA, often allowing the IFN-treated cell to survive virus infection. In subsequent rounds of infection in adjacent cells, IFN production and action continue to compromise the yield of virus and spare cells from its lethal action. Expression of the dsRNA-dependent 2–5 (A) synthetase/2–5A/RNase L system is transient, with a 2′–5′-phosphodiesterase regulating the level of 2–5A. Not all viruses are blocked by its action, but picornaviruses are particularly vulnerable. Normal regulation of cell growth, differentiation and development may require this enzyme system.

PKR, a dsRNA-dependent protein kinase

PKR (protein kinase RNA), a serine-threonine kinase found at low basal levels in cells, is another enzyme induced to high levels by IFN and activated by dsRNA. PKR has emerged as an important regulator of cellular processes. Due to its effects on both transcription and translation, PKR plays a key role in the development of an antiviral state against several different virus families, and in the regulation of cell growth, differentiation and transformation. When PKR is activated by dsRNA in the course of virus infection it autophosphorylates and in turn phosphorylates and thereby inactivates the translation initiation factor eIF-2α. This mechanism is not intrinsically specific for viral protein synthesis; none the less, when transient global inhibition of protein synthesis occurs, inhibition of translation of viral mRNA is particularly vulnerable, but the cell may recover. PKR plays a key role in both IFN induction, with IκB as its substrate, and in IFN action, where eIF2α serves as a major substrate. The importance of PKR in establishing an antiviral state is gleaned from the myriad reactions that viruses have evolved to circumvent its activation (see below). The 2–5 (A) synthetase/2–5 A/RNase L and PKR systems account for many, but not all, antiviral states induced by IFN.

Mx proteins

The type I IFN-inducible Mx proteins were first thought to be unique in their action against influenza viruses (*Orthomyxoviridae*). Indeed, transgenic mice expressing the *Mx* gene constitutively are resistant to influenza virus, but not to other viruses. In certain cell systems Mx proteins are active against viruses in the *Paramyxo-*, *Rhabdo-*, and *Bunyaviridae* families. The *Mx* gene is highly conserved in mammals, birds and fish, and responds to IFN through the ISRE and produces proteins that are distributed in the cytoplasm or nucleoplasm, depending upon the species of cell. Not all Mx proteins produce an antiviral state, as for example in chicken cells. Mx proteins are GTPases that belong to the dynamin family of GTP-binding proteins, a microtubule-associated enzyme with ATP- and GTP-hydrolyzing activity. The Mx protein is thought to block influenza virus replication because it forms a tight complex that encircles viral nucleoprotein and functionally impairs primary transcription of genomic RNA. Understanding the molecular basis of Mx protein action with its dependence on compartmentalization in the cell may lead to targeting treatments for specific viruses. Since mice lacking functional *Mx* genes appear healthy, no obvious function for this gene has been found beyond its role in the antiviral state.

The Mx system provides an example of what poetically may be termed Koch's postulates at the

molecular level. Cells lacking the *Mx* gene do not develop an antiviral state against influenza virus when treated with type I IFN. However, cells that naturally contain the *Mx* gene express its product when exposed to IFN and develop an antiviral state. When the *Mx* gene is isolated from these cells and expressed constitutively in cells otherwise lacking it, they become intrinsically resistant to influenza virus without exposure to IFN.

Other proteins associated with antiviral states induced by IFN

Many other IFN-induced proteins are being characterized to determine their contribution, if any, to the generalized antiviral state. For example, dsRNA-specific adenosine deaminase (dsRAD) induced by type I IFN catalyzes deamination of adenosine to inosine in dsRNA, but not in ssRNA. These changes might destabilize an RNA helix during virus replication and impart unwindase activity, or target the dsRNA for degradation. Neither possibility has yet been demonstrated as an antiviral mechanism.

Another type I IFN-induced protein, ISG-15, may adversely affect processing of human immunodeficiency virus (HIV) RNA, whereas p202 protein may reduce the activity of NFκB and other transcription factors, thereby adversely affecting the replication of viruses dependent on them. A family of IFN-induced IFN-regulatory factors (IRF-1 to -7, ICSBP, ISGF3λ, Pip) share binding affinity for ISRE/ISRE-like elements and function as transcription factors that may positively (IRF-1) or negatively (IRF-2) regulate ISRE-containing genes. To the extent that these genes may function to induce an antiviral state they are important in the global regulation of that state. One IFN-γ induced gene, IP-10, encoding a chemokine, may assume importance in the control of HIV infection because of the newly assigned role of the chemokine family as a coreceptor for HIV infection of CD4 cells. IFN-γ also activates macrophages. These produce a variety of toxic products and immune system reactants that may contribute to an antiviral state. One such product, an inducible nitric oxide (NO) synthase is reported to inhibit the replication of herpes- and poxviruses. Genes encoding the major histocompatibility antigens also are activated by IFNs, and may contribute to the killing of virus-infected cells by cytotoxic T lymphocytes. Another IFN-γ-induced protein illustrates IFN action directed at a higher organism. Human IFN-γ induces in fibroblasts high levels of indoleamine 2,3-dioxygenase. This tryptophan-degrading enzyme reduces intracellular tryptophan levels, thus compromising the growth of *Toxoplasma gondii*, an obligate intracellular protozoon.

Mechanisms of Interferon Action

Interferon is the only natural product known that can block the replication of viruses once they have entered the cell. Considering the myriad genes activated by IFN and the diverse nature of the viruses affected, it is not surprising that IFN may exert its antiviral action at different steps in the replication of different viruses, and by a range of means in different cells. Alterations occur in the physical and biochemical nature of the plasma membrane following exposure to IFN; however, inhibition of the early steps in virus replication, i.e. viral attachment, entry and uncoating, are not usually affected. In some cell types, entry of the virus is slowed, and in others, IFN action may block a late step in the maturation or release of virus. In cells treated with IFN and infected with transcriptase-containing [−]strand RNA viruses, the first synthetic step in replication, primary transcription from the input genome, is often compromised. Transcripts escaping this early block may succumb to an inhibition of translation. The combination in sequence provides a formidable barrier to viral replication. For [+]strand RNA viruses the inhibition of viral protein synthesis is the dominant mechanism of IFN action. The 2–5 (A) synthetase and PKR systems both provide mechanisms for inhibiting protein synthesis, and, along with the Mx system, are likely to be responsible for much of the antiviral action of IFN.

The plethora of other gene products induced by IFN provides the potential for other mechanisms, some quite specific in their action for a particular combination of host cell and virus. DNA viruses sensitive to the action of IFN are known to form dsRNA during replication, enabling them to activate any of the dsRNA-dependent systems and mimic RNA viruses in sensitivity.

Some IFN inducible genes can be activated directly by dsRNA, presumed to form during virus replication, through common sequences in the ISRE. Activation by dsRNA may involve phosphorylation events regulated by PKR, and in some cases involve activation of NFκB. Although several transcription factors may not be involved, IRF-1 is needed. IFN is not an intermediate in these inductions, nor is ISGF-3 required. This means that particular genes may be activated by novel transcription factors during infection without the necessity of awaiting the production and release of IFN and its mandatory feedback action through receptors on the cell surface. Direct activation of certain IFN-inducible genes by dsRNA provides a unique early warning/response to virus infection.

When cells are treated simultaneously with types I and II IFN the antiviral state that develops may be 10- to 40-fold greater than that produced by either type of IFN acting singly, or expected from additive effects. Type I IFN by itself induces ISGF-3α (STAT1/STAT2) which when complexed with the DNA binding protein p48 (ISGF-3γ) to form ISGF-3 (STAT1/STAT2/p48) translocates to the nucleus where it binds to ISRE with high affinity to cooperate in the transcription of IFN-stimulated genes (ISGs). The amount of ISGF-3 formed after type I IFN treatment may be limited by the basal levels of p48 intrinsic to the cell, and hence limit the magnitude of ISGs activated and the antiviral state. However, IFN-γ induces p48 (ISGF-3γ) so that in cells exposed to both types of IFN p48 is no longer limiting and elevated amounts of ISGF-3 are produced. From this view, the two types of IFN act in concert to produce maximal activation of IFN-stimulated genes and the enhanced antiviral state that follows. The production of both types of IFN at the site of virus infection and the ensuing synergy may mirror the situation in nature where the IFN system is presumed to abort most virus infections or ameliorate the disease.

In summary, to generate an antiviral state IFN must first bind to specific high affinity cellular receptors in the plasma membrane. The extent of this binding and/or basal or induced levels of transcription factors may constitute rate limiting steps in the spectrum of genes activated and the magnitude of the transcriptional response to IFN.

Antiantiviral Mechanisms

Viruses have evolved elaborate means of evading the action of interferon. Many simply turn off cellular macromolecular synthesis so rapidly that IFN is never produced, or leave little time for it to act. PKR, with its capacity to inactivate eIF2 and thus inhibit protein synthesis, emerges as a common target for many viruses that have developed antiantiviral mechanisms, a tribute to the important role this kinase plays in body defenses in addition to its central role as a regulator of cell proliferation. Adeno-, Epstein–Barr and human immunodeficiency viruses block activation (autophosphorylation) of the dsRNA-dependent PKR through production of small RNAs with secondary structure. The helical portions of these small viral RNAs bind to, but do not activate, PKR; however, they prevent activation by larger dsRNA that the virus may produce. Vaccinia and reoviruses synthesize dsRNA-binding proteins that preferentially sequester dsRNA, making it unavailable for activation of dsRNA-dependent enzymes. In addition, vaccinia virus synthesizes a decoy of eIF2 and thus blocks PKR-mediated phosphorylation inactivation of this translation initiation factor. Herpes simplex virus produces a protein that blocks the phosphorylation of eIF2 by PKR. All the anti-PKR mechanisms are designed to preserve cellular protein synthesis so that virus protein synthesis will not be compromised. Poliovirus and influenza virus use two other anti-antiviral mechanisms. The former produces a protease which degrades the PKR, and the latter activates a cellular protein, p58, which, in concert with some stress proteins, binds to PKR and prevents its activation. Adenovirus EIA protein competes with STATs for binding to ISRE and hence blocks transcriptional activation of ISGs. Hepatitis C virus (*Flaviviridae*) encodes a nonstructural protein that binds to, and inhibits, PKR. Other viruses employ different mechanisms. Poxviruses encode IFNGR-like molecules, which as decoys bind to IFN-γ, aborting its action. Equally inventive from the IFN induction view is hepatitis B virus: its core protein, and/or a terminal portion of its polymerase, prevents the induction of IFN. Undoubtedly other antiantiviral mechanisms will be revealed.

Clinical Use against Infections

In spite of their discovery as antiviral agents, the major impetus to develop IFNs as products of biotechnology and move them into the clinics came from their antitumor activity. The first therapeutic use of IFN by physicians was sanctioned by the Food and Drug Administration, based on its effectiveness against hairy cell leukemia, probably of viral origin (HTLV-II). Approval soon followed for the clinical use of IFN against hepatitis B virus (HBV, *Hepadnaviridae*), where IFN is the therapeutic agent of choice. With over 200 million carriers of HBV in the world, and the strong causal relationship between HBV infection and hepatocellular carcinoma, IFN could have a formidable impact on a serious virus-mediated disease leading to malignancy. IFN-α also emerges as the only available treatment for hepatitis C virus (*Flaviviridae*).

Infections (genital warts) caused by human papilloma viruses have been treated successfully by intralesional, topical and parenteral therapy with IFN. It also is used as adjunctive therapy in the reoccurrence of juvenile laryngeal papillomas following surgery.

Recombinant human IFN-α (rHuIFN-α) and IFN-β_{ser} have shown significant activity against rhinovirus infections in volunteers in both therapeutic and prophylactic situations, yet in natural infection in a family environment rIFN-α was not effective against

other respiratory viruses (influenza, parainfluenza or corona).

Synergistic effects have been reported between IFN and viral-specific antibody both *in vitro* and *in vivo*. Ocular infections with the picornavirus, causing acute hemorrhagic conjunctivitis, and with herpes simplex virus suggest that the combination of endogenously produced IFN and antibody may be more efficacious than either agent alone. A shared time during infection with these two antiviral agents, as the concentration of IFN wanes and that of antibody waxes, may represent the time for this synergistic action.

The progression of lesions from activated latent herpesviruses can be ameliorated, or their initiation prevented, by IFN treatment – an important consideration given the widespread distribution of this family of viruses and its propensity to break from latency in the stressed or immunocompromised host. The intrinsic sensitivity of herpes viruses to IFN portends its use in treating the most recently discovered human herpes virus HHV8 as the likely etiologic agent of Kaposi's sarcoma.

Successes with IFN as a clinically effective antiviral generally have not paralleled the dramatic effects observed *in vitro*. Once IFN enters the body it becomes but one actor in a large company of cytokines, some of which IFN can induce. Much remains to be learned about the optimal use of this potent cytokine *in vivo* before its full potential as an antiviral agent can be realized. For example, studies indicate that IFN may be more effective when used in combination with chemical antiviral agents, antibodies, dsRNA, or other IFNs – as in synergy.

Polyribonucleotides, in the form of a complex of polylysine and carboxymethylcellulose with poly(rI)·poly(rC) [poly ICLC], have been used clinically as inducers of IFN with the potential to activate the antiviral state they may illicit. This drug is resistant to serum dsRNases, and may mimic IFN induction and its action following natural infection. In a mouse model system poly ICLC enhances the antimalarial activity of chloroquine. Clinical results from these biological response modifiers that both induce and activate the IFN system should be enlightening.

Some bacterial infections result in the production of IFN, and are themselves adversely affected by its action through mechanisms not well understood but thought to involve the immune defense system, including enhanced phagocytic activity. Several protozoan parasites induce IFN in their mammalian host, and exogenously added IFN-γ, but not IFN-α/β, appears to alter the course of infection. The induction of a tryptophan-degrading enzyme by IFN may be involved. Further studies are needed to clarify the role of IFN-γ in resistance to infection by protozoa like *Toxoplasma* or *Leishmania*.

A note of caution must accompany the use of IFN as an antiviral agent. In some virus diseases in which IFN is induced to extremely high titers, the prognosis for recovery is inversely related to the titer of circulating IFN. Infection with the arenavirus causing Argentine hemorrhagic fever is an example. Also, prolonged therapy with IFN may induce neutralizing antibodies against some forms of the protein. However, new evidence indicates that proper preparation and storage of IFN will eliminate IFN–IFN and IFN–human serum albumin aggregates that appear to be antigenic.

Future Perspectives

The use of IFN as an antiviral agent *in vivo* has yet to live up to expectations based on *in vitro* studies. Perhaps we have not yet mastered the optimal means of inserting a potent biological response modifier like IFN into a system where perturbations to cytokine crosstalk must be carefully regulated. None the less, the IFN system has served as a prototype cytokine and continues to reveal new facets of gene regulation. The future seems bright for fine-tuning the use of IFN for clinical use. Several areas warrant further study. A more comprehensive understanding of the IFN system is needed at the molecular level to define the functions of most of the genes activated by IFN, dsRNA directly, or by drugs, and at the biological level to expand its role as an antiviral agent and biological response modifier. Subtle differences in the action of members of the IFN-α family need to be resolved. This may lead to molecular tailoring for the activation of specific IFN-stimulated genes and the design of IFNs for specific virus infections. A full appreciation of the extent to which synergy between types I and II IFNs functions in natural infection is needed for prudent application in the clinic. The three-dimensional structure/function analysis of IFNs and their receptors should provide further insight into the first step in IFN action and allow for designer IFNs. Continued progress in elucidating genetic-based defects in IFN-mediated signal transduction should serve as a guide for intervention through gene therapy. Mounting evidence points to significant antiviral and immunomodulatory effects from the administration of IFN at low doses (10–1000 units) to oral/mucosal systems. Thus, low doses of IFN-α administered orally to children who had measles, significantly ameliorated all symptoms of the disease. This seemingly novel means of stimulating the mucosal immune system may actually represent a natural pathway used to amplify and disseminate the action of IFN, and presages increased use of this mode

of administration. The production of IFN by the administration of chemical inducers of IFN orally or topically provides other novel means of activating the IFN system *in vivo*, as does its expression through the introduction into tissues of plasmid DNA encoding IFN genes.

The future calls for further study on the molecular subtleties of IFN structure/function, ligand–receptor interaction, signal transduction participants and pathways, expression of IFN-stimulated genes and their spectrum of actions, and the optimization of IFN use as one of many cytokines that function together. New avenues of research portend a bright future for the use of IFNs in the clinic and as the antiviral agents their discoverers envisioned over four decades ago.

See also: **Interference; Pathogenesis: Animal viruses; Immune response: Cell mediated immune response, General features; Vectors: Animal viruses; Antivirals.**

Further Reading

Baron S, Coopenhaver DH, Dianzani F, Fleischmann Jr WR, Hughes Jr TK, Klimpel GR, Niesel DW, Stanton GJ (eds) (1992) *Interferon: Principles and Medical Applications*. The University of Texas Medical Branch at Galveston, TX.

Commemorative Issue (1987) A Tribute to Jean Lindenmann Celebrating the 30th Anniversary of the Discovery of Interferon. *J. Interferon Res.* 7 (5).

De Maeyer E and De Maeyer-Guignard J (1988) *Interferon and Other Regulatory Cytokines*. New York: Wiley.

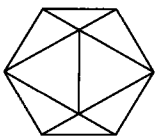
Fields BN, Knipe DM, Howley PM *et al* (eds) (1996) *Fields Virology*, Vol 1, 3rd edn. New York: Lippincott-Raven

Sen GC and Lengyel P (1992) The interferon system: A bird's eye view of its biochemistry. *J. Biol. Chem.* 267: 5017.

Sen GC and Ransohoff RM (1977) *Transcriptional Regulation in the Interferon System*. Austin, TX: Landes/ Chapman & Hall.

Stark GR, Kerr IM, Williams BRG, Silverman RH and Schreiber RD (1998) How cells respond to interferons. *Annual Rev. Biochem.* 67: 227.

IRIDOVIRIDAE – INVERTEBRATE



Richard Webby and **James Kalmakoff**, University of Otago, Dunedin, New Zealand

Introduction

Iridoviruses are icosahedral, double-stranded (ds) DNA viruses belonging to the family *Iridoviridae*. Members of this family are able to infect protozoa, algae, invertebrates and vertebrates. The viruses are ubiquitous in nature and have been found on all continents of the world and have been isolated from a variety of insect species. The genus *Iridovirus* consists of the small insect viruses (120–140 nm) with blue to purple iridescence, whereas the genus *Chloriridovirus* consists of the large insect virus group (>180 nm) with a yellow–green iridescence. The type species for the *Iridovirus* and Chloriridovirus groups are *Chilo* iridescent virus (CIV) (type 6) and IV3, respectively. Two other genera infecting vertebrates in the *Iridoviridae* family are *Ranavirus* and *Lymphocystivirus*.

Taxonomy and Classification

The first iridovirus (IV) to be described was isolated from the larvae of the crane fly *Tipula paludosa*, discovered by Claude Rivers in 1954. The systemic infection conferred a blue coloration to the larvae and the term iridescent virus was coined. Since then similar viruses have been isolated from different parts of the world and different invertebrate species. Iridoviruses have been isolated from invertebrates indigenous to all the major continents. The nomenclature of these viruses can be confusing as they were sometimes referred to by their historical designation, based on host of isolation, or by their type number. In the interim typing scheme viruses were assigned type numbers depending on the chronological order of their isolation. Since *Tipula* iridescent virus (TIV) was the first discovered it has been designated iridovirus type 1 (IV1), whereas CIV, being the sixth to be reported, was iridovirus type 6 (IV6). The problems associated with both these nomenclature systems have led to the suggestion that they should be replaced with geographical descriptors. TIV would thus become Plowden IV, Plowden being the nearest major town to the site of isolation. This system is analogous to that used for many virus families,

of administration. The production of IFN by the administration of chemical inducers of IFN orally or topically provides other novel means of activating the IFN system *in vivo*, as does its expression through the introduction into tissues of plasmid DNA encoding IFN genes.

The future calls for further study on the molecular subtleties of IFN structure/function, ligand–receptor interaction, signal transduction participants and pathways, expression of IFN-stimulated genes and their spectrum of actions, and the optimization of IFN use as one of many cytokines that function together. New avenues of research portend a bright future for the use of IFNs in the clinic and as the antiviral agents their discoverers envisioned over four decades ago.

See also: **Interference; Pathogenesis: Animal viruses; Immune response: Cell mediated immune response, General features; Vectors: Animal viruses; Antivirals.**

Further Reading

Baron S, Coopenhaver DH, Dianzani F, Fleischmann Jr WR, Hughes Jr TK, Klimpel GR, Niesel DW, Stanton GJ (eds) (1992) *Interferon: Principles and Medical Applications*. The University of Texas Medical Branch at Galveston, TX.

Commemorative Issue (1987) A Tribute to Jean Lindenmann Celebrating the 30th Anniversary of the Discovery of Interferon. *J. Interferon Res.* 7 (5).

De Maeyer E and De Maeyer-Guignard J (1988) *Interferon and Other Regulatory Cytokines*. New York: Wiley.

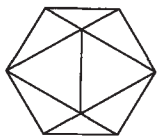
Fields BN, Knipe DM, Howley PM *et al* (eds) (1996) *Fields Virology*, Vol 1, 3rd edn. New York: Lippincott-Raven

Sen GC and Lengyel P (1992) The interferon system: A bird's eye view of its biochemistry. *J. Biol. Chem.* 267: 5017.

Sen GC and Ransohoff RM (1977) *Transcriptional Regulation in the Interferon System*. Austin, TX: Landes/ Chapman & Hall.

Stark GR, Kerr IM, Williams BRG, Silverman RH and Schreiber RD (1998) How cells respond to interferons. *Annual Rev. Biochem.* 67: 227.

IRIDOVIRIDAE – INVERTEBRATE



Richard Webby and **James Kalmakoff**, University of Otago, Dunedin, New Zealand

Introduction

Iridoviruses are icosahedral, double-stranded (ds) DNA viruses belonging to the family *Iridoviridae*. Members of this family are able to infect protozoa, algae, invertebrates and vertebrates. The viruses are ubiquitous in nature and have been found on all continents of the world and have been isolated from a variety of insect species. The genus *Iridovirus* consists of the small insect viruses (120–140 nm) with blue to purple iridescence, whereas the genus *Chloriridovirus* consists of the large insect virus group (>180 nm) with a yellow–green iridescence. The type species for the *Iridovirus* and Chloriridovirus groups are *Chilo* iridescent virus (CIV) (type 6) and IV3, respectively. Two other genera infecting vertebrates in the *Iridoviridae* family are *Ranavirus* and *Lymphocystivirus*.

Taxonomy and Classification

The first iridovirus (IV) to be described was isolated from the larvae of the crane fly *Tipula paludosa*, discovered by Claude Rivers in 1954. The systemic infection conferred a blue coloration to the larvae and the term iridescent virus was coined. Since then similar viruses have been isolated from different parts of the world and different invertebrate species. Iridoviruses have been isolated from invertebrates indigenous to all the major continents. The nomenclature of these viruses can be confusing as they were sometimes referred to by their historical designation, based on host of isolation, or by their type number. In the interim typing scheme viruses were assigned type numbers depending on the chronological order of their isolation. Since *Tipula* iridescent virus (TIV) was the first discovered it has been designated iridovirus type 1 (IV1), whereas CIV, being the sixth to be reported, was iridovirus type 6 (IV6). The problems associated with both these nomenclature systems have led to the suggestion that they should be replaced with geographical descriptors. TIV would thus become Plowden IV, Plowden being the nearest major town to the site of isolation. This system is analogous to that used for many virus families,

Table 1 Invertebrate iridescent viruses[a]

Type/ alternative name	*Host of isolation*	*Status of species*
IV1/TIV	*Tipula paludosa*	ICTV recognized species for which material is available
IV2/SIV	*Sericesthis pruinosa*	
IV3[b]	*Aedes taeniorhynchus*	
IV6/CIV	*Chilo suppressalis*	
IV9/WIV	*Wiseana cervinata*	
IV10	*Witlesia sabulosella*	
IV16/CzIV	*Costelytra zealandica*	
IV18	*Opogonia* sp.	
IV21	*Heliothis armigera*	
IV22	*Simulium* sp.	
IV23/BbIV	*Heteronychus arator*	
IV24	*Apis cerana*	
IV28	*Lethocerus columbiae*	
IV29	*Tenebrio molitor*	
IV30	*Heliothis zea*	
IV31	*Armadillidium vulgare*	
IV32	*Porcellio diatatus*	
AgIV	*Anticarsia gemmatalis*	Nonrecognized species for which material is available
PjIV	*Popillia japonica*	
IV4[b]	*Aedes cantans*	ICTV recognized species for which material is unavailable
IV5[b]	*Aedes annulipes*	
IV7[b]	*Simulium ornatum*	
IV8[b]	*Culicoides* sp.	
IV11[b]	*Aedes stimulans*	
IV12[b]	*Aedes cantans*	
IV13[b]	*Corethralla brakeleyi*	
IV14[b]	*Aedes detritus*	
IV15[b]	*Aedes detritus*	
IV17	*Pterostrictus madidus*	
IV19	*Odontria striata*	
IV20	*Simocephalus expinosus*	
IV25	*Tipula* sp.	
IV26	*'Mayfly'*	
IV27	*Nereis diversicolor*	
IV33[b]	*Chironomus plumosa*	

[a] Williams T, Chinchar G, Darai G, Kalmakoff J and Seligy V (1996) Report of the ICTV study group for the Iridoviridae. ICTV Executive Committee Meeting, Jerusalem August 1996

[b] Members of the genus *Chloriridovirus*.

including *Reoviridae, Bunyaviridae, Rhabdoviridae* and *Arenaviridae,* but this scheme offers little in terms of taxonomic relationships.

To date, more than 30 invertebrate iridescent viruses have been recognized and assigned a type (**Table 1**). Not all of the different isolates listed in **Table 1** are distinct viruses. For instance, type 9 was 100% homologous to type 18 by both DNA and serological comparisons, and these two viruses are probably the same. Type 19, which was isolated from the coleopteran host *Odontria striata,* is also very likely to be the same virus as type 16, which was isolated from a similar coleopteran host *Costelytra zealandica*. Both of these examples of redundancies in iridovirus classification are a direct consequence of the wide host range of IVs. In both situations the homologous viruses were isolated from the same location but from different hosts. Taking this into

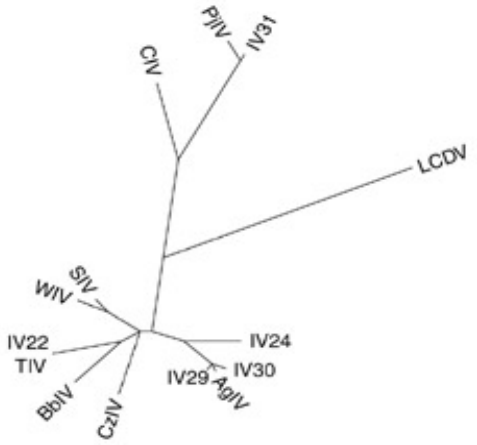


Figure 1 Phylogenetic tree of the genus *Iridovirus*. The tree was constructed from the nucleotide sequence of a 355 bp fragment of the major capsid protein gene using the Neighbor–Joining method. The vertebrate lymphocystis disease virus (LCDV) was used as an outgroup to estimate the tree root.

Table 2 Division of the genus *Iridovirus* based on molecular techniques

Group I[a]	*Group II*[a]	*Group III*[a]
IV31 (IV32)	CIV	TIV
PjIV	IV28 (IV21)	SIV
		WIV (IV10, IV18)
		CzIV (IV19)
		IV22 (IV25)
		IV23
		IV24
		IV29
		IV30
		AgIV

[a] Isolates in brackets represent variants of the same species.

consideration, and the fact that no material is available for many others, the number of species recognized by the International Committee on Taxonomy of Viruses (ICTV) can be significantly reduced. Recent findings also suggest that current stocks of IV types 21 and 28 (again probably variants of the same species) have become contaminated with CIV and should no longer be recognized as separate species.

The problems associated with the classification of the *Iridovirus* genus have only recently been addressed using molecular approaches. A broad comparative study using restriction endonuclease (REN) profiles, polymerase chain reaction (PCR) and DNA hybridization revealed that the genus can be divided into three further clusters. The high level of sequence homology in the major capsid protein (MCP) gene has also led to the development of a PCR/sequencing approach to classify these viruses. Consensus PCR primers were designed to amplify a 500 bp fragment of the *MCP* gene containing both conserved and variable regions. PCR products from a range of diverse invertebrate IVs were amplified and sequenced and the resulting nucleotide sequence used to construct a phylogenetic tree (**Fig. 1**). These results support the three divisions within the genus *Iridovirus*. A summary of the result of these studies is shown in **Table 2.** The groupings observed in the MCP-derived tree show no correlation to host of isolation, which is not surprising considering the wide host range of the virus family. The tree does, however, reflect the geographical distribution of virus isolates, with a correlation between closely related viruses and the regions of their geographic isolation. The true indication of virus relationships will remain unknown until more genes are compared in this way, or entire genomes sequenced. This PCR/sequencing technique in conjunction with restriction endonuclease profiles offers a simple tool to identify novel isolates. Preliminary results of solution DNA–DNA reassociation reactions indicate that this technique may be of limited taxonomic value for all but the most related of isolates. This is evident from the low homologies observed between related viruses from the same genus group. For example, *Wiseana* iridescent virus (WIV) and TIV, viruses clustered within the same group by molecular techniques, have DNA–DNA homologies of less than 5%. No homology was detected between WIV and CIV, viruses belonging to different genus groups.

Virion and Genome Structure

The first 'spherical' virus shown by shadowing and electron microscopy to have icosahedral symmetry was TIV (**Fig. 2**) in 1958. An icosahedron consists of 20 equilateral triangular faces each of which can consist of smaller subunits called capsomeres. Disruption of TIV gave triangular and pentagonal subunit aggregates. Each pentasymmetron contained 31 subunits and each trisymmetron contained 55 subunits. This suggested a possible triangulation number of $T = 147$ (1472 subunits). Negative staining shows the particle size to be 120 nm.

The genome is a single copy, linear, dsDNA, which varies in size between 150 and 280 kbp depending on viral species. It is cyclically permuted with terminal redundancy and has internal repetitive DNA sequences. CIV has a genome size of 210 kbp with a terminal redundancy (the amount of DNA duplicated

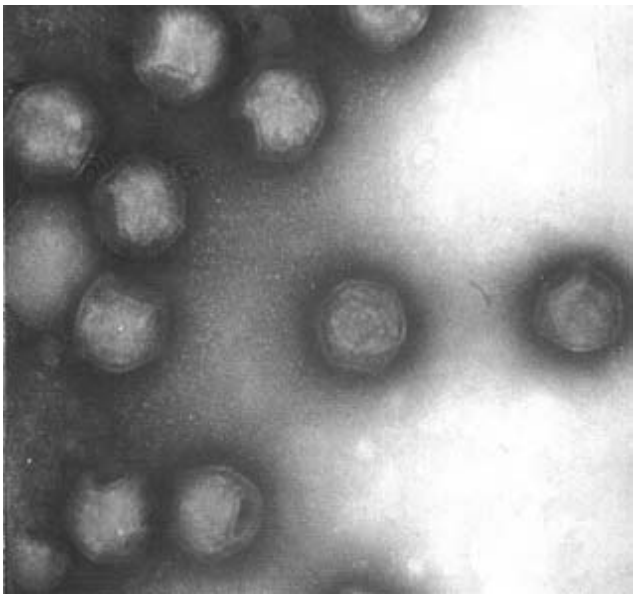


Figure 2 Electron micrographs of negative-stained TIV particles.

at each end of the genome) of 12% and the cyclic permutation (the range of fragments that formed the ends) was 100%, i.e. no unique ends. These genome structures are common to some prokaryotic viruses such as bacteriophage T4, but have not been demonstrated in any other eukaryotic viruses.

Virion Composition

The virions contain 11–18% DNA packed within an electron-dense core in association with six or more DNA binding proteins. The GC content of the DNA is about 30%. This DNA core is surrounded by an internal 4 nm thick lipid membrane (about 4–9% of the virus weight) in which phospholipids are abundant. The composition of this lipid membrane is independent of the host of propagation, but does differ from virus species to virus species. The exact mechanisms of viral lipid sequestering are unknown, although evidence suggests that the membrane is synthesized *de novo* during viral replication rather than by the acquisition of host membranes.

The third outer concentric domain is a proteinaceous icosahedral capsid which makes up 50% of the viral mass. It is intimately associated with the viral core by proteins that span the lipid membrane. Iridoviruses have been shown to have 25–70 structural polypeptides ranging in molecular weight from 12 to 150 kDa depending on the method used. They all have a major protein of 50–55 kDa which has been estimated to comprise 45% of the total proteins. Various enzymes have been found to be associated with virus particles. These include a protein kinase, an alkaline protease, a deoxyribonuclease, an RNA polymerase, a nucleotide phosphorylase and an ATPase. Not all of these enzymes have been shown to be virally encoded. There are reports that the protein kinase phosphorylates the structural proteins. Studies on TIV have shown that none of the virion structural proteins appear to be glycosylated. The DNA of the iridoviruses is not methylated unlike other members of the *Iridoviridae* which infect vertebrates. Some isolates of TIV contain two DNA components L (176–247 kbp) and S1 (10.8 kbp) within the DNA core.

Virus Genes

Sequence data for the family *Iridoviridae*, apart from lymphocystis disease virus (LCDV) which has been completely sequenced, are limited. Excluding the *MCP* gene, the only invertebrate IV species for which genes have been described are CIV and TIV. The complete *MCP* genes have been sequenced from IV22, TIV, CIV, WIV and *Costelytra zealandica* iridescent virus (CzIV). These share significant amino acid similarities with values ranging from 65.4% between CIV and IV22 to 93.7% between TIV and IV22. The only other gene described for TIV codes for a protein of 96 kDa which has homologies to various DNA binding proteins. This, and the presence of a glycosaminoglycan-like proteolytic cleavage site, has led to the speculation it may be involved in TIV structure. Additional genes from CIV code for proteins that share database homologies to zinc finger proteins, a nonhistone chromosomal high-mobility-group (HMG) protein, a GTP phosphohydrolase, a helicase, a DNA-dependent RNA polymerase subunit, a ribonucleoside-diphosphate reductase, and a protein that contains conserved motifs of *Baculoviridae* apoptosis inhibitors (IAP). Sequencing of 60 170 bp of the CIV genome revealed the presence of 112 possible reading frames with similarities in gene arrangements to LCDV. Phylogenetic comparisons of the CIV helicase and RNA polymerase genes suggest that these two genes have been acquired from eukaryotic host cells more recently than those of other cytoplasmic DNA viruses. This does, however, rely on unsubstantiated assumptions concerning evolutionary rates between virus families and host cells.

Virus–Host Interaction

The host range of some iridoviruses such as TIV appears to be wide, since they are able to infect dipteran, lepidopteran and coleopteran hosts, whereas others, e.g. CzIV are more restricted. The mode of transmission for these viruses has not been elucidated.

Many routes of infection have been suggested, including oral ingestion, infection through the spiracles, transovarial transmission and the contamination of eggs. The oral ingestion of virus appears to be one of the most obvious routes, but generally it is thought that these viruses are not very infectious *per os*, the virus being degraded shortly after entering the midgut. In the foregut, the peritrophic membrane is a barrier to infection. Transovarial infection appears to be the method of transmission for the chloriridovirus group. Other possible routes include cannibalism, wounding or infection via other parasites (such as nematodes). Cannibalism of infected cadavers would result in a massive uptake of concentrated virus. Wounding, which is common among aggressive species, would mimic the intrahemocelic injection that is used to infect insects in the laboratory.

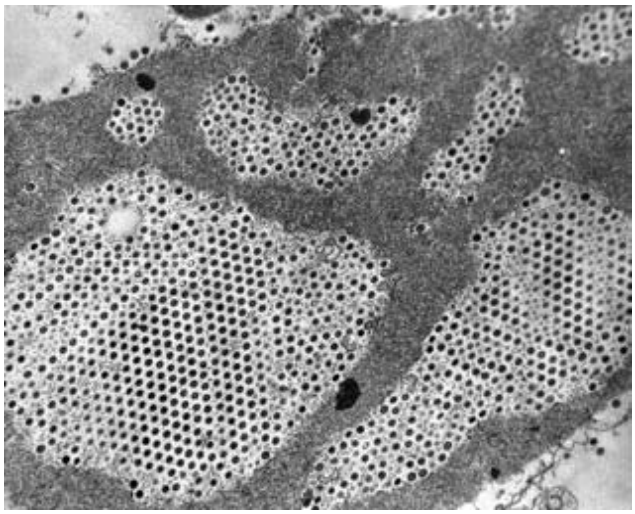


Figure 3 Viral crystalline array of TIV in the cytoplasm of a virus-infected cell.

Serology

Serological methods include serum neutralization, complement fixation, ELISA, latex agglutination, immunoperoxidase staining and radioimmunoassay. In general, serum neutralization is impractical for screening because of bacterial contamination of triturated larvae. Complement fixation is relatively insensitive when larval extracts are used and is not quantitative. Latex agglutination was found to be 50 times less sensitive than ELISA, and radioimmunoassay is technically demanding. The ELISA assay is probably the best serological technique available for virus detection, but has the disadvantage of being only semiquantitative. Moreover, many iridescent viruses were propagated in experimental laboratory hosts such as *Galleria mellonella*, and it is possible that there has been crosscontamination between iridescent viruses. For example, there were antigenic differences in TIV propagated in *Lymantria dispar* or *Tipula paludosa* when compared with TIV propagated in *G. mellonella*. Despite these problems, serology has been used to compare different isolates of iridoviruses and some general conclusions can be made. The members of the chloriridovirus genus (iridescent viruses types 3, 4, 5, 8, 11, 12, 14 and 15) appear to be serologically related to each other, but not to any members of the iridovirus genus. Within the iridovirus genus TIV and SIV have been shown to be virtually identical by most serological tests, including immunodiffusion, immunoprecipitation, and immunoelectron microscopy, although complement fixation and neutralization tests successfully discriminated between them. Other isolates tested showed relatedness to varying degrees with the exception of CIV and iridovirus type 24. Iridoviruses types 1, 2, 9, 10, 16, 18, 21, 22, 23, 24, 25 and 28 all showed common antigens and formed a broad serological group. Three groups of closely related viruses could be distinguished: (1) types 1, 22 and 25, (2) types 21 and 28, and (3) types 9 and 18. Analysis by Western blotting and the purification of individual polypeptides has shown that the antigenic relationships between the iridoviruses is very complex and some of the earlier findings have to be revised. For example CIV was shown not to be as antigenically distinct from the other iridoviruses (type 9 and type 16) as previously reported.

Inclusion Bodies

The major common feature of all iridescent viruses is the iridescent, or opalescent, blue–green coloration of heavily infected larvae or purified pellets of the virus. This coloration is caused by closely packed paracrystalline arrays of virus particles within heavily infected tissues causing Bragg reflections of light. The color of the iridescence is determined by the interparticle spacing. For TIV the virus particles are packed in a face-centered array with an interparticle distance of 250 nm, or about twice the size of virions observed by negative staining. The production of iridescence in infected larvae does not always occur, probably because paracrystalline virus arrays do not always form. This has been shown both *in vivo* and *in vitro* and may be due to the virus budding continuously from infected cells. Massive amounts of virus can result from the infection of larvae with the highest concentration in the fat body and epidermis. It has been suggested that iridescence may be a trivial characteristic of these viruses as it bears no relationship to any functional feature of the virus and is due only to physical structure.

By electron microscopy, viroplasmic centers can be seen as membrane-free pockets of cytoplasm 18–24 h after infection (**Fig. 3**). These contain 'dark matrix' where DNA synthesis is occurring and 'light matrix' where ribosomes are synthesizing viral proteins and where virion assembly occurs.

Pathology

The pathogenesis of these viruses is poorly understood since little is known about how the virus gains access to the initial site of replication. When administered orally these viruses have low infectivity which has led to suggestions that under natural conditions cannibalism, wounding, and parasitism by nematodes may play roles in transmission. The exception may be an iridovirus from *Scapteriscus vicinus* (Southern mole cricket) which has been shown to cause infections *per os*. The primary sites of replication are probably the hemocytes and fat bodies. The rate of pathogenesis is temperature-dependent and at optimum temperatures the virus spreads through the insect to produce a systemic infection. Larvae become blue and flaccid about 5–10 days after infection. The cytopathology has mainly been studied in hemocytes or cells in culture. Virus particles enter cells by pinocytosis or by phagocytic engulfment into membrane-bound vesicles within 1.5 h of inoculation. A general breakdown of the virus occurs within the vesicles where presumably they are uncoated, and viroplasmic centers appear in the cytoplasm after 4 h. Release of virus occurs by cell lysis or exocytosis and the virus is dispersed into the hemolymph. The cytoplasm has been described as the site of virus assembly, morphogenesis and DNA synthesis. However, the necessity for a functional cell nucleus has been proposed for frog virus 3. Infection with CIV rapidly induces the formation of syncytia which is independent of viral replication and due to components of the invading CIV particle.

Replication

The replication of frog virus 3 (FV3), a vertebrate IV, has been used as a model for the replication of other members of the *Iridoviridae*. In this model there are nuclear and cytoplasmic sites of DNA replication. After entry of the virus into the cell, the viral DNA is transported to the nucleus where transcription is initiated by the host RNA polymerase. The parental DNA is then used for DNA synthesis which results in the formation of genomic and greater than genomic length DNA. This DNA is then transported to the cytoplasm where it undergoes further DNA synthesis resulting in large concatemeric complexes. This concatemeric DNA is packaged into larger than genomic lengths by a 'headful' mechanism giving rise to the terminally redundant and cyclically permuted genome.

With CIV infection host cell macromolecular synthesis is rapidly shut down and this appears to be caused by unknown viral structural protein(s). The viral inner membrane proteins, which can be solubilized by octylglucoside, contain all the factors important in the initial stages of infection, including a cell-fusion activity (independent of capsid proteins), an inhibitory protein which switches off host cell synthesis and an activating factor which stimulates the transcription of immediate early viral genes. Cellular DNA polymerase, thymidine kinase and RNA polymerase levels increase during infection. The DNA is then transported to the cytoplasm where further DNA replication occurs producing high-molecular-weight concatemeric complexes. Studies carried out with WIV have shown that genes are transcribed in a temporal cascade that results in the formation of distinct immediate, delayed-early and late mRNA species. Furthermore, it was shown that the early mRNA is shut-off during late phase infection illustrating that the expression of the viral genome is controlled at the transcriptional level in a manner similar to other DNA viruses such as the herpesviruses.

The application of the FV3 replication model to invertebrate IVs may not be entirely correct. There are differences in genome structure that indicate some features of the FV3 model will need to be modified. The genome of FV3 is highly methylated unlike that of TIV and WIV and other invertebrate IVs. The expression of the FV3 genome may involve a methylation-based control mechanism not present in invertebrate IVs. The nature of the terminal redundancy is also different between invertebrate and vertebrate viruses. In CIV and WIV (and other iridoviruses) the ends of the DNA can represent any part of the genome and are therefore 'random' whereas in FV3 the DNA ends are restricted to 30% of the genome. This suggests that either the DNA packaging mechanism or the nature of the complex concatemeric DNA structure is different. The bacteriophage T4 has 'random' ends to its cyclically permuted terminally redundant genome. Furthermore, bacteriophage T4 has been shown to initiate replication from at least seven origins of replication, a similar number being found in CIV. During WIV infection the DNA in the cytoplasm appears to undergo the branched recombination/replication structure proposed for bacteriophage T4. This would suggest that the DNA replication of invertebrate IVs may be more closely related to bacteriophage T4 than that proposed for FV3.

Economic Importance

Conventional wisdom has it that the iridoviruses are not good biological control agents since they do not cause a lethal infection and are difficult to transmit in the field. However, the iridovirus isolated from southern mole crickets, *Scapteriscus vicinus*, operates in an epizootic fashion, killing greater than 70% of the collected mole crickets. When the virus is added to the diet of laboratory-reared nymphs, at least 65% became infected. This virus also infects *Trichoplusia ni* larvae and insect cell cultures and may be a pathogenic strain of iridovirus.

CIV might be of agricultural importance since it has been shown to infect the green rice leafhopper, *Nephtholettix cincticeps*, and is lethal for the leafhopper, *Colladonus montanus*, the vector of a mycoplasma agent of stone fruits. CIV could be used to eliminate insects which act as vectors in transmitting plant diseases.

WIV infects *Wiseana* spp. a major soil-dwelling pasture pest in New Zealand and infection rates can be as high as 30% based on the iridescence of the infected larvae. The actual infection rate could have been much higher since iridescence is a poor indicator of infection. Infection of the larvae results in cessation of feeding and death within 14 days. A similar situation applies to CzIV, a potential pathogen of coleopteran larvae. However, naturally occurring epizootics of WIV have not be reported since 1969.

There is an exciting potential in the field of genetic modification of these viruses. The terminal redundancy and cyclic permutation can be used to introduce toxins or to modify the virus easily. There is no necessity to find nonessential regions to insert genes since the DNA contains at least 112% of the genome and the duplication is at random. Furthermore the promoter for the coat protein gene would seem to be a good candidate site since this gene is highly expressed during virus infection, up to 25% of the weight of an infected insect being virus.

Ecology

Evans and Entwistle have discussed the division of the invertebrate *Iridoviridae* into the large (*Chloriridovirus*) and small (*Iridovirus*) insect iridescent viruses, and their evolution. They suggested that, based on ecologically different routes of evolution, *Iridovirus* with its wider host range is the older genus and *Chloriridovirus*, which has evolved exclusively in association with the aquatic diptera, is a more specialized derivative. The iridoviruses are thought to have only a horizontal route of transmission, whereas the chloriridoviruses have a vertical route of transmission in addition to larval cannibalism. It has been suggested that the internal membrane structure gives these viruses greater stability in aquatic environments. This idea has been extrapolated to suggest that insect viruses were the first viruses and that they later adapted to life in vertebrates which fed on the insects. WIV and CzIV, isolated from indigenous sympatric hosts in New Zealand, are genetically closely related and probably share a common ancestor. The WIV genome is 24 kbp larger than the CzIV genome and the region of nonhomology between the WIV and CzIV genomes is also 24 kbp in size. It is possible that in the course of evolution WIV gained that extra piece of DNA or CzIV lost it. In considering insect viruses in general, it has been postulated that the evolution of the virus group is probably not older than the host taxon itself. Using this assumption one could postulate that since the Coleoptera (late permian) are more ancient than the Lepidoptera (middle cretaceous) then CzIV is probably older than WIV, in which case WIV acquired the extra piece of DNA as it diverged from CzIV. Since CzIV has a limited host range, one could speculate that the piece of DNA which was gained by WIV coded for the host range determinants. This idea can also cover CIV which has a genome of 209 kbp, 17 kbp larger than WIV, and CIV has a larger host range than WIV. It would be worthwhile to return to the sites of the original WIV and CzIV isolations to see whether there have been any changes in these viruses in the years since the original isolation. One iridovirus isolate, TIV, appears to have diverged into two evolutionary lines. The TIV virion comprises two DNA components, L and S1. Recently an isolate from Ireland was found to contain only the L component. Since this isolate was from the same host as the original isolate, one might speculate that time and differences in climate and environmental conditions have enabled a variant TIV strain to evolve. Of interest would be the natural occurrence of the iridoviruses from isopods and nematodes and their relationship with soil insects. Equally interesting is the ecology of the isolates from aquatic environments. For instance, *Corethrella brakeleyi* is a small chaoborid found in Louisiana, a predator of first-instar mosquito larvae which can be infected with an iridovirus, but no transmission occurs to mosquitoes or vice versa. In New Zealand a copepod was found to be infected with an iridovirus and in the same pool an epizootic of an iridovirus was occurring among mosquitoes, yet the two viruses appeared to be unrelated.

A major drawback to the ecological study of IVs is the historical use of iridescence to monitor infected insects. Within an infected population only a small

percentage of individuals will show this phenomenon. Williams found that within a population of blackfly larvae from the River Ystwyth, Wales, external signs of IV infections were extremely rare, whereas infection rates as high as 37% were seen when a PCR-based diagnosis approach was used. This suggests that many earlier studies on the impact of IV infections on insect populations may be misleading and grossly understated. Further studies on the same blackfly populations also indicated distinct patterns of covert (inapparent) and patent (lethal) infections. Although diverse viral isolates were apparent by REN profile polymorphism, it seems that the two patterns of infections are not caused by different IV species, with identical viruses being associated with covert and patent infections. Changes in the ratios of covert and patent infections coincided with changes in population density, water flow, temperature and growth rates. Similarly, seasonal changes and host densities have been shown to be critical to the persistence of IV infections within mixed woodlice populations.

Now that specific and sensitive DNA probes are available to detect iridoviruses from field samples, the ecology offers many Cinderella-like opportunities.

See also: **Frog virus 3 (*Iridoviridae*); Lymphocystis disease virus (*Iridoviridae*).**

Further Reading

Darai G (ed.) (1990) *Molecular Biology of Iridoviruses.* Boston, Dordrecht and London: Kluwer Academic Publishers.

Evans HF and Entwistle PF (1987) Viral diseases In: Fuxa JR and Tanada Y (eds) *Epizootiology of Insect Disease,* p. 257. New York: John Wiley.

Ward VK and Kalmakoff J (1991) Invertebrate *Iridoviridae.* In: Kurstak E (ed.) *Viruses of Invertebrates,* p. 197. New York: Marcel Dekker.

Williams T (1997) The Iridoviruses. *Adv. Virus Res.* 46: 345.

Willis DB (ed.) (1985) *Iridoviridae. Curr. Top. Microbiol. Immunol.* 116: 173.

Isfahan Virus *see* **Chandipura, Piry and Isfahan Viruses**

J

JAPANESE ENCEPHALITIS VIRUS (*FLAVIVIRIDAE*)

Akira Igarashi, Nagasaki University, Nagasaki, Japan

History

Acute encephalitis which is different from Von Economo's encephalitis (type A) was described as type B encephalitis as early as 1981 in Japan. A large epidemic occurred in 1924, and the causative agent was isolated from fatal human brain in 1935. The disease sporadically occurred in late summer to early autumn in Japan. Epidemiological studies on the disease clarified the transmission cycle by vector mosquito *Culex tritaeniorhynchus* and virus amplification in swine and avians. The names of the disease and its causative virus, 'Japanese encephalitis (JE)' and 'JE virus', were derived from the first virus isolation in Japan. However, JE and JE virus exist in much wider Asian monsoon areas in the east, through southeast, to south Asia, where rice cultivation and swine raising are common.

Taxonomy and Classification

From its natural transmission cycle and antigenic crossreactivity, JE virus was classified in the group B arboviruses, which contains many human and veterinary pathogens. According to the virion morphology and single-stranded positive-sense RNA genome, group B arboviruses were later classified in the genus *Flavivirus* of the family *Togaviridae*. Because of its unique genomic organization, structural and replication strategy, this genus then became the genus *Flavivirus* of an independent family *Flaviviridae*.

Properties of the Virion

The JE virus is an enveloped spherical particle of 38–50 nm in diameter. Nucleocapsid symmetry is probably cubic, but a report showed helical structure in the virion preparation. Peplomers have not been clearly identified.

Properties of the Genome

The genome of JE virus is single-stranded, positive-sense linear RNA molecule of 45S. Its 3′ end does not carry a poly(A) tract, but is postulated to form a stem–loop secondary structure, to which some cellular regulatory proteins could interact. The entire or partial nucleotide sequence has been analyzed for several strains of JE virus. The total number of nucleotides is 10 976 (95 in the 5′ noncoding region, 10 296 in a long open reading frame (ORF), and 585 in the 3′ noncoding region). Similar to other flavivirus genomes, the ORF codes seven virus specific proteins (C, PreM/M, E, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5).

Properties of Virus Proteins

JE virion contains three structural proteins: membrane protein (M), core protein (C), and envelope glycoprotein (E). The M protein consists of 75 amino acids with a calculated molecular weight (mol. wt) of 8329 and is hydrophobic, and inside the lipid envelope. The C protein consists of 136 amino acids of mol. wt 13 859, is rich in basic amino acids (20% Lys and Arg) and interacts with genomic RNA. The E protein consists of 500 amino acids of mol. wt 53 334, contains a single potential *N*-glycosylation site (Asn-X-Ser) at amino acid number 448, and is exposed on the surface of the virion.

Multiple E protein epitopes with various biological properties and crossreactivities have been identified by monoclonal antibodies, such as JE-specific, flavivirus crossreacting and subgroup crossreacting ones. Passive immunization of the neutralizing and JE-specific monoclonal antibody was protective in mice from lethal challenge of JE virus.

Physical Properties of the Virion

The sedimentation coefficient of infective mature virion is around 200S with a density of 1.23 g cm^{-3} in sucrose-D_2O or 1.20 g cm^{-3} in potassium tartrate. Besides mature virion, slowly sedimenting hemagglutinin (SHA) and soluble complement fixing antigen (SCF) are present in infected mouse brain homogenate and infected cell culture fluid. The SHA is associated with the E protein and the SCF with the nonstructural protein NS1, respectively.

JE virus infectivity is readily lost by heat, acid, lipid solvents and detergents. Lipid solvents (ether and chloroform), or ionic detergents (sodium deoxycholate and sodium dodecyl sulfate) inactivate both infectivity and hemagglutinating activity (HA). Although nonionic detergents (Triton X100, or Nonidet P40) destroy virion structure and infectivity, HA is retained.

Replication of Genomic RNA

Replication of JE virus RNA appears to take place in close relationship with membrane structures, particularly the outer nuclear membrane as shown by the localization of radiolabeled virus-specific RNA and viral RNA polymerase. Virus growth was inhibited in cells enucleated before virus infection, but proceeded at reduced level in the cells enucleated after 6 h of infection. Sucrose gradient sedimentation analysis of radiolabeled virus-specific RNA revealed that the 8–12S RNA was labeled earlier than the RNase-resistant 23S replicative form. The implication of this 'intermediate RNA' is not clear. Positive-sense genomic RNA codes virus-specific nonstructural proteins, some of which (probably NS3 and NS5) were found in the membrane and 'nuclear' fractions which are associated with high viral RNA polymerase activity. This enzyme activity was inhibited by antisera against NS3 and NS5, indicating their role in viral RNA replication. By strand-specific probes, progeny positive-sense viral RNA was found primarily in the membrane fraction, in contrast to the negative-sense virus-specific RNA which was present in the supernatant fraction of the infected cells.

A single polyprotein is translated from a long ORF of virion RNA and is cotranslationally processed to the final products. Two forms of the nonstructural glycoprotein, NS1, were found in virus-infected cells, probably arising from different modes of cleavage of the polyprotein. Both forms of the NS1 and E protein were secreted into the medium of infected mosquito cells. The E protein has to be associated with the PreM protein for their proper transport and secretion. The M protein is post-translationally cleaved from its glycosylated precursor (PreM) in the Golgi bodies.

Mature virions accumulate in cytoplasmic vacuoles and lumen of smooth endoplasmic reticulum in the infected cells and tissues.

The cytopathic effect of JE virus infection is generally mild, possibly because of slow growth rate.

Host Range and Virus Propagation

JE virus is transmitted among various vertebrate hosts by vector mosquitoes. A number of mammals (swine, equine, canine), avians and reptiles are infected with JE virus in nature. Horses suffer from fatal encephalitis by JE virus infection, whereas infections in swine are generally inapparent, except for stillbirth or abortion in pregnant swine and aspermia in the boar.

The most important vector mosquito in the epidemic cycle is *C. tritaeniorhynchus* and related species which breed in watered rice fields.

Mice have been used most extensively for animal experiments on JE, and suckling mice are the most sensitive animals to propagate JE virus, particularly by intracerebral inoculation. Persistent infection of JE virus in pregnant mice was reported to result in vertical transmission of the virus into progenies.

A number of primary and continuous cell cultures can support the growth of JE virus, such as hamster, porcine, monkey, chicken, as well as cells of mosquito origin. They have been used for virus infectivity assay by plaque formation or cytopathic effect, and also for virus isolation.

Genetics

Only a single serotype has been identified among JE virus isolates, although variation was recognized by serological and molecular studies. Hemagglutination inhibition (HI) and neutralization (N) tests combined with antigen adsorption procedure could distinguish at least two subtypes, Nakayama and JaGAr-01 (including Beijing-1), among JE virus isolates in Japan. On the other hand, all 12 strains isolated in Thailand and examined so far belonged to other subtypes. By oligonucleotide fingerprint analysis, and recently by nucleotide sequencing of PreM gene, differences among JE virus strains with different isolation histories were demonstrated at the genomic level. The strains isolated in the same geographical areas are generally similar, particularly those isolated in the same year.

Recombination was not definitely demonstrated among different JE virus strains possessing different phenotypes.

Serologic Relationships and Variability

JE virus crossreacts with most of the flaviviruses in the HI and less in the complement fixation (CF) test. A particularly close relationship was observed with Murray Valley encephalitis, West Nile and St Louis encephalitis viruses. This relationship was also shown by the homology of their nucleotide sequence, and these viruses constitute a subgroup or complex in the genus *Flavivirus*, family *Flaviviridae*, although each virus can be differentiated by the N test.

Epidemiology

Because of the mode of transmission cycle in nature, infection of JE virus depends on the activity of vector mosquitoes. The epidemic season is late summer (August) to early autumn (September) in temperate areas like Japan, Korea and China, or the rainy season in tropical or subtropical areas. Only one out of several hundred infected humans manifests apparent encephalitis and the majority remain as inapparent infection. In the areas where human vaccination is not widely performed and JE immunity is conferred by natural infection, the most frequently affected age groups are the children under 14 years old. In contrast, adults (particularly elderly people) are frequently affected in Japan where human vaccination has been performed widely.

After 1966 in Japan, the number of human JE cases decreased dramatically and the low endemicity with less than 100 annual cases has been maintained from 1972 until present. It is generally agreed that this decrease is due to mass vaccination of humans and reduced vector population. The latter was the result of wide spraying of pesticides in watered rice fields by the farmers to protect the crop. Recently, field-caught *C. tritaeniorhynchus* was found to be highly resistant to pesticides, particularly organophosphorus compounds, and the mosquito population is increasing. Still, the number of human JE cases is kept at a low level in Japan probably because of sustained vaccination and spatial dissociation between the *Culex*–swine cycle of JE virus transmission and human dwellings.

In the Republic of Korea, the number of human cases has decreased significantly since 1981, with virtually no officially recognized JE cases after 1983. This change is reported as the result of mass vaccination of humans.

In China, extensive human vaccination with hamster kidney cell culture-derived JE vaccine has been performed. However, more than 10 000 sometimes 20 000 JE cases are sill reported every year, indicating insufficient vaccine supply.

In contrast, the magnitude of JE epidemics has apparently been increased in Vietnam and Thailand after the 1960s, in India and Nepal after 1978, and in Sri Lanka after 1985. It was suggested that this change was the result of economic, particularly agricultural, development to meet the demand of food supply by population explosion.

JE is endemic in tropical Asia like Philippines, Malaysia and Indonesia and spill over of JE in surrounding areas has been reported, for example, in Pakistan, Saipan Island and Badu Island in the Torres Strait between Papua New Guinea and Australia.

Transmission and Tissue Tropism

Infection of JE virus in humans occurs through infective vector mosquitoes. Humans are relatively insensitive to JE virus infection, and dead-end hosts in the natural transmission cycle of JE virus, without significant viremia, and no appreciable transmission from human to human or vectors.

In infected vertebrates, JE virus appears in the blood (viremia) before invasion to its final target organ, the central nervous system (CNS). The viremia level is determined by virus growth in extraneural tissues (initial inoculation site, regional lymph nodes, liver, kidney and muscles). Viremia subsides as humoral antibodies are produced, and further clearance takes place by the reticuloendothelial system.

Pathology

The pathogenicity of JE virus depends on its neuroinvasion, and is determined by both host and viral factors, as well as virus dose and route of infection. Generally, young animals are more susceptible than old ones, and the difference is more pronounced by peripheral than intracerebral inoculation of the virus. Neuroinvasiveness differs from strain to strain and is related to the level of viremia. Analysis by monoclonal antibody-escaped mutants showed that certain amino acid replacements on the E protein result in the loss of neuropathogenicity, indicating that the interaction of the virion surface and host cell membrane is a determinant factor of neuroinvasion. Comparative nucleotide sequence analysis on Chinese attenuated vaccine strain (SA14-14-2) and its parental virulent strain identified multiple nucleotide and amino acid substitutions. Analysis of the progeny virus derived from infectious cDNA showed that a single substitution from Glu to Lys at amino acid position 138 on the E protein is related to the attenuation phenotype.

In the CNS, JE virus grows efficiently in the neurons, and their destruction is directly related to the clinical manifestation of encephalitis.

Clinical Features of Infection

JE is a typical acute encephalitis with sudden onset of high fever and headache after incubation for 1–2 weeks. After a few days of prodromal stage, full symptoms of encephalitis appear with impaired consciousness. Nuchal rigidity is present in all cases, with frequent signs and symptoms of chills, vomiting, malaise, photophobia, pareses and tremors. Cerebrospinal fluid (CSF) findings are moderately elevated pressure and protein content, and lymphocytic pleocytosis with normal sugar level. The peripheral blood picture shows moderate leukocytosis with relative lymphopenia. The case fatality rate is high, around 25–50%, and most of the deaths occur around 5–9 days after onset. Respiratory dysfunction, abnormal reflexes, seizures, prolonged fever, albuminuria, infectious virus and absence of anti-JE antibodies in the CSF are correlated with poor prognosis. There is no specific treatment for JE except supportive care. Grave sequelae with neurological and psychiatric disorders are observed in approximately half of survivors.

Pathology and Histopathology

Three major pathological findings in the CNS have been described: (1) perivascular cuffing (infiltration of small round inflammatory cells around small vessels); (2) neuronophagia (degeneration and necrosis of neurons and their uptake by phagocytes); and (3) foci (accumulation of small round cells probably microglia and lymphocytes). These changes are observed in the gray matter of the brain. Immunofluorescent staining reveals the presence of JE antigen in the neurons but not in glia cells.

Immune Response

IgM class antibodies are found in the acute phase serum and CSF after JE virus infection. Demonstration of these early antibodies, particularly in CSF, is diagnostic and indicative of CNS involvement. Serum IgM antibodies are produced also in systemic inapparent infection.

Because of crossreactions with other flaviviruses, classical serology by the HI test on paired sera sometimes shows a pattern of secondary flavivirus infection. This pattern is characterized by high-titered IgG antibodies which crossreact with almost all flavivirus antigens, and is frequently observed in the areas where multiple flavivirus infections, particularly dengue virus infections, coexist. Such patterns are difficult to interpret and provide the diagnosis of just flavivirus infection. Since IgM class antibodies are produced transiently and are more specific to recently infecting virus type, the HI test was performed on IgM fractions separated by sucrose gradient centrifugation, or on serum specimens before and after 2-mercaptoethanol treatment. The IgM antibody assay was simplified by radioimmunoassay or ELISA.

Prevention and Control

Formalin-inactivated and highly purified vaccine has been developed from infected mouse brain in Japan, and its efficacy and safety have been demonstrated in the field as well as laboratory studies. Sufficient level of N antibodies was produced when two doses of vaccine were given with a one week to one month interval followed by a booster shot in the next year. The protective level of N antibodies gradually declines over 3–4 years but can promptly be augmented by a booster shot. In passively immunized mice, N antibodies detectable at 1:10 serum dilution was protective against the lethal challenge of 10^4 LD_{50} JE virus, which is the maximal amount of the virus inoculated by a single mosquito bite. The present recommendation for JE vaccination in Japan is initial immunization with two doses with 1–2 week intervals followed by a booster shot in the next year. Protective immunity should be maintained by booster immunization every 3–4 years.

Another formalin-inactivated vaccine, prepared from infected hamster kidney cell cultures, has been extensively used in China. An international standard to cover both the Japanese and Chinese inactivated vaccines has been worked out by the Expert Advisory Committee of the World Health Organization (WHO).

To cover various JE virus strains with different antigenic subtypes, bivalent vaccine was prepared by combining the classical Nakayama vaccine and Beijing-1 vaccine. Mice immunized with the bivalent vaccine produced antisera which neutralized almost all strains to similar levels. Since a single strain is preferred for vaccine production and quality control, the National Institute of Health of Japan changed the vaccine strain from Nakayama to Beijing-1 because of a wider range of neutralizing capacity of anti-Beijing-1 sera than anti-Nakayama sera produced in mice.

Swine immunization with live-attenuated vaccines in limited areas in Japan provided apparently successful results with a reduced number of human JE cases in the following years. However, swine immunization has not been extensively used because of the high turnover rate of the swine population and the limited

window period of live-attenuated vaccine administration, which is ineffective in the presence of maternal antibodies.

Several live-attenuated JE vaccine strains were developed in China, and one of them (SA14-14-2) produced anti-JE antibodies in a number of children and horses without adverse reactions.

Overwintering in Temperate Climatic Areas

No definite answer has been obtained for the overwintering mechanism of JE virus in temperate areas. Two alternative possibilities have been postulated: overwintering of the virus locally and reintroduction of the virus from tropical or subtropical areas. It is generally agreed that the epidemic cycle of *Culex*–swine amplification of JE virus is not directly involved in virus survival in nature and some basic cycle must be present. The possibility of virus survival in vertebrate animals was analyzed by serological survey and virus isolation. Bats were shown to be infected with JE virus for a prolonged period and produced sufficient viremia to infect vector mosquitoes. Some viruses (including JE virus) were isolated from bats in nature; however, the connection between bat infection and the *Culex*–swine epidemic cycle has not been demonstrated. Experimental infection of JE virus in some reptiles demonstrated virus survival during the winter season and appearance of viremia the following spring. However, no evidence was obtained that the reptile was involved in the natural transmission of JE virus. Neither has the implication of JE antibodies among turtles been clarified. Also, the attempts to isolate JE virus from overwintering *C. tritaeniorhynchus* turned out to be negative. Under experimental conditions, JE virus was transovarially transmitted from infected female *Aedes* mosquitoes to progenies. This finding should be substantiated by demonstrating that natural *Aedes* mosquitoes are frequently infected with JE virus and connected to the epidemic virus transmission cycle. Attempts to demonstrate JE virus reintroduction by infected migratory birds or vector mosquitoes have also been negative.

A recent survey of swine abortion in the low endemic northern island of Japan (Hokkaido) demonstrated that JE virus infection occurs focally (not continuously) on several separate swine farms. This result supports the possibility that JE virus overwinters in isolated domestic areas. By the reverse transcription polymerase chain reaction (RT-PCR) persisting JE virus genome was detected in peripheral blood or spleen cells on infected swine. The finding should be supported by the facts that such persisting viral genome could lead to the epidemic cycle of JE virus transmission.

Further Perspectives

Control of JE epidemics in developing Asian countries is a high-priority health problem. In order to meet the supply of inexpensive JE vaccine in large quantity, development of the second generation vaccine by molecular technologies has been postulated by WHO. Initial success toward this objective has been the expression of the E gene by the recombinant yeast, *Escherichia coli*, baculovirus and vaccinia virus. However, antigenicities of the gene products were generally low and inappropriate as candidate vaccines. This phenomenon may be related to the fact that most of the E protein epitopes are conformation dependent. The E gene product, with almost similar reactivities with a battery of monoclonal antibodies as authentic E protein, was obtained by a recombinant which possessed not only E but also its upstream PreM and a part of the C gene. This gene product was expressed on the surface of the recombinant virus-infected cells. In the recombinant vaccinia virus-infected cells subviral particles similar to SHA and carrying E and PreM protein was secreted extracellularly. Animals immunized with such recombinant baculovirus-infected cells, recombinant vaccinia virus, or subviral particles produced anti-JE N antibodies and were protected from lethal challenge of the virus. Further studies require purification of the gene products and safety assessment to use live recombinant vaccinia virus for humans in the face of smallpox eradication. Recent immunization of mice with a DNA molecule coding JE virus PreM and E proteins produced anti-JE N antibodies and conferred protection against lethal challenge by JE virus.

The overwintering mechanism (see earlier) of JE virus should be clarified using modern molecular technologies together with the connection to epidemic cycle of JE virus transmission.

The endemicity of JE in tropical Asia should be investigated.

The biological functions of several virus-specific nonstructural proteins should also be investigated in the future.

See also: **Dengue viruses (*Flaviviridae*); Latency; Nervous system viruses; Persistent viral infection; Vaccines and immune response.**

Further Reading

Hammon WMcD, Kitaoka M and Downs WG (eds) (1971)

Immunization for Japanese Encephalitis. Tokyo: Igaku Shoin.

Schlesinger S and Schlesinger MJ (eds) (1986) *The Togaviridae and Flaviviridae*. New York: Plenum Press.

Monath TP and Heinz FX (1996) Flaviviruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 961. Philadelphia: Lippincott–Raven.

JC AND BK VIRUSES (*PAPOVAVIRIDAE*)

Richard J Frisque, Department of Biochemistry and Molecular Biology, Penn State University, University Park, Pennsylvania, USA

History

In 1971, two laboratories reported the isolation of a human polyomavirus; subsequent biological and biochemical characterization revealed that the two isolates were previously unrecognized and closely related viruses. Duard Walker's group in Madison, Wisconsin isolated JC virus (JCV) from the brain tissue of a patient with the rare demyelinating disease progressive multifocal leukoencephalopathy (PML). At about the same time, Sylvia Gardner's laboratory in London, UK recovered BK virus (BKV) from the urine of a renal allograft recipient undergoing immunosuppressive therapy. The names of both viruses were derived from the initials of these patients.

JCV has now been isolated from the brain tissue of a large number of PML patients, and it is considered the etiologic agent of this usually fatal disease. BKV has been associated with cystitis and acute respiratory disease in children, ureteral stenosis in renal allograph patients and hemorrhagic cystitis in bone marrow transplant recipients. Both viruses have been detected in human tumors. In most individuals JCV and BKV establish asymptomatic infections in the kidneys and viruria is frequently observed.

The naming of JCV strains has generally relied on their geographic origin followed by a number, e.g. JCV(Mad1) was the first (prototype) strain of JCV isolated in Madison, Wisconsin. Naming of most BKV and some JCV strains has utilized the initials of the person from whom the variant was obtained, e.g. BKV(AS) indicates a BKV isolate from an individual with the initials AS.

Classification

JCV and BKV belong to the genus *Polyomavirus* of the family *Papovaviridae*. Additional members of this group include the closely related monkey virus SV40 and the more distantly related type species, mouse polyomavirus. Initially, these small DNA tumor viruses were classified together on the basis of the size and structure of their capsid, the antigenic similarities of their viral proteins and the site of their multiplication (nucleus) within the infected cell.

Properties of the Virion

The polyomavirus capsid is 38–44 nm in diameter, is made up of 72 capsomers, and has an icosahedral symmetry. The virion lacks an envelope and is resistant to ether and heat treatment. Red blood cells are agglutinated by the JC and BK virus particles.

Properties of the Genome

The JCV and BKV genomes (**Fig. 1**) are double-stranded, covalently closed circular DNA molecules that include approximately 5000 nucleotide pairs (np). Their G + C content of 40–41% is similar to that of the host chromosomes. The two viral DNAs share 75% sequence identity with one another, and 69–70% identity with the SV40 genome. The non-coding regulatory regions of the three viruses span about 400 nucleotides. The *cis*-acting sequences specifying the transcriptional control elements have diverged to the greatest extent, whereas those making up the core origins of DNA replication are highly conserved. It is within these latter sequences that replication initiates and then proceeds bidirectionally around the circle and terminates at a point approximately 180° away.

The coding region of these viral DNAs is divided into two nearly equal parts, based on whether the viral genes are expressed before (early) or after (late) DNA replication. The early and late mRNAs are transcribed from opposite strands of the DNA; their start sites are heterogeneous and are located near the replication origin.

Immunization for Japanese Encephalitis. Tokyo: Igaku Shoin.

Schlesinger S and Schlesinger MJ (eds) (1986) *The Togaviridae and Flaviviridae*. New York: Plenum Press.

Monath TP and Heinz FX (1996) Flaviviruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 961. Philadelphia: Lippincott–Raven.

JC AND BK VIRUSES (*PAPOVAVIRIDAE*)

Richard J Frisque, Department of Biochemistry and Molecular Biology, Penn State University, University Park, Pennsylvania, USA

History

In 1971, two laboratories reported the isolation of a human polyomavirus; subsequent biological and biochemical characterization revealed that the two isolates were previously unrecognized and closely related viruses. Duard Walker's group in Madison, Wisconsin isolated JC virus (JCV) from the brain tissue of a patient with the rare demyelinating disease progressive multifocal leukoencephalopathy (PML). At about the same time, Sylvia Gardner's laboratory in London, UK recovered BK virus (BKV) from the urine of a renal allograft recipient undergoing immunosuppressive therapy. The names of both viruses were derived from the initials of these patients.

JCV has now been isolated from the brain tissue of a large number of PML patients, and it is considered the etiologic agent of this usually fatal disease. BKV has been associated with cystitis and acute respiratory disease in children, ureteral stenosis in renal allograph patients and hemorrhagic cystitis in bone marrow transplant recipients. Both viruses have been detected in human tumors. In most individuals JCV and BKV establish asymptomatic infections in the kidneys and viruria is frequently observed.

The naming of JCV strains has generally relied on their geographic origin followed by a number, e.g. JCV(Mad1) was the first (prototype) strain of JCV isolated in Madison, Wisconsin. Naming of most BKV and some JCV strains has utilized the initials of the person from whom the variant was obtained, e.g. BKV(AS) indicates a BKV isolate from an individual with the initials AS.

Classification

JCV and BKV belong to the genus *Polyomavirus* of the family *Papovaviridae*. Additional members of this group include the closely related monkey virus SV40 and the more distantly related type species, mouse polyomavirus. Initially, these small DNA tumor viruses were classified together on the basis of the size and structure of their capsid, the antigenic similarities of their viral proteins and the site of their multiplication (nucleus) within the infected cell.

Properties of the Virion

The polyomavirus capsid is 38–44 nm in diameter, is made up of 72 capsomers, and has an icosahedral symmetry. The virion lacks an envelope and is resistant to ether and heat treatment. Red blood cells are agglutinated by the JC and BK virus particles.

Properties of the Genome

The JCV and BKV genomes (**Fig. 1**) are double-stranded, covalently closed circular DNA molecules that include approximately 5000 nucleotide pairs (np). Their G + C content of 40–41% is similar to that of the host chromosomes. The two viral DNAs share 75% sequence identity with one another, and 69–70% identity with the SV40 genome. The non-coding regulatory regions of the three viruses span about 400 nucleotides. The *cis*-acting sequences specifying the transcriptional control elements have diverged to the greatest extent, whereas those making up the core origins of DNA replication are highly conserved. It is within these latter sequences that replication initiates and then proceeds bidirectionally around the circle and terminates at a point approximately 180° away.

The coding region of these viral DNAs is divided into two nearly equal parts, based on whether the viral genes are expressed before (early) or after (late) DNA replication. The early and late mRNAs are transcribed from opposite strands of the DNA; their start sites are heterogeneous and are located near the replication origin.

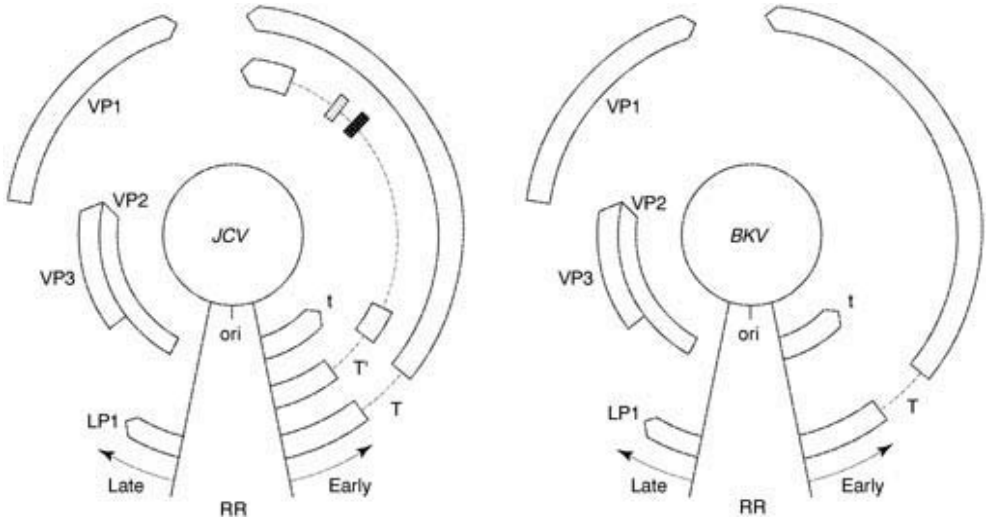


Figure 1 Comparison of the JCV and BKV genomes. The organization of these human polyomavirus genomes is nearly identical. The inner circles represent the double-stranded DNA genomes (~5 kb), the outer arrows denote the encoded viral proteins. The genomes are divided into three parts. The early region specifies five (JCV) or two (BKV) regulatory proteins produced by translation of alternatively spliced early mRNAs. T′ on the JCV map represents three proteins which share their first two exons and have unique carboxy-terminal exons (T'_{165}, open box; T'_{136}, box with lines; T'_{135}, shaded box). The existence of BKV T′ proteins and JCV and BKV early leader peptides (JELP and BELP) is predicted but has not been confirmed. The late region specifies four proteins required for capsid assembly. The regulatory region (RR) contains the *cis*-acting signals which control viral DNA replication (ori) and transcription.

Properties of Virus Proteins

Based on DNA sequence information, mRNA and protein analyses, and comparisons with SV40, the JCV and BKV genomes are predicted to encode seven to ten proteins. The early proteins are translated from mRNAs that are generated by alternative splicing of a single precursor message. In addition to the regulatory large T and small t antigens, three additional JCV early proteins, T'_{165}, T'_{136} and T'_{135}, have recently been detected in lytically infected and transformed human cells. BKV proteins corresponding to these T′ species are predicted based on immunoprecipitation experiments involving BKV-transformed cells. Amino-terminal sequences of all early proteins are shared, and carboxy-terminal sequences are unique. They are first produced early in a lytic infection, and they are the only proteins expressed in cells oncogenically transformed by the viruses. The multifunctional SV40 large T antigen has been studied extensively and is known to play critical roles in viral DNA replication, transcription and oncogenesis. Several of these functions have been assigned to domains within the corresponding JCV (**Fig. 2**) and BKV proteins. The small t and T′ proteins are less well understood, but they appear to augment large T activity under certain conditions. Four late proteins have been identified, three capsid proteins, VP1, VP2 and VP3, and LP1 (or agnoprotein) which may be involved in virion assembly. One additional SV40 protein, SELP (SV40 early leader protein) has been identified, but has no known function. Its expression is the result of a switch in early mRNA start site utilization that occurs during a lytic infection. Expression of the corresponding JCV (JELP) and BKV (BELP) polypeptides is predicted, but has not been demonstrated.

Geographic and Seasonal Distribution

Serological surveys indicate that JCV and BKV are widely distributed in the human population, and that most people are infected before reaching adolescence. Recent sequence analyses have identified predominant strains of JCV excreted in the urine of individuals living in North America, Europe, Africa and Asia. These isolates can be assigned to various types and subtypes based on nucleotide differences found primarily within their VP1 coding regions.

Seasonal variations in incidence rates have not been reported, probably because a primary infection is difficult to detect (due to its subclinical nature and occurrence early in the life of its host), and because it

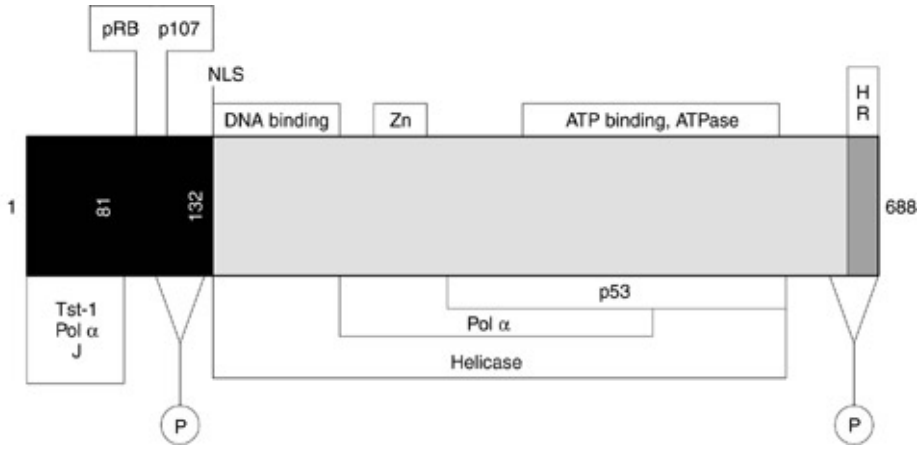


Figure 2 Predicted functional domains of the JCV T antigen. Identification of functional domains of the 688 amino acid (aa) JCV T antigen is based on mutational analyses and sequence comparisons with the SV40 T antigen. Similar domains are expected to be encoded within the BKV T protein. The first 81 aa are shared with the other four JCV early proteins, and the first 132 aa (shaded box) are shared with the three T′ proteins. The carboxy-terminal 33 aa (hatched box) overlap those of T'_{165}. NLS, nuclear localization signal; Zn, metal binding region; HR, host range domain; Polα, binding region for DNA polymerase α-primase; J, sequences homologous to the J domain of DnaJ proteins; P, serine and threonine phosphorylation sites.

often results in viral (and antibody) persistence that may preclude reinfection.

Host Range and Virus Propagation

JCV and BKV are strictly human viruses; there is no evidence to suggest that an animal reservoir exists. Inoculation of rodents (with JCV and BKV) and primates (with JCV) often results in the formation of a variety of tumors; infectious virions are not produced. JCV has a propensity for causing tumors in neural tissue, whereas BKV induces tumors in a variety of tissues.

The host range of the polyomaviruses in culture is restricted to a limited number of cells. This restriction is exaggerated in the case of JCV and has impeded the study of this opportunistic pathogen. JCV multiplies inefficiently in human primary B lymphocytes, embryonic kidney, amnion, transitional epithelial, Schwann and adult brain cells, and in glial and B cell lines. Significant replication of the virus has been demonstrated in tonsillar stromal cells, but propagation is most efficient in primary human fetal glial (PHFG) cells or transformants derived from this heterogeneous cell population. Even in these latter cells, however, the lytic cycle is prolonged and virus yields are generally low. BKV demonstrates a wider host range; virion production occurs in a number of primary and established human and monkey cell types and is most efficient in human fetal glial and kidney cells.

Measurement of virus titers is most readily determined using a rapid and inexpensive hemagglutination assay. A viral plaque assay is also available for BKV, but not for nondefective strains of JCV. Additional means of determining virus titers, such as a fluorescent cell focus assay, are not convenient for routine use.

Evolution and Genetics

Serological investigations have demonstrated that sera collected from individuals prior to the use of SV40-contaminated polio and adenovirus vaccines contained antibodies to JCV and BKV. This finding eliminated any suspicion that the high prevalence of anti-JCV and anti-BKV antibodies in today's population is due to exposure to SV40 in vaccines. Based on the extent to which the human and monkey viruses have diverged, it is evident that the viruses have not recently evolved from one another.

Alterations to the JCV and BKV genomes readily occur during passage of the viruses in their host and/or in cell culture. Sequence hypervariability involves the deletion and amplification of promoter–enhancer signals (**Fig. 3**). In the prototype strain of JCV, these transcriptional control elements are represented by a tandem duplication of 98 nucleotides, which is identical in genomes cloned directly from the PML patient's brain or from Mad1 virus propagated in cell culture. A large number of additional JCV DNAs have been cloned from the brain tissue of other PML patients. Although their promoter–enhancer elements

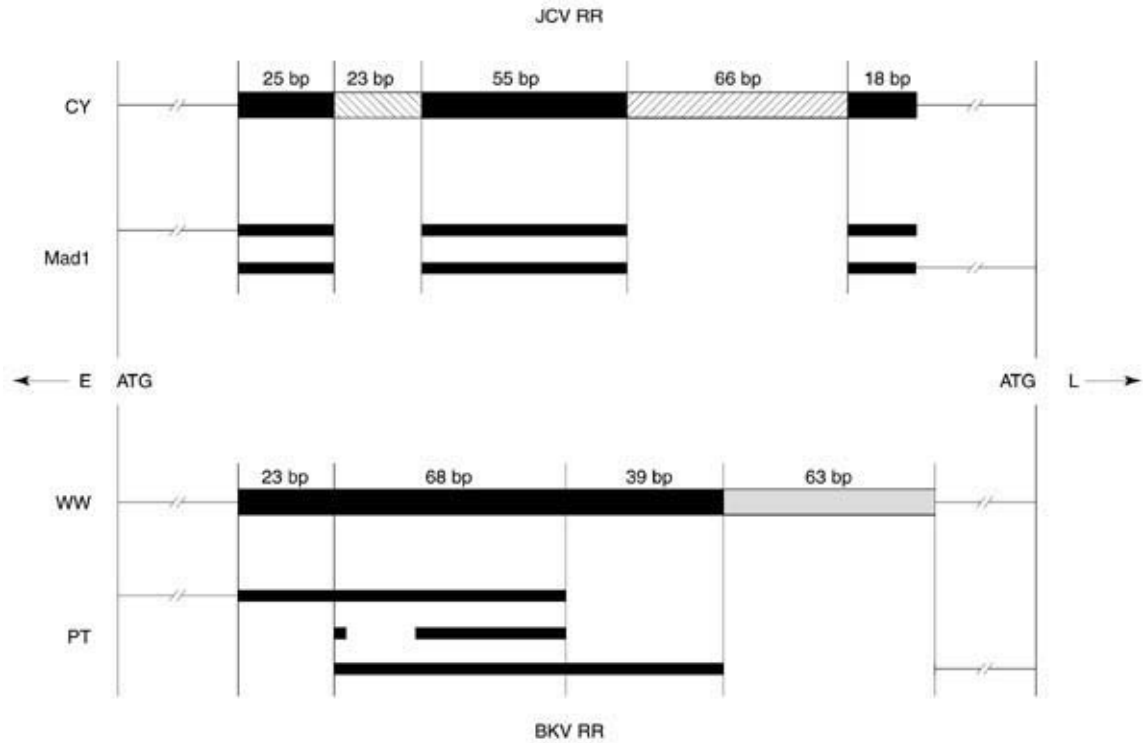


Figure 3 Comparisons of the JCV and BKV archetype and rearranged transcriptional control regions. The transcriptional control region (TCR) within the regulatory region (RR) of the prototype Mad1 strain of JCV contains a 98 bp tandem repeat composed of duplicate copies of 25 (includes TATA box), 55 and 18 bp blocks of DNA. These sequences, and an additional 23 and 66 bp sequence, are present as single copies in the promoter–enhancer of the archetype CY strain of JCV. The TCR of the prototype PT strain of BKV contains a 23 (includes TATA box) and 39 bp block of DNA and a triplication of a 68 bp sequence (the central repeat has a deletion of 18 bp). These sequences, and an additional 63 bp sequence, are present as single copies in the TCR of the archetype WW strain of BKV. The Mad1 and PT sequences are identified by lines below the CY and WW sequences and are read left to right. A gap within these lines represents a deletion relative to the archetype sequence; parallel lines represent multiple copies of that particular region. The initiation codons (ATG) for the early (E) and LP1 (L) proteins are indicated.

differ in size and sequence, the tandem repeats are clearly related to those of Mad1. Passage of these isolates *in vitro* often generates additional regulatory region alterations which may favor the viruses' growth in cell culture. In contrast to these rearranged (or PML) variants, JCV DNA obtained directly from the kidney or urine of normal individuals exhibits little sequence heterogeneity. A representative of this latter type of genome has been called 'archetype', a form from which all known rearranged JCV variants could have evolved. The presence of unique 23 and 66 np sequences, and the absence of a large tandem duplication distinguishes the archetype promoter–enhancer region from those of other JCV DNAs.

An archetype form of BKV DNA is also routinely cloned directly from human urine specimens. Rearrangement of the BKV promoter–enhancer sequences has been demonstrated in DNAs obtained from virus passaged in cell culture. The transcriptional control regions of these isolates contain large tandem duplications or triplications which often involve a 68 np sequence. It now appears that, like JCV, the presence of tandem repeats in the BKV regulatory region may occur in strains that already exist *in vivo* and are not simply the result of passage in culture.

Multiple typing schemes have been developed for JCV and BKV based on sequence differences within specific regions of the viral genomes; naming of these genotypes has been a source of confusion in the literature. Archetype and rearranged types of JCV and BKV are differentiated by alterations within the transcriptional control regions. This scheme has been used to distinguish the proposed circulating form of the virus (archetype) from the forms which evolve anew in each infected host or in cell culture (rearranged). A second typing scheme recognizes nucleotide differences which have accumulated in the VP1

gene during long-term association of the virus with geographically separated human populations. A scheme proposed prior to the discovery of archetype strains which attempted to classify rearranged variants into two groups is outdated and should be discarded.

Serologic Relationships and Variability

Genus- and species-specific antigens have been identified on and within the JCV and BKV capsids. Members of the polyomavirus genus share a cross-reacting antigenic determinant that is not exposed on the virion surface. Antibodies directed against this determinant are produced by immunizing rabbits with disrupted virions or purified VP1. At least one species-specific antigenic determinant is found on the surface of the JCV, BKV and SV40 capsids which allows them to be distinguished from one another by serological means. Antibodies to this antigen are generated during an infection by the viruses of their natural hosts.

Antigenic similarities between the large and small T proteins of these three viruses can be demonstrated by a variety of immunological methods. Polyclonal antiserum raised against the early antigens of one virus generally crossreacts with those of the other two viruses, whereas monoclonal antibodies are less likely to recognize all three sets of proteins. Cytotoxic T lymphocytes (CTL) also have the ability to identify shared and distinct epitopes of T antigen presented on the surface of JCV- and SV40-transformed cells; most CTL clones directed against determinants of the SV40 protein lyse cells expressing the JCV protein.

One major serotype of JCV (represented by Mad1) appears to circulate in the population, although a distinct antigenic variant JCV(Mad11) has been recognized. The Mad1 and Mad11 VP1 proteins only differ at amino acid position 66 (aspartic acid vs. histidine) which lies within a region (amino acids 40–80) predicted to be an antigenic epitope. Recently, two genotypic variants have been identified with deletions in their VP1 gene. It is not known whether these alterations signal the existence of additional JCV serotypes. BKV has been grouped into four serotypes. Sequence differences observed between amino acids 61 and 83 of their VP1 proteins are predicted to have altered a dominant epitope on the capsid surfaces, resulting in antigenic variability.

An important consequence of having multiple JCV and BKV serotypes circulating in the population might be the underestimation of the prevalence of these viruses in their human host if a single viral strain is used as the source of antigen in serological testing.

Epidemiology

Until recently, investigators relied on seroepidemiology as a means of acquiring information about the distribution and natural history of JCV and BKV. Such surveys suggested that humans are the natural reservoir for JCV and BKV. Most people are infected before reaching adolescence, and greater than 70% of adult sera contain antibodies to one or both of these viruses. These studies have been based on the detection of hemagglutination-inhibiting antibodies, because they can be measured easily and quickly, and because their titers correlate well with neutralizing antibody titers. Enzyme-linked immunosorbent assays (ELISA) have also been used to screen human sera for evidence of exposure to these viruses. Concerns remain, however, regarding the specificity of these assays, since crossreactivity of anti-JCV and anti-BKV antibodies may occur when purified viral proteins are used as the source of the antigen. Epidemiological approaches are being extended using molecular techniques such as the polymerase chain reaction (PCR), which greatly increases the ability to detect and analyze JCV and BKV in body fluids and tissue samples.

Transmission and Tissue Tropism

Very little is known about how the human polyomaviruses are transmitted to their host, although it is suspected that virus may enter via a respiratory or oral route. The only recognized release of JCV and BKV is through the urine, and this occurs transiently in many individuals over the age of 20. Studies in urban versus rural areas of Japan indicate that population density and environmental conditions may affect the transmission of JCV. Genotypic analysis suggests that up to 50% of the population may become infected when JCV is transmitted from a family member.

Little information is available regarding the syndrome of the primary infection. The initial infection generally occurs during childhood and is largely asymptomatic. BKV infection has been linked to cystitis and an acute respiratory illness among immunocompetent children; clinical illness has not been clearly associated with a primary JCV infection. Preliminary data indicate that JCV and BKV may infect tonsillar tissue *in vivo*, and tonsillar stromal cells do support JCV replication in culture.

Once virus has multiplied at the primary site of infection, lymphocytes are thought to transport it to the brain, urinary tract and lymphoid tissues. It has also been speculated that lymphocytes might contribute to the rearrangement process which alters the

archetype promoter–enhancer sequences and results in the appearance of variants with new tissue tropic properties. Upon reaching the kidney, archetype virus establishes a persistent infection which is often manifested by transient viruria both in immunocompetent and immunocompromised individuals. Because virus is frequently detected in the urine of pregnant women, attempts have been made to uncover evidence for a transplacental route of transmission for the human polyomavirues; such evidence has not been forthcoming.

After a primary infection or the reactivation of a persistent kidney infection, JCV, and perhaps BKV, may reach the brain where they remain latent or, in the case of JCV, might actively replicate to cause PML. The predominant form of virus detected in normal and diseased brain contains a rearranged transcriptional control region. Recent PCR findings suggest that the Type 2 genotype of JCV is disproportionately represented in the brains of PML patients; no evidence is available to suggest differences in the pathogenic behavior of BKV genotypes.

The influence of the JCV and BKV regulatory signals on cell tropism has been tested *in vitro*, and the results indicate that the BKV signals have a broader range and are more active than those of JCV. In addition, the rearranged transcriptional control elements are more potent than the archetype signals. These studies indicate that the JCV promoter–enhancer elements function best in cells of glial origin, thus leading some investigators to improperly designate JCV as a strictly neurotropic virus.

Pathogenicity

An association between BKV and human disease most frequently involves pathologic changes in the urinary tract following reactivation of a persistent infection in immunodeficient individuals; they rarely represent a life-threatening event. BKV is also detected in lymphocytes and brain, but pathogenic consequences appear to be rare. BKV variants isolated from tissue or urine may have single or multiple copies of the promoter–enhancer sequences. Although these strains vary in their lytic behavior *in vitro*, data supporting differences in their pathogenic potential are lacking.

JCV causes PML, a fatal degenerative disease of the central nervous system (CNS). There is evidence to suggest that PML occasionally results as a consequence of primary infection. Such a possibility is supported by cases involving very young children with innate immune deficiencies. Examination of one such case indicates that promoter–enhancer rearrangements occur early in the infection, that multiple variants are generated and that the virus becomes established in multiple tissues. However, most cases of PML appear to result from the reactivation of a latent or persistent infection during a decline in immune status. Usually only one rearranged variant is detected in these patients.

The following series of events might occur in a reactivation case of PML. After an initial exposure to archetype virus, latent and persistent infections may be established in a healthy individual. It is unclear whether rearranged genomes arise before the initial immune response or only later after some impairment to T cell function. However, both forms are eventually found in most people and they can be detected at different preferred sites in the body. The host–parasite relationship may remain fairly stable during most of an individual's life, with only occasional episodes of viruria to signal the presence of JCV. Disruption of this relationship could occur during periods of prolonged and severe immune deficiency, and JCV might then actively replicate in the CNS to yield PML.

Although once considered an extremely rare disease, PML is now being seen with increasing regularity, due primarily to an increase in the numbers of patients comprising two at-risk groups; people undergoing immunosuppressive therapy and victims of the AIDS pandemic. It is estimated that PML is the cause of death in up to 5% of the individuals in this latter population. The relatively high incidence of PML in AIDS patients may be due to more than just the host's inability to maintain effective immune surveillance. The HIV *tat* protein has been shown to *trans*-activate the JCV late promoter *in vitro*. *In vivo*, *tat* might influence the pathogenic potential of JCV either through a direct interaction with a TAR-like element found in JCV late mRNAs or through indirect effects on specific inflammatory cytokines which might enhance JCV gene expression.

Clinical Features of Infection

PML presents as an insidious, subacute disease and indications of infectious inflammatory disease are often minor or absent. Signs and symptoms depend on the location of the lesions in the CNS, with prominent early manifestations including motor disturbances, mental changes and visual and speech impairment. Over the course of the disease, these neurological problems generally progress and new ones are observed as additional sites are affected. At the time of diagnosis, many patients exhibit partial or complete paralysis. Mental problems may have advanced to dementia and visual defects to cortical blindness. The

survival times of most PML patients following diagnosis is often less than six months.

Pathology and Histopathology

Lesions within the subcortical white matter of the PML brain are seen as multiple foci of demyelination that range in size from 1 mm to several centimeters in diameter. The pathognomonic feature is the altered oligodendrocyte. The nucleus of this cell is two to three times the expected size, and it usually stains deeply basophilic with hematoxylin. The normal chromatin pattern is lost, and the nucleus is filled with virions and viral antigens.

The altered oligodendrocytes are most prominent within small early lesions and near the borders of larger, more advanced lesions. Within the central areas of older PML lesions, the oligodendrocytes are missing and macrophages and reactive astrocytes are common. Giant, bizarre astrocytes with pleomorphic, hyperchromatic nuclei are often found as well, and these cells strongly resemble malignant astrocytes of pleomorphic glioblastomas.

Immune Response

Most people produce antibodies against JCV and BKV; significant antibody titers may persist for life due to the ability of these viruses to establish persistent infections. Reactivation of these infections is not prevented by high levels of antibody, but does occur during conditions that lead to impairment of T-cell function, for example during AIDS, autoimmune diseases, immunosuppressive therapy used for treating malignancy or preventing rejection of transplants, pregnancy and old age. In addition to a general depression of cell-mediated immunity, a severe, more specific deficiency in cellular immune response to JCV may be a determining factor in whether PML becomes the final outcome of reactivation.

Once PML is diagnosed, most patients only survive a few months. In some individuals, however, the disease progresses more slowly, resulting in significantly longer survival times. In such cases, evidence of immunosuppressive therapy or underlying immune deficiency disease may be absent. These long-surviving patients often demonstrate signs of active cellular inflammatory response and perivascular cuffing with plasma cells and lymphocytes. The ability of these individuals to mobilize some cellular immunity against the infection presumably accounts for the slower advance of the disease.

Prevention and Control of Human Polymavirus Infections

Considering the early age at which people are infected by JCV and BKV, the prevalence with which these viruses circulate in the population, and the ability of these viruses to establish persistent infections in immunocompetent hosts, it appears unlikely that we will soon be in a position to prevent or eliminate primary or reactivation infections by the human polyomaviruses. At present, efforts to alter the outcome of severe opportunistic infection through the identification of effective means of diagnosis and treatment should be encouraged.

The most serious polyomavirus infection in humans, PML, is caused by JCV. There is no proven treatment for this disease, and the prognosis for PML patients has generally been poor. If some degree of success in therapy is to be achieved, top priorities must include attempts to make an early diagnosis, to shore up the host immune response and to inhibit viral multiplication with antiviral agents. Although sporadic success has been reported using antiviral agents such as nucleic acid base analogs (e.g. cytosine arabinoside [Ara-C]) and β interferon, a recent study conducted under the auspices of the NIH Neurological AIDS Research Consortium failed to demonstrate significant clinical benefit for patients receiving Ara-C therapy. Given that PML nearly always occurs in severely immunocompromised individuals, there are two groups of patients for which an effective strategy is being developed. Individuals undergoing immunosuppressive therapy to control autoimmune disease or to prevent allograph rejection have demonstrated functional resistance if removed from immunosuppression. In addition, immune reconstitution in HIV-infected patients with PML by combined antiretroviral therapy has resulted in prolonged remission of PML. This host resistance in combination with an agent designed to inhibit viral DNA replication might further improve the prognosis. Potential candidates include camptothecin, a DNA topoisomerase inhibitor, and cidofovir, an acyclic nucleoside phosphonate analogue, which have been shown to block replication of primate polyomaviruses in cultured cells.

Future

A key to the successful treatment of PML will be the ability to make an early diagnosis before extensive damage is done to the CNS. At present the only definitive means of diagnosis is by histopathological examination of diseased tissue obtained by biopsy or at autopsy. Noninvasive procedures, although not specific for PML, are useful in their ability to exclude

other potential causes of the presenting signs and symptoms. These procedures make use of the electroencephalogram, computer tomography and magnetic resonance imaging. Several laboratories have employed PCR to detect JCV in cerebral spinal fluid, and although some questions remain concerning the levels of sensitivity and specificity of this approach, it holds promise as a diagnostic tool.

A number of questions need definitive answers before we have a significant understanding of the pathogenesis of PML. For example, how is the virus transmitted from person to person and what is the site of primary infection? When does JCV make its way to the brain once it is in the body? Are lymphocytes involved in rearrangement of the archetype transcriptional control region. Does the adaptation from a kidney to a brain variant result in a virulent form of JCV and under which conditions might such an event occur? What role does the host immune system play in the establishment and maintenance of a persistent JCV infection?

See also: **Diagnostic techniques: Detection of viral antigens, nucleic acids and specific antibodies, isolation and identification by culture and microscopy; Human immunodeficiency viruses (*Retroviridae*): Anti-retroviral agents, General features, Molecular biology; Immune response: Cell mediated immune response, General features; Nervous system viruses; Persistent viral infection; Polyomaviruses – murine (*Papovaviridae*): General features, Molecular biology; Simian virus 40 (*Papovaviridae*); Tumor viruses – human.**

Acknowledgement

This work was supported by Public Health Service Grant CA-44970 from the National Cancer Institute and American Cancer Society Grant RPG-95-051-03-VM.

Further Reading

Berger JR and Concha M (1995) Progressive multifocal leukoencephalopathy: the evolution of a disease once considered rare. *J. NeuroVirol.* 1: 5.

Dörries K (1996) Virus–host interactions and diagnosis of human polyomavirus-associated disease. *Intervirology* 39: 165.

Frisque RJ and White FA III (1992) The molecular biology of JC virus, causative agent of progressive multifocal leukoencephalopathy. In: Roos RP (ed.) *Molecular Neurovirology*, pp 25–158, Totowa, NJ: The Humana Press.

Jin L and Gibson PE (1996) Genomic function and variation of human polyomavirus BK (BKV). *Rev. Med. Virol.* 6: 201–214.

Raj G and Khalili K (1995) Transcriptional regulation: lessons from the human neurotropic polyomavirus, JCV. *Virology* 213: 283.

Tornatore C, Amemiya K, Atwood W, Conant K and Major EO (1994) JC virus: current concepts and controversies in the molecular virology and pathogenesis of progressive multifocal leucoencephalopathy. *Rev. Med. Virol.* 4: 197.

Junin Virus *see* **Lassa, Junin, Machupo and Guanarito Viruses**

L

LASSA, JUNIN, MACHUPO AND GUANARITO VIRUSES (*ARENAVIRIDAE*)

Joseph B. McCormick, Epidemiology and Biostatistics Unit, Institute Pasteur, Paris, France

History

Lymphocytic choriomeningitis (LCM) virus, the first identified arenavirus, was isolated by Lillie and Armstrong in 1933 from the cerebrospinal fluid of a patient suspected of having St Louis encephalitis. The virus was again isolated in 1935 from patients with aseptic meningitis, and finally by Traub in 1935 from laboratory mice. More than 20 years passed before Junin virus, the next member of this taxon to be identified, was isolated from patients with Argentine hemorrhagic fever in 1957. Machupo virus from patients with Bolivian hemorrhagic fever was similarly identified in 1964. A third arenavirus from South America which is pathogenic for humans is Guanarito virus, isolated from patients in Venezuela in 1991. Yet another pathogenic arenavirus, Sabia virus, this time from Brazil, was isolated from a fatally ill individual. Several other arenaviruses have also recently been isolated from rodent species in South America, but none of these have yet been associated with human illness. Lassa virus was the first arenavirus isolated from Africa, in 1969, and remains the only arenavirus pathogenic for humans from Africa, although several other arenaviruses have also been identified from Africa. In total, 19 arenaviruses have been identified worldwide, but only six are known to be pathogenic for humans.

Classification

The 19 members in the *Arenavirus* genus of the *Arenaviridae* have traditionally been placed in categories of Old and New World arenaviruses (**Table 1**), based on geographic locations. More recently Tacaribe complex viruses (in the Western hemisphere) have been more completely placed in phylogenetic relationship with each other based on a sequence of about 600+nucleotides in the nucleoprotein. The present information suggests three related groups: lineage A contains Pichinde, Parana, Flexal and Tamiami viruses; lineage B contains Junin, Machupo, Amapari, Guanarito, Sabia and Tacaribe viruses; lineage C contains Latino and Oliveros viruses. More recently still, further genetic analysis has led to the suggestion that Pichinde and Oliveros viruses are most closely associated with Old World arenaviruses. The likelihood that arenaviruses and their rodent hosts have coevolved was suggested some time ago, and now seems a possible hypothesis to begin to test through the parallel use of rodent genetics, alongside the understanding of the genetic relationships between arenaviruses.

The six arenaviruses presently know to be pathogenic for humans (LCM, Lassa, Junin, Machupo, Guanarito and Sabia viruses) will be the primary subjects of this entry.

Properties of the Virion

The arenaviruses are enveloped, pleomorphic, membrane viruses ranging in diameter from 50 to 300 nm, with a mean diameter of 110–130 nm. The virion density in sucrose is 1.17 g ml^{-1}. They contain two segments of single-stranded RNA tightly associated with a nucleocapsid protein. This is enclosed in a membrane consisting of two glycosylated proteins (or in some cases a single glycosylated protein). The genome consists of two segments of single-stranded RNA, both contain two genes encoded in an ambisense structure. The small RNA segments encodes for the glycoprotein precursor (GPC) and for the nucleoprotein (NP). The NP and GPC genes are encoded in nonoverlapping reading frames with origins at the 3′ and 5′ ends of the molecule,

Table 1 Arenaviruses: basic biological information

Virus	*Human disease*	*Geographic distribution*
Lymphocytic choriomeningitis	Lymphocytic choriomeningitis	Europe, Asia, Western Hemisphere
Lassa	Lassa fever	West Africa
Mopeia	Human infection, no disease known	Southern Africa/Mozambique, Zimbabwe, Rep. of South Africa
Mobala	Human infection, no known disease	Central African Republic
Ippy	Unknown	Central African Republic
Lineage B[a]		
Junin	Argentine hemorrhagic fever	Argentina
Machupo	Bolivian hemorrhagic fever	Bolivia
Guanarito	Venezuelan hemorrhagic fever	Venezuela
Sabia	Hemorrhagic fever	Brazil
Amapari	None	Brazil
Tacaribe	None	Trinidad
Lineage A[a]		
Parana	None	Paraguay
Tamiami	None	Florida
Pichinde	None	Colombia
Flexal	None	Brazil
Lineage C[a]		
Latino	None	Bolivia
Oliveros	None known	Argentina
Not yet placed[a]		
Pirital	None known	Venezuela
White Arroyo	None known	North America

[a] In current phylogenetic scheme suggested for Tacaribe complex viruses.

respectively. The N gene is encoded by the 5′ half of the viral complementary RNA sequence corresponding to the 3′ half of the viral RNA molecule. The GPC gene is encoded by the 5′ half of the viral RNA molecule. Similarly, the large strand of RNA codes for the RNA-dependent viral RNA polymerase and a smaller ring-finger protein involved in replication. The arenaviruses are virtually indistinguishable from each other morphologically, and all share the characteristic granules noted in electron micrographs. These granules appear to be the results of the binding of the zinc-binding ring-finger protein of arenaviruses with the nuclear fraction of ribosomal proportion. The nuclear ribosomal protein (PO) appears in the virion, while other ribosomal proteins do not. This suggests that the granules, believed to be nonspecific inclusion of ribosomes into the virion, may rather be a specific process related to virion replication and assembly. These granules give rise to the family name *Arenaviridae*, derived from *arenos*, the Latin word for sand, based on their electron micrographic appearance as grains of sand.

Geographic and Seasonal Distribution

Junin, Machupo and Guanarito viruses occur in Argentina, Bolivia and Venezuela, respectively. Junin and Guanarito are endemic in their respective areas. Junin virus primarily infects workers during the corn harvesting season, by disturbance of the rodent host, *Callomys callosus*, which lives in the corn fields. Guanarito virus infection is endemic, and its epidemiology may resemble that of Lassa virus in Africa, which occurs throughout the year; however, insufficient data presently exist to confirm this impression. Machupo virus occurred in epidemic fashion in the 1960s in a circumscribed area of Bolivia. It was associated with the transient marked increase in the population of *Calomys* rodents, which are normally field rodents but because of overpopulation moved into human dwellings in search of food. Elimination of the rodents in the towns stopped the epidemic, and few further cases have since been reported (though a few cases were reported in 1996). Lassa virus infection is endemic in West Africa from Senegal to Cameroon

and perhaps other areas not yet explored. There are increases in Lassa infection during the dry seasons, perhaps because of increased virus stability in lower humidity, but other, as yet unknown, factors may also be involved.

Host Range and Virus Propagation

All of the New World arenaviruses have rodent reservoir hosts with the exception of Tacaribe virus, which was isolated from bats in Trinidad (**Table 1**). A hallmark of the arenaviruses is their intimate biological relationship with rodents, resulting in lifetime infection and chronic virus excretion. Many arenaviruses have more than one rodent host, although usually a single species will predominate as the reservoir in nature.

The hosts of LCM virus (LCMV) have included *Mus* species and hamsters. Guinea pigs are also capable of transmitting the virus in laboratory settings. Machupo virus often renders its major natural host, *Calomys callosus*, essentially sterile by causing the young to die *in utero*. Machupo virus also induces a hemolytic anemia in its rodent host, with significant splenomegaly, often an important identifier of infected rodents in the field. The major rodent hosts for Junin virus are *Calomys* species. Transmission of Junin virus from rodent to rodent is generally horizontal, and not vertical, and is believed to occur through contaminated saliva and urine. The *Calomys* rodents are affected by the virus, with up to 50% fatality among infected suckling animals, and stunted growth in many others. Both Junin and Machupo viruses induce a humoral immune response when transmitted to their suckling natural rodent hosts, which may have neutralizing antibody in the face of persistent infection. Guanarito virus has been isolated from *Zygodontomys brevicauda*, though its detailed biology remains to be learned.

The only known reservoir of Lassa virus in West Africa is *Mastomys natalensis*, one of the most commonly occurring rodents in Africa. At least two species of *Mastomys* (diploid types with 32 and 38 chromosomes) inhabit West Africa, and both have been found to harbor the virus. All species are equally susceptible to silent persistent infection, as seen when LCMV infects mice. This presumably occurs as in LCM infection, from a selective deletion of the thymic T cell response to the virus. All of the arenaviruses pathogenic for humans will also infect and produce illness in a wide range of primates. However, it is not known whether such infections occur in nature, as is known for Ebola virus for example. In addition human infection plays no biological role in the life cycle and ecology of the arenaviruses.

Virus Propagation

The original isolation of LCMV was made in suckling mice, which have been important in isolation and characterization of several of the arenaviruses. The arenaviruses are, however, easily cultivated in a wide variety of mammalian cell monolayers. The Vero E6 remains the cell of choice for primary isolation and cultivation, but arenaviruses also replicate in baby hamster kidney cells, as well as in a number of specialized cells such as continuous macrophage lines, endothelial cells, fibroblasts and a variety of mouse cell lines, with specific MHC markers used as targets for immunological studies. The infected cells may produce a cytopathic effect (CPE) beginning on days 4–7 of incubation. However, not all arenaviruses produce CPE, especially on primary isolation. For diagnosis, cells may be harvested after 48–72 hours and assayed for antigen by immunofluorescent antibody (IFA) or ELISA. Virus plaquing techniques may also be used for the arenaviruses.

Genetics

While advances have been made in determining the genetic relationship between different arenaviruses (see Classification), the level of genetic variability within species of arenaviruses is not well characterized, though it must be added that little genetic data on field isolates exist on which to make this judgement. It would appear that the frequency of variability at the amino acid level, as judged by B cell epitope variability, is not high. Thus the variability in B cell epitopes among Lassa viruses isolated from humans or rodents over a 10 year period in a circumscribed area was not substantial, suggesting that B cell epitopes are under limited immune pressure in their rodent hosts. No data are available on the variability of T cell epitopes among Lassa viruses. The South American arenaviruses may be under both B and T cell immune pressure in their rodent hosts, though no data are available on this issue. Reassortment, demonstrated only in the laboratory, may also be a means of genetic variability, but its occurrence in nature and therefore its importance is unknown.

Evolution

The *Arenaviridae* are distributed over five continents and can be divided into three 'coevolutionary' groups, Lassa complex in Africa, LCM in North America, Europe and South America, and the Tacaribe complex in South America. Today's arenaviruses probably descended from an ancestral virus which subsequently differentiated in parallel with the evolution of the Cricetid rodents persistently infected by arena-

viruses. It seems likely, therefore, that the present distribution and evolution of these viruses are directly related to the distribution and evolution of the earliest Cricetid rodents and their descendants, which now make up the natural hosts of most of this family of viruses (Tacaribe virus has a bat host). The coevolution of these viruses will undoubtedly continue and depend primarily on mutations, selected by the persistently infected host's immune pressure, and perhaps on reassortment in the rodent host.

Serological Relationships and Variability

Some monoclonal antibodies raised against African arenaviruses react with arenaviruses of broadly separated geographic origins and others only with local strains. However, other data suggest considerable antigenic stability of arenaviruses in a circumscribed area. The most variable antigenic sites are on GP1 and NP proteins. Thus, a monoclonal antibody to the GP1 and to the NP of Lassa Josiah strain from Sierra Leone identified only local isolates from Sierra Leone or from the immediately adjacent countries. On the other hand some epitopes on both NP and GP2 proteins were conserved among the known African arenaviruses. The GP2 proteins revealed two patterns of conservation. One epitope reacted only with West African strain viruses, while a different epitope reacted with all representative arenaviruses, including South American ones. This observation is in agreement with analysis of sequence homology among the GPC precursors of arenaviruses which showed extensive conservation of sequences in GP2 proteins. Other data, such as neutralization tests, are not yet available or sufficiently reliable to assess these relationships further.

Epidemiology

The fundamental determinant of the ecology of hemorrhagic fevers is the occurrence of persistent virus infection in rodents. Who becomes infected, and when, are functions of the behavior of the persistently infected rodent, and the cultural and occupational patterns of human populations. The arenavirus hemorrhagic fevers are primarily rural and semirural diseases; however, some evidence exists that under certain conditions (poverty, overcrowding) their rodent reservoirs may also establish urban habits. The rodents infect the environment via urine, feces and saliva. Interactions between rodents and humans are peridomestic or in agricultural areas. However, details of the rodent population dynamics, behavior and the natural history of the persistent virus infection in the feral rodent hosts are only poorly understood. The Old World arenaviruses, LCM and Lassa, produce persistent infection in their rodent hosts without significant detrimental effects. The South American viruses, in contrast, may cause illness and death in newborn rodents, or may induce persistence. The modes of transmission from rodent to human are not precisely known. Direct contact by humans, with cuts and scratches on hands and feet, with articles and surfaces contaminated by virus may be a more important and consistent mode of transmission. Transmission to mucosal surfaces may also occur under some circumstances.

Lassa fever

Lassa fever occurs in West Africa, but with a wide geographic area from Northern Nigeria to Guinea, encompassing perhaps 100 million population. At least two species of *Mastomys* occupy West Africa, and both have been found to harbor the virus. These rodents, especially the species with 32 chromosomes, are highly commensal with humans. The movement of *Mastomys* within a village is very limited, and their average lifespan is about 6 months, with little seasonal fluctuation in their breeding pattern. From 5% to as many as 70% have been found to be infected with virus in some village houses. Therefore most virus transmission takes place in and around the homes. All age groups and both sexes are affected and antibody prevalence increases with age. Risk factors for human infection include contact with rodents, direct contact with ill persons infected with Lassa virus, presence of a large household rodent population, and human practices such as catching, cooking and eating rodents and indiscriminant storage of food. In many endemic areas Lassa fever is a common cause of hospitalization. The death rate in systematically studied, untreated hospitalized patients with Lassa fever is 16%, very similar to that described for Junin and Machupo infections. Nosocomial transmission occurs in Africa from contact with infected patients, or through improper use or sterilization of needles, sometimes leading to an outbreak. Person-to-person spread of Lassa virus in households is common, a unique characteristic of Lassa virus in relation to other arenaviruses (no data are yet available on Guanarito virus from Venezuela, or Sabia virus from Brazil), and is usually associated with direct contact or care of someone with a febrile illness, or possibly with sexual contact with a spouse during the incubation or convalescent phases of illness. Illness to infection ratios are 10–25% in some endemic areas of West Africa. Antibody prevalence ranges from less than 1% to over 40% in some villages.

Argentine hemorrhagic fever (AHF)

AHF, caused by Junin virus, was recognized in the 1950s in the northwestern part of the Buenos Aires Province in Argentina, an area of very fertile farmland and therefore of great economic importance. The total number of cases reported over a 30 year period is about 21 000. AHF is a seasonal disease with peak yearly incidence in May. The average number of cases from 1981 to 1986 was 360 per year. Although all ages and sexes are susceptible, nevertheless the major group affected is the male working population, explained by the habits of the rodent hosts for Junin virus, *Calomys musculinus* and *Calomys laucha*. These animals are not peridomestic, but rather occupy grain fields, and this is the major reason for the affected population of field workers. Infection also occurs infrequently in other rodents: *Mus musculus, Akodon azarae* and *Oryzomys flavecens*. Transmission from rodent to rodent is horizontal, not vertical, and is thought to occur via contaminated saliva and urine. It is believed that the major routes of virus transmission from humans is through contact with virus-infected dust and grain products and subsequent infection through cuts and abrasions on the skin, or through airborne dust generated primarily by killing and scattering of rodents during mechanized farming. The disease has spread over the 30 years or so since its recognition from an area of 16 000 km^2 and a quarter of a million persons to an area greater than 120 000 km^2 containing a population of over 1 million persons. Furthermore, the incidence in the older affected areas appears to wane after 5–10 years. Overall antibody prevalence is 12%, with a typical predominance in agricultural workers. One-third of the seropositive individuals have no history of typical illness, suggesting that the case:infection ratio is about 2:3.

Bolivian hemorrhagic fever (BHF)

The only known reservoir for the virus is *Callomys callosus*, a Cricetid rodent that is found in the highest density at the borders of tropical grassland and forest. The distribution of this rodent appears to include the eastern Bolivian plains, as well as northern Paraguay and adjacent areas of western Brazil. The disease was recognized in 1959, and by 1962 more than 1000 cases had been identified in a confined area of two provinces, with a 22% case fatality ratio. The largest known epidemic of BHF, involving several hundred cases, occurred in the town of San Joaquin in 1963 and 1964. This outbreak occurred because of a marked increase in the *Callomys* population, and the subsequent invasion of homes in the town by these rodents. Although the *Callomys* appears to be capable of living both in the areas surrounding the towns and in the towns themselves (where the most efficient transmission of virus would appear to occur), they favor a nonperidomestic habitat where contact with humans is much reduced. It appears that the situation in San Joaquin was unusual, and such an event has not been observed again after nearly 25 years. There has not appeared to be any increase in the geographic areas affected by BHF in the last decade, and few cases have been reported.

Venezuelan hemorrhagic fever

The epidemiologic pattern of this disease has not yet been well characterized. In one survey the antibody prevalence in the population in the endemic area in central Venezuela was 2.6%. The virus was isolated from the cane rat *Zygodontomys brevicauda*. The occurrence of person-to-person transmission has not yet been demonstrated. The low frequency of infection in family contacts and lack of disease in hospital workers caring for patients suggest that person-to-person spread is uncommon. The pattern of infection includes all ages and sexes, suggesting that transmission occurs in and around houses, similar to Lassa fever and BHF and unlike AHF.

Transmission and Tissue Tropism

The primary mode of arenavirus transmission is from human contact with rodent urine or blood. This probably occurs primarily when individuals come into contact with surfaces or material recently contaminated by rodent urine, or when they trap a rodent and handle the carcass. Some evidence suggests that rodent blood or urine might be aerosolized by machinery during mechanized harvesting of corn in Argentina, with consequent transmission of Junin virus to people working near the machinery; however, no detailed studies of specific risk factors exist. In Africa, the rodents are highly commensal and probably deposit virus-laden urine in many areas of the houses they inhabit. The people tend to walk barefooted, and, based on the epidemiologic pattern of somewhat sporadic infection in households, it seems likely that contact with infectious urine is the primary source of contamination. In addition, in parts of Africa people catch and eat rodents, putting them in direct contact with rodent tissue, blood and secretions during the preparation. Finally, for some of the viruses, particularly Lassa virus, there is person-to-person transmission primarily through contact with the blood or secretions of an infected, ill person. Whatever the mode of transmission, it would seem that the reticuloendothelial system is probably a primary target of replication of the

arenaviruses, though they replicate well in many organs, including liver, adrenal gland, placenta, lung and many other organs.

Pathogenesis

The most common sites of initiation of human infection by the arenaviruses is not yet known, although they seem likely to be cuts and abrasions in the skin. Following the initiation of infection, all arenaviruses progress to generalized multiorgan infections, especially of the reticuloendothelial system. Thereafter, however, the pathogenesis of the different infections is variable.

Lassa fever

The 1–3 week incubation that follows infection suggests an unknown primary replication site, probably within the reticuloendothelial system. Route and titer of infecting dose may be important determinants of outcome, as may the virus strain. For example, death rates in nosocomial outbreaks where parenteral exposure is substantial are usually higher than community-acquired infections.

The degree of organ damage in fatal human infections is mild, which is sharply at variance with the clinical course and collapse of the patient. Indeed, there are few clues to the pathogenesis of Lassa fever in standard pathological studies. Liver damage is variable, with concomitant cellular injury, necrosis and regeneration. Nevertheless, serum aspartate aminotransferase (AST) levels over 150 iu ml^{-1} are correlated with poor outcome, and an ever-increasing level is also associated with increased risk of death. Alanine aminotransferase (ALT) is only marginally raised, and the ratio of AST:ALT in natural infections and in experimentally infected primates is as high as 11:1. Furthermore, prothrombin times, glucose and bilirubin levels are near normal, excluding biochemical hepatic failure. An increasing Lassa viremia is also associated with increasing case fatality. In addition to the liver, high virus titers occur in many other organs without significant pathologic or functional lesions, perhaps reflecting blood rather than parenchymal levels of virus.

Some patients develop severe pulmonary edema and adult respiratory distress syndrome, gross head and neck edema, pharyngeal stridor and hypovolemic shock. This pattern is consistent with edema due to capillary leakage, rather than cardiac failure and impaired venous return. Endothelial cell dysfunction has been demonstrated in primates experimentally infected by Lassa fever, in that there is apparently a marked decrease in prostacyclin production by endothelial cells. Loss of integrity of the capillary bed presumably causes the leakage of fluids and macromolecules into the extravascular spaces and the subsequent hemoconcentration, hypoalbuminemia and hypovolemic shock. Proteinuria is common, occurring in two-thirds of patients.

Edema and bleeding may occur together or independently. Since there is minimal disturbance of the intrinsic, and almost none of the extrinsic, coagulation system, and there is no increase in fibrinogen breakdown products, disseminated intravascular coagulation (DIC) is excluded. Furthermore, platelet and fibrinogen turnover in experimental primate infections are normal. Though platelet numbers are only moderately depressed, in severe disease platelet aggregation is almost completely abolished by a circulating inhibitor. The origin of this inhibitor is not known; however, it cannot be reproduced with viral material nor can it be blocked by antibodies to Lassa virus. In the platelet it blocks dense granule and ATP release and thus abolishes the secondary wave of *in vitro* aggregation, while sparing the arachidonic acid metabolite-dependent primary wave. The inhibitor of platelet function also interferes with the generation of the FMLP-induced superoxide generation in polymorphonuclear leukoeytes probably through a similar mechanism.

AHF and BHF

Despite the different degrees of bleeding, there are sufficient similarities between the course of disease in AHF, BHF and Lassa fever to speculate that there exists a similar pathophysiologic pathway underlying all of the diseases. Organ function, other than the endothelial system, appears to remain intact, and the critical period of shock is brief, lasting only 24–48 hours. Hepatitis is mild, and renal function is also well maintained. Bleeding is more pronounced with AHF and BHF than Lassa fever, but it is not the cause of shock and death. Capillary leakage is significant, with loss of protein and intravascular volume being much more pronounced than loss of red cells. Proteinuria is significant, and dehydration with hemoconcentration appears to be an important process. The shock is not associated with evidence of disseminated intravascular coagulation, and even though there are petechiae suggesting some direct endothelial damage, no clear evidence of virus replication in, and damage of, endothelium has been demonstrated. Thus clinical observations suggest that vascular endothelial dysfunction may be the basis of subsequent circulatory failure in AHF and BHF. Persistent hypovolemic shock in the face of intravascular volume expanders suggests that it is due to the loss of endothelial function and leakage of fluid into

extravascular spaces. This is supported by the tissue edema frequently observed, and more directly by the pulmonary edema which may result from vigorous fluid therapy of the shock. These events lead to irreversible shock and death in the most severely ill patients. Two other observations have been made in AHF: high levels of interferon in severely/fatally ill patients, and a decrease in complement. These are general phenomena observed in other severe infectious processes and are consistent with the events described above. Neither would dictate a substantially different pathophysiologic explanation of these diseases.

Clinical Features

Lassa fever

Lassa fever begins after 7–18 days of incubation, with fever, headache and malaise. Aching in the large joints, pain in the lower back, a nonproductive cough, severe headache and sore throat are common. Many patients also develop severe retrosternal or epigastric pain. Vomiting and diarrhea occurs in between a half and two-thirds of patients. In more severely ill patients complete prostration may occur by the 6th to 8th day of illness. Patients with Lassa fever appear toxic and anxious, and in the absence of shock, the skin is usually moist from diapheresis. There is an elevated respiratory rate and pulse. The systolic blood pressure may be low. There is no characteristic skin rash; petechiae and ecchymoses are rare, nor is jaundice a feature of Lassa fever. Conjunctivitis is common, but rare conjunctival hemorrhages portend a poor prognosis. Seventy percent of patients have pharyngitis, often exudative, but few if any petechiae, and ulcers are rare. Mucosal bleeding occurs in 15–20% of all patients, and although associated with severe disease it is almost never of a magnitude to produce shock by itself. Edema of the face and neck are commonly seen in severe disease. About 20% of patients have pleural or pericardial rubs heard late in disease at the beginning of convalescence. The abdomen may be diffusely tender. The ECG may be abnormal, primarily with elevated T waves, but without a characteristic pattern. Moderate or severe diffuse encephalopathy with or without general seizures is characteristic in severe disease. Severe Lassa fever can progress rapidly between the 6th and 10th days to respiratory distress with stridor due to laryngeal edema, central cyanosis, hypovolemic shock and clinical signs of encephalopathy, sometimes with coma and seizures. Tremors are often seen in the hours just before death. Acute, focal neurological signs rarely occur, with the exception of VIIIth nerve deafness seen in convalescence. Residual ataxia is common. Lassa fever is also a pediatric disease affecting all ages of children. The disease appears to be even more difficult to diagnose by clinical criteria in children than in adults because its manifestations are so general. In very young babies marked edema may be seen, associated with very severe disease. In older children the disease may manifest as a primary diarrheal disease or as pneumonia or simply as an unexplained prolonged fever. The case fatality in children is 12–14%.

The mean white count in early Lassa fever may be low or normal, with a relative lymphopenia, but late neutrophilia may supervene in severe disease. Proteinuria is very characteristic. A serum AST (SGOT) level of >150 iu ml^{-1} is associated with a case fatality of 50%, and there is a correlation between an ever-increasing level and an increased risk of death. A viremia of $>1 \times 10^3$ $TCID_{50}$ ml^{-1} is associated with an increasing case fatality. Both factors together carry a risk of death of 80%.

Pharyngitis, proteinuria and retrosternal chest pain have a predictive value for Lassa fever in febrile hospitalized patients of 81% and a specificity of 89%. Likewise, a triad of pharyngitis, retrosternal chest pain and proteinuria (in a febrile patient) correctly predicted Lassa fever 80% of the time (in an endemic area). Both triads have sensitivities of 50% for detecting cases of Lassa fever. Significant complications of Lassa fever include a two- to threefold increased risk of maternal death from infection in the third trimester, and a fetal/perinatal loss of 84% that does not seem to vary by trimester. Another significant complication is that of acute VIIIth nerve deafness, with nearly 30% of patients suffering an acute loss of hearing in one or both ears. Other complications which appear to occur much less frequently are uveitis, pericarditis, orchitis, pleural effusion and ascites.

AHF and BHF

These diseases have an insidious onset of malaise, fever, general myalgia and anorexia. Lumbar pain, epigastric pain, retro-orbital pain, often with photophobia, and constipation occur commonly. Nausea and vomiting frequently occur. Temperature is high, reaching 40°C or above. Unlike LCM and Lassa fever, AHF and BHF do not usually lead to respiratory symptoms and sore throat. On physical examination patients appear toxic. Conjunctivitis, erythema of the face, neck and thorax are prominent. Petechiae may be observed in the axillae by the 4th or 5th days of the illness. There may be a pharyngeal enanthem, but pharyngitis is uncommon. Relative bradycardia is

often observed. The disease may begin to subside after 6 days of illness; if not, the second stage of illness supervenes, and most commonly manifests as epistaxis and/or hematemesis or acute neurological disease. The bleeding may be from mucosal surfaces or into the skin, with petechiae and hemorrhagic rash, with preceding increase in packed red cell volume. Pulmonary edema is common in severely ill patients. The appearance of intractable shock is a serious sign which becomes irreversible in some patients, and accounts for the majority of deaths from AHF and BHF. Fifty percent of AHF and BHF patients also have neurologic symptoms during the second stage of illness, such as tremors of the hands and tongue, progressing in some patients to delirium, oculogyrus and strabismus.

A low white blood cell count (under 1000 mm^{-1}) and a platelet count under 100 000 are invariable. Proteinuria is common and microscopic hematuria also occurs. Alterations in clotting functions are minor, and DIC is not a significant part of the diseases. Liver and renal function tests are only mildly abnormal.

Venezuelan hemorrhagic fever

Little information is available on the spectrum of disease caused by Guanarito virus infection. Hospitalized patients with severe diseases are febrile on admission, with prostration, headache, arthralgia, cough, sore throat, nausea/vomiting, diarrhea and hemorrhage. The bleeding includes epistaxis, bleeding gums, menorrhagia and melena. On physical examination, patients with severe disease appear toxic, and usually dehydrated. They are described as having one or more of a series of signs, including pharyngitis, conjunctivitis, cervical lymphadenopathy, facial edema and petechiae. Thrombocytopenia and neutropenia are common at admission. Case fatality of a single group of 15 hospitalized patients was over 60%; however, the single serum survey available suggests that overall mortality:infection ratio is much lower.

Pathology and Histopathology

Lassa fever

The most frequently and consistently observed microscopic lesions in fatal human Lassa fever are focal necrosis of the liver, adrenal glands and spleen. The liver damage is variable in the degree of hepatocytic necrosis. The liver demonstrates cellular injury, necrosis, and regeneration, with any or all present at death. A substantial macrophage response occurs, with little if any lymphocytic inflammatory response. Nevertheless, fatal cases do not exhibit sufficient hepatic damage to implicate hepatic failure as a cause of death. Similarly, moderate splenic necrosis is a consistent finding, primarily involving the marginal zone of the periarteriolar lymphocytic sheath. Diffuse focal adrenocortical cellular necrosis has been less frequently observed. Although high virus titers occur in other organs, such as brain, ovary, pancreas, uterus and placenta, no significant lesions have been found. Thus few clues to the pathogenesis of Lassa fever are found in standard pathological studies. It is clear that the outcome in Lassa fever is associated with the degree of virus replication; nevertheless the effect of replication is not major tissue destruction, but a more subtle biological effect on vascular endothelium, and perhaps other key cells or organs.

AHF and BHF

AHF and BHF are typical hemorrhagic diseases and have very similar pathologic features, some of which differ from Lassa fever. Patients with AHF manifest a skin rash and petechiae, and gross examination of organs at necropsy also show petechiae on the organ surfaces. Ulcerations of the digestive tract have been described, but the bleeding is not massive. Microscopic examination shows a general alteration in endothelial cells, mild edema of the vascular walls, with capillary swelling and perivascular hemorrhage. Large areas of intra-alveolar or bronchial hemorrhage are often seen with no evidence of inflammatory process. Pneumonia with necrotizing bronchitis or pulmonary emboli is observed in half of the fatal necropsied cases. Hemorrhage and a lymphocytic infiltrate have been observed in the pericardium, occasionally with interstitial myocarditis. The lymph nodes are enlarged and congested with reticular cell hyperplasia. Splenic hemorrhage is common. Medullary congestion with pericapsular and pelvic hemorrhages are frequently seen. Acute tubular necrosis occurs in about half of the fatal cases, but adrenal necrosis has not been reported.

Venezuelan hemorrhagic fever

Only a limited number of post-mortem dissections have been performed. Observations included pulmonary edema with diffuse hemorrhages in the parenchyma, and subpleural, focal hepatic hemorrhages with congestion and yellow discoloration, cardiomegally with epicardial hemorrhages, splenic and renal swelling, and bleeding into numerous cavities, including stomach, intestines, bladder and uterus.

Immune Response

There is a brisk B cell response to Lassa virus, with a classic primary IgG and IgM antibody response to virus early in the illness. This event does not, however, coincide with virus clearance, and high viremia and high IgG and IgM titers often coexist in both humans and primates. Indeed virus may persist in the serum and urine of humans for several months after infection, and possibly longer in occult sites, such as renal tissue. Neutralizing antibodies to Lassa virus are absent in the serum of patients at the beginning of convalescence, and in most people they are never detectable. In a minority of patients some low titer serum neutralizing activity may be observed several months after resolution of the disease. Passive protection with antibody to Lassa virus has been demonstrated in animals given selected antiserum at the time or soon after inoculation with virus, but clinical trials of human plasma given after onset of illness have shown little or no protective effect. Thus the clearance of Lassa virus in acute infection appears to be less dependent of antibody formation, and presumably depends more on the cell immune response. This is supported by recent experience with experimental Lassa vaccines in primates. In recent studies of LCM infection there is clearly apoptosis of T cells and probably B cells during acute infection in the mouse model. Such a scenario must be entertained for acute Lassa infection, and could help explain the fulminant course in some patients. Such work will need to be performed in primate models or during studies of human disease in endemic areas. Neutralizing and complement fixing antibody to Junin and Machupo are usually detectable 3–4 weeks after the onset of illness. Indirect fluorescent antibodies may be detected at the end of the second week of illness. The efficacy of convalescent plasma has been demonstrated in the therapy of Junin infection (see Therapy). The effectiveness of the plasma has been demonstrated to be associated with the level of Junin virus-neutralizing antibodies. The IFA test is the most commonly performed test for diagnosis by antibody detection for AHF and BHF. Little data are available on Guanarito virus, which appears to induce an antibody response. However, more like AHF and BHF, antibodies to Guanarito virus seem to appear later in illness.

Prevention and Control

Rodent control

The ideal method of prevention for these rodent-borne diseases is to prevent contact between rodents and humans. The effectiveness of this was shown in the outbreaks of BHF in the 1960s. However, the prospects of rodent control in preventing AHF are not so bright. The human–rodent encounter resulting in AHF occurs during the crop harvests, and with present technology it is difficult to imagine how control of noncommensal feral rodents could be accomplished. The best choice may be better protection of the agricultural worker from contact with rodent secretions and blood. Similarly the control of rodents as a broad approach to preventing Lassa fever is not realistic. The improvement of housing and food storage could reduce the domestic rodent population, but such changes are not easily made. Rodent trapping in an individual village where transmission is high has demonstrated as much as a fivefold reduction in the rate of virus transmission. However, such a program is only applicable in villages with exceptional transmission rates, and would certainly not be applicable to large areas.

Vaccines

The live attenuated Junin vaccine has now been shown to be not only effective, but has had a dramatic effect in reducing the number of cases of AHF seen each year in the endemic area. A vaccine against Lassa fever has been made by cloning and expressing the Lassa virus glycoprotein gene into vaccinia virus. This vaccine has proven highly successful in preventing severe disease and death in challenged monkeys. Only the glycoprotein gene is protective in the two species of monkeys thus far tested, and the basis of protection is not neutralizing antibody, which does not develop, but more likely the cell-mediated immune response.

Drug prophylaxis

In the event of identifiable exposure to Lassa virus (and possibly other pathogenic arenaviruses) in a hospital or laboratory setting, the prophylactic use of orally administered ribavirin is recommended.

Therapy

Significant advances have been made in the therapy of Junin and Lassa virus infections over the last decade. Convalescent plasma is effective in the treatment of Junin virus during the first 8 days of illness, but not Lassa virus. Ribavirin is effective in reducing the viremia and mortality of Lassa fever particularly when given in the first week of illness.

Lassa fever

Ribavirin can prevent death in Lassa fever when given at any point in the illness, but it is more effective when given early, and intravenously. Thus, patients with

risk factors for severe disease who were treated within the first 6 days of illness experienced a 5–9% case fatality. Those with the same risk factors, in whom treatment was initiated more than 6 days after the onset of illness, had a case fatality of 20–47%. Regardless, the case fatality was significantly less for ribavirin-treated patients in all categories than for either nontreated patients or for those treated with plasma alone. As such it seems reasonable to assume that it would also be an effective measure in the event of laboratory or hospital exposure to the disease. The pathogenesis of the infection is less reversible later in illness. Furthermore, patients treated with ribavirin had significant declines in viremia regardless of outcome, whereas patients who were untreated or treated with plasma and who died showed no decrease in viremia, consistent with the observation that outcome, and presumably the result of therapy, is closely related to the inhibition of virus replication. Therefore patients coming late in disease will require more effective clinical management of physiologic dysfunction, and perhaps other drugs which may be used to stabilize the shock state sufficiently long to allow recovery and improve survival (see Pathogenesis).

AHF

A randomized trial of patients with AHF demonstrated that convalescent-phase plasma reduced the mortality from 16% to 1% in the patients who were treated in the first 8 days of illness. The efficacy of the plasma was directly related to the concentration of neutralizing antibodies of the plasma.

The success of this therapy was not without a price, however, which was the development of a late neurological syndrome in about 10% of cases. Thus far there is no correlation between the day of therapy nor the dose of neutralizing antibodies given and the occurrence of late neurological syndrome. Passive antibody therapy depends on collection of plasma from persons known to have had the disease, testing the plasma (or screening the donor) for antibodies to blood-borne agents such as hepatitis, and proper storage until its use. In addition, the advent of the acquired immunodeficiency syndrome (AIDS) and the other diseases transmissible by blood products means that further screening is required before use.

BHF

Recent experience of successful treatment of two patients with intravenous ribavirin suggests that further efforts to evaluate the effectiveness of ribavirin in this disease will be worthwhile.

Future Perspectives

An important area of future research includes a more thorough understanding of the viral and host components of the virus-clearing and protective immune response in humans. A second area of substantial interest is the nature of the persistent infection in the rodent host. A more complete understanding of the pathogenesis of Lassa fever may provide insight not only to that disease, but to basic elements of how the host response may be detrimental as well as beneficial to the host. Finally, a more thorough comprehension of how to control the more widespread arenavirus diseases, either through vaccination, or preferably rodent control, is essential.

See also: **Lymphocytic choriomeningitis virus (*Arenaviridae*): General features; Encephalitis viruses (*Flaviviridae*): Encephalitis viruses and related viruses causing hemorrhagic disease, Tick-borne encephalitis and Wesselsbron viruses.**

Further Reading

Bishop DHL and Auperin DD (1987) Arenavirus gene structure and organization. *Curr. Top. Microbiol. Immunol.* 133: 5.

Fisher-Hoch SP (1993) Arenavirus pathophysiology. In: Salvato M (ed.) *The Arenaviruses,* p. 386. New York: Plenum Press.

Fisher-Hoch SP, McCormick JB, Auperin D *et al.* (1989) Protection of rhesus monkeys from fatal Lassa fever by vaccination with a recombinant vaccinia virus containing the Lassa virus glycoprotein gene. *Proc. Natl Acad. Sci. USA* 85: 317.

McCormick JB (1990) The arenaviruses. In: Fields BN *et al* (eds) *Virology,* 2nd edn, p. 124S. New York: Raven Press.

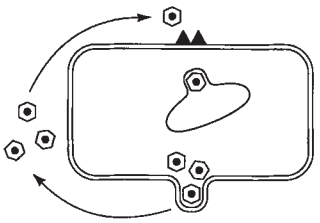
McCormick JB and Fisher-Hoch SP (1990) Viral hemorrhagic fevers. In: Warren KS and Mahmoud AAF (eds) *Tropical and Geographical Medicine,* 2nd edn, p. 700. New York: McGraw-Hill.

McCormick JB, Webb PA, Scribner CL *et al* (1986) Lassa fever: effective therapy with ribavirin. *N. Engl. J. Med.* 314: 20.

McCormick JB, Webb PA, Krebs JW, Johnson KM and Smith K (1986) A prospective study of the epidemiology and ecology of Lassa fever. *J. Infect. Dis.* 155: 437.

LATENCY

Jack G Stevens, University of California, Los Angeles, California, USA



Introduction

Latent infections, as they relate to animal viruses, represent one of two general mechanisms by which virus exists for extended periods in an infected host. The other mechanism, which involves continued replication of infectious virus, is termed a chronic infection. Latent infections are defined as those in which complete copies of the viral genetic information persist for extended periods by processes not requiring the continued production of infectious virus. Whichever mechanism is employed, it is important to note that unique methods of evading the immune response have evolved. In the case of latent viruses, the mechanism usually involves restrained expression of the viral genome so that immunologically relevant molecules displayed on the viral or cellular surface during the course of productive infection are not synthesized.

In all instances sufficiently documented to date, the phenomenon of latency has been limited to DNA-containing viruses or to RNA-containing viruses employing a DNA intermediate in their replicative cycle. In all these systems, the viral DNAs are conserved either by integration into the host cell chromosome (at random or specific sites) or by maintenance of plasmids (usually, but not always, as multiple copies of individual genomes). In most of the systems studied, when technologies of sufficient sensitivity were employed, the latent viral DNA was found to be transcribed and protein products were often produced. Among these products are the 'transforming proteins' associated with neoplastic alterations of latently infected cells.

There are at least two biologically important consequences of latent infection. The most notable is a later 're-activation' or reappearance of infectious virus within the individual harboring the latent infection. This event may result in the appearance of clinically apparent features, which may or may not resemble those associated with the disease evident during initial infection. The disease resulting from re-activation is often accentuated in immuno-compromised hosts; indeed, immunosuppression may play a role in the re-activation process itself. The second important consequence of the latent infection is neoplastic alteration of the latently infected cell, and the subsequent appearance of tumors. Essentially all latent agents accomplish the former; only some are responsible for the latter. Finally, although the hypothesis that latent infections can alter important functional properties of differentiated cells has been historically an intriguing one, there is no consistent evidence supporting this notion.

Viruses that Establish Latent Infections

A list of viruses capable of existing in the latent state is presented in **Table 1**. Several general statements can be made about this group. First, the kinds of cells involved are many, ranging from immature lymphocytes to terminally differentiated neurons. It should also be stressed that with many viruses, the kinds of cells harboring latent infection are likely to extend beyond those presently appreciated or postulated. Second, features of latent infection now shown only in cell culture systems (for example, integrated sequences of the HIV process) are also likely in the future to be demonstrated in cells taken from infected individuals. Third, in some latent infections, perhaps most notably those involving herpes simplex virus and papillomaviruses, viral-encoded proteins have not been detected in cells harboring the viral DNA. However, transcripts containing potential open reading frames have been found in the former, and transcripts encoding proteins that have been detected in transfected and lytically infected cells have been identified in the latter. Finally, it should be emphasized that in some instances the relative roles played by chronic and latent infection in maintenance of the persistent infection are not clearly defined. For example, even though latent infections are well appreciated in hepadnavirus and cytomegalovirus infections, a persistent viremia is also characteristic of hepadnavirus infection, and cytomegalovirus is often shed from the urine for extended periods.

The Herpesvirus Model

Additional features characteristic of latent infections can be identified through a more detailed consideration of latent infections engendered by the herpes simplex virus and Epstein–Barr virus (EBV). The herpesviruses, particularly herpes simplex virus, are the classic examples of latent agents, and latent infections are basic to their natural history.

Table 1 Latent infection in the intact host by viruses infecting humans

Virus group	*Cell type involved*	*State of DNA*	*Viral proteins present?*
Adenoviruses	B lymphocytes?	Probably integrated	Probably
Hepadnavirus	Hepatocytes, lymphocytes	Integrated	Probably
Herpesviruses			
(a) Herpes simplex 1 and 2	Neurons	Episomal	No
(b) Varicella-Zoster	Satellite cells and neurons reported	?	?
(c) Cytomegalovirus	Lymphocytes? macrophages?	?	?
(d) Epstein–Barr	B lymphocytes, epithelial cells	Usually episomal	Yes
(e) Human herpesviruses 6, 7, 8	Probably T lymphocytes	?	?
Papillomaviruses	Keratinizing epithelial cells	Integrated and episomal	No
Parvoviruses	Epithelial cells	Integrated	No
Polyomaviruses	?	Probably integrated	Probably
Retroviruses			
(a) Human T-cell leukemia viruses	T lymphocytes	Integrated	No
(b) Human immunodeficiency virus (HIV)	CD4+ T lymphocytes, probably macrophages	Episomal and probably integrated	No

Herpes simplex virus

The relevant pathogenesis of herpes simplex virus involves infection and viral replication at the body surface, followed by the passage of the virus (or, more probably, the viral nucleocapsid) to the regional sensory ganglion by fast axonal transport in the associated sensory nerve. At this juncture, two mutually exclusive events occur in the infected neuronal somas. Either (1) the five immediate early regulatory proteins encoded within the viral genome are expressed, thus initiating the 'lytic cascade' of gene expression which results in viral replication and cell death, or (2) the latency-associated transcription unit (see below) is expressed at high copy number and the latent infection is established. In the latter instance, present evidence indicates that no viral proteins are expressed, and the viral DNA does not replicate. In an individual sensory ganglion initially infected by herpes simplex virus, then, some neurons produce infectious virus and are destroyed, while others simultaneously harbor viral DNA, survive and thus become latently infected. The experiments that established these features took two general forms related to the genetic constitution of the virus employed. In one group of experiments, viruses compromised for replication were employed. These viruses were defective in genes expressing thymidine kinase, the virion-associated transcription factor αTIF, the immediate early regulatory protein ICP0, or the immediate early regulatory protein ICP4. In all instances, latent infections were established, indicating that extensive genetic expression and viral replication are not required for establishment of the latent infection. Studies with the ICP4 negative mutants were particularly instructive, since cells infected with these agents do not replicate viral DNA and transcription is qualitatively drastically limited. In the other groups of experiments, replicating viruses were employed. Neurons found to express viral proteins did not transcribe from the latency-associated transcript promoter, and vice versa. In addition, experiments of several designs indicated that viral DNA did not replicate during establishment of the latent infection. In studies of a different design, neurons in ganglia with no evidence of prior acute viral infection were later found to be latently infected. Taken together, these studies indicate that viral DNA replication and expression of viral proteins are not associated with establishment of the latent infection. Thus, the 20 or so viral plasmids known to be present in each latently infected neuron consist of input DNA, and the neuron controls establishment of the latent infection. How this occurs is unknown, but specific transcription factors seem likely to play important roles. Expression of these factors may well be related in some way to the different, mutually exclusive, surface markers that have been identified on sensory neurons destined to become latently infected compared to those that are lytically infected. Thus, most neurons that are latently infected are SSEA-3 antigen positive and LD-2 antigen negative; those lytically infected may be either SSEA-3 or LD-2 antigen positive.

Recent studies of the molecular aspects of herpes simplex virus latency have been based largely on the discovery some 5 years ago of latency-associated transcripts. These transcripts are encoded from the long terminal repeat region of the viral genome, and the principal unit appears to be an 8.5 kb transcript the synthesis of which initiates downstream from, and on the opposite DNA strand to, that encoding the immediate early regulatory protein ICP0. This transcript extends across the ICP0 coding region and terminates in the short terminal repeat region just downstream from the termination of the ICP4 (also an immediate early regulatory gene). Several derivatives of the latency-associated transcription unit are present in latently infected cells, the most prominent being a 2.0 kb stable intron present in more than 10^4 copies per nucleus of each latently infected neuron. The 8.5 kb transcription unit and its derivatives appear to be the only transcripts present in latently infected cells, and genetic experiments involving deletion mutants have shown that these RNAs play no role in either establishment or maintenance of the latent infection in mice or rabbits. Both of these major events can take place with no expression from the viral genome.

To restate and emphasize the important features of herpes simplex virus which have just been presented, establishment and maintenance of the latent infection appear to be neuronal functions. Multiple copies of input viral DNA are maintained in sensory neurons as single-copy episomes which express latency-associated transcripts. There is no evidence that any of these transcripts are translated into proteins, and a principal derivative persists as a stable intron in neuronal nuclei.

The latent infection is maintained for the life of the infected individual, and essentially all persons seropositive for herpes simplex virus 1 harbor latent infection in sensory neurons in trigeminal ganglia. In a subset of these individuals, clinically apparent reactivations occur periodically, and virus, or at least viral genetic information, is thought to pass intraaxonally to neuroepithelial junctions where infection of epithelial cells results in productive infection and, ultimately, lesions. The molecular basis for the reactivation is not understood, but in the rabbit ocular model in which re-activation from trigeminal ganglia

is efficiently induced by iontophoresis of epinephrine through the cornea, some derivative of the 8.5 kb latency-associated transcription unit plays a significant role. Thus, viruses with deletions in the transcription unit do not re-activate following the induction process. Additional viral genes active in this process have yet to be identified. Finally, although it has been known for over 20 years that *in vitro* cultivation of latently infected ganglia would result in 're-activation' of the virus, it is not known whether the same process occurs in latently infected ganglia *in situ*.

Epstein–Barr virus

The process just described is considerably more simple than that undergone by the related EBV. The major biologic difference relates to the fact that while herpes simplex virus latently infects a terminally differentiated cell, EBV establishes this infection in B lymphocytes (and probably pharyngeal epithelial cells) which subsequently divide. Obviously, in this case, a method has to be derived for replicating the approximately 50 single-copy episomes present in these latently infected cells, and distributing them to daughter lymphocytes. In addition, the fact that EBV is oncogenic implies that viral gene products play a role in the neoplastic process. These phenomena would predict that the viral protein products now known to be expressed in latently infected cells would, in fact, be present.

As presently understood, the virus infects via the nasopharynx, productively infecting epithelial cells in this area, and secondarily infecting B lymphocytes, which then harbor latent virus for the life of the individual. Whether or not these cells have the basic properties of 'immortalized' B cells which carry EBV genomes in culture systems is not known. Although virus is shed from the nasopharynx for extended periods, the best evidence indicates that this is not a requirement for maintenance of a latently infected B lymphocyte. However, the converse may well be true. That is, B lymphocytes may re-activate in the nasopharynx, supplying virus that maintains the nasopharyngeal infection. Alternatively, the long-term active infection of the nasopharynx may be a chronic infection not requiring re-infection for maintenance.

The 'classic' latently infected immortalized B cell in culture expresses some nine EBV-encoded proteins six of which [the Epstein–Barr nuclear antigens (EBNA)] are translated from one basic transcript which undergoes very long-range splicing events with some derivatives being bi- or poly-cystronic. Only one of the EBNA proteins, EBNA-1, appears to be crucial to the latent phase, since it is the only one known to be universally present in cells harboring the EBV genome. This molecule binds to the EBV DNA origin of replication employed for plasmid replication by the host-cell-encoded DNA polymerase and enables molecules to replicate and persist as episomes. For completeness, it should be noted that a different origin of DNA replication and the viral-encoded rather than the host-cell-encoded polymerase, are employed for the EBV lytic infection. Other EBNA molecules are known to function in the cellular immortalization process, or possess functions unknown at present. The other three proteins (LMP-1, 2a, 2b) target the cellular cytoplasmic membranes, and LMP-1 is known to be involved in the immortalization process. From all of this it is clear that, unlike the process with herpes simplex virus, EBV viral genetic expression is crucial to establishment and maintenance of the latent infection.

The process of EBV re-activation is better understood than is the process described for herpes simplex virus. As with herpes simplex virus, *in vitro* cultivation of latently infected EBV cells may result in re-activation and replication of infectious virus in some lymphocytes. A number of other stimuli, including *n*-butyrate, the phorbol ester tumor promoter 12-O-tetradecanol phorbol 13-acetate (TPA) and calcium ionophores may increase the number of cells producing virus. Whatever the inducing agent, it seems likely that a common pathway exists involving initial expression of the ZEBRA protein [from the *BamZ* leftward open reading frame number one (BZLF-1)] from the viral genome. Expression of this gene is repressed (by unknown, but obviously very important, mechanisms) in latently infected cells. Induction of the protein is followed by expression of early genes, and the entire viral replicative cycle may then follow. The precise nature of this induction is unknown, but probably involves some mechanism of direct transcriptional activation occurring in conjunction with cellular transcription factors, since ZEBRA is a DNA-binding protein with homologies and binding-site properties similar to the *fos–jun* family of cellular-derived oncogenes.

Summary and Future Perspectives

An overview of latent infections as they relate to virus infection of human beings has been presented. Although, as **Table 1** indicates, viruses comprising several major groups are capable of persisting in this state, in essentially all cases the latent genome persists as DNA. As the discussion of herpesviruses emphasizes, beyond this fundamental property the molecular characteristics of the latent state vary greatly. For

example, genomes may be integrated or exist as episomes, transcripts and proteins may or may not be present, latently infected cells may or may not divide, and the latent state may be accompanied by alterations in basic cellular properties, including the induction of neoplasia.

Increasingly, the general medical significance of latent viral infections is being recognized. For example, latent viral infections are fundamental to the natural history of such major syndromes as AIDS and viral hepatitis. Adequate control of these and other diseases in which latent infections play significant roles can be intelligently planned and achieved when a more precise basic understanding of the latent infection is obtained.

See also: **Epstein-Barr virus (*Herpesviridae*): General features; Herpes simplex viruses (*Herpesviridae*): General features; Retroviral Oncogenes; Persistent viral infection; Transformation: Animal viruses.**

Further Reading

Ahmed R and Stevens JG (1990) Viral Persistence. In: Fields BN *et al.* (eds) *Virology*, 2nd edn. p. 241. New York: Raven Press.

Boulinguez S and Rogez S (1997) Herpes simplex virus: mechanism of latency and reactivation phases [in French]. *Ann. Dermatol. Venereol.* 124(5): 409.

Garcia-Blanco MA and Cullen BR (1991) Molecular basis of latency in pathogenic human viruses. *Science* 254: 815.

Margolis TP *et al.* (1992) Pathways of viral gene expression during acute neuronal infection with HSV-1. *Virology* 189: 150.

Rogers RP, Strominger JL and Speck SH (1992) Epstein–Barr virus in B lymphocytes: viral gene expression and function in latency. *Adv. Cancer Res.* 58: 1.

[This article is reproduced from the 1st edn (1994).]

Lelystad Virus *see* **Arteriviruses (Arteriviridae)**

Lumpy Skin Disease *see* **Poxviruses**

LUTEOVIRUS (*LUTEOVIRIDAE*)

W Allen Miller, Department of Plant Pathology, Iowa State University, Ames, Iowa, USA

Taxonomy

Luteoviridae are spherical, phloem-limited, aphid transmitted plant viruses, containing a positive-sense genomic RNA. In 1998, the International Committee on the Taxonomy of Viruses (ICTV) raised the luteovirus group to the level of family. The new *Luteoviridae* family now contains three genera: the genus *Luteovirus* (formerly known as luteovirus subgroup I), the genus *Polerovirus* (formerly known as luteovirus subgroup II), and the genus *Enamovirus* which consists solely of pea enation mosaic virus-1. This entry refers only to the luteoviruses and poleroviruses. Another entry discusses the genus *Enamovirus*. Members of the *Luteovirus* and *Polerovirus* genera have distinctly different genome organizations, cytopathological effects and serological properties. The differences and similarities between the two genera will become evident throughout this entry.

A list of *Luteoviridae* and their vectors is shown in **Table 1**. Barley yellow dwarf virus (BYDV), beet western yellows virus (BWYV) and potato leafroll virus (PLRV) are the best characterized, so most discussion will focus on these. Several viruses on this list may be strains of a single virus, because *Luteoviridae* (1) are difficult to identify by sympto-

example, genomes may be integrated or exist as episomes, transcripts and proteins may or may not be present, latently infected cells may or may not divide, and the latent state may be accompanied by alterations in basic cellular properties, including the induction of neoplasia.

Increasingly, the general medical significance of latent viral infections is being recognized. For example, latent viral infections are fundamental to the natural history of such major syndromes as AIDS and viral hepatitis. Adequate control of these and other diseases in which latent infections play significant roles can be intelligently planned and achieved when a more precise basic understanding of the latent infection is obtained.

See also: **Epstein-Barr virus (*Herpesviridae*): General features; Herpes simplex viruses (*Herpesviridae*): General features; Retroviral Oncogenes; Persistent viral infection; Transformation: Animal viruses.**

Further Reading

Ahmed R and Stevens JG (1990) Viral Persistence. In: Fields BN *et al.* (eds) *Virology*, 2nd edn. p. 241. New York: Raven Press.

Boulinguez S and Rogez S (1997) Herpes simplex virus: mechanism of latency and reactivation phases [in French]. *Ann. Dermatol. Venereol.* 124(5): 409.

Garcia-Blanco MA and Cullen BR (1991) Molecular basis of latency in pathogenic human viruses. *Science* 254: 815.

Margolis TP *et al.* (1992) Pathways of viral gene expression during acute neuronal infection with HSV-1. *Virology* 189: 150.

Rogers RP, Strominger JL and Speck SH (1992) Epstein–Barr virus in B lymphocytes: viral gene expression and function in latency. *Adv. Cancer Res.* 58: 1.

[This article is reproduced from the 1st edn (1994).]

Lelystad Virus *see* **Arteriviruses (Arteriviridae)**

Lumpy Skin Disease *see* **Poxviruses**

LUTEOVIRUS (*LUTEOVIRIDAE*)

W Allen Miller, Department of Plant Pathology, Iowa State University, Ames, Iowa, USA

Taxonomy

Luteoviridae are spherical, phloem-limited, aphid transmitted plant viruses, containing a positive-sense genomic RNA. In 1998, the International Committee on the Taxonomy of Viruses (ICTV) raised the luteovirus group to the level of family. The new *Luteoviridae* family now contains three genera: the genus *Luteovirus* (formerly known as luteovirus subgroup I), the genus *Polerovirus* (formerly known as luteovirus subgroup II), and the genus *Enamovirus* which consists solely of pea enation mosaic virus-1. This entry refers only to the luteoviruses and poleroviruses. Another entry discusses the genus *Enamovirus*. Members of the *Luteovirus* and *Polerovirus* genera have distinctly different genome organizations, cytopathological effects and serological properties. The differences and similarities between the two genera will become evident throughout this entry.

A list of *Luteoviridae* and their vectors is shown in **Table 1**. Barley yellow dwarf virus (BYDV), beet western yellows virus (BWYV) and potato leafroll virus (PLRV) are the best characterized, so most discussion will focus on these. Several viruses on this list may be strains of a single virus, because *Luteoviridae* (1) are difficult to identify by sympto-

Table 1 *Luteoviridae* classification

Virus	*Abbreviation*	*Vector*[a]
Luteovirus genus		
Barley yellow dwarf-PAV	BYDV-PAV	*Rhopalosiphum padi, Sitobion avenae*
Barley yellow dwarf-MAV (rice giallume)	BYDV-MAV	*S. avenae*
Probable *Luteovirus*		
Soybean dwarf[b] (strawberry mild yellow edge)	SBDV	*Aulacorthum solani*
Subterranean clover redleaf	SCRLV	*A. solani*
Polerovirus genus		
Cereal yellow dwarf-RPV (formerly BYDV-RPV)	CYDV-RPV	*R. padi*
Beet western yellows (malva yellows) (turnip mild yellows)	BWYV	*Myzus persicae*
Beet mild yellowing	BMYV	*M. persicae*
Cucurbit aphid-borne yellows	CABYV	*M. persicae, Aphis gossypii*
Potato leaf roll (Solanum yellows) (tomato yellow top)	PLRV	*M. persicae*
Probable *polerovirus*		
Barley yellow dwarf-RMV	BYDV-RMV	*Rhopalosiphum maidis*
Carrot red leaf	CRLV	*Cavariella aegopodii*
Cereal yellow dwarf virus – GPV (formerly BYDV-GPV)	CYDV-GPV	*Schizaphis graminum*
Groundnut rosette assistor	GRAV	*Aphis craccivora*
Genus uncertain (*Luteovirus* or *Polerovirus*)		
Barley yellow dwarf-SGV	BYDV-SGV	*Schizaphis graminum*
Bean leaf roll = pea leaf roll (pea leaf roll) (Michigan alfalfa) (legume yellows)	BLRV	*M. persicae*
Chickpea stunt associated	CpSaV	*M. persicae*[c]
Sweet potato leaf speckling	SPLSV	*Macrosiphum euphorbiae*
Tobacco necrotic dwarf	TNDV	*M. persicae*
Solanum yellows	SYV	*M. persicae*
Indonesian soybean dwarf	ISDV	*Aphis glycines*
Enamovirus genus		
Pea enation mosaic virus-1	PEMV-1	*M. persicae*

Synonyms are in parentheses. Probable members of genera have been assigned by the author, not the ICTV.
[a]For each virus, the best characterized vector is shown. In some cases, several aphid species serve as vectors.
[b]Has been proposed to be in a third subgroup owing to structural genes that are most closely related to those of poleroviruses, and absence of ORF 6.
[c]Less than 10% transmission efficiency.
Modified from D'Arcy *et al* (1999).

matology, (2) are difficult to purify, (3) show varying degrees of serological crossreactivity, and (4) vary in host range, depending on the isolate. For example, soybean dwarf (SBDV) and subterranean clover red leaf (SCRLV) viruses are so closely related serologically that they may be strains of the same virus. However, a Mississippi isolate of SCRLV is unable to infect soybeans.

In contrast to potentially synonymous viruses, BYDV has been split into two viruses. Originally, five serotypes were defined by the different aphid vectors that transmit them (**Table 1**). Furthermore, isolates of a serotype can vary greatly in symptom severity in a given host, and one isolate can cause widely varying symptoms in different hosts. Because of major differences in genome organization and cytopathology, BYDV serotypes have been divided among luteoviruses and poleroviruses. BYDV-PAV and BYDV-MAV are closely related strains of BYDV, the only official luteovirus; while the former BYDV-RPV is now called cereal yellow dwarf virus-RPV and assigned to the *Polerovirus* genus.

Geographic Distribution

BWYV, PLRV, BYDV and carrot red leaf virus (CRLV) have worldwide distributions. BYDV or cereal yellow dwarf virus (CYDV) have been reported virtually wherever wheat, barley or oats are grown. Tobacco necrotic dwarf virus (TNDV) and SBDV are

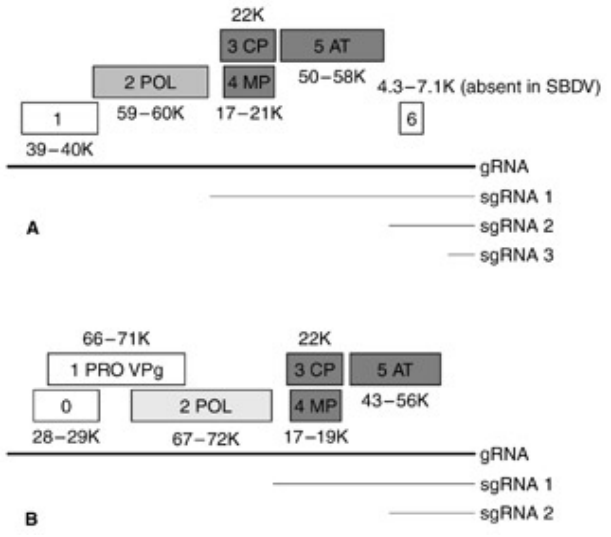


Figure 1 Genome organizations of *Luteoviridae:* (**A**) *Luteovirus*; (**B**) *Polerovirus*. Molecular weights of proteins encoded by each ORF are indicated in kilodaltons (K). Unshaded, no homology to known proteins; checkered, highest homology to diantho- and carmoviruses; light shading, highest homology to sobemoviruses; dark shading, highest homology to other *Luteoviridae*. Functions of proteins are indicated where known: PRO, putative protease; VPg, genome-linked protein; POL, RNA-dependent RNA polymerase; CP, coat protein; AT, aphid transmission; MP, probable movement protein. Positions of subgenomic RNAs (thinner lines) are indicated below genomic RNA (bold line).

limited to Japan. Isolates of SCRLV, which have been considered synonymous to SBDV, occur in Australia and the southern USA, but the American isolate does not infect soybean. Groundnut rosette assistor virus (GRAV) occurs only in Africa.

Host Range

Most luteoviruses have limited host ranges. BWYV stands out with its very wide range of dicotyledonous hosts, although very few isolates actually infect beets. In contrast, the more common beet-infecting luteovirus is beet mild yellowing virus (BMYV). Because PLRV, which is limited to the *Solanaceae*, and BWYV both have the same vector, host range is not determined merely by the host range of the vector. BYDV and CYDV infect most if not all members of the *Graminae*. CRLV is the only luteovirus known to infect the *Umbelliferae*.

Virus Structure and Composition

Luteoviridae particles are icosahedral, $T = 3$, with a diameter of 24–30 nm. They contain 180 subunits of a 22 kDa coat protein. A small, undetermined number of coat protein subunits contain an additional 50–53 kDa polypeptide fused to the C-terminus, produced by translational readthrough of the coat protein gene stop codon. The virion is 28% RNA by weight. It contains a 5.5–6 kb genomic RNA. A genome-linked protein (VPg) with reported molecular weights of either 7 kDa (PLRV) or 17 kDa (CYDV) is attached covalently to the 5′ end of the genomic RNA of poleroviruses. BYDV RNA lacks a VPg or any other known modification. The 3′ end of the genomic RNA contains neither a poly(A) tail nor a tRNA-like structure.

Replication

Genome organization

Luteoviridae have a positive-sense RNA genome encoding 5–6 open reading frames (ORFs) (**Fig. 1**). Different genome organization is a major distinction between the *Luteovirus* and *Polerovirus* genera (**Fig. 1**). Poleroviruses have a gene (ORF 0) at the 5′ end of the genome that encodes a 28–29 kDa protein (P0) which is absent in luteoviruses. Luteoviruses contain an ORF encoding a 4–7 kDa protein (P6) that is absent in poleroviruses. SBDV, while resembling luteoviruses in untranslated sequences, RNA-dependent RNA polymerase (RDRP), and genome organization, lacks ORF 6 and is more homologous to poleroviruses in the structural genes.

Protein functions

The functions of P0 and P6 are unknown. The products of ORFs 0, 3, 4, 5 and 6 are unnecessary for RNA replication in protoplasts, but some of them can increase the accumulation of viral RNA. The product of ORF 2 is expressed only as a C-terminal fusion with ORF 1 (P1–2). ORF 2 encodes the RDRP component of the replicase. P1 of poleroviruses contains the catalytic triad of amino acids conserved among cysteine proteases. Downstream of this region, but upstream of the frameshift site, P1 also contains the VPg amino acid sequence. Thus, the VPg is made available presumably by proteolytic cleavage of P1 and/or P1–2. P3 is the major coat protein (CP). A minor portion of the coat proteins produced in infection consists of a fusion of P3 and P5 made by translational readthrough of the ORF 3 stop codon. P4 is necessary for systemic infection of plants by BYDV, but not by BWYV. Evidence supports a role in cell-to-cell movement for P4 of PLRV: it is associated with plasmodesmata and fragments of this phosphorylatable protein have noncovalent, nonspecific single-stranded nucleic acid binding properties. The P3–5 translational readthrough product is often

found cleaved about midway in the P5 domain. The N-terminal portion of P5 is necessary and sufficient (when fused to coat protein on virions) for aphid transmission. The C-terminal portion of P5 may be involved in virus movement within the plant, at least for BWYV.

Transcription

Although only the genomic RNA is encapsidated, subgenomic (sg) RNAs are produced by RNA-templated transcription in the infected cell. ORFs 3, 4 and 5 are translated from subgenomic mRNA 1 (sgRNA1) (**Fig. 1**). In BYDV, smaller sgRNAs 2 and 3 are also produced. These RNAs may serve a regulatory function. sgRNA2 is predicted to be an efficient message for ORF 6 and functions as such *in vitro*. In cells infected with PLRV or cucurbit aphid-borne yellows virus (CABYV), sgRNA2 may also be a message for small ORFs. Presence of the ST9-associated RNA increases the abundance of sgRNA2 in BWYV-infected cells (below).

All sgRNAs are 3′-coterminal with the genomic RNA and are present in abundance that is roughly inversely proportional to their size. The 5′ termini of sgRNAs and genomic RNA begin with common sequences. In poleroviruses, sobemoviruses and dianthoviruses this sequence is ACAAAA. In luteoviruses the consensus is AGUGAAGA. These sequences or their complements are likely involved in replicase promoters. The ACAAA motif is unusually abundant throughout the genomes of all luteoviruses.

Translation

Luteoviridae employ a plethora of rare translational events to control gene expression. BYDV RNA lacks a 5′ cap (m^7GpppN). A sequence located, surprisingly, in the 3′ end of the genome facilitates efficient cap-independent translation. The polymerase gene (ORF 2) is expressed only via a '−1' ribosomal frameshift that occurs during translation of ORF 1 in the region where the two ORFs overlap. At a low frequency, elongating ribosomes slip back one nucleotide, moving from ORF 1 to ORF 2, resulting in a low abundance fusion protein comprising these two ORFs. Ribosomal frameshifting facilitates synthesis of low levels of polymerase, an enzyme needed in only small quantities, and high levels of its N-terminal region, P1, whose function by itself is unknown. Some of the *cis*-acting signals that mediate this event – a shifty heptanucleotide followed by a highly structured sequence – are similar to those which induce −1 frameshifting in translation of the polymerases of corona viruses and retroviruses. However, in the case of BYDV, sequences located several kilobases downstream in the 3′ untranslated region of the genome, are also necessary for frameshifting. This differs dramatically from other known frameshift signals.

Translation initiates at the start codons of ORFs 3 and 4 by leaky scanning on sgRNA1. This mechanism may also apply to translation of the overlapping ORFs 0 and 1 from genomic RNA of poleroviruses. ORF 5 is expressed by occasional in-frame read-through of the coat protein stop codon, giving rise to the P3–5 fusion protein. A nucleotide tract harboring 8–16 multimeric CCXXXX repeats just downstream of the ORF 3 amber stop codon, as well as a sequence about 750 nt downstream, are necessary for read-through.

Luteoviridae-associated RNAs

A 322 nucleotide satellite RNA (satCYDV RNA, formerly satRPV or sBYDV RNA) was discovered in an Australian isolate of CYDV-RPV. satCYDV RNA replicates by a symmetrical rolling circle mechanism. Hammerhead self-cleavage structures exist in the plus (encapsidated) and minus strands of the RNA. satCYDV RNA reduces CYDV-RPV helper virus levels and ameliorates disease symptoms in infected oat plants. It is not supported by BYDV, but its replication is supported by BWYV in dicotyledonous hosts. Thus, a component provided by poleroviruses is necessary for replication, and the host range of the helper virus may define the satellite's host range.

Additional RNAs have been found associated with a severe isolate of BWYV called ST9 and with CRLV. These 3 kb RNAs encode RDRP sequences that are more similar to luteovirus RDRPs than those of poleroviruses, to which BWYV and CRLV belong. They can replicate independently in protoplasts, but depend on their helper viruses for encapsidation and efficient spread in plants. Both of the associated RNAs enhance accumulation of their helper viruses. This typifies a phenomenon in *Luteoviridae* in which RNAs harboring luteovirus-like and polerovirus-like RDRP genes, enhance each others' accumulation. These cross-enhancing interactions range from mixed infections of BYDV and CYDV viruses, to complete interdependence of two such RNAs in pea enation mosaic virus (PEMV). PEMV, the sole member of the *Enamovirus* genus, consists of two particles. Each PEMV RNA can replicate autonomously in plant cells, but both are required to form a viable agent in the field. PEMV1 has a polerovirus-like RDRP while the PEMV2 RDRP resembles those of luteoviruses and umbraviruses.

Evolution

As discussed above, the 5′ halves of the genomes of the *Luteoviridae* have two separate origins (**Fig. 1**). The RDRP genes of luteoviruses are most similar to the RDRP genes of the umbra- carmo-, tombus- and dianthoviruses. The RDRP genes of poleroviruses are more closely related to those of the sobemoviruses than to those of luteoviruses. In contrast, the genes in the 3′ halves of the genomes of all *Luteoviridae,* are more similar to each other than to genes of any other virus family or genus. Hence, these must be the genes that confer the biological properties that make the *Luteoviridae* a distinct family. Because of the divergent origins of luteoviral genomes it is obvious that *Luteoviridae* do not descend from a common ancestor. Instead, horizontal exchanges of genes must have occurred to generate the two main genera.

Additional divergence between halves of the genome exists within genera. BWYV and BMYV have around 90% amino acid sequence identity in the structural genes, but less in the polymerase genes and only 25% and 34% identity in ORFs 0 and 1, respectively. Yet BMYV and CABYV are 42% and 49% identical in these ORFs but only 63% identical in the CP and 39% identical in the P5 domain. Similarly, Mexican and Californian isolates of CYDV-RPV are 90% identical to the New York CYDV-RPV isolate in structural genes, but show no homology in ORF 0 detectable by northern blot or polymerase chain reaction (PCR).

The sharpest point of divergence in sequence homology both within and between subgroups is at or near the region that separates the RDRP and structural genes. This is also the site of the promoter for sgRNA1. Thus it is possible that recombination occurs by replicase strand-switching at sgRNA promoters.

Comparison of luteovirus sequences with other viruses has revealed striking evolutionary relationships. The genome organization of PEMV1 RNA is similar to that of poleroviruses, except that it lacks ORF 4, and ORF 5 is only 33 kDa. All the ORFs of RNA1 show clear similarity to those of other *Luteoviridae,* which is why it has been placed in this family. In contrast, while ORFs 1 and 2 of PEMV2 RNA resemble those of the luteoviruses, they are most closely related to the umbraviruses which, like PEMV2 RNA, lack structural genes. Thus PEMV2 has been placed in the *Umbravirus* genus. PEMV may have originated as a mixed infection of an umbravirus and a polerovirus, with the former RNA providing a movement protein gene that confers the ability to spread beyond the phloem, allowing mechanical transmission.

Serologic Relationships

Luteoviridae have been compared extensively by ELISA. They can be clustered into the following subsets based on high serological crossreactivity: (1) BYDV-MAV, BYDV-PAV and BYDV-SGV; (2) CYDV-RPV, BYDV-RMV and RGV; (3) SBDV, SCRLV and BLRV; and (4) TNDV, PLRV and TYTV. BWYV reacts at least slightly with antibodies to nearly all *Luteoviridae.* All antibodies to BWYV crossreact with BMYV.

Epidemiology

The spread of *Luteoviridae* depends primarily on the movement of aphids that carry them. The mere presence of a large population of aphids is not sufficient to predict virus spread. The species of aphid and the predominant strain of virus are important factors. For instance, if a large population of *Sitobion avenae* accumulates in an oat field infected only with CYDV-RPV, these aphids are unlikely to spread the virus because *S. avenae* is a poor vector for CYDV-RPV. *Luteoviridae* are spread by aphids that colonize their host, rather than those that exhibit short probing behavior before moving on. The aphid must feed on phloem tissue to acquire and spread the virus. Because the virus must circulate and then accumulate in the accessory salivary gland of the aphid, a latent period of 8–24 h is necessary between the time the virus is acquired and the time it can be transmitted.

Because the virus is persistent, aphids can travel thousands of miles in jet streams over several days, and still transmit the virus. This is important because crops can become infected by a different strain of the virus than is found in the weeds in or near the field. On the other hand, neighboring crops and weeds often serve as reservoirs. Irrigated corn, weeds and nonweedy wild grasses can serve as symptomless hosts of BYDV or CYDV, from which the viruses are transferred to wheat, barley or oat crops by aphids. The timing of aphid infestation is important. A relatively small population of aphids can cause significant damage by infecting a crop when seedlings are young. When older plants are inoculated, the disease causes less damage.

Transmission and Tissue Tropism

Aphid transmission

With the exception of PEMV, *Luteoviridae* are transmitted to plants only via aphid vectors. Protoplasts can be inoculated by electroporation and whole plants have been infected by inoculation with *Agrobacterium* harboring a full-length clone of the BWYV

genome in the Ti plasmid. Thus, the aphid does not supply a factor required for infection, rather its feeding likely serves as a very efficient delivery system. *Luteoviridae* are transmitted in a persistent, circulative manner, i.e. they circulate in the hemocele of the aphid. Once an aphid acquires a luteovirus, that virus can be transmitted throughout the life of the aphid, even after moltings. *Luteoviridae* do not replicate in aphids.

Luteoviridae have highly specialized relationships with their aphid vectors. This allowed differentiation of BYDV/CYDV strains based on their predominant aphid vectors (**Table 1**). Heterologous encapsidation in mixed infections can allow transmission of a genomic RNA of one strain by a vector of another. This phenomenon explains transmission of some virus complexes. CRLV allows the non-*Luteoviridae* carrot mottle umbravirus (CMoV) to be transmitted by *Cavariella aegopodii* in a persistent fashion because some CMoV genomic RNAs are encapsidated in particles containing CRLV coat protein. Similarly, groundnut rosette umbravirus is not transmissible in the absence of groundnut rosette assistor virus (GRAV) which, by itself, causes little damage.

The mechanism of virus transmission and vector specificity has been examined ultrastructurally by Gildow. He showed that the virus is specifically transported across three barriers: the hindgut epithelium, and the basal lamina and plasmalemma of the accessory salivary gland. Particles are then released into the phloem via the salivary duct as the aphid feeds. The particles appear to cross both the hindgut epithelium and the accessory salivary gland plasmalemma via receptor-mediated endocytosis in coated vesicles. *Luteoviridae,* but not other plant viruses, can cross the hindgut into the hemocele even in nonvector aphid species. However, only those virions that can be transmitted by the aphid can cross the plasmalemma of the accessory salivary gland. A highly abundant protein, called symbionin, that is produced by endosymbiotic bacteria (*Buchnera* spp.) in the hemocele, binds the readthrough domain of the virus particle. This protein resembles the chaperonin groEL, and may be involved in maintaining particle stability in the hemolymph.

Tissue tropism

Luteoviridae are generally confined to the phloem, including phloem parenchyma, companion cells and sieve elements. The exception is PEMV which can infect mesophyll cells and spread systemically, allowing it to be transmitted mechanically. The blockage of sieve tubes and degeneration of vascular tissue may account for the disease symptoms, although severity of disease symptoms does not always correlate with the amount of virus accumulating in the plant. Under laboratory conditions, protoplasts derived from mesophyll, epidermis, xylem and undifferentiated tissue can be infected with *Luteoviridae*. Thus, *Luteoviridae* do not require conditions found only in phloem cells, but rather their limitation to the phloem may be a result of their lack of ability to move from cell-to-cell into other tissues. Some evidence indicates that in whole plants, PLRV can be found in low levels in mesophyll cells.

Pathogenicity

Symptomatology

Symptoms caused by *Luteoviridae* are often difficult to identify clearly. In general, *Luteoviridae* cause stunting of plants, yellowing, reddening or 'rolling' of leaves, and an increase in leaf brittleness. Because symptoms may be confined to stunting or a change in leaf color that is not an obvious mosaic or mottle, luteovirus infection can be confused with nutritional deficiencies or other environmental stresses. Environmental factors also affect symptoms. Cool temperatures and high light maximize changes in leaf color. The most common symptom caused by BYDV or CYDV is stunting. As their names imply, they cause yellowing in many lines of barley. They can induce bright reddening of leaf tips in oats, corn and other grasses. This can be confused with the effects of frost. In oats, BYDV causes a marked increase in sterile florets. This, combined with reduced kernel weight caused by BYDV in oats and barley, greatly reduces grain yield. Symptoms can vary between hosts. A BYDV isolate called PAV129 is virtually symptomless in wheat from which it was isolated initially, but it is very severe in oats, including cultivars that are resistant to other isolates. BWYV induces bright yellowing of leaves on sugar beets, lettuce and spinach. In addition to leaf curling, PLRV causes net necrosis, a blackening of the vascular tissue in the tuber. Unlike most plant viruses, TNDV can kill its host in natural infections, as can BYDV under laboratory conditions.

Cytopathology

The cytopathology of *Luteoviridae* has been characterized best for BYDV and BWYV. Cells infected by luteoviruses show: (1) extreme distortion of the nucleus, and aggregation and accumulation of densely staining, heterochromatin-like material; (2) accumulation of new virus particles in the cytoplasm; and (3) single-membraned vesicles containing fibrils in the cytoplasm. Cells infected by poleroviruses show: (1)

relatively normal nuclei at first, until the heterochromatin slowly disintegrates; (2) accumulation of new virus particles around the nucleolus; and (3) cytoplasmic vesicles containing fibrils enclosed in a second membrane.

Prevention and Control

Several strategies have been used to manage *Luteoviridae*. These include: (1) avoidance of aphids through careful monitoring of aphid populations and timing of planting; (2) use of insecticides or parasites to reduce aphid populations; and (3) use of crops that are genetically resistant to *Luteoviridae*. For control of BMYV in England, an equation involving the abundance of vector *Myzus persicae* in the autumn and the number of frost days in the winter is used to predict the arrival of aphids the following spring. If the predicted infestation is high, seeds pretreated with insecticide are recommended. An additional spray warning system is used after aphids have begun to colonize plants. If aphids exceed a threshold, growers are advised to spray insecticides. For BYDV hosts, sowing fall and spring cereals after major aphid migrations will reduce the chances of aphid colonization. However, this strategy reduces the time available for the crop to reach maturity and thus may reduce yield. The use of insecticides to control *Luteoviridae* is cost-effective only in areas of extremely intensive agriculture, such as wheat fields in England and potato fields in the northwestern USA. Parasitic wasps have proved to be effective biological control agents, reducing BYDV spread in South America, but their ability to control aphids is often limited.

A BYDV resistance gene, *Yd2*, from Ethiopian barley has been introduced into some commercial lines of barley. This has greatly reduced losses in California and elsewhere. However, this resistance has broken down in some cases, and is linked to undesirable traits. Tolerance to BYDV in oats is a quantitative trait. Resistance to BYDV has been introduced into wheat from *Thinopyron* species by alien gene introgression. However, the lines obtained to date still carry too many nonagronomic traits from the wild relative to be released as agronomically acceptable seed.

Plants that have been genetically engineered to express the coat protein of PLRV show delayed disease development and reduced symptoms when challenged with PLRV. Highly virus resistant Russet Burbank potatoes and oats have been obtained by transformation with the polymerase genes (ORFs 1 and 2) of PLRV and BYDV, respectively. Owing to the recombinogenic nature of the *Luteoviridae* genome on an evolutionary time scale, and the potential synergistic interactions between genomes, forethought must be used in designing and deploying *Luteoviridae* genes as resistance transgenes.

Economic Significance

The *Luteoviridae* family is one of the more economically significant plant virus families because BYDV, CYDV, PLRV and BWYV are ubiquitous, and cause significant losses in major crops. Because they often occur every year and are often not diagnosed, it is impossible to put an accurate dollar value on the losses due to *Luteoviridae*. Natural BYDV infection can cause average annual losses of 11–33%, and in some areas up to 86%. If BYDV caused 5% losses in 1989, it would have been valued at $US387 million for wheat, $US48 million for barley and $US28 million for oats. BYDV usually causes more obvious damage to oats and barley than to wheat. But due to the extensiveness of wheat production, the reduction in yield caused by BYDV, however slight, probably adds up to a significant monetary loss. The losses to BYDV in wheat are probably greater in North Africa, China and in tropical regions.

Infection of sugar beet with BMYV at an early stage in plant development can decrease root yield by 30%. Losses valued as high as £13 000 000 occur in BMYV epidemics in the UK. BWYV caused increasingly severe damage to sugar beets in California between 1951 and 1968. Beginning in 1968, the use of more resistant varieties of sugar beets and phytosanitary cropping practices greatly reduced the incidence of BWYV infection. The ensuing increase in yield, credited largely to the reduction in BWYV incidence, was estimated at $US167 million for a 5 year period in the state of California alone. Artificial inoculation of oilseed rape with BWYV resulted in 25% yield loss. BWYV has also been reported to cause significant losses in spinach, lettuce, turnips, cabbage, many brassicas, pea and flax at various locations around the world.

Groundnut rosette disease, caused by the complex of groundnut rosette virus and GRAV, is one of the most severe limitations to peanut production in Africa. The net necrosis symptom of PLRV makes potatoes unmarketable. Thus, yield loss in infected plants can be 100%. The value of the losses is so high that growers are anticipated to be willing to pay a premium for commercially produced transgenic potatoes with engineered resistance to PLRV. This, combined with engineered resistance to other viruses and insects in this potato variety, should reduce the need to apply high levels of insecticides that are

currently used to control the aphid vector of PLRV and other insect pests.

See also: **Dianthoviruses (*Tombusviridae*); Pea enation mosaic virus (*Luteoviridae*); Satellite RNAs and Satellite viruses; Sobemoviruses; Synergism: Plant viruses; Umbraviruses; Vectors: Plant viruses.**

Acknowledgement

The author thanks Mark Stevens, Randy Beckett and Mike Mayo for contributing valuable information to this chapter.

Further Reading

D'Arcy CJ and Burnett PA (eds) (1995) *Barley Yellow Dwarf: 40 Years of Progress*. St Paul: APS Press.

D'Arcy CJ and Mayo M (1997) Proposals for changes in luteovirus taxonomy and nomenclature. *Arch. Virol.* 142: 1285.

Mayo MA and Ziegler-Graf V (1996) Molecular biology of luteoviruses. *Adv. Virus Res.* 46: 413.

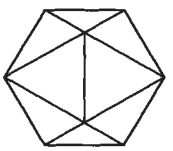
Miller WA, Dinesh-Kumar SP and Paul CP (1995) Luteovirus gene expression. *Crit. Rev. Plant Sci.* 14: 179.

Miller WA, Brown CM and Wang S (1997) New punctuation for the genetic code: luteovirus gene expression. *Semin. Virol.* 8: 3–13.

D'Arcy CJ, Domeier LL, Mayo MM (1999) Luteoviridae. In: Fauquet et al? (eds) *Virus Taxonomy: Seventh Report of the International Committee on Taxonomy of Viruses*. San Diego: Academic Press. In press.

Smith HG, Barker H and Jones P (eds) (1998) *Luteoviruses*. Wallingford: CAB International.

LYMPHOCYSTIS DISEASE VIRUS (*IRIDOVIRIDAE*)



Christian A Tidona and **Gholamreza Darai**, Institute for Medical Virology, University of Heidelberg, Heidelberg, Germany

History

Lymphocystis disease virus (LCDV) is the causative agent of lymphocystis disease (LD), which has been reported to occur in over 100 different fish species in fresh water and salt water worldwide. These include species that are of particular importance for fisheries and fish farming. LCDV infection frequently appears in *Pleuronectidae* (flatfish), such as *Platichthys flesus* (flounder), *Pleuronectes platessa* (plaice), and *Limanda limanda* (dab). LD of fish was first described in 1874 by Lowe. The term lymphocystis cell was used when Woodcock (1904) considered it a life stage of a parasite infecting flatfish. The viral aetiology was proposed by Weissenberg in 1914. The first electron microscopic observation of the virus was made in 1962 by Walker.

Taxonomy and Classification

LCDV is classified in a separate genus (*Lymphocystivirus*) within the family *Iridoviridae*. Iridoviruses are relatively large icosahedral cytoplasmic deoxyriboviruses that have been isolated from insects and vertebrates. LCDV type 1 (LCDV-1) occurs in flounder and plaice; LCDV type 2 (LCDV-2) was isolated from dab.

Properties of the Virion

The nonenveloped LCDV particles accumulate in the cytoplasm of the infected lymphocystis cells (**Fig. 1A**). The virions are characterized by an icosahedral morphology with diameters of 227.5 ± 12.5 nm (flounder; **Fig. 1B**), 198.8 ± 12.9 nm (plaice), and 200.5 ± 12 nm (dab) respectively. The outer capsid of the virion is separated from the electron-dense concentric core by an intermediate lipid layer (**Fig. 1C, D**).

Properties of the Virus Genome

The LCDV particles contain a single linear double-stranded DNA molecule that has been characterized

currently used to control the aphid vector of PLRV and other insect pests.

See also: **Dianthoviruses (*Tombusviridae*); Pea enation mosaic virus (*Luteoviridae*); Satellite RNAs and Satellite viruses; Sobemoviruses; Synergism: Plant viruses; Umbraviruses; Vectors: Plant viruses.**

Acknowledgement

The author thanks Mark Stevens, Randy Beckett and Mike Mayo for contributing valuable information to this chapter.

Further Reading

D'Arcy CJ and Burnett PA (eds) (1995) *Barley Yellow Dwarf: 40 Years of Progress*. St Paul: APS Press.

D'Arcy CJ and Mayo M (1997) Proposals for changes in luteovirus taxonomy and nomenclature. *Arch. Virol.* 142: 1285.

Mayo MA and Ziegler-Graf V (1996) Molecular biology of luteoviruses. *Adv. Virus Res.* 46: 413.

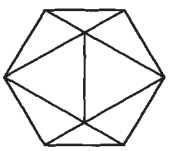
Miller WA, Dinesh-Kumar SP and Paul CP (1995) Luteovirus gene expression. *Crit. Rev. Plant Sci.* 14: 179.

Miller WA, Brown CM and Wang S (1997) New punctuation for the genetic code: luteovirus gene expression. *Semin. Virol.* 8: 3–13.

D'Arcy CJ, Domeier LL, Mayo MM (1999) Luteoviridae. In: Fauquet et al? (eds) *Virus Taxonomy: Seventh Report of the International Committee on Taxonomy of Viruses*. San Diego: Academic Press. In press.

Smith HG, Barker H and Jones P (eds) (1998) *Luteoviruses*. Wallingford: CAB International.

LYMPHOCYSTIS DISEASE VIRUS (*IRIDOVIRIDAE*)



Christian A Tidona and **Gholamreza Darai**, Institute for Medical Virology, University of Heidelberg, Heidelberg, Germany

History

Lymphocystis disease virus (LCDV) is the causative agent of lymphocystis disease (LD), which has been reported to occur in over 100 different fish species in fresh water and salt water worldwide. These include species that are of particular importance for fisheries and fish farming. LCDV infection frequently appears in *Pleuronectidae* (flatfish), such as *Platichthys flesus* (flounder), *Pleuronectes platessa* (plaice), and *Limanda limanda* (dab). LD of fish was first described in 1874 by Lowe. The term lymphocystis cell was used when Woodcock (1904) considered it a life stage of a parasite infecting flatfish. The viral aetiology was proposed by Weissenberg in 1914. The first electron microscopic observation of the virus was made in 1962 by Walker.

Taxonomy and Classification

LCDV is classified in a separate genus (*Lymphocystivirus*) within the family *Iridoviridae*. Iridoviruses are relatively large icosahedral cytoplasmic deoxyriboviruses that have been isolated from insects and vertebrates. LCDV type 1 (LCDV-1) occurs in flounder and plaice; LCDV type 2 (LCDV-2) was isolated from dab.

Properties of the Virion

The nonenveloped LCDV particles accumulate in the cytoplasm of the infected lymphocystis cells (**Fig. 1A**). The virions are characterized by an icosahedral morphology with diameters of 227.5 ± 12.5 nm (flounder; **Fig. 1B**), 198.8 ± 12.9 nm (plaice), and 200.5 ± 12 nm (dab) respectively. The outer capsid of the virion is separated from the electron-dense concentric core by an intermediate lipid layer (**Fig. 1C, D**).

Properties of the Virus Genome

The LCDV particles contain a single linear double-stranded DNA molecule that has been characterized

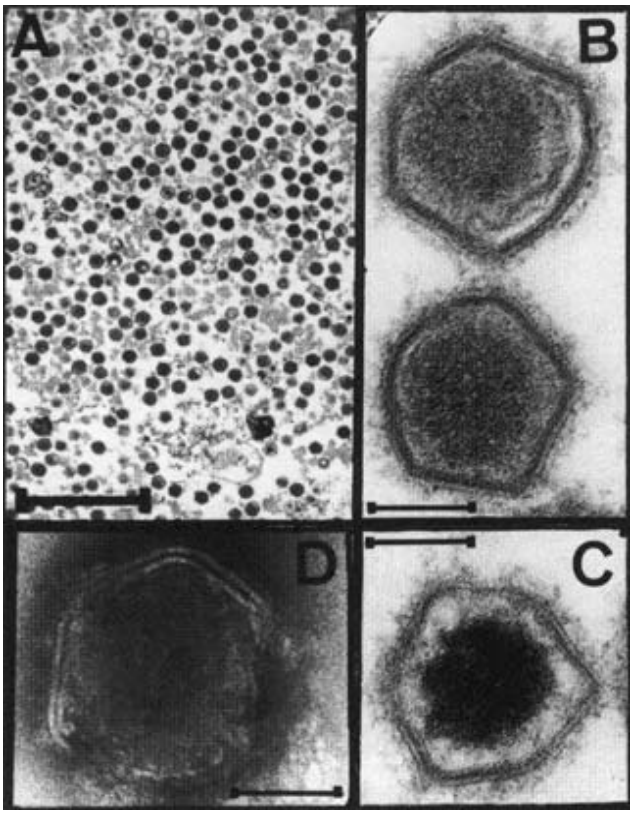


Figure 1 Electron micrographs of lymphocystis disease virus particles. (**A**) Ultrathin section of the cytoplasm of a lymphocystis cell exemplifying the huge amount of virus particles present in infected cells (bar = 1 μm). (**B**) The fine structure of the intact virion is best revealed in ultrathin sections (bar = 100 nm). (**C**) The concentric electron-dense core inside the virion is often separated from the capsid by an electron-translucent region (bar = 100 nm). (**D**) Negative staining of an LCDV virion. Due to osmotic shock the capsid of the virion is broken and partly dissociated into two layers, while the core seems to remain compact and stable (bar = 100 nm).

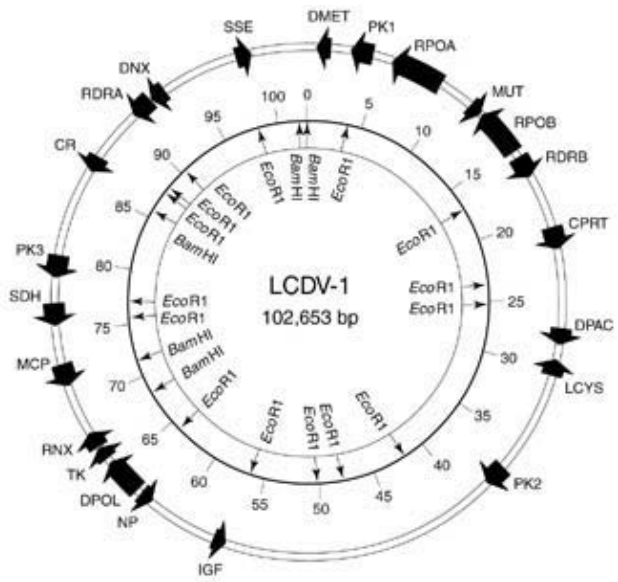


Figure 2 Physical map of the LCDV-1 genome. Inner circle: the arrows indicate target sites for the restriction endonucleases *Eco*RI and *Bam*HI and the figures indicate nucleotide positions given in kilobase pairs (kb). Outer circle: the arrows represent putative genes identified by significant amino acid sequence homology to well-characterized proteins of other species. Abbreviations of putative genes: DMET, DNA methyltransferase; PK1,-2,-3, protein kinases 1–3; RPOA/B, subunits of the DNA-dependent RNA polymerase; MUT, mutT protein homologue; RDRA/B, subunits of the ribonucleoside diphosphate reductase; CPRT, cysteine proteinase; DPAC, putative DNA packaging factor; LCYS, collagen type IX homologue; IGF, insulin-like growth factor homologue; NP, putative nucleoprotein; DPOL, DNA polymerase; TK, thymidine kinase; RNX, putative ribonuclease; MCP, major capsid protein; SDH, steroid dehydrogenase; CR, tumor necrosis factor receptor homologue; DNX, putative restriction endonuclease; SSE, structure-specific endonuclease.

by molecular cloning, physical mapping and nucleotide sequence analysis. The structure of the genome of LCDV was found to be circularly permuted and terminally redundant. LCDV DNA is sensitive to lambda 5′-exonuclease and to *Escherichia coli* 3′-exonuclease III without preference for any specific terminal DNA restriction fragment, indicating the circular permutation of the viral genome. Isolated LCDV DNA molecules appear to be heterogeneous in size and the average terminal redundancy was found to be approximately 50%. Because of the genomic features, the physical map of the viral genome is circular (**Fig. 2**).

The genome of LCDV-1 is 102 653 bp in length and contains 110 largely nonoverlapping putative viral genes. The base composition of the genomic DNA of LCDV-1 was found to be 29.1% G+C and 70.9% A+T.

A characteristic genomic property common to LCDV and frog virus 3 (FV3), which is another vertebrate iridovirus, is the high degree of methylation at cytosine residues. Comparison of cleavage patterns of LCDV DNA after treatment with the restriction endonucleases *Msp*I and *Hpa*II indicated that CCGG sequences are completely methylated at the inner C. The content and distribution of the 5-methylcytosine in the LCDV-1 DNA was determined by high-performance liquid chromatography (HPLC) and nearest neighbor analysis. These studies revealed that 22% of all C residues are methylated, including 74% in CpG, 1% in CpC and 2–5% in CpA dinucleotide sequences.

Replication of the virus genome and assembly into virus particles occurs exclusively in the cytoplasm of the infected cell.

Viral Proteins

LCDV virions contain at least 33 polypeptides with apparent molecular weights ranging from 14 to 220 kDa. LCDV polypeptide patterns are similar when virus isolates from different host species (e.g. flounder and plaice) are compared. However, the polypeptide patterns of the two LCDV strains show significant differences when compared with each other. The predominant structural component of the virus particles, comprising 40–45% of the total particle polypeptide, was shown to be the major capsid protein (MCP) with a molecular weight of about 50 kDa.

Viral Enzymes

Enzymatic activities such as protein kinase, ATPase, RNase, DNase, thymidine kinase and protein phosphatase have been reported to be associated with purified LCDV particles. Putative LCDV-1 gene products with enzymatic activity were identified by amino acid sequence homology analysis. These include the two major subunits of the viral DNA-dependent RNA polymerase, DNA polymerase, at least three different protein kinases, two subunits of the ribonucleoside diphosphate reductase, thymidine kinase and DNA methyltransferase (**Fig. 2**).

Geographic and Seasonal Distribution

LCDV is geographically distributed worldwide. LD epidemics frequently occur in wild flatfish in the North Sea as well as in sea bream (*Sparus aurata*) aquaculture in the Mediterranean and the Red Sea. A high rate (14–17%) of incidence of the disease was observed by Möller, in 1979, in the Gullmarsfjord of the Swedish West coast and in the Irish Sea. Similarly high rates of incidence have been documented in areas of the North Sea such as Öresund and Kattegat. LD epidemics in different fish species have also been reported from the Eastern USA, Florida and Bahamas, South America, Trinidad, Hawaii, South Pacific, Indochina and Singapore.

The occurrence of LD seems to be seasonally dependent. In the North Sea, for example, a significant increase of LD in fish can be observed during summer and autumn. However, other factors such as pollution, stress and changes in salt concentration may increase the susceptibility of fish to LCDV infection.

Host Range and Viral Propagation

The only well-characterized members of the genus *Lymphocystivirus* have been isolated from flatfish, predominantly *Pleuronectes platessa* (plaice), *Platichthys flesus* (flounder), and *Limanda limanda* (dab). LCDV can be experimentally propagated *in vivo* after subdermal inoculation of *Lepomis macrochirus* (bluegill).

Since the discovery of LD in 1874 by Lowe, many attempts have been made to propagate LCDV *in vitro*, with limited success. For example, the LCDV strain Leetown (ATCC VR-342) can be propagated at 21°C in BF-2 cell cultures (ATCC CCL 91) derived from bluegill fry. However, the titer yield at 21–28 days postinfection is usually very low.

Evolution

The amino acid sequence of the MCP was shown to be a suitable target for the study of iridovirus evolution because it contains highly conserved domains but is sufficiently diverse to distinguish closely related iridovirus isolates. Based on the amino acid sequence of the MCP, the iridoviruses were found to be phylogenetically at least distantly related to the family *Phycodnaviridae* and African swine fever virus. Within the iridovirus family the different genera infecting insect and vertebrate hosts appear to have diverged at the basis of iridovirus evolution.

Transmission and Tissue Tropism

The route of transmission of LCDV is not known. LCDV infection usually occurs within the connective tissues of fins and skin. The enhanced susceptibility of fish to LCDV infection in high-density aquaculture suggests that LCDV may be transmitted by direct contact between diseased and healthy individuals. The involvement of ectoparasite vectors has also been postulated.

Clinical Features of Infection

LD rarely causes death; the major impact on the infected animals is the production of unsightly raspberry-like external lesions which appear as disseminated white to grey nodules on fins and skin. There have been reports of ocular lesions and involvement of gills and internal organs associated with a much higher mortality rate. The disease does not usually elicit an inflammatory response. Often the infections are self-limiting and disappear spontaneously.

Pathology and Histopathology

The infected animals develop clusters of extremely hypertrophied fibroblasts or osteoblasts, called lymphocystis cells. Individual cells may undergo a 50 000–

100 000-fold increase in size and reach a diameter of up to 2 mm. Infected cells are characterized by an enlarged nucleus, basophilic cytoplasmic inclusions and a hyaline capsule surrounding the individual cells. As the infection progresses and the virus replicates, the nucleus becomes vacuolated and undergoes necrosis while the virus progeny accumulates in the cytoplasm and the capsule thickens.

Future Perspectives

The major obstacle in the analysis of the molecular mechanisms underlying LCDV infection, replication and pathogenesis is the lack of an efficient cell culture system for virus propagation. However, the knowledge of the complete primary structure of the viral genome now allows the *in vitro* expression and functional analysis of individual viral gene products.

See also: **Frog virus 3 (*Iridoviridae*); *Iridoviridae* – invertebrate.**

Further Reading

Darai G (ed.) (1990) *Molecular Biology of Iridoviruses*. Boston: Kluwer.

Tidona CA and Darai G (1997) The complete DNA sequence of lymphocystis disease virus. *Virology* 230: 207.

Tidona CA, Schnitzler P, Kehm R and Darai G (1998) Is the major capsid protein of iridoviruses a suitable target for the study of viral evolution? *Virus Genes* (Suppl.) 16: 59.

LYMPHOPROLIFERATIVE DISEASE VIRUS OF TURKEYS (*RETROVIRIDAE*)

Arnona Gazit and **Abraham Yaniv**, Department of Human Microbiology, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv, Israel

History

The lymphoproliferative disease (LPD) of turkeys is a naturally occurring lymphotumoral disorder characterized by enlargement of the spleen, thymus and liver, with focal lesions in most organs. The disease was first recognized in the UK, by Biggs *et al* in 1974, as a new disease entity distinct from reticuloendotheliosis (RE), which is also a leukotic condition in turkeys. Subsequently, sporadic outbreaks of LPD were also reported to occur in other European countries and in Israel. In 1978, a retrovirus, designated lymphoproliferative disease virus (LPDV), was isolated from afflicted turkeys.

Etiology

A retroviral etiology for LPD was first suspected based on the finding of C-type particles in plasma pellets and in tissues and lesions of birds with natural or experimentally induced disease, as well as the demonstration that preparations of these materials produced the disease when inoculated into poults. Further evidence for a retroviral etiology was based on the presence of budding C-type particles in tissue sections of LPD tumors and on molecular evidence showing that LPDV information is present and expressed in LPD tumor cells. Transfection of the cloned LPDV provirus into turkey lymphocytes and their injection into autologous turkeys resulted in the induction of LPD symptoms. This indicated that LPDV is a replication-competent virus, capable of inducing the disease in the absence of a defective oncogene-containing retroviral counterpart.

General Features and Classification

The mature LPDV viral particles measure 90–120 nm in diameter and have an electron-dense core with a less dense intermediate layer bounded by an outer envelope. As with a typical retrovirus, LPDV virions possess a buoyant density of 1.17 g ml^{-1} in sucrose gradients. They contain 35S plus-strand viral RNA and an RNA-dependent DNA polymerase (RT) that is preferentially activated by magnesium ions. The virion structural proteins are the two envelope glycoproteins of 76 and 41 kDa, the matrix (MA) protein of 20 kDa and several core proteins, including the capsid (CA) protein of 28 kDa, a core protein of 31 kDa, the nucleocapsid (NC) protein and the protease (PR) of 13–15 kDa each.

liferative disease virus of turkeys represents a distinct class of avian type-C retrovirus. *Gene* 122: 349.

Sarid R, Chajut A, Gak E *et al* (1994) Genome organization of a biologically active molecular clone of the lymphoproliferative disease virus of turkeys. *J. Virol.* 204: 680.

Yaniv A, Sarid R, Chajut A *et al* (1995) The lymphoproliferative disease virus (LPDV) of turkeys. *Isr. J. Vet. Med.* 50: 87.

LYMPHOCYTIC CHORIOMENINGITIS VIRUS (*ARENAVIRIDAE*)

Contents

General Features

Raymond M. Welsh, Department of Pathology, University of Massachusetts Medical Center, Worcester, Massachusetts, USA

History

Lymphocytic choriomeningitis virus (LCMV) is an etiological agent for human 'acute aseptic meningitis' and 'grippe-like' infections and is maintained in nature by life-long persistent infections of mice (*Mus musculus*). Three strains still being studied today were initially isolated in North America in the 1930s. During an attempt to recover and passage virus from a suspected human case of St Louis encephalitis, C. Armstrong and R. Lillie isolated LCMV (Armstrong strain) from a monkey that developed a lymphocytic choriomeningitis. E. Traub next reported the isolation of a serologically indistinguishable virus contaminating his mouse colony (Traub strain), and T. Rivers and T. Scott isolated similar viruses from human meningitis patients (one being the WE strain). Subsequently, many other isolations from humans and animals were made, and a clear cause-and-effect relationship between LCMV and about 8% of the North American cases of human nonbacterial meningitis was established.

The LCMV infection of the mouse soon became an important model system for studying viral immunology. A distinguishing feature of the LCMV infection was that it established a long-term persistent infection in mice infected *in utero* or shortly after birth, whereas adult mice inoculated with LCMV either cleared the virus with lasting immunity or, in the case of an intracranial infection, died of a lethal meningoencephalitis. Antiviral antibody was difficult to detect in the persistently infected mice, and this led Burnet and Fenner to postulate that exposure to viral antigen before the maturation of the immune system resulted in mice becoming immunologically tolerant to LCMV and thus unable to clear the infection. It was this system that provided the basis for Burnet's theories of immunological tolerance that have stood the test of time. Subsequent work by Oldstone and Dixon, however, demonstrated that persistently infected mice did make antiviral antibody which was difficult to detect because of excess viral antigen. The antiviral antibody traveled in the circulation complexed to virus and complement, and these circulating as well as tissue-bound 'immune complexes' contributed to a progressive degenerative disease involving glomerulonephritis, arteritis, and chronic inflammatory lesions. In the acute infection both the clearance of the virus and the lethal meningoencephalitis were shown to be due mostly to the cytotoxic T lymphocyte (CTL) response. Antiviral CTL were first demonstrated in the LCMV model, and Zinkernagel and Doherty used this model to demonstrate the important concept of major histocompatibility complex (MHC)-restriction in CTL recognition. Persistently infected mice were eventually shown to have 'split-tolerance' to LCMV, in that although they could mount an antibody response to LCMV they could not generate the LCMV-specific CTLs which were needed to clear the infection.

Classification

LCMV is the prototype virus of the genus *Arenavirus* in the *Arenaviridae* family of RNA viruses, and although it has some homology with all arenaviruses,

it is most closely related to the African virus Lassa and is classified as an Old World arenavirus. Its ambisense genome consists of two single-stranded RNAs each encoding two genes of opposite polarity and separated by a double-stranded hairpin structure. The small S RNA (1.1×10^3 kDa) in the virion encodes in the negative-sense a 63 kDa ribonucleoprotein (NP) and in the positive- or message-sense a 75 kDa cell-associated glycoprotein (GPC), which is cleaved into two virion glycoproteins (GP1 (44 kDa) and GP2 (35 kDa)). The large L RNA (2.1×10^3 kDa) in the virion encodes in the negative-sense a 200 kDa RNA-dependent RNA polymerase (L) and in the positive-sense a smaller 10 kDa zinc-binding protein (Z) which binds to the ribonucleoprotein complex. LCMV replicates in the cytoplasm and buds from the plasma membrane, incorporating host lipids into the viral membrane. It is pleomorphic, with sizes reported from 50 to 300 nm. Some of the virions, which may or may not be infectious, contain ribosome-like structures, giving the virion the characteristic arenavirus appearance.

Geographic and Seasonal Distribution

LCMV infections of mice and man have been well established in Europe and in North and South America, but they are not well documented elsewhere. LCMV is considered an Old World arenavirus brought to the Americas by its host, *Mus musculus*. There is some evidence that human infections occur more commonly in the winter and spring.

Host Range and Virus Propagation

The natural host for LCMV is the mouse, but it can be transmitted to humans, hamsters, guinea pigs, rats, dogs, swine, monkeys, chimpanzees and chick embryos. It commonly causes long-term persistent infections in mice and hamsters, and this has provided a source for human infections. The receptor for LCMV is not yet clearly identified, but it is likely to be ubiquitous, as LCMV grows in a wide variety of tissues *in vivo* and in most tested cells in culture. For instance, LCMV has been propagated and plaque assayed in 3T3, baby hamster kidney (BHK), Detroit-98, HeLa, JLSV-9, L-929, MDBK, MDCK, Vero, and vole cell lines. Vero, L-929, and BHK cells are most commonly used for plaque assays and BHK cells have been the cell of choice for most biochemical analyses because of the relatively high yields of virus (ca 10^8 PFU ml^{-1}) and the lack of secreted endogenous retrovirus contaminants. LCMV also grows in lymphocytes and macrophages, and the latter can be used as stimulator or target cells in T cell proliferation or cytotoxicity assays, respectively.

Genetics

Like other single-stranded RNA viruses, LCMV mutates frequently, and these mutants vary in their tropism and disease-producing potential. A single passage of a cloned LCMV variant into mice will soon segregate into clear neurotropic and turbid viscerotropic plaque variants, which can be recovered from the brain and spleen, respectively. A single amino acid change in the LCMV glycoprotein (residue 260) can convert the immunostimulatory Armstrong strain of LCMV into an immunosuppressive (clone 13) variant, and these genotypes rapidly intraconvert during *in vivo* passage. Several strains of LCMV have been sequenced at least in part, and the highest level of sequence homology is at the 3′ termini of the S and L virion RNA. These are presumed polymerase binding sites which are well conserved throughout the arenavirus family. Different arenaviruses crossinterfere via a defective interfering virus mechanism. The preservation of these polymerase binding sites may allow for this heterotypic interference. The NP and GP of the Armstrong and WE strains share 90% amino acid homology.

The presence of two virion RNAs allows for high frequency 'recombination' due to reassortment of viral genomes. The technique of generating reassortants has led to the assignment of virus-encoded proteins to the appropriate RNA and has facilitated the mapping of genes required for disease-producing potential. The ease of producing reassortants in the laboratory suggests that they also occur in nature and probably play roles in enhancing the genetic diversity of arenaviruses.

Evolution

Arenaviruses do not show substantial sequence homology with any other virus group, but the homology within members of this family suggests a common origin for all arenaviruses. LCMV is most closely related to Lassa virus, which, like LCMV, has its origins in the Eastern hemisphere. Each arenavirus favors a specific rodent host, and selective evolutional pressure on these viruses must have been conferred by their adaption to their respective hosts in forms that established persistent infections. Of interest is that LCMV and other arenaviruses undergo rapid evolution as they form persistent infections. These persistent infections *in vitro* and *in vivo* result in the extensive production of defective interfering virus and of attenuated relatively noncytopathic plaque variants

which may help in the maintenance of persistent infections by preventing virus-induced cell death.

Serological Relationships and Variability

Antisera to Lassa virus and to all members of the Tacaribe complex crossreact to some extent with LCMV by complement fixation and immunofluorescence assays but not at the level of viral neutralization. Few monoclonal antibodies show crossreactivity between LCMV and other Tacaribe complex viruses, but several crossreact with the more closely related Lassa virus. Protective immunity between LCMV and Lassa virus in guinea pigs is reciprocal and may reflect crossreactive T cell responses. Different strains of LCMV are not easily distinguishable by antisera but can be distinguished by some monoclonal antibodies.

Epidemiology

Human LCMV infections occur without sexual bias and at all ages, but most frequently in the 20- to 30-year age group. A longitudinal study in the United States from 1941 to 1958 implicated LCMV infections in about 8% of patients diagnosed with suspected viral meningitis, and serological studies have suggested an incidence of LCMV infection in 5–15% in the general population. Most of these infections are probably mild or subclinical. Laboratory infections with LCMV are relatively common, and several cases have been in laboratories working with the WE strain, which was reisolated from one laboratory worker and identified serologically with monoclonal antibodies.

Transmission and Tissue Tropism

LCMV has been experimentally transferred to humans by intramuscular injection, but the normal route of infection is probably via the respiratory tract after exposure to mouse secretions. LCMV is shed at high titer in mouse feces and urine and is probably not transmitted by arthropod vectors. Another source of infection is the Syrian hamster (*Mesocricetus auratus*), which, like the mouse, can harbor a long-term persistent infection. Several cases of LCMV in different geographic areas have been linked to a colony of persistently infected hamsters distributed throughout the United States. Horizontal person-to-person transmission is rare, but LCMV can cross the placenta and infect the fetus.

Pathogenicity

LCMV strains appear to differ in their pathogenicity in humans, but conclusive analyses of strain virulence differences in humans have not been done. Several human infections have occurred in laboratories working with the WE strain, and the WE strain has been reisolated from a laboratory worker with meningitis and clearly identified. There appear to be fewer anecdotal reports of human infection with the Armstrong strain, but one confirmed laboratory infection did occur in 1995. The WE strain is much more virulent than the Armstrong strain in hamsters and guinea pigs, and reassortant analyses have mapped the ability to cause lethal infections in guinea pigs to the L RNA of the WE strain. The United States Center for Disease Control recommends Biosafety Level 2 practices for most studies with LCMV in mice but Biosafety Level 3 practices for work with hamsters. This is based on the presumption that LCMV becomes more virulent for humans as it passes through hamsters. Although this has not been formally proven, this precaution would appear necessary due to the high number of clinical infections in individuals exposed to persistently infected hamsters.

LCMV has been used in a number of pathogenesis studies in mice and is dependent on age of host, route of infection and virus dose (**Fig. 1**). Viral strain variant differences exist in the encephalitis model, in which 'docile' or viscerotropic variants fail to kill mice, whereas 'aggressive' or more exclusively neurotropic variants do. However, a docile variant for one strain of mouse may be aggressive to another strain of mouse, and the susceptibility of mice to these variants seems to be linked to both major histocompatibility complex (MHC)- and non-MHC genes. One reason for 'docility' is the immunosuppressive nature of some LCMV strains, particularly when inoculated into mice at high dose. Lethal encephalitis does not occur when the T cell response is severely compromised. Immunosuppressive variants of LCMV tend to replicate to very high levels in the visceral organs and may mediate immunosuppression by replicating in leukocytes or simply by inducing a clonal exhaustion of T cells. T cells activated during the LCMV infection become very susceptible to apoptosis on T cell receptor signalling (activation-induced cell death). This is the mechanism for a virus-induced transient immune deficiency associated with the failure of virus-activated T cells to proliferate in response to mitogenic signals and may also be a mechanism for clonal exhaustion, which occurs during a presumed overwhelming T cell receptor stimulation under the conditions of high antigen load.

One noteworthy pathogenic feature of LCMV is its ability to cause a loss in cellular specialized or 'luxury' functions required not for cell survival but for homeostasis of the whole organism. Persistent LCMV infection results in reduced neurotransmitter enzyme activity in cultured neuroblastoma cells *in vitro* and

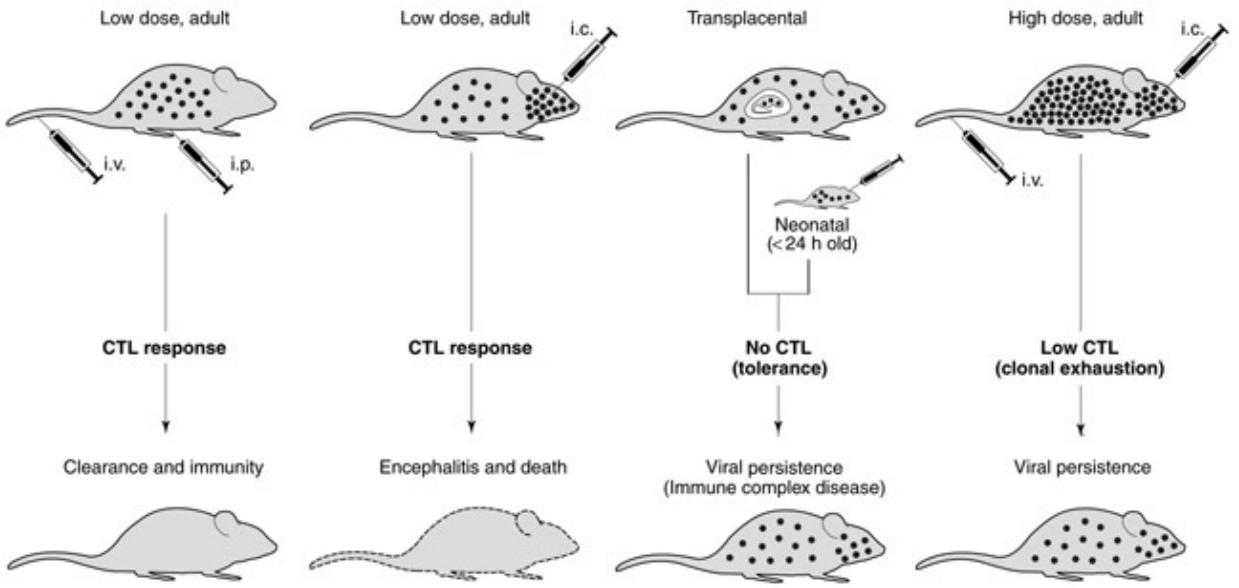


Figure 1 Pathogenesis of the LCMV infection is dependent on the age of the host, the route of infection and the dose of the virus.

reduced levels of growth and thyroid hormones in mice. Reduced growth hormone synthesis is associated with a runting syndrome in young infected mice. LCMV infects *in vivo* the cells which produce growth hormone and thyroid hormone and causes significant reductions in levels of mRNA encoding these hormones but not other hormones and proteins such as thyroid stimulating hormone, actin, and collagen.

Clinical Features of Infection

The LCMV infection of humans can be an inapparent or subclinical infection, or it can present as a non-meningeal grippe-like ailment, an aseptic meningitis, or a more severe meningoencephalitis. The incubation period is 1–2 weeks or longer, and the disease may come in two or three waves. The grippal type is characterized by fever, malaise, lethargy, weakness, myalgia, arthralgia, headache, photophobia, anorexia and nausea. Some patients develop a rash, arthritis, parotitis or orchitis. The grippal type is likely the most common form of disease. The meningeal type is often preceded by the grippal type and presents with stiff neck, vomiting, irritability and Brudzinski's and Kernig's signs. The meningoencephalomyelitic form, which is relatively rare, is associated with confusion, hallucinations, papilledema and weakness progressing to paralysis. Patients usually recover without lasting sequelae. Death is very rare, and there have been only nine fatal cases documented since 1942. There are also some reports of human transplacental infection, resulting in fetal abortion or malformation.

Pathology and Histopathology

Virus can be recovered from the blood, cerebrospinal fluid, urine, and nasopharyngeal secretions during the human LCMV disease. Leukopenia is a common feature of the infection, but there is a pleocytosis in the cerebral spinal fluid in the meningeal stages, and histological analyses of diseased brain tissue in lethal cases of LCMV have revealed meningeal perivascular inflammation and many lymphocytes and monocytes in the arachnoid. This is consistent with studies in the mouse which have demonstrated a lymphocytic infiltration of the meninges. Extensive studies in the mouse model of LCMV-induced acute meningoencephalitis have indicated that virus-specific MHC class 1-restricted CD8+ CTL are the major mediators of the lethal disease, and this may also be the mechanism for the human disease. The mouse model has also shown that antibody–virus–complement immune complexes can be pathological entities, and these could be involved in some of the human arthritic symptoms and the rash, when present.

Immune Response

Human cases of LCMV are characterized by a rise in antibody titer after infection, followed by lasting immunity. Little work has been done on human cellular immunity to infection. However, the LCMV

infection of the mouse has provided the most extensive analyses of the cellular immune response to any virus disease. The infection is characterized by an early stage 1–5 days postinfection and a later stage 6–10 days postinfection. The early stage involves the log-phase replication of the virus and a variety of antigen-nonspecific responses including the liberation of interferon α (IFN-α), IFN-β, tumor necrosis factor α (TNF-α), the activation and proliferation of NK cells and a depression in bone marrow hematopoiesis. The IFN and probably some of the TNF is directly and rapidly induced in cells by the LCMV infection. IFN and possibly other cytokines stimulate the activation and proliferation of NK cells, which substantially increase in number and accumulate in virus-infected tissue. The depressed bone marrow function, which is likely due to the effects of inhibitory cytokines such as IFN and to the ability of activated natural killer (NK) cells to lyse or suppress hematopoietic precursor cells, may be the cause of the leukopenia. Genetic or antibody-mediated depletion of IFN-α/β function enhances the synthesis of LCMV, but antibodies to NK cells do not alter the course of the infection. During this early stage there is a pronounced IFN-induced increase in class 1 MHC antigen expression throughout the body, an increase in the susceptibility of the cells to CTL, and a decrease in their susceptibility to NK cells. IFN-mediated protection of uninfected and LCMV-infected target cells may make them resistant to surveillance by NK cells.

The second phase of the response is associated with the expansion of clones of virus-specific T and B cells, a decrease in the production of virus, an increase in the production of IFN-γ and interleukin 2 (IL-2), and an increase in the activation of macrophages. The major factor in the control of the LCMV infection, in the development of encephalitis, and in the development of immunopathological lesions throughout the body is the generation of CD8+ CTL, which can develop in CD4+ T cell-depleted mice. The IFN-induced upregulation of class 1 MHC antigens conditions cells in the host to be good targets for CD8+ T cells, which recognize viral peptides in the context of class 1 antigens. Some LCMV immunodominant T cell epitopes have been identified, and, in the context of synthetic peptides, vaccinia virus recombinants, or DNA vaccines, they can immunize mice against LCMV. During the peak of the T cell response to LCMV infection, the host becomes transiently immune deficient as the T cells fail to proliferate in response to mitogens but instead undergo apoptosis. Apoptosis is also observed in T cell populations *in vivo* as the immune response silences, but CTL precursors remain both at a high frequency and stable for the lifetime of the animal. The preservation of these memory CD8+ CTL probably does not require the persistence of viral antigen but does require the presence of CD4 cells, which may provide them with growth or survival factors. Very high doses of LCMV can overwhelm the T cell response, causing clonal exhaustion of CTL and resulting in a persistent infection.

Antiviral antibody plays only a minor role in the acute LCMV infection and is not needed to clear the infection. However, in the absence of a CTL response, such as in congenitally infected mice, antibody–virus–complement immune complexes mediate the development of glomerulonephritis, arteritis, and other inflammatory lesions. Usually the LCMV infection *per se* is relatively mild, and it is the immune response to the virus that causes the damage in the infected mouse.

Prevention and Control of LCMV

LCMV has not been a sufficiently significant human pathogen to warrant special public health measures for its control. However, the disease can be reduced in frequency by ridding houses of mice, by ensuring that pet hamsters are not infected, and by adhering at least to Biosafety Level 2 procedures in the laboratory. Supportive therapy without antiviral chemotherapy is recommended for patients experiencing LCMV infection.

Future

Studies with LCMV have in the past provided much of the conceptual basis for viral immunology and pathogenesis, including (1) the theory of immunological tolerance, (2) the concept of virus-induced immunopathology, (3) the analysis of immune complex disease, (4) the discovery of virus-specific CTL and demonstration of their roles in viral clearance and immunopathology, (5) the discovery of MHC-restriction in T cell recognition, (6) the discovery and analysis of NK cell activation and proliferation and (7) the concept that sublethal viral infections can abrogate specialized 'luxury' functions of cells and lead to metabolic disturbances. In the future the LCMV infection will likely continue to be an important model, and it is now being used for the development of vaccines directed against T cell epitopes, for examining the clearance of virus by T cell immunotherapy, for elucidating mechanisms of virus-induced immunosuppression and tolerance, for analyzing the regulatory roles of cytokines in the development of the NK cell and T cell response, for determining the relationships between CD8+ and CD4+ T cell function during infection, and for

determining factors contributing to the development and maintenance of immunological memory. LCMV variants can produce, in mice, syndromes that very closely resemble acquired immunodeficiency syndrome, and it is possible to manipulate this system to get a persistent infection without a CTL response at all, a persistent infection associated with immunosuppression and a low level CTL response, or an acute infection with a strong CTL response which clears the infection. Exploiting the biology of these systems should continue to provide basic concepts fundamental to viral immunology and pathogenesis.

See also: **Apoptosis and virus infection; Cytokines; Defective interfering viruses; Immune escape mechanisms; Immune response: Cell mediated immune response, General features; Interference; Interferons: General features; Lassa, Junin, Machupo and Guanarito viruses (*Arenaviridae*); Persistent viral infection.**

Further Reading

Lehmann-Grube F (1986) Lymphocytic choriomeningitis virus. In: Braude AI, Davis CE and Fierer J (eds) *Infectious Diseases and Medical Microbiology*, p. 1076. Philadelphia: W. B. Saunders.

Oldstone MB, von Herrath M, Evans CF and Horwitz MS (1996) Virus-induced autoimmune disease: transgenic approach to mimic insulin-dependent diabetes mellitus and multiple sclerosis. *Curr. Top. Microbiol. Immunol.* 206: 67.

Peters CJ, Buchmeier BJ, Rollin PE and Ksiazek TG (1996) Arenaviruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, Vol. 2, p. 1521. Philadelphia: Lippincott-Raven.

Welsh RM, Lin M-Y, Lohman BL *et al* (1977) $\alpha\beta$ and $\gamma\delta$ T-cell networks and their roles in natural resistance to viral infections. *Immunol. Rev.* 159: 79.

Zinkernagel RM and Doherty PC (1997) The discovery of MHC restriction. *Immunol. Today* 18: 14.

Molecular Biology

Peter J Southern and **Barbara J Meyer**, Department of Microbiology, University of Minnesota, Minneapolis, Minnesota, USA

Introduction

Lymphocytic choriomeningitis virus (LCMV) is the prototype of the *Arenaviridae* family and has been studied extensively since the 1930s as a model of both acute and persistent viral infection in laboratory mice. The outcome of infection is determined by both viral and host parameters: the strain and dose of virus, the strain and age of the mouse and the route of infection. Mouse infections are highly reproducible and, in this property, LCMV represents one of the most diverse and accessible systems for the study of *in vivo* infections. Analysis of *in vitro* infections has revealed considerable information on the basic virus life cycle; major contributions have also been derived for the related arenaviruses, Pichinde and Tacaribe. The application of cloning and sequencing techniques has allowed definition of the complete genetic potential and provided invaluable probe reagents for hybridization studies. Comparative sequence analyses for several members of the arenavirus family (Pichinde, LCMV-two strains and one variant, Tacaribe, Lassa and Junin viruses) have created a foundation from which to explore the molecular basis of host and tissue tropism and the pathogenesis of virus-induced diseases.

Properties of the Virion

The virions are enveloped spherical particles with a diameter in the range of 50–300 nm; variability in both size and shape has been observed depending upon the method of virion purification. The surface of the virions is covered with club-shaped spikes that project 5–10 nm beyond the envelope. These spikes are thought to be composed of one or both of the viral glycoproteins. Within virions, viral genomic RNAs are organized in helical nucleocapsid structures that have been visualized as arrays of 'nucleosome-like' subunits in circular configurations ranging between 40° and 1300 nm in length. Virion particles display a grainy interior appearance when viewed in the electron microscope and this gives rise to the name 'arena' which is derived from the Latin *arenosus*, sandy. This appearance is seemingly due to the encapsidation of host ribosomes acquired during budding from the plasma membrane of virus-infected cells; the ribosomes are not required for infectivity.

LCM virions can be readily purified from the supernatant medium of infected tissue culture cells using polyethylene glycol precipitation followed by density gradient centrifugation. Buoyant densities have been reported as follows: in sucrose 1.17–1.18 g cm^{-3}, in cesium chloride 1.19–1.20 g cm^{-3} and in amidotrizoate compounds, which are used most commonly, 1.14 g cm^{-3}. The virions are relatively unstable *in vitro* and are rapidly inactivated outside the pH range of 5.5–8.5 or by heating at 56°C. In addition, the virions are very sensitive to inactivation by either UV or γ irradiation. Solvents or detergents that disrupt the integrity of the virion

determining factors contributing to the development and maintenance of immunological memory. LCMV variants can produce, in mice, syndromes that very closely resemble acquired immunodeficiency syndrome, and it is possible to manipulate this system to get a persistent infection without a CTL response at all, a persistent infection associated with immunosuppression and a low level CTL response, or an acute infection with a strong CTL response which clears the infection. Exploiting the biology of these systems should continue to provide basic concepts fundamental to viral immunology and pathogenesis.

See also: **Apoptosis and virus infection; Cytokines; Defective interfering viruses; Immune escape mechanisms; Immune response: Cell mediated immune response, General features; Interference; Interferons: General features; Lassa, Junin, Machupo and Guanarito viruses (*Arenaviridae*); Persistent viral infection.**

Further Reading

Lehmann-Grube F (1986) Lymphocytic choriomeningitis virus. In: Braude AI, Davis CE and Fierer J (eds) *Infectious Diseases and Medical Microbiology*, p. 1076. Philadelphia: W. B. Saunders.

Oldstone MB, von Herrath M, Evans CF and Horwitz MS (1996) Virus-induced autoimmune disease: transgenic approach to mimic insulin-dependent diabetes mellitus and multiple sclerosis. *Curr. Top. Microbiol. Immunol.* 206: 67.

Peters CJ, Buchmeier BJ, Rollin PE and Ksiazek TG (1996) Arenaviruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, Vol. 2, p. 1521. Philadelphia: Lippincott-Raven.

Welsh RM, Lin M-Y, Lohman BL *et al* (1977) $\alpha\beta$ and $\gamma\delta$ T-cell networks and their roles in natural resistance to viral infections. *Immunol. Rev.* 159: 79.

Zinkernagel RM and Doherty PC (1997) The discovery of MHC restriction. *Immunol. Today* 18: 14.

Molecular Biology

Peter J Southern and **Barbara J Meyer**, Department of Microbiology, University of Minnesota, Minneapolis, Minnesota, USA

Introduction

Lymphocytic choriomeningitis virus (LCMV) is the prototype of the *Arenaviridae* family and has been studied extensively since the 1930s as a model of both acute and persistent viral infection in laboratory mice. The outcome of infection is determined by both viral and host parameters: the strain and dose of virus, the strain and age of the mouse and the route of infection. Mouse infections are highly reproducible and, in this property, LCMV represents one of the most diverse and accessible systems for the study of *in vivo* infections. Analysis of *in vitro* infections has revealed considerable information on the basic virus life cycle; major contributions have also been derived for the related arenaviruses, Pichinde and Tacaribe. The application of cloning and sequencing techniques has allowed definition of the complete genetic potential and provided invaluable probe reagents for hybridization studies. Comparative sequence analyses for several members of the arenavirus family (Pichinde, LCMV-two strains and one variant, Tacaribe, Lassa and Junin viruses) have created a foundation from which to explore the molecular basis of host and tissue tropism and the pathogenesis of virus-induced diseases.

Properties of the Virion

The virions are enveloped spherical particles with a diameter in the range of 50–300 nm; variability in both size and shape has been observed depending upon the method of virion purification. The surface of the virions is covered with club-shaped spikes that project 5–10 nm beyond the envelope. These spikes are thought to be composed of one or both of the viral glycoproteins. Within virions, viral genomic RNAs are organized in helical nucleocapsid structures that have been visualized as arrays of 'nucleosome-like' subunits in circular configurations ranging between 40° and 1300 nm in length. Virion particles display a grainy interior appearance when viewed in the electron microscope and this gives rise to the name 'arena' which is derived from the Latin *arenosus*, sandy. This appearance is seemingly due to the encapsidation of host ribosomes acquired during budding from the plasma membrane of virus-infected cells; the ribosomes are not required for infectivity.

LCM virions can be readily purified from the supernatant medium of infected tissue culture cells using polyethylene glycol precipitation followed by density gradient centrifugation. Buoyant densities have been reported as follows: in sucrose 1.17–1.18 g cm^{-3}, in cesium chloride 1.19–1.20 g cm^{-3} and in amidotrizoate compounds, which are used most commonly, 1.14 g cm^{-3}. The virions are relatively unstable *in vitro* and are rapidly inactivated outside the pH range of 5.5–8.5 or by heating at 56°C. In addition, the virions are very sensitive to inactivation by either UV or γ irradiation. Solvents or detergents that disrupt the integrity of the virion

envelope rapidly eliminate infectivity in viral preparations.

Several distinct enzymatic activities have been associated with purified virions: poly(A) and poly(U) polymerases thought to be derived from the encapsidated host ribosomes, a serine/threonine protein kinase of unknown origin that can phosphorylate the viral nucleocapsid protein, NP, and a viral-encoded RNA-dependent RNA polymerase which is essential for viral RNA transcription and replication in infected cells. An *in vitro* assay to monitor viral polymerase activity yielded variable and unconvincing results for LCMV whereas parallel incubations with disrupted purified Pichinde virions produced clear evidence of polymerase activity.

Properties of the Genome

The genome of LCMV comprises two single-stranded RNA molecules, designated L and S, that exhibit ambisense coding arrangements. The only significant homology between the genomic RNAs is located at the 3′ termini where there is a highly conserved sequence of approximately 30 nucleotides; the complement of these sequences is found at the 5′ termini. Thus, individual RNAs could form panhandle type structures and intermolecular annealing could generate homodimeric or heterodimeric forms. The L and S RNAs are not present in equimolar amounts and, within virions and infected cell RNA samples, the S RNA is always more abundant. Hybridization studies with strand-specific probes have established that genomic complementary RNA can also be detected in virions. RNA extracted from highly purified virions normally contains host-derived 28S and 18S ribosomal RNAs together with a heterogeneous collection of RNAs in the 4–7S size range. A viral subgenomic mRNA, encoding the Z protein, is a component of this low molecular weight virion RNA fraction but there is no evidence to suggest that Z mRNA should be considered a genomic RNA.

The S RNA (3376 nucleotides) contains the coding sequences for the major structural proteins: the internal nucleocapsid protein, NP, and the surface glycoprotein precursor, GPC. The NP and GPC coding regions are separated by an intergenic non-coding region that has the potential to form a stable stem-loop structure (21 base pairs in the stem and four bases in the loop). The L RNA (7220 nucleotides) contains the coding sequences for the L and Z proteins. There is an intergenic region of almost 200 nucleotides separating the coding regions of the L RNA that can be predicted to assume various complex stem–loop structures. Precise information is still lacking because the intergenic region contains a 30-base C-rich sequence that has proved extremely refractory to direct RNA sequencing and this region is invariably deleted in cDNA clones. The existence of the Z coding region and the ambisense nature of the L RNA segment were completely unknown prior to M. Salvato's sequencing studies of the LCMV genomic L RNA. An equivalent coding region has been detected at the 5′ end of the Tacaribe L segment but there is considerable uncertainty about the role of Z protein in the infectious process.

Properties of the Proteins

The nucleoprotein, NP (558 amino acids, observed molecular weight 63 000), is the first newly synthesized viral protein to be detected in acutely infected cells, and its appearance corresponds to the beginning of the exponential phase of the replicative cycle. NP is complexed with genomic RNAs within cells and virions in the form of ribonucleoprotein (RNP) complexes. By analogy with other viral systems, it is probable that levels of intracellular NP influence the relative rates of transcription and replication and it has been suggested that NP coating of the intergenic regions might serve to disrupt secondary structures and allow synthesis of full-length replication intermediates. The vast majority of mature NP is identical to the primary translation product but there are indications that modified species may also exist. Both virions and infected cell extracts routinely contain small amounts of reproducible NP breakdown fragments. How and why these fragments are generated remain unknown but it is conceivable, given limited coding capacity, that NP-breakdown fragments fulfil the roles played by minor structural proteins in other viral systems. Also, a phosphorylated form of NP has been observed at late times in acute infection raising the possibility that this modification may influence viral gene expression. There is currently no information on whether the kinase detected in purified virions is responsible for intracellular phosphorylation of NP.

The glycoprotein precursor, GPC (498 amino acids, observed molecular weight 70 000–75 000) is cleaved by a trypsin-like protease to release the mature glycoprotein species: GP-1 (40 000–46 000 molecular weight) and GP-2 (molecular weight 35 000). GP-1 constitutes the N-terminal portion of GPC and the actual cleavage site is located between residues 262 and 263 of the precursor. Cleavage apparently occurs in the Golgi or post-Golgi compartment and requires prior glycosylation of GPC. There are five predicted sites for N-linked glycosylation in GP-1 and two sites in GP-2. From deglycosylation studies it appears that all sites can accept carbohydrate but, because GP-1

migrates as a heterogeneous species on sodium dodecyl sulfate (SDS)-denaturing polyacrylamide gels, all sites may not be utilized all the time. Neutralizing antibodies are directed primarily against conformational epitopes within GP-1, and epitope recognition is dependent on both glycosylation and retention of disulfide bonds. Recent protein cross-linking studies in M.J. Buchmeier's laboratory have begun to elucidate the structural organization of the mature glycoproteins in virions. GP-1 is a peripheral membrane protein, whereas GP-2 is an integral membrane protein; both species are assembled as native homotetramers. There is no evidence yet for any interaction between GP-1 and GP-2 and, although such an interaction is anticipated, it is currently unclear how GP-1 remains associated with the membrane. GP-2 can be linked to NP in the interior of virions using a membrane-permeable cross-linker, and this interaction is likely to be important in virion assembly.

There is no evidence for any modification to the L-encoded proteins but reagents necessary for systematic analyses are only now becoming available. The L protein is a minor constituent of virions and reproducible detection was problematic until the development of anti-peptide antibodies specific for sequences predicted from the L open reading frame. Conventional procedures to form hybridomas had failed to produce any antibody with reactivity against the L protein – presumably because of the low relative abundance of L when compared with the major structural proteins. It has been assumed that the L protein (2211 amino acids, predicted molecular weight 254 529 and observed molecular weight approximately 200 000) constitutes part or all of the viral RNA-dependent RNA polymerase. By analogy with the polymerases of negative-strand viruses, the L protein may possess multiple enzymatic activities.

The Z-protein-encoding region contains 90 amino acids and predicts a primary translation product of molecular weight approximately 10 000. Antibodies to examine Z protein accumulation and distribution were first raised against a fusion protein expressed in *Escherichia coli* but subsequent studies have also utilized anti-peptide antibodies. The Z protein has been detected as a relatively abundant component of virions and complexes between Z and NP have been generated in cross-linking studies. Z may therefore be a matrix-like protein. Alternatively/additionally, Z may be involved in transcriptional regulation and, in support of this possibility, the predicted Z protein sequence includes a potential zinc-finger motif, and zinc binding has been formally demonstrated. The presence of Z mRNA within virions would provide a mechanism for Z protein to be present at the earliest stages of infection, much sooner than could be achieved from *de novo* transcription of the genomic complementary L RNA template.

Replication

Transcription and replication are confined to the cytoplasm of virus-infected cells and require the activity of the virus-encoded RNA-dependent RNA polymerase. This enzyme is largely unaffected by addition of actinomycin D or α-amanitin. Viral RNP complexes are known to be associated with the nuclear membrane and there remains an unexplained dependence on the presence of the host cell nucleus for efficient viral replication. The process of RNA replication involves synthesis of full-length genomic complementary RNAs (anti-genomes) that then serve as templates for synthesis of genomic RNAs. The precise mechanism for priming the initiation of replication is currently unclear but D. Kolakofsky and his colleagues, working with Tacaribe, have proposed a mechanism based on primer annealing at the 3′ end of the template RNA followed by slippage backwards so that the 5′ nucleotide of the chain to be synthesized is no longer base-paired. The source of the primer is not known. This model attempts to account for the presence of an extra, nontemplated G residue detected at the 5′ end of genomic S RNA segments. When first observed, the extra G was thought to be a cloning artifact, but independent verifications strengthen the interpretation that the 5′ extra G is a genuine structural feature. There is still some ambiguity about the nature of the 5′-terminal nucleotide present on genomic RNA segments and, because of a clear link with initiation of RNA replication, this problem is now under active investigation.

Transcription

NP mRNA is transcribed from the 3′ end of the genomic S RNA and is therefore of genomic complementary polarity. The GPC mRNA is transcribed from the 3′ end of the genomic complementary S RNA and therefore has the same polarity as the genome. This transcription profile is imposed by the ambisense coding arrangement and suggests a temporal component to transcription regulation that has been supported by experimental observations: NP mRNA is synthesized immediately after infection while GPC mRNA is not synthesized until RNA replication has been initiated. Recent transcription mapping studies have determined that the S-derived subgenomic mRNAs have 5′ cap structures and extend for one to seven nucleotides beyond the ends

of the RNA templates. The origin(s) of the 5′ caps and nontemplated bases are currently unknown. It is possible that LCMV may utilize a cap-snatching mechanism or that primer synthesis and capping can be performed by the viral polymerase. The 3′ termini of the subgenomic mRNAs derived from the S RNA segment are located at heterogeneous positions in the intergenic region. The mRNAs are not polyadenylated so the possible presence of a terminal hairpin structure could provide an alternative mechanism to stabilize the 3′ termini from ribonuclease degradation in the cytoplasm. Essentially identical results have been reported for the related arenavirus, Tacaribe, suggesting that 5′ nontemplated bases and 3′-terminal heterogeneity may be general characteristics of at least the S-derived mRNAs.

The L-protein-encoding region is located at the 3′ end of the genomic L RNA segment and therefore can be transcribed immediately in the infected cell to produce a genomic complementary mRNA. The open reading frame for the L protein spans 6633 nucleotides from the total length of 7220 for the genomic L segment. The 5′ end of the L RNA segment contains the Z-encoding region (Z mRNA is approximately 0.5 kb) and the subgenomic Z mRNA has the same polarity as the genomic L RNA. At present, there is no sequence information relating to the termini of L-derived mRNAs but it is highly likely that the general features observed for S mRNAs will be duplicated for the L mRNAs.

Polymerase Activity

An *in vitro* assay has been developed to monitor the LCMV polymerase activity but, although the enzyme is presumed to be present in virions, the activity was much more readily detectable in cytoplasmic extracts harvested from acutely infected cells. Both genomic and subgenomic mRNAs could be synthesized *in vitro* but the labelled products detected probably represented elongation of preinitiated chains. A short virus-specific RNA was repeatedly synthesized in the polymerase assays and, although unassigned when the experiments were being performed, this product can be retrospectively identified as Z mRNA. A subsequent study of the Tacaribe polymerase demonstrated that addition of short oligonucleotide primers could significantly increase the efficiency of RNA synthesis *in vitro*.

Progression of Acute Infections

LCMV infects and replicates efficiently in a wide variety of cell types from many different species. The viral receptor has not been identified but, based on the known diversity of susceptible cells, the receptor must be a highly conserved and widely distributed molecule. Infections with LCMV are not normally cytolytic. After an initial period of active viral transcription, replication, synthesis of virus-encoded proteins and release of progeny virions, viral gene expression is downregulated and the cells progress towards a state of persistent infection. Production of progeny virions is reduced significantly and the accumulation of viral glycoproteins in the cell membrane is correspondingly diminished. Assuming that the L protein represents part or all of the polymerase protein, the *in vitro* assay for polymerase activity demonstrated that increasing intracellular concentrations of L protein correlated with a significant reduction in the level of enzymatic activity. This could represent part of the regulatory mechanism influencing the progression from acute to persistent infection. The accumulation of L protein observed at late times in an acute infection might lead to the formation of aberrant RNA–L-protein complexes and thereby remove RNA templates from a replicating pool. Similar observations have been made with the vesicular stomatitis virus polymerase to suggest that the intracellular concentration of viral polymerase may have a critical influence on the state and progression of the infection.

In tissue culture cells, the persistent infection is perhaps best viewed as a dynamic state. Cells contain high levels of cytoplasmic viral antigen (NP) and intracellular viral RNAs (both genomic and subgenomic mRNAs) and some degree of viral replication appears necessary to prevent dilution of the persistent infection at cell division. However, uninfected cells can be recovered from persistently infected cultures by single cell cloning and the intracellular viral RNA content of individual cells in the culture has been found to vary widely. Infectious virus is periodically released to reinitiate infection in any susceptible cells in the culture. Viral RNAs with internal deletions [potential defective-interfering (DI) RNAs] have been detected in persistently infected tissue culture cell lines but there is no convincing evidence that these RNAs are causal to the persistent infection. Apparently full-length genomic RNAs are the most abundant viral RNA species detected in total cell RNA preparations from persistently infected cells.

LCMV Strains and Reassortant Viruses

Several different strains of LCMV (Armstrong or Arm, WE, Pasteur, Traub and UBC) have been defined on the basis of distinct biological properties, monoclonal antibody recognition profiles, RNA fingerprinting, nucleic acid cross-hybridization and finally nucleotide

sequencing. Considerable effort has been invested into determining the molecular basis for strain-specific properties, and significant insight has been derived from the creation of reassortant viruses. Using this approach, Oldstone and colleagues were able to map LCMV virulence in adult guinea pigs to the L RNA segment and assign tissue tropism and virulence in mice to the S RNA segment. They also observed, as has been noted in other viral systems with segmented genomes, that reassortment between two nonpathogenic parental strains can generate viruses with novel and unexpected pathogenic potential.

Variant Viruses

Infection of neonatal BALB/c mice with LCMV results in viral replication in multiple tissues and a life-long persistent infection is established (see *In situ* hybridization below). Virus can readily be recovered from such persistently infected mice and it was widely known that the recovered viruses could differ in plaque morphology depending upon the tissue source (for example, predominantly clear plaques were recovered from brain whereas turbid plaques were generally recovered from spleen). Ahmed made a critical observation when he realized that the spleen-derived virus displayed fundamentally different biological properties. All wild-type LCMV strains induce a lethal immune-mediated choriomeningitis when inoculated intracerebrally into adult immunocompetent mice, but an equivalent infection with the spleen-derived virus (Clone 13) resulted in an asymptomatic persistent infection. It was subsequently shown that infection with Clone 13 fails to induce a cytotoxic T-cell (CTL) response in adult mice despite the fact that Clone 13-infected cells are recognized efficiently by CTLs induced by the parental LCMV Arm strain. Comparisons of the genomic RNA sequences for Clone 13 and Arm have revealed remarkably few changes (five differences from a total of 10 600 nucleotides causing two amino acid changes – residue 260 of GPC, Phe in Arm and Leu in Clone 13, and residue 1079 in L, Lys in Arm and Glu in Clone 13). The change in GPC is located in close proximity to the protease cleavage site of GPC but it is by no means clear how CTL induction may be disrupted. Curiously, LCMV strains other than Arm have Leu at residue 260 in GPC and yet are fully proficient at CTL induction. The situation is clearly complex because independent isolation of 'Clone 13-like' viruses from the parental Arm stock has consistently reproduced the Phe to Leu change but Clone 13 revertants that reacquire the ability to induce CTLs all retain Leu at position 260. Thus, perhaps both amino acid differences between Arm and Clone 13 are required for the phenotypic change. This conclusion is reinforced by early reassortant experiments that indicated an involvement for the L segment. It is probable that a critical event must occur immediately after infection with Clone 13 because low-dose infection or infection by intraperitoneal rather than intravenous injection does allow the generation of a Clone 13 CTL response.

Persistent Infections and *in situ* Hybridization

The availability of molecular clones corresponding to the genomic L and S RNA segments has permitted systematic hybridization analyses of viral-encoded RNAs throughout the time course of both tissue culture and mouse infections. Adaptation of conventional techniques to perform *in situ* hybridization on whole-body sections of mice has provided an efficient method to monitor the distribution of viral RNAs in persistently infected mice. Furthermore, this procedure has provided a dramatic illustration of the clearance of viral nucleic acid sequences following syngeneic cell transfer of spleen cells from an LCMV-immune mouse into a persistently infected recipient. In analyzing persistent infections of mice between birth and 6 months of age, there appeared to be a discrepancy between the titer of infectious virus that could be recovered and the intracellular accumulation of viral RNAs. Initially, virus titers are high [10^6 plaque-forming units (PFU) per g of tissue or per ml of serum], and viral nucleic acid is essentially undetectable but, by about 2 weeks after infection, virus titers have fallen (10^2–10^3 PFU per g of tissue or per ml of serum), and nucleic acid sequences have accumulated and are retained at consistently high intracellular levels. This is suggestive of the emergence of DI RNAs but, although LCMV DI particles have been implicated in several different studies, no rigorous correlation has yet been established between biological interference and molecular changes in either the protein and/or RNA components of virions. A complex population of heterogeneously sized viral RNAs has been detected within multiple tissues of persistently infected mice but, although these RNAs are unique to the persistent infection *in vivo*, it is not clear if they are causally linked to the persistent infection. One consistent observation is that the ratio of L-derived to S-derived RNAs is further skewed towards S during persistent infections.

Expression of LCMV Genes in Novel Environments

The construction of cDNA genes corresponding to the NP- and GPC-encoding regions has permitted

expression studies in several different vector systems (plasmids, vaccinia virus and baculovirus). The vaccinia vectors have been used extensively by Whitton and Oldstone to map CTL epitopes within NP and GPC. Each viral structural protein contains a few linear amino acid sequences that represent major CTL determinants, but recognition is profoundly influenced by MHC haplotype.

Several independent lines of transgenic mice expressing LCMV cDNA genes have been produced in the laboratories of Oldstone and Zinkernagel. These mice display a T-cell nonresponsive (tolerant) phenotype towards the viral transgene products. However, infection of these transgenic mice with LCMV appears to overwhelm the non-responsive state leading to 'autoimmune-like' tissue damage in locations where the transgenes are expressed. Many details relating to the induction of the pathogenic immune response still remain to be clarified. The homologous nature of the system (expression of genes from a mouse virus in mice with the possibility to challenge with the original virus) should produce substantial new information on the nature of virus-induced immune responses and pathogenic events in the LCMV-infected LCMV transgenic mice.

See also: **Defective interfering viruses; Lassa, Junin, Machupo and Guanarito viruses (*Arenaviridae*); Persistent viral infection; Vectors: Animal viruses.**

Further Reading

Borrov P. (1997) Mechanism of viral clearance and persistence. *Journal of Viral Hepatitis* 4 (supp 2): 16–24.

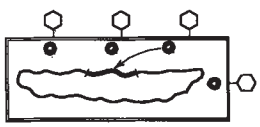
Howard, CR (1986) Arenaviruses. *Perspectives in Medical Virology*, vol. 2, Amsterdam: Elsevier.

Oldstone MBA (ed.) (1987) *Current Topics in Microbiology and Immunology*, vols 133 and 134. Berlin: Springer-Verlag.

Salvato MS (ed.) (1993) *The Arenaviridae*. New York: Plenum Press.

[This article is reproduced from the 1st edn (1994).]

LYSOGENY AND PROPHAGE



Max Gottesman, Institute of Cancer Research, Columbia University College of Physicians and Surgeons, New York, USA

Arnos Oppenheim, Department of Medical Genetics, Hebrew University, Hadassah Medical School, Jerusalem, Israel

Introduction

The phenomenon termed lysogeny is discussed here, mainly through the perspective of the coliphage λ in the family *Siphoviridae*, genus 'λ-like viruses'. Lambda lysogeny was the first to be understood in detail, and remains the best studied example of the process. Furthermore, it is probable that the principles derived from the study of λ lysogeny will be applicable, *mutatis mutandis*, to other lysogenic phage.

General Features

The chromosome of coliphage λ is 48 514 bp, containing about 50 genes, and is fully sequenced. The λ chromosome is present in the virion as double-stranded DNA, with 12 bp 5′ cohesive overhangs. The order of genes in the virion is unique, and the viral DNA is injected through the tail in a polar fashion. The injected DNA is rapidly circularized and negatively supercoiled by the bacterial enzymes DNA ligase and gyrase, respectively.

Unlike the virulent T phages, λ is a temperate phage; a portion of infected cells survive. Most of the survivors become lysogens, and carry the λ as a stable, mostly inert, prophage integrated in the bacterial chromosome. The lysogenic state – the presence of a prophage – can be detected in several ways. Lysogens are immune; they are not killed by superinfecting λ virions. The inert prophage is carried like a set of bacterial genes, and is passed on from generation to generation. However, certain treatments induce, or activate, the prophage. The induced prophage excises from the bacterial chromosome, replicates, packages itself, and lyses its host, releasing several hundred phage particles.

Because of its ability to lysogenize, λ makes a turbid plaque on a lawn of sensitive bacteria growing

100 000-fold increase in size and reach a diameter of up to 2 mm. Infected cells are characterized by an enlarged nucleus, basophilic cytoplasmic inclusions and a hyaline capsule surrounding the individual cells. As the infection progresses and the virus replicates, the nucleus becomes vacuolated and undergoes necrosis while the virus progeny accumulates in the cytoplasm and the capsule thickens.

Future Perspectives

The major obstacle in the analysis of the molecular mechanisms underlying LCDV infection, replication and pathogenesis is the lack of an efficient cell culture system for virus propagation. However, the knowledge of the complete primary structure of the viral genome now allows the *in vitro* expression and functional analysis of individual viral gene products.

See also: **Frog virus 3 (*Iridoviridae*); *Iridoviridae* – invertebrate.**

Further Reading

Darai G (ed.) (1990) *Molecular Biology of Iridoviruses*. Boston: Kluwer.

Tidona CA and Darai G (1997) The complete DNA sequence of lymphocystis disease virus. *Virology* 230: 207.

Tidona CA, Schnitzler P, Kehm R and Darai G (1998) Is the major capsid protein of iridoviruses a suitable target for the study of viral evolution? *Virus Genes* (Suppl.) 16: 59.

LYMPHOPROLIFERATIVE DISEASE VIRUS OF TURKEYS (*RETROVIRIDAE*)

Arnona Gazit and **Abraham Yaniv**, Department of Human Microbiology, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv, Israel

History

The lymphoproliferative disease (LPD) of turkeys is a naturally occurring lymphotumoral disorder characterized by enlargement of the spleen, thymus and liver, with focal lesions in most organs. The disease was first recognized in the UK, by Biggs *et al* in 1974, as a new disease entity distinct from reticuloendotheliosis (RE), which is also a leukotic condition in turkeys. Subsequently, sporadic outbreaks of LPD were also reported to occur in other European countries and in Israel. In 1978, a retrovirus, designated lymphoproliferative disease virus (LPDV), was isolated from afflicted turkeys.

Etiology

A retroviral etiology for LPD was first suspected based on the finding of C-type particles in plasma pellets and in tissues and lesions of birds with natural or experimentally induced disease, as well as the demonstration that preparations of these materials produced the disease when inoculated into poults. Further evidence for a retroviral etiology was based on the presence of budding C-type particles in tissue sections of LPD tumors and on molecular evidence showing that LPDV information is present and expressed in LPD tumor cells. Transfection of the cloned LPDV provirus into turkey lymphocytes and their injection into autologous turkeys resulted in the induction of LPD symptoms. This indicated that LPDV is a replication-competent virus, capable of inducing the disease in the absence of a defective oncogene-containing retroviral counterpart.

General Features and Classification

The mature LPDV viral particles measure 90–120 nm in diameter and have an electron-dense core with a less dense intermediate layer bounded by an outer envelope. As with a typical retrovirus, LPDV virions possess a buoyant density of 1.17 g ml^{-1} in sucrose gradients. They contain 35S plus-strand viral RNA and an RNA-dependent DNA polymerase (RT) that is preferentially activated by magnesium ions. The virion structural proteins are the two envelope glycoproteins of 76 and 41 kDa, the matrix (MA) protein of 20 kDa and several core proteins, including the capsid (CA) protein of 28 kDa, a core protein of 31 kDa, the nucleocapsid (NC) protein and the protease (PR) of 13–15 kDa each.

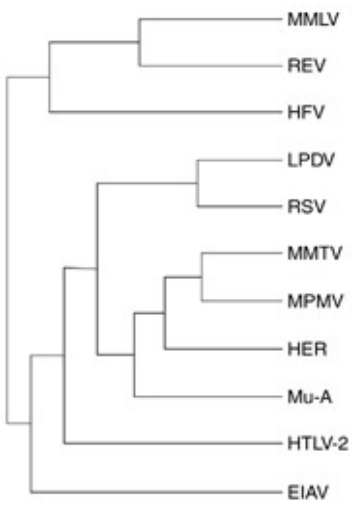


Figure 1 Evolutionary tree of LPDV. The deduced amino acids of the *pol* genes of the following viruses were aligned: Moloney murine leukemia virus (MMLV); reticuloendotheliosis virus (REV); human foamy virus (HFV); lymphoproliferative disease virus, LPDV; Rous sarcoma virus (RSV); mouse mammary tumor virus (MMTV); Mason–Pfizer monkey virus (MPMV); human endogenous virus (HER); mouse intracisternal A particle (Mu-A); human T-cell leukemia virus (HTLV-2); equine infectious anemia virus (EIAV).

According to cross-nucleic acid hybridization assays and nucleic acid sequence analysis, LPDV is a representative of a new and distinct class of avian retroviruses that can be readily distinguishable from the avian sarcoma–leukemia viruses (ASLVs) and the RE viruses. Sequence analysis of the *pol* gene and its comparison to the *pol* genes of other retroviruses established that LPDV is evolutionarily most related to the avian sarcoma–leukemia viruses (**Fig. 1**) and belong in the Alpharetrovirus genus of the *Retroviridae* family.

Viral Genome Organization and Properties of Viral Proteins

The LPDV RNA genome is 7143 bp long. The LPDV provirus is flanked by two host-derived 6 bp direct repeats formed during integration of the viral DNA into the host DNA. The LPDV proviral genome is bordered by two long terminal repeats (LTRs) of 354 bp each, which are bordered by 7 bp imperfect inverted repeats (5′-TGTGTG/CGCTACA-3′). The LTR comprises three sections: U3 of 236 bp, R of 22 bp, and U5 of 96 bp. The U3 region is a potent transcriptional element containing a TATA box, an upstream CAAT box in an inverted position (ATTGG), and two 12 bp imperfect direct repeats which constitute two CArgG motifs, suggesting their role as serum response elements (SREs). In addition, negative regulatory elements were found to reside at the 5′ end of the U3.

The overall structure of the LPDV LTR is similar to that of ASLV genomes. The primer binding site, which serves as the primer for the synthesis of minus-strand DNA, is a 18 bp motif, complementary to the chicken $tRNA^{Trp}$, as is the primer binding site of ASLV. The polypurine tract, which presumably serves as a primer for the synthesis of the plus-strand viral DNA, is nearly identical to those of ASLV genomes. By sequence analysis, the coding region of the entire viral genome contains the three major retroviral structural genes, *gag*, *pol* and *env*, with the overall topography of 5′-LTR-*gag*-*pro*-*pol*-*env*-LTR-3′, similar to the genomic organization of a typical replication-competent retrovirus (**Fig. 2**).

Each of these three open reading frames (ORFs) are phased differently. According to amino acid sequence analysis, the *gag* ORF is 2243 bp long and encodes the Gag polyprotein precursor of 724 amino acids, which is acylated rather than myristoylated. The Gag polyprotein is cleaved by the viral protease into the various mature virion proteins. The N-terminal protein is the membrane-associated MA protein of 155 amino acids which, comparable with other retroviruses, should function by interacting with the inner face of the viral lipid envelope. Adjacent to the MA protein is the core virion protein, p31, which is a protein of 234 amino acids by sequence analysis. Of note, p31 contains proline-rich stretches that are similar to conserved prolin-rich elements found in the analogous products of other retroviruses, suggesting functional and evolutionary relationships of MA–CA junction proteins. The subsequent protein, p28, comprised of 232 amino acids, is the major CA protein. Then follows the NC protein of approximately 103 amino acids, a highly basic nucleic acid-binding protein which is presumably tightly complexed with the genomic viral RNA within the mature virion. Similar to the NC protein of ASLV, this protein contains two copies of the finger motifs, the Cys-His boxes. This is in contrast to the murine retroviruses whose NC proteins contain only one copy of the Cys-His box.

The viral protease is encoded by a distinct ORF (designated *pro*) and is expressed as a Gag-Pro polyprotein of 843 amino acids long, which is produced by ribosomal frameshift. The frameshift site is a heptanucleotide GGGAAAC, identical to that of the Mason–Phizer monkey virus (MPMV) and its related simian viruses. Amino acid sequence analysis of the N-terminus of the viral protease established it as a 116 amino acid long protein, autocatalytically cleaved 95 nt downstream of the frameshift site. It belongs to the aspartic proteinase class and as such

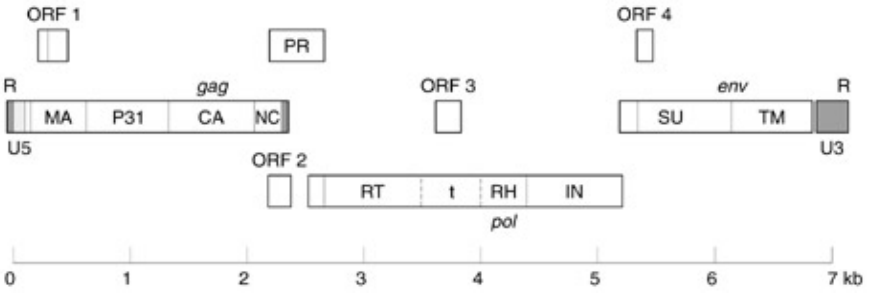


Figure 2 Coding regions of the LPDV genome deduced from ORF analysis of its nucleotide sequence.

contains two conserved hydrophobic elements pertaining to the enzyme active site, Asp-Thr-Gly and Ile-Leu-Gly-Arg.

The *pol* ORF of LPDV is transcribed as a Gag-Pro-Pol polyprotein of 1676 amino acids, produced by ribosomal frameshifting at the heptanucleotide sequence motif AAAUUUA, which is identical to that of ASLV genomes. This polyprotein is cleaved by the viral protease into the RT and the integrase (IN) proteins. There is a high genetic relatedness between the RT and IN of LPDV and those of ASLVs.

The *env* gene of LPDV is expressed via the *env* transcript whose splice donor site is located within the U5 domain; its splice acceptor site is located within the *env* ORF, 48 codons upstream to the Env initiation codon. The Env polyprotein is processed through proteolytic cleavage at the basic stretch, Arg-Glu-Lys-Arg, into the surface (SU) and transmembrane (TM) glycoproteins. These Env proteins are glycosylated through *N*-linked glycosylation of aspargine residues, 10 sites in the SU and four sites in the TM domains.

In addition to these four main ORFs, there are four short ORFs, overlapping the *gag*, the *pol* and the *env*. Whether or not any of these short ORFs are functional remains to be determined.

No viral oncogene resides within the LPDV genome.

Host Range and Propagation

In nature, LPD is observed only in turkeys. The age of onset is between 8 and 10 weeks of age, with a peak around 16 weeks of age. The cumulative mortality losses in a flock might reach 15–25%. Various strains of turkeys exhibit different susceptibility levels, ranging from low (4–8%) to high (25–36%).

Under experimental conditions, the chicken is the only other domestic bird which is susceptible to LPDV infection, developing both viremia and LPD.

Attempts to propagate LPDV in a variety of cell cultures have so far been unsuccessful.

Pathogenicity and Clinical Features

LPD is usually observed in small outbreaks. LPDV can spread horizontally between poults in contact. The incubation period for the natural disease is unknown, but is suggested to be as short as 7 weeks. The clinical course of the disease is acute, with practically no premonitory signs of the disease prior to death. In experimentally induced LPD, lesions are preceded by a viremia of C-type particles which develops 2 weeks after infection and persists in some birds for at least 8–10 months. Typical LPD lesions develop 3–6 weeks after infection. An unusual feature of the disease is that poults infected at 4 weeks of age develop a high incidence of the disease, whereas those infected at 1 day of age develop viremia in the absence of any clinical signs of the disease.

Clinically, the most affected organ is the spleen, followed by the thymus, pancreas and liver. Other organs such as the kidneys, gonads, lung and peripheral nerves can also be affected, but usually to a lesser extent.

The characteristic gross lesion is splenomegaly (**Fig. 3**). The affected spleen can reach 40–60 g. Its color is whitish or pale pink. The thymus and liver are also enlarged. The affected organs contain miliary gray-white foci which, in advanced cases, become confluent, forming large infiltrated grayish-white

Figure 3 Morphology of an LPDV-affected spleen as compared with a normal one.

lesions with irregular borders. Histologically, the characteristic microscopic lesions of LPD are infiltrative foci which consist of a pleomorphic population of mature and premature different-sized lymphoid cells such as lymphocytes, lymphoblasts, plasma cells, reticular cells and fusiform giant cells. The numerous mitoses of the infiltrated lymphoid cells suggest a proliferative character of the tumoral process.

In experimental cases, the surviving birds exhibit regressing lesions, suggesting that the tumoral process can be abrogated or reversed in resistant birds.

Anemia is frequently present; some affected birds are leukocytotic, others are leukopenic. Elevated IgG concentration has also been recorded. Up to 11 weeks postinfection, suppression of cell-mediated but not humoral immunity is detected.

Organotropism studies of LPDV showed that the replication of LPDV is confined to lymphoid organs. Cells residing in the bone marrow are the first to sustain virus expression. Thereafter, significant levels of LPDV expression can be detected in the thymus, and then in the spleen and the bursa of Fabricius. Among the lymphoid organs, the thymus acts as the main target for virus expression throughout the malady. Although virus expression takes place in all lymphoid organs, transformed foci are not detected in the bursa of Fabricius.

The mechanism by which LPDV exerts its leukemogenic potential has not yet been determined. Sequence analysis of the viral genome established LPDV as being devoid of a viral oncogene. Using inverse polymerase chain reaction (PCR), the viral genome was shown to be randomly integrated within the DNA of transformed foci in the afflicted lymphoid organs, thus excluding insertional mutagenesis as the mechanism responsible for LPD induction. It is hypothesized that the lymphoproliferative process of LPD is a premalignant stage that after several months, might develop into frank tumors through seldom-occurring insertional mutagenesis. It remains to be determined whether LPDV might encode a transactivator that could induce the expression of a cellular gene capable of stimulating lymphoproliferation.

Diagnosis

Owing to lack of overt clinical manifestations, early detection of LPDV is essential for preventing the rapid horizontal spread of the disease that can decimate flocks.

The main disease with which LPD can be confused is RE. The laboratory tests that are used to differentiate between the two infections are:

- *Virus isolation* LPDV cannot be isolated *in vitro*, either in embryonated eggs or in cell cultures. In contrast, REV can be grown in various types of cell cultures.
- *Antigen detection* LPDV can be specifically demonstrated in high-speed pellets of plasmas from affected birds, in buffy-coat cells and in frozen sections of affected spleen, employing an indirect ELISA or an indirect immunofluorescence test using LPDV-specific antiserum.
- *RT assay* A differential diagnostic test of LPDV based on the divalent cation preference of the viral RT was developed. Viral particles, obtained from high speed pellets of plasma are subjected to a standard templated RT reaction in the presence of either Mg^{2+} or Mn^{2+}. In contrast to the RT of REV that is catalyzed by Mn^{2+}, the RT of LPDV is active in the presence of Mg^{2+}.
- *Polymerase chain reaction* The availability of the nucleotide sequence of the LPDV genome enables specific diagnosis of LPDV employing the PCR. A simple, rapid, sensitive and highly specific PCR capable of providing differential diagnosis of LPD soon after infection was described.
- *Serology* Recently, a sensitive Western test for the detection of LPDV antibodies in sera of affected birds was developed by using bacterially synthesized highly immunogenic major viral proteins (p31 and CA) as antigens.

Future Perspectives

The molecular events underlying LPDV replication and oncogenesis are still obscure. Search for a permissive cell line will enable the investigation of LPDV infection at its molecular level. Furthermore, identification of putative LPDV-induced tumors in survivors of afflicted turkey flocks will likely enable studies addressing the molecular mechanism of LPDV oncogenicity. The development of an effective vaccine will help to eradicate LPDV infection and spread.

See also: **Diagnostic techniques: Isolation and identification by culture and microscopy; Diantho-viruses (*Tombusviridae*); Avian type C retroviruses (*Retroviridae*); Retroviruses – type D (*Retroviridae*); Retroviruses of drosophila: The gypsy paradigm.**

Further Reading

Biggs PM (1991) Lymphoproliferative disease of turkeys. In: Calnek *et al* (eds) *Diseases of Poultry* 9th edn, p. 456. Ames: Iowa State University Press.

Chajut A, Sarid R, Yaniv A *et al* (1992) The lymphopro-

liferative disease virus of turkeys represents a distinct class of avian type-C retrovirus. *Gene* 122: 349.

Sarid R, Chajut A, Gak E *et al* (1994) Genome organization of a biologically active molecular clone of the lymphoproliferative disease virus of turkeys. *J. Virol.* 204: 680.

Yaniv A, Sarid R, Chajut A *et al* (1995) The lymphoproliferative disease virus (LPDV) of turkeys. *Isr. J. Vet. Med.* 50: 87.

LYMPHOCYTIC CHORIOMENINGITIS VIRUS (*ARENAVIRIDAE*)

Contents

General Features

Raymond M. Welsh, Department of Pathology, University of Massachusetts Medical Center, Worcester, Massachusetts, USA

History

Lymphocytic choriomeningitis virus (LCMV) is an etiological agent for human 'acute aseptic meningitis' and 'grippe-like' infections and is maintained in nature by life-long persistent infections of mice (*Mus musculus*). Three strains still being studied today were initially isolated in North America in the 1930s. During an attempt to recover and passage virus from a suspected human case of St Louis encephalitis, C. Armstrong and R. Lillie isolated LCMV (Armstrong strain) from a monkey that developed a lymphocytic choriomeningitis. E. Traub next reported the isolation of a serologically indistinguishable virus contaminating his mouse colony (Traub strain), and T. Rivers and T. Scott isolated similar viruses from human meningitis patients (one being the WE strain). Subsequently, many other isolations from humans and animals were made, and a clear cause-and-effect relationship between LCMV and about 8% of the North American cases of human nonbacterial meningitis was established.

The LCMV infection of the mouse soon became an important model system for studying viral immunology. A distinguishing feature of the LCMV infection was that it established a long-term persistent infection in mice infected *in utero* or shortly after birth, whereas adult mice inoculated with LCMV either cleared the virus with lasting immunity or, in the case of an intracranial infection, died of a lethal meningoencephalitis. Antiviral antibody was difficult to detect in the persistently infected mice, and this led Burnet and Fenner to postulate that exposure to viral antigen before the maturation of the immune system resulted in mice becoming immunologically tolerant to LCMV and thus unable to clear the infection. It was this system that provided the basis for Burnet's theories of immunological tolerance that have stood the test of time. Subsequent work by Oldstone and Dixon, however, demonstrated that persistently infected mice did make antiviral antibody which was difficult to detect because of excess viral antigen. The antiviral antibody traveled in the circulation complexed to virus and complement, and these circulating as well as tissue-bound 'immune complexes' contributed to a progressive degenerative disease involving glomerulonephritis, arteritis, and chronic inflammatory lesions. In the acute infection both the clearance of the virus and the lethal meningoencephalitis were shown to be due mostly to the cytotoxic T lymphocyte (CTL) response. Antiviral CTL were first demonstrated in the LCMV model, and Zinkernagel and Doherty used this model to demonstrate the important concept of major histocompatibility complex (MHC)-restriction in CTL recognition. Persistently infected mice were eventually shown to have 'split-tolerance' to LCMV, in that although they could mount an antibody response to LCMV they could not generate the LCMV-specific CTLs which were needed to clear the infection.

Classification

LCMV is the prototype virus of the genus *Arenavirus* in the *Arenaviridae* family of RNA viruses, and although it has some homology with all arenaviruses,

expression studies in several different vector systems (plasmids, vaccinia virus and baculovirus). The vaccinia vectors have been used extensively by Whitton and Oldstone to map CTL epitopes within NP and GPC. Each viral structural protein contains a few linear amino acid sequences that represent major CTL determinants, but recognition is profoundly influenced by MHC haplotype.

Several independent lines of transgenic mice expressing LCMV cDNA genes have been produced in the laboratories of Oldstone and Zinkernagel. These mice display a T-cell nonresponsive (tolerant) phenotype towards the viral transgene products. However, infection of these transgenic mice with LCMV appears to overwhelm the non-responsive state leading to 'autoimmune-like' tissue damage in locations where the transgenes are expressed. Many details relating to the induction of the pathogenic immune response still remain to be clarified. The homologous nature of the system (expression of genes from a mouse virus in mice with the possibility to challenge with the original virus) should produce substantial new information on the nature of virus-induced immune responses and pathogenic events in the LCMV-infected LCMV transgenic mice.

See also: **Defective interfering viruses; Lassa, Junin, Machupo and Guanarito viruses (*Arenaviridae*); Persistent viral infection; Vectors: Animal viruses.**

Further Reading

Borrov P. (1997) Mechanism of viral clearance and persistence. *Journal of Viral Hepatitis* 4 (supp 2): 16–24.

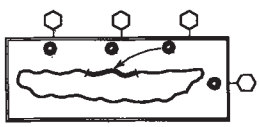
Howard, CR (1986) Arenaviruses. *Perspectives in Medical Virology*, vol. 2, Amsterdam: Elsevier.

Oldstone MBA (ed.) (1987) *Current Topics in Microbiology and Immunology*, vols 133 and 134. Berlin: Springer-Verlag.

Salvato MS (ed.) (1993) *The Arenaviridae*. New York: Plenum Press.

[This article is reproduced from the 1st edn (1994).]

LYSOGENY AND PROPHAGE



Max Gottesman, Institute of Cancer Research, Columbia University College of Physicians and Surgeons, New York, USA

Arnos Oppenheim, Department of Medical Genetics, Hebrew University, Hadassah Medical School, Jerusalem, Israel

Introduction

The phenomenon termed lysogeny is discussed here, mainly through the perspective of the coliphage λ in the family *Siphoviridae*, genus 'λ-like viruses'. Lambda lysogeny was the first to be understood in detail, and remains the best studied example of the process. Furthermore, it is probable that the principles derived from the study of λ lysogeny will be applicable, *mutatis mutandis*, to other lysogenic phage.

General Features

The chromosome of coliphage λ is 48 514 bp, containing about 50 genes, and is fully sequenced. The λ chromosome is present in the virion as double-stranded DNA, with 12 bp 5′ cohesive overhangs. The order of genes in the virion is unique, and the viral DNA is injected through the tail in a polar fashion. The injected DNA is rapidly circularized and negatively supercoiled by the bacterial enzymes DNA ligase and gyrase, respectively.

Unlike the virulent T phages, λ is a temperate phage; a portion of infected cells survive. Most of the survivors become lysogens, and carry the λ as a stable, mostly inert, prophage integrated in the bacterial chromosome. The lysogenic state – the presence of a prophage – can be detected in several ways. Lysogens are immune; they are not killed by superinfecting λ virions. The inert prophage is carried like a set of bacterial genes, and is passed on from generation to generation. However, certain treatments induce, or activate, the prophage. The induced prophage excises from the bacterial chromosome, replicates, packages itself, and lyses its host, releasing several hundred phage particles.

Because of its ability to lysogenize, λ makes a turbid plaque on a lawn of sensitive bacteria growing

on an agar surface. A plaque grows from a single phage killing a bacterium. The phage progeny diffuse through the agar, creating a circular, cleared area where additional bacteria have been killed. The turbid center of the λ plaque represents the lysogenic survivors growing amid a sea of λ virions. Lambda mutants unable to lysogenize are recognized by a clear plaque phenotype. Clear mutants kill all infected cells. Similarly, λ makes clear plaques on host mutants which cannot by lysogenized by λ. Finally, under conditions where λ lysogenizes 100% of infected cells, it makes no plaques at all.

The λ virion genetic map consists of blocks of functionally related genes, ordered with respect to time of expression after infection. Genes that are expressed very early are located near the center of the virion map and encode regulatory functions. The next blocks of genes to be transcribed include regulatory functions, as well as functions for DNA replication, recombination and integration. At the right end of the virion map are late genes involved in cell lysis, whereas the left virion end carries late genes encoding packaging, head and tail proteins. Joining of the two virion DNA ends after infection creates a single late gene operon, expressed from a single promoter.

The genetic map also links λ functions and the site upon which they act. For example, the λ O gene activates the λ origin of replication (*ori*) which lies within O. Cleavage of circular λ DNA to form the virion chromosome is accomplished by genes lying adjacent to *ter*, the cleavage site. This linkage reflects function–site specificity. Lambda recombines extensively with related (*lambdoid*) phage. Linkage reduces the chances of forming recombinants with functions incompatible with their sites of action.

The λ prophage is usually present as a single copy. Phage integration results from recombination between two unique sites, *attP* and *attB*, located on the phage and bacterial chromosomes, respectively. The *attB* site is located near the bacterial *gal* operon. The *attP* site is approximately in the center of the virion map. As a result of integration, therefore, the λ prophage gene order is a unique circular permutation of the virion genetic map. Indeed, the change in gene order upon λ integration provided the first evidence for circular DNA in bacteria.

Lysogeny is advantageous to λ because it offers a means of survival under adverse conditions. Free phage is sensitive to environmental stresses, to which a prophage, carried in a lysogen, is resistant. The frequency of lysogenization generally increases with conditions disfavoring lytic phage growth. If the bacteria are growing vigorously, and can support phage propagation, λ tends to enter the lytic rather that the lysogenic pathway. Conversely, infection of starved bacteria leads to lysogenization rather than cell killing. After several rounds of lytic growth, the supply of bacteria becomes limited and further lytic growth is disadvantageous. The surviving bacteria, which are attacked simultaneously by many phage, will tend to be lysogenized by infecting λ.

The prophage responds to changes in the environment that portend the death of the lysogen. In particular, agents that cause DNA damage will induce the prophage, allowing the newly assembled virus particles to escape the dying cell. Supporting the lysogenic lifestyle is expensive, and λ has evolved a complex genetic system to allow efficient lysogeny and induction. The phage genes directly involved in lysogenization, and which are dispensable for the lytic response, can be grouped in two classes: (1) genes required for establishing the lysogenic state; and (2) genes required for maintaining the prophage in a quiescent (repressed) state. These include genes needed for the regulation of other lysogeny-specific genes, for the integration of the prophage into the chromosome or for the excision of the prophage after induction. Mutation of either gene class prevents lysogeny, but for different reasons. Mutations blocking prophage repression lead to a clear plaque phenotype. Phage unable to integrate form turbid plaques (they can establish repression) but the surviving cells are not lysogenic. They are, instead, abortive lysogens. The infected cells carry repressed but unintegrated prophage. The repressed prophage cannot replicate autonomously, and are diluted away with cell division. Phage mutants unable to excise form turbid plaques containing stable lysogens. Although induction of these lysogens leads to cell death, viable phage particles are not released. The induced prophage remains bound to the bacterial chromosome, present in a gene order that cannot be appropriately expressed or packaged.

Mutations

Phage mutations that affect repression

Phage mutants that cannot repress lytic functions form clear plaques (λ*c* mutants). The mutants fall into two categories: (1) those which never lysogenize; and (2) others which rarely lysogenize. The latter class can be complemented by wild-type phage, or by phage mutant in a different complementation group.

The phenotypic difference between the two mutant classes reveals the components of the lysogenic response. A single prophage gene product is required to maintain λ in the quiescent state. This protein, the cI repressor, binds to two operator sites, oL and oR, that control two promoters, pL and pR. The pL and

pR promoters initiate transcription early in the lytic pathway, and the expression of the λ lytic functions depends upon their activity. By blocking pL and pR, the repressor prevents the transcription of most of the λ chromosome, maintaining λ as a prophage. The repressor also attaches to the operators of superinfecting phage, blocking the expression of their lytic genes as well; it therefore maintains λ prophage and makes the lysogen immune to superinfection.

The *c*I gene is the structural gene for λ repressor, hence the inability of λ*c*I mutants to form lysogens. Conditional *c*I mutations, such as the temperature-sensitive *cIts857* allele, have been isolated. λ*cI857* makes turbid plaques and forms lysogens at permissive (32°C) temperature. At the nonpermissive (42°C) temperature, λ*cI857* forms clear plaques and kills all infected cells. Lysogens obtained at 32°C can be maintained at that temperature. Shifted to 42°C, the repressor protein denatures, and the prophage is induced.

Two other clear-plaque mutants, λ*c*II and λ*c*III, define functions required for the initial synthesis of repressor in infected cells. The *c*II and *c*III genes are not needed to maintain λ as a prophage and, indeed, are not expressed in lysogens.

The observation that two λ functions, *c*II and *c*III, were needed only to initiate *c*I expression suggested that repressor positively regulates its own synthesis. An experiment showing that repressor was necessary and sufficient for *c*I expression was performed by Eisen, Periera de Silva and Jacob. These workers constructed a lysogen bearing a defective λ*c*I857 prophage that could not express lytic genes or genes *c*II and *c*III. The lysogen could be grown at 32°C or 42°C. As expected, it was immune to superinfection at the former but not at the latter temperature. However, lysogens grown at 42°C and then returned to 32°C did not regain immunity. Repressor synthesis had stopped in the absence of active repressor, and could not restart at the permissive temperature. Because prophage express repressor and few other functions, Eisen and his colleagues concluded that repressor was an obligatory activator of *c*I expression in lysogens.

Other λ mutants that form clear plaques define the sites of action of the *c*I and *c*II products. These mutations are not conditional, i.e. ambers or *ts*, indicating that they do not lie in the structural genes for proteins. The *cY* mutation defines a promoter, pRE, which is activated by *c*II, and which transcribes *c*I. Like *c*II mutants, λ*cY* mutants cannot initiate repressor synthesis on infection, but can form stable lysogens if provided with *c*I repressor by a complementing phage at the time of infection. The pRM mutation inactivates the repressor autoregulatory site. The pRM promoter is responsible for *c*I transcription in lysogens. The λpRM mutants form lysogens, but these are unstable. Unable to stimulate its own synthesis, repressor formed upon infection dilutes away, and the lysogen eventually induces.

The complementation pattern of the λ*c* mutants is distinctive. Complementation is tested by mixing mutant phage together, and spotting on a susceptible host. In the following complementation matrix, '−' indicates a clear spot and '+' indicates complementation, i.e. a turbid spot and the formation of lysogens.

	λ*c*I	λ*c*II	λ*c*III	λpRE	λpRM
λ*c*I	−	+	+	−	−
λ*c*II	+	−	+	+	+
λ*c*III	+	+	−	+	+
λpRE	−	+	+	−	+
λpRM	−	+	+	+	−

A consideration of the regulatory principles of λ can explain the above pattern. For example, λ*c*I cannot complement a λpRE mutant. A λ*c*I mutant can never lysogenize, and a λpRE mutant can only lysogenize if it can initiate repressor synthesis. Although the λ*c*I mutant can supply *c*II and *c*III products, these cannot act on the mutant pRE. What is required to initiate repressor synthesis in a pRE mutant is *c*I product, and this cannot be expressed by the λ*c*I mutant. In contrast, a λ*c*II, λ*c*III or λpRM mutant can all supply *c*I product to a λpRE mutant. The λ*c*II × λpRE and the λ*c*III × λpRE complementation assays are reciprocal. Lysogens carrying either member of the complementing pair can be recovered. In the case of complementing pairs that include λ*c*I or λpRM, it is the other partner that lysogenizes; stable λ*c*I or λpRM lysogens cannot form.

The final class of clear-plaque mutants inactivate the oL and oR operators. These mutants are virulent; unlike the λ*c* mutants they are not subject to immunity, and form plaques on λ lysogens. Naturally, they cannot lysogenize, and cannot be complemented by co-infection with a wild-type λ.

The λ*cro* product influences that balance between lysis and lysogeny. Cro protein, like cI repressor, binds to oL and oR. However, Cro inhibits *c*I transcription. Cro is essential for λ lytic growth. A λ*cI857cro* mutant fails to form plaques at 32°C. Infected cells overexpress cI; every infected cell is lysogenized. Cro also inhibits pL and pR, starting a

few minutes after infection. This inhibition is essential for λ development. Thus, *λcI857cro* does not grow at 42°C. In the absence of both cI and Cro, overexpressed pL and pR operon genes prevent phage development.

The analysis of heteroimmune phage, like the characterization of *λc* mutants, helped to define the regulatory loops in repressor synthesis. The various lambdoid phage have repressor and operators that function in a fashion similar to λ. The repression system can be phage-specific, however. For example, phage 434 forms lysogens that are resistant to 434 but sensitive to λ. Conversely, phage 434 forms plaques on λ lysogens. The biochemistry of this specificity is known. Lambda repressor does not bind to 434 operators and 434 repressor does not bind λ operators. The two phages are, nevertheless, remarkably similar, and readily recombine. By repeatedly recombining λ and 434, a phage that is almost entirely λ, but still has the repression profile of 434, was created. This phage, *λimm434*, defines a small continuous region (the immunity region) encoding the genes required for immunity specificity. These are the *cI* and *cro* genes of 434 and their sites of action at the oL and oR operators.

Host mutations that affect repression

Escherichia coli mutations that influence the lytic–lysogenic decision have been isolated and characterized, although their mechanism of action often proves elusive. Mutations inactivating the CRP–cAMP system cause λ to enter the lytic pathway. Based on the behavior of *crp* or *cya* mutants, it is likely that λ tends to lyse rapidly-growing cells because they have low intracellular cAMP levels. No CRP–cAMP-dependent λ promoter has been detected, suggesting that CRP–cAMP may regulate a host function required for lysogenization.

Mutations in *rnc* (RNaseIII) or *him/hip* also favor the λ lytic response. The genes *him* and *hip* encode the two subunits of integrative host factor (IHF) a low molecular weight histone-like protein. IHF binds in the DNA minor groove at specific sites in λ, and introduces a bend that exceeds 140°. The translation of the λ *c*III gene is reduced in *rnc* hosts, whereas *c*II translation is inhibited by *him* or *hip* mutations.

Mutations in the host *hfl* genes have the opposite effect on λ lysogeny; infection of *hfl* mutants leads exclusively to lysogenization. HflB (also designated FtsH) was shown to be a membrane bound, ATP-dependent protease that is responsible for the instability of *c*II protein. Transcription of *hflB* increases following heat shock. HflB degrades sigma^{32} as well as *c*II protein; both reactions are inhibited by *c*III protein.

Phage mutants defective in integration or excision

In addition to establishing and maintaining repression, the λ lysogenic pathway demands that the prophage be inserted in the bacterial chromosome. The integrated prophage must be stably inherited, i.e. not readily lost upon bacterial replication. Furthermore, upon induction, the activated prophage must detach rapidly, and replicate autonomously.

Lambda integration and excision is mostly promoted by phage-encoded functions. The host recombination pathways do not meet the needs of λ. The host pathways are relatively inefficient and are not polar. If phage integration occurred by recombination between extensive homologous segments in λ and *E. coli*, the resulting prophage would segregate frequently.

Lambda *int* mutants were isolated as turbid plaque formers that failed to lysogenize. The *int* mutation defines a function; *int* mutants can be complemented to form stable lysogens. The *λint* lysogens appear normal, and lyse after induction, but the lysate carries few viable phage particles. Thus Int is required for both integration and excision. In contrast, *xis* mutants integrate with wild-type efficiency, but fail to yield phage particles on induction. Neither the *int* nor the *xis* mutations affect the ability of λ to undergo generalized recombination. The Int and Xis proteins recognize sequences in *attP* and *attB* and promote site-specific recombination between them. Interestingly, λ also encodes functions that promote generalized recombination but the mutations in these genes, *redα*, *redβ* or *orf*, do not affect λ lysogeny.

Mutations in the λ *c*II gene also inhibit phage integration. In addition to promoting *c*I transcription, cII is needed for the transcription of *int* in infecting phage.

Phage mutations in the *λatt* site form turbid plaques and fail to lysogenize. Some of the mutants carry part of *λatt*, and arise by Int-promoted recombination between *λatt* and sites resembling *λatt* that lie in the phage *b* region. These *λb* mutants cannot be complemented by wild-type phage. Instead, co-infection leads to the formation of multiple lysogens, carrying tandem helper and *λb* mutant prophage.

Host mutants that block λ integration and excision

Only one type of host mutation is known that, by itself, directly affects λ integration and excision. *E.*

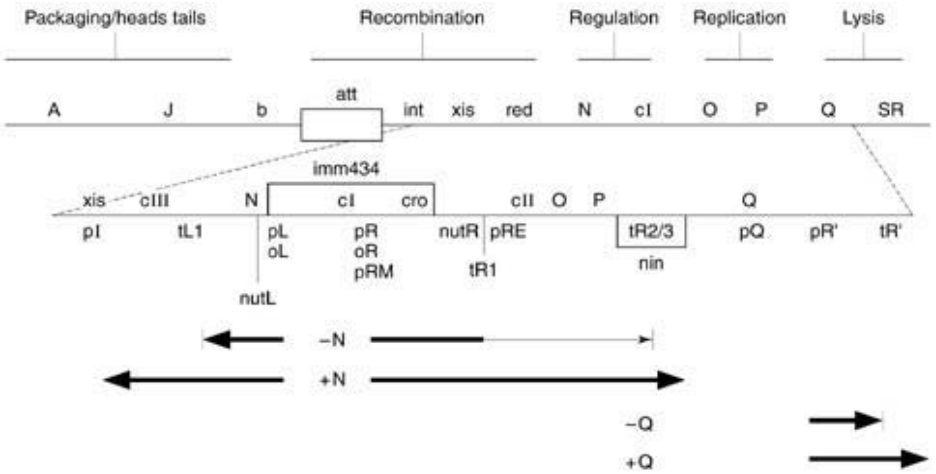


Figure 1 Map of lambda (not drawn to scale). Top line: the complete genome showing the organization by functional units and the location of a few key genes. Middle line: an expansion showing the organization of the λ immunity region and the extent of the nonhomology with phage 434. Bottom two lines: transcription patterns in the presence and absence of N. (Reproduced with permission, Cold Spring Harbor Laboratories.)

coli him or *hip* mutants do not support λ site-specific recombination. Recall that IHF plays a second role in the lysogenic response; it is required for *c*II translation, and thus for repressor expression.

The reconstitution of a purified cell-free λ integration-excision system confirms the genetic analysis. Int, Xis and IHF are sufficient to promote λ site-specific recombination. The *attP* site must be present in a supercoiled DNA molecule; *attB* is reactive in linear form. Under special circumstances, a second host factor, Fis, stimulates the excision reaction, but the physiological relevance of this is not clear.

How λ Establishes Lysogeny

After infection or induction, the transcription of the λ chromosome initiates at two promoters, pL and pR (**Fig. 1**). In the absence of phage protein synthesis, the pL transcript ends just promoter-distal to gene N, at the tL1 transcription termination site. The pR transcript is attenuated at site tR1, located just promoter-distal to *cro*, and terminates completely at tR2 and tR3, located in the *nin* region.

This pattern of abortive transcription is altered by the λ N gene product. The λ N protein suppresses transcription termination, allowing transcription to proceed outward into the phage chromosome. This mechanism of positive gene regulation is referred to as antitermination, and has now been described in higher eucaryotic systems. The expression of HIV appears to be regulated in a similar way by the product of the viral TAT gene. The mechanism of antitermination in λ is fairly well understood. N protein binds to sites in the pL and pR operon transcripts called *nut*. N, *nut* RNA, RNA polymerase and a set of host 'Nus' proteins, form a termination-resistant transcription complex. The λ *nut* sequence is not found in the *E. coli* chromosome, so the antitermination activity of N is restricted to the phage.

The extension of the pL and pR transcriptions under the influence of N activates other genes, including a second antitermination function, gene Q. The mechanism of action of Q is quite different from N. Unlike N, Q is a DNA-binding protein with a binding site in the pR′ promoter region. Transcription initiated at pR′ pauses after transcribing 16–17 nucleotides of the λ late transcript. This pause permits the modification of the transcribing RNA polymerase by Q protein. Terminators in the pR′ operon are suppressed, allowing expression of the λ late genes. The *c*III gene is transcribed from pL and *c*II from pR. The *c*II protein activates three promoters that are related not only by sequence but by function as well; all are required for successful lysogeny. The pRE promoter transcribes *c*I and the paQ promoter transcribes leftward (toward pR) from within Q. Like mutations in pRE (*c*Y), mutations in paQ present as a clear plaque phenotype. It is likely that convergent transcription from pRE and from paQ reduce the activity of pR, and thus the expression of lytic functions.

The third promoter activated by *c*II producer is pI, located within *xis* and transcribing leftward into *int*. The expression of *int* after infection derives exclusively from pI. This is surprising, as *int* is included in

the pL transcript. The failure of *int* to be expressed from pL is due to the instability of the pL transcript which, under the influence of N, extends into the *b* region. Within the *b* region, and present in the pL transcript, is the *sib* site. The pL transcript is cleaved at *sib* by RNaseIII and then rapidly degraded from the cleaved 3′ end into *int* by two exoribonucleases, polynucleotide phosphorylase and RNaseII. The pI transcript lacks *nut* and is not acted upon by N. It terminates before *sib*, and is relatively resistant to degradation.

The coordinate expression of *c*I and *int* by *c*II is important for successful lysogeny. On induction, however, rapid prophage excision is essential for the lytic response. Unlike infecting phage, *int* gene expression does not derive from the *c*II-dependent pI promoter. Instead, Int is expressed from pL. The relevant difference between the pL transcripts of infecting phage and induced prophage lies at the 3′ end. Integration at *attP* results in a genetic permutation that places the *b* region at the extreme prophage right. Thus, the prophage pL transcript does not include *sib*, and is stable.

The regulation of gene expression by sequences 3′ to the gene is called retroregulation. By making Int expression dependent on *c*II, λ reduces the chances of integration during the lytic cycle, when *c*II levels are likely to be low.

The *xis* gene is expressed exclusively from pL. In addition to promoting prophage excision, Xis inhibits integration. This inhibition, in theory, might be of benefit to excised and replicating prophage. Unlike Int, Xis is extremely unstable. In the lysogenic pathway, an infecting phage synthesizes phage functions for only a few minutes and is then repressed. Initially, an equilibrium between inserted and free phage may be present. As Xis decays, this equilibrium is shifted towards the integrated state, as Xis is required for excision.

The *c*II protein binds to pRE and pI at two TTGC sequences located in the −35 regions of the two promoters. *c*II is thought to bind as a multimer. The concentration of *c*II may be the most critical factor in determining the frequency of lysogenization. It is likely that high multiplicities of infection favor lysogenization because elevated *c*II concentrations are achieved early.

Maintenance of the λ Prophage

The λ oR region

The right operator region of λ is the site of interplay of two regulatory factors, the *c*I and Cro proteins. In addition, it includes two divergent promoters with antagonistic functions, pR and pRM. The pR promoter is repressed in a lysogen, is fully active early in the lytic response, and then is dampened later during lytic development. The pRM promoter is active in a lysogen and is repressed in the lytic pathway. *In vitro* studies show that the two promoters interfere with each other. The *c*I and Cro proteins determine which promoter is recognized productively by RNA polymerase.

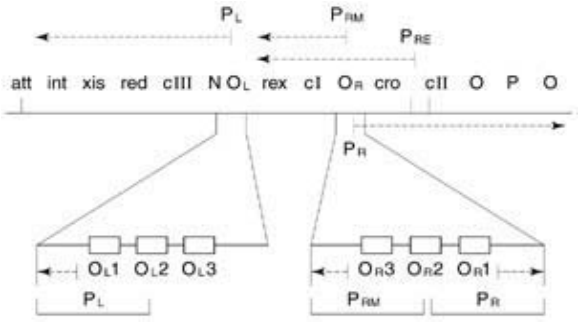


Figure 2 Regulation of λ *c*I and early lytic transcription. Transcription of early λ genes is initiated at pL and pR, and is subject to repression by *c*I repressor acting at oL and oR, respectively. Transcription of *c*I can be initiated either at pRM or pRE. Transcripts are indicated by dashed lines. The expanded diagrams illustrate spatial relationships between pL and three repressor binding sites in oL, and among pRM, pR and three repressor-binding sites in oR. Cro protein also binds at oR and oL to reduce transcription from pR, pRM and pL. (Reproduced with permission, Cold Spring Harbor Laboratories.)

Despite the complexity of the regulatory network at oR, the mechanism of oR control is understood in some detail (**Fig. 2**). There are three 17 bp repeated sequences in oR to which *c*I repressor binds. The repeats have dyad symmetry and recognize a repressor dimer. Each subunit of dimer binds one half-site.

The repeats are not identical, and have different affinities for *c*I protein *in vitro*. The oR1 sequence has the highest affinity, and is filled at the lowest *c*I concentrations. Although the oR2 and oR3 elements have equal 'intrinsic' affinities for *c*I, *c*I bound at oR1 interacts cooperatively with *c*I bound at oR2. Thus oR2 binds *c*I before oR3.

Cooperativity between *c*I dimers has another important consequence. It causes small decreases in *c*I concentration to have a major effect on repression. Unlike the *lac* operon, therefore, the induction of which is linear with respect to inducer concentration, induction of λ prophage is rapid and complete.

When *c*I is bound at oR1 and oR2, RNA polymerase is occluded from binding to pR. At the same time, the activity of pRM is enhanced. In part,

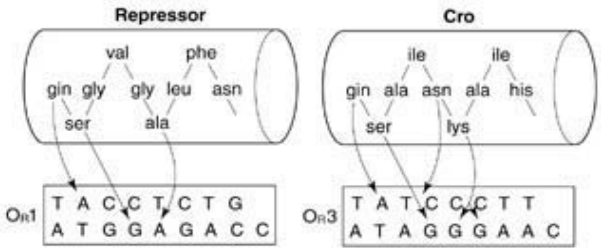


Figure 3 Pattern of amino acid–base pair interactions at oR. Shown are contacts between the amino acids of the *cI* repressor and Cro recognition helices and oR1 and oR3 half-sites, respectively. (Reproduced with permission, Blackwell Scientific Cell Press.)

this positive autoregulation results from the exclusion of RNA polymerase from pR. Additionally, the *cI* protein is a direct activator of transcription at pRM. Contacts between *cI* bound at oR2 and RNA polymerase bound at pRM enhance the rate of open complex formation about sevenfold. In theory, *cI* at high concentrations would bind oR3 and inhibit pRM. In the lysogen, *cI* is bound to oR1 and oR2; binding at oR3 has not been demonstrated.

The three mutations that confer virulence to λ lie in oR1, oR2 and oL. The oR1 and oR2 mutations reduce the binding of *cI* at oR, leading to constitutive pR expression. Constitutive pL expression provides N, which is required for λ late gene expression.

The order of addition of Cro to the oR sites is the opposite of *cI*. Cro binds as a dimer first to oR3, where it represses pRM. At higher concentrations of Cro, oR2 and oR1 are filled, reducing pR activity as well. Cro is a smaller protein than *cI*; cooperative interactions between Cro dimers bound at oR do not occur. At physiological levels of Cro, pR retains considerable activity.

The cI repressor

The *cI* protein has been studied in great detail. Its structure has been determined both by crystallographic analysis of *cI* and *cI*–operator DNA complexes, and by nuclear magnetic resonance (NMR) spectroscopy. These studies, as well as experiments using chemical probes, have demonstrated the contact points between *cI* and the operator.

The *cI* gene product is a 26 kDa, 236 amino acid protein that is organized in two distinct domains. The amino domain (residues 1 through 92) contacts operator DNA. Residues 93 through 132 form an unstructured bridge that connects the amino domain to the carboxyl domain (residues 133 through 236). The carboxyl domain is responsible for cooperative interactions between adjacent *cI* dimers. The carboxyl domains of *cI* monomers also interact, stabilizing the *cI* dimer. Interactions between the amino domains also contribute to dimer stability.

Prophage induction occurs when the two domains are separated by proteolytic cleavage between bridge region residues Ala111 and Gly112. Without the cooperative interactions provided by the carboxyl domain portion, the affinity of amino domain for oR is insufficient to sustain repression. Wild-type λ is induced by DNA-damaging agents, which activate RecA and induce the SOS response. The activated RecA binds to *cI* monomer and stimulates an intramolecular autocleavage reaction that involves an attack by the Lys192 residue on the Ala111–Gly112 peptide bond. Mutations in *cI* that prevent induction, called *ind,* lie at the cleavage site or at the RecA binding site.

The amino domain is folded into five α helices. When *cI* repressor is bound to the operator, the second α helix lies above the DNA major groove and serves to position α helix 3, the recognition helix, within the major groove. The recognition α helices of the *cI* monomers each sit in the major groove on the same DNA face and one DNA helical turn apart. Three amino acids in the recognition α helix, Gln, Ser and Ala, make contacts with oR base pairs (**Fig. 3**). A Gln residue, located just before α helix 2, contacts DNA phosphate residues. Finally, the first six residues of repressor, which are flexible, reach behind the DNA helix to make contacts with bases and the phosphate backbone within the operator on the opposite helix face.

The *cI* p^c mutations lie in or adjacent to residues in *cI* that are closest to RNA polymerase bound at pRM. The mutations are thought to prevent the contacts between *cI* and RNA polymerase that promote the formation of open complex at pRM. Although incapable of stimulating RNA polymerase at pRM, *cI* p^c binds to oR DNA and represses pR.

Cro

The structure of Cro is very similar to that of *cI*, as expected for proteins that bind variants of the same DNA sequence. As in *cI*, the Cro α helix 2 positions the recognition α helix 3, which inserts within the DNA major groove. The Gln and Ser residues of the *cI* α helix 2 are conserved in Cro, where they make the same base contacts. Instead of Ala, the Cro recognition helix includes an Asn and a Lys residue that make additional base contacts. The differences in α helix 2 account for the preference of *cI* for oR1 and of Cro for oR3.

Integration and Excision

The integration and excision of λ is a highly efficient recombination process. As shown by the analysis of mutations, it involves two phage-encoded functions, Int and Xis, which act upon the two recombining sites, the phage *attP* and the bacterial *attB* sites. The host IHF function is required; a second host function, Fis is stimulatory.

The amount of homology between *attP* and *attB* is small (15 bp) and the reaction is dependent upon the recognition of specific *att* sequences by Int and Xis. *attP* is considerably more complex than *attB*. Int recognition elements in *attP* are found from about −150 bp to +90 bp relative to the crossover region. There is only one *attB* site in *E. coli,* although *attB*-deleted strains can be lysogenized at low frequency by Int-promoted recombination at secondary *attB*-like sites.

The 40 kDa Int protein has several biochemical activities. It binds DNA with two distinct sequences. It is a topoisomerase type I, and can also act as a resolvase on *attP–attB* Holliday junctions.

The *att* sites are displayed in **Fig. 4.** The *attP* site consists of several regions with different roles in the site-specific recombination reaction. The arms of *attP* bind Int with high affinity at a consensus sequence, C/AAGTCACTAT, that is repeated several times. In the center of *attP* is the core region, consisting of a 15 bp *O* sequence that is also found in *attB*. Flanking *O* are second consensus Int binding sequences, CAACTTNNT. A single Int molecule binds to both arm and core sequences. Because the binding of Int to the latter is weak, Int bound to the arm sites must be brought close to the core. This is accomplished by binding *attP* DNA; IHF binds to specific *attP* sites and introduces a strong bend. The binding of Int to the core is IHF and DNA helix face dependent.

Int cleaves within the *O* regions of *attP* and *attB* to create a 7 bp overhang. The initial cleavage is in the top strand of *O*. Strand exchange creates a Holliday structure that is resolved by single-strand exchange between bottom strands. The Int cleavage reaction passes through an (*O*)-phosphotyrosine ester intermediate between Tyr342 of Int and the 3′-PO_4 terminus of the cleaved *O* sequence. In this respect, Int resembles other topoisomerase I enzymes, which also form an (*O*)-phosphotyrosyl linkage. Although intermediates in Int-promoted recombination can be isolated through genetic or biochemical manipulations, the reaction is normally concerted.

Note that as a result of recombination between *attP* and *attB,* two *att* sites are created that are different from either parent. The *attL* site, which lies to the left of the prophage, carries the right arm of *attP;* the *attR* site, lying to the right of the prophage, carries the *attP* left arm. Recombination between *attL* and *attR,* which leads to prophage excision and reformation of *attP,* requires Xis function. The binding sites of Xis and Fis are also shown in **Fig. 4.** How Xis blocks integrative recombination and promotes excisive

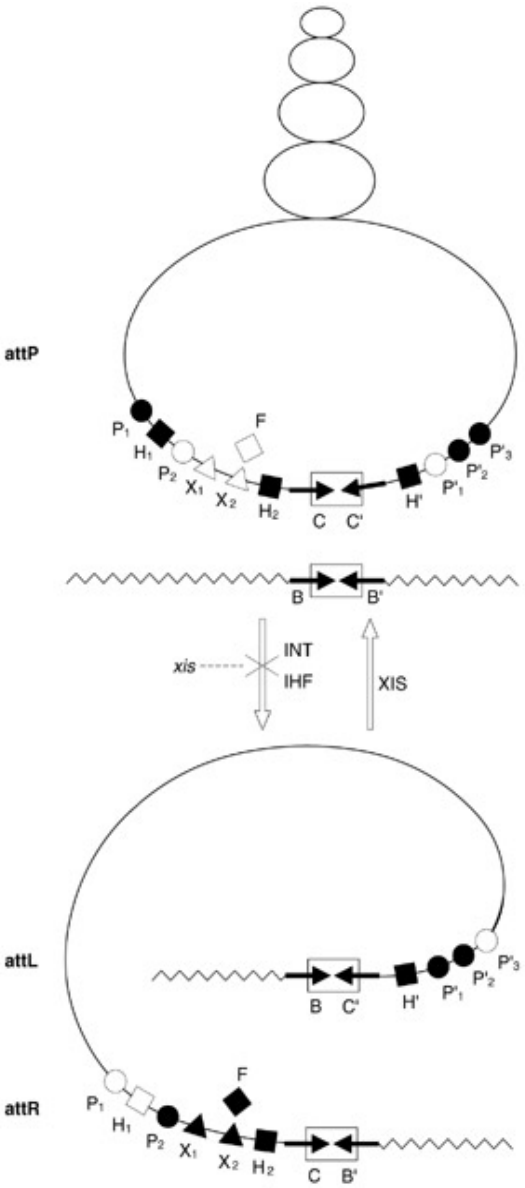


Figure 4 Integrative and excisive recombination pathways. The protein-binding sites for arm-type Int (○), core-type Int (→), IHF (□), Xis (△) and FIS (◇) are indicated by filled symbols when that site is occupied by its cognate protein to make a competent recombination partner for integrative (⇓) or excisive (⇑) recombination. Proteins required for each reaction (Int, IHF and Xis) are in bold. Inhibition by Xis indicated. (—), supercoiled λ chromosome; (〰), bacterial chromosome. (Reproduced with permission.)

recombination is not entirely clear, although the rationale for the effect is evident.

The outcome of phage λ infection is determined by specific proteolysis of the N antiterminator and the *c*II activator. Controlled protein degradation in regulatory cascades has been shown to be conserved in all organisms, controlling diverse processes such as growth and apoptosis.

See also: **Coliphage lambda (*Siphoviridae*).**

Further Reading

Hendrix R, Roberts J, Stahl F and Weisberg R (eds) (1983) *Lambda II*. New York: Cold Spring Harbor Laboratory.

Ptashne M (1992) *A Genetic Switch*. Cambridge, MA: Blackwell Scientific/Cell Press.

M

MACHLOMOVIRUSES (*TOMBUSVIRIDAE*)

Steven A Lommel, Department of Plant Pathology, North Carolina State University, Raleigh, North Carolina, USA

History

Maize chlorotic mottle virus (MCMV) was first isolated from maize in Peru and named in 1973 and has since been reported in Argentina and Mexico. In 1976 MCMV was recovered from field corn along the Nebraska–Kansas border in the USA, where it is now endemic. In 1990, a severe outbreak of the virus was reported in winter corn breeding plots on the Island of Kauai Hawaii, USA. In 1978, it was shown that MCMV in co-infections with any corn infecting member of the *Potyviridae* produced a synergistic interaction resulting in a lethal disease termed corn lethal necrosis (CLN).

Taxonomy and Classification

MCMV is the type species and the only member of the *Machlomovirus* genus. Based on significant amino acid sequence homology in the viral replicase, the *Machlomovirus* genus has been placed within the family *Tombusviridae*. Recently, panicum mosaic virus, also known as Saint Augustine decline, has been sequenced and shown to be similar in genome organization and sequence homology to MCMV. It is likely, however, that a new genus will be created for panicum mosaic virus. The monotypic *Machlomovirus* genus is defined by a virus with 30 nm icosahedral virions composed of 180 copies of a 25 kDa capsid protein packaging a single genomic RNA of 4437 nucleotides. The key taxonomic features distinguishing this genus from other genera in the *Tombusviridae* are capsid protein sequence and structure more closely related to members of the *Sobemovirus* genus, and an additional 5′ proximal open reading frame which largely overlaps the pre-readthrough domain of the replicase.

Properties of the Virion

The virus is a 30 nm sphere when observed under a transmission electron microscope after negative staining. The virions appear smooth and icosahedral in shape. Based on electron micrographic observations and sequence similarity of the capsid protein with that of southern bean mosaic sobemovirus, the virion is hypothesized to be a $T = 3$ icosahedral virion formed by 180 copies of the 25 kDa capsid protein subunit. The virion packages the 4437 nucleotide single-stranded RNA genome.

Properties of the Genome

The structure of the *Machlomovirus* genome is deduced from the complete nucleotide sequence of MCMV. The genome is composed of one positive-polarity, single-stranded RNA of 4437 nucleotides. The genomic RNA is infectious as naked nucleic acid. The genomic RNA is capped at the 5′ terminus with m^7GpppA and is not 3′-terminally polyadenylated. The RNA contains four open reading frames (ORFs). The first ORF initiates at the first methionine codon, 118 nucleotides from the 5′ terminus. The ORF is capable of encoding a 32 kDa polypeptide which is synthesized *in vitro*. This first ORF largely overlaps, in a different reading frame, the second ORF, which initiates at nucleotide 137 and is capable of encoding a 111 kDa polypeptide. This ORF is punctuated by an in-frame amber termination codon creating a pre-readthrough ORF encoding a 50 kDa polypeptide. Both the 50 kDa and 111 kDa polypeptides are expressed *in vitro*. Similarly, the internal ORF capable of encoding a 33 kDa polypeptide is interrupted by an in-frame opal termination codon yielding a pre-readthrough 9 kDa polypeptide (**Fig.**

1). Neither of these predicted polypeptides have been observed *in vitro*. The fourth and 3′ proximal ORF encodes the 25 kDa capsid protein. The capsid protein encoding ORF overlaps the 3′ half of the preceding ORF in a different translational reading frame.

Physical Properties

MCMV virions sediment as a single species with a sedimentation coefficient ($s_{20,w}$) of 109 S and have an equilibrium density of 1.365 g ml^{-1} in CsCl. The weight of a virion is calculated to be 5.9 mDa. MCMV in plant sap has a thermal inactivation point of 80–85°C.

Replication

Little is known regarding the specifics of *Machlomovirus* replication. It is assumed that MCMV replicates by producing a negative-sense complementary copy of the viral RNA. The viral replicase most likely recognizes a structural feature on the viral sense RNA enabling it to initiate synthesis of the full-length complementary strand. After completion of complementary strand synthesis, the viral replicase then recognizes the 3′ terminus of the complementary strand to initiate synthesis of progeny genomic RNA. This replication strategy dictates that prior to replication, viral replicase must be expressed from the original infecting genomic RNA. Consistent with this assumption, the putative replicase ORFs are located at the 5′ terminus of RNA, making them easily accessible to translation. The 3′-terminal capsid protein ORF is expressed *in vivo* from a 1.1 kb subgenomic RNA. Presumably, viral replicase recognizes an internal sequence including an 11 nucleotide element upstream of the capsid protein ORF initiation codon. This sequence element is identical to the 3′ terminus of the full-length complementary strand. Once bound, the replicase synthesizes the sense capsid protein subgenomic RNA. Based on significant similarities in the genome organization and homology of various proteins with members of the *Carmovirus* genus, it was expected that MCMV would produce a second subgenomic RNA to facilitate the expression of the internal ORF yielding the 9 kDa and 33 kDa polypeptides. However, no second subgenomic RNA has been detected.

Geographic and Seasonal Distribution

MCMV has been reported in several countries dispersed throughout the Western Hemisphere. To date, these countries include Argentina, Mexico, Peru and the USA. MCMV is most likely present but not yet reported in other Western Hemisphere countries as well. Based on the fact that vector *Diabrotica* species are strictly New World, MCMV has the potential to be present and become established in any maize-growing region within the Western Hemisphere. The recent report from Hawaii that MCMV can be transmitted by thrips significantly increases the potential range of the virus.

In Nebraska and Kansas, MCMV has been traditionally restricted to the Republican River valley which borders these two states. In Kauai, Hawaii the virus is present in the winter nursery maize breeding plots. MCMV is endemic in the Lima, Huaral, Chancay, Lurin and Canete valleys in the Department of Lima, Peru.

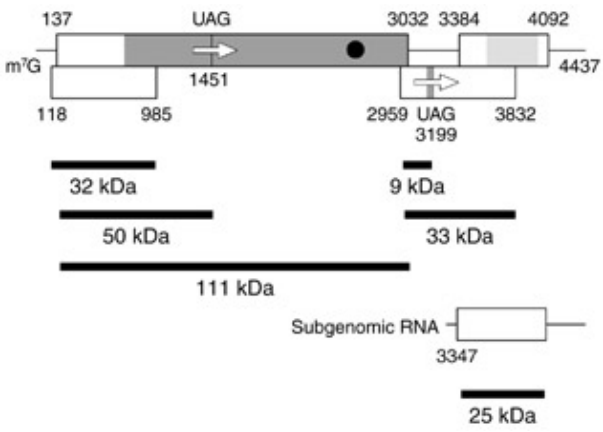


Figure 1 MCMV genome organization, polypeptides expressed and identification of conserved protein domains. The genomic and single subgenomic RNA are depicted as solid lines with open reading frames (ORFs) identified as open rectangles. Numbers above or below the borders of the ORFs identify initiation and termination codons defining the ORF. White horizontal arrows identify in-frame termination codons that must be periodically suppressed to allow expression of the readthrough polypeptides. Black bars below the RNAs represent viral encoded polypeptides predicted from the ORFs and, with the exception of the 9 kDa and 33 kDa readthrough polypeptides, proteins identified *in vitro*. Gray shaded region within the major ORF encoding the 111 kDa polypeptide identify domains with significant amino acid sequence similarity to polymerase proteins in the *Tombusviridae*. The black circle identifies the location of the canonical 'GDD' motif. The gray shaded region at the 3′ end of the ORF encoding the 9 kDa protein identifies an area of amino acid conservation with similar proteins involved in cell-to-cell movement from the carmoviruses. The light gray shaded region in the ORF encoding the 25 kDa capsid protein indicates where amino acid conservation occurs with *Sobemovirus* capsid proteins.

Host Range and Virus Propagation

Maize is the only known natural host for MCMV and it is readily mechanically transmitted to maize as well

as other indicator hosts. Depending on the maize genotype infected, the reaction to the virus can range from highly resistant to extremely susceptible. The inbred corn line N28ht is quite susceptible and produces excellent symptoms to MCMV and is the preferred propagation host. The experimental host range of the virus is restricted to the *Gramineae (Poaceae)*. The virus does not cause symptoms and probably does not infect dicots.

Genetics

The 4.4 kb MCMV genomic RNA is sufficient for replication in maize protoplasts. The genome contains four ORFs capable of encoding polypeptides of 111, 33, 32 and 25 kDa. The first ORF encountered on the 5′ end of the genome encodes a 32 kDa polypeptide (**Fig. 1**). No mutation studies have been performed on this ORF, nor does the 32 kDa polypeptide possess amino acid sequence similarity with any other known viral protein. The function, if any, of this protein is not known. The major ORF encoding the 111 kDa polypeptide is punctuated by an in-frame amber termination codon directing the synthesis of a 50 kDa pre-readthrough product. The readthrough portion of the 111 kDa polypeptide contains the canonical Gly-Asp-Asp (GDD) motif present in all RNA-dependent RNA polymerases. In addition, extensive amino acid sequence similarity exists between the carboxyl-half of the 50 kDa and the 111 kDa polypeptides and the replicases encoded by all of the species within the *Tombusviridae* (**Fig. 1**). As noted with other viruses within the *Tombusviridae*, neither the 50 kDa nor 111 kDa polypeptides possess an identifiable helicase motif. The internal ORF encoding a 33 kDa polypeptide is also interrupted by an in-frame opal termination codon yielding a pre-readthrough polypeptide of 9 kDa. The 9 kDa polypeptide, and particularly the carboxyl-terminus, possesses a high degree of amino acid sequence similarity to other small polypeptides encoded by *Carmoviruses* (**Fig. 1**). These small proteins are implicated in facilitating cell-to-cell movement of the virus infection. The putative 33 kDa readthrough protein has no identifiable sequence similarity to any other protein. The 3′ proximally located ORF encodes the 25 kDa capsid protein.

Serologic Relationships and Variability

To date, three different MCMV serotypes have been identified: Kansas serotypes 1 and 2 and the Peru serotype. The serotypes are differentiated by agar double-diffusion analysis. The Kansas serotypes 1 and 2 are prevalent in Kansas and have been identified as the virus that appeared in Hawaii.

Epidemiology

In Kansas and Nebraska, MCMV infections reoccur in the same locations within corn fields year after year. The areas of infection are discrete and little plant-to-plant spread seems to occur within a single growing season. These observations have led to the hypothesis that the virus is maintained in the soil from season to season. There are conflicting reports as to whether MCMV can survive in maize residue during maize-free periods. The virus has been shown to overwinter in plowed corn stubble. Alternatively, it is hypothesized that the larval stages of the corn rootworms, which have been shown to successfully transmit the virus, are capable of harboring infectious virus during the host-free periods.

The current thought is that MCMV can be introduced into geographically distinct maize growing regions by the introduction of an infected plant or by seed transmission. Once present, the virus can then persist in the region by infection in maize or by overwintering in the larval stages of the vector when the maize host is not present.

Transmission and Tissue Tropism

The virus is easily mechanically transmitted in the laboratory. Extensive research has been conducted on how MCMV is transmitted and maintained in nature, particularly during maize-free periods. MCMV can be transmitted by six species of beetle, belonging to the family *Chrysomelidae;* the cereal leaf beetle (*Oulema melanopa*), the corn flea beetle (*Systena frontalis* and *Chaetocnema pullicaria*), the southern corn rootworm (*Diabrotica undecimpunctata*), the northern corn rootworm (*D. longicornis*) and the western corn rootworm (*D. virgifera*). In Hawaii, MCMV has been shown to be transmitted by the thrip *Frankliniella williamsi*. MCMV can be, and is, seed transmitted at a very low level.

Pathogenicity

In Peru, losses in floury and sweet maize varieties to MCMV have been reported to be on the average of 10–15%. In experimental plots, inoculated plant yields were reduced by up to 59%.

When MCMV co-infects maize with any potyvirus, the synergistic interaction causing CLN occurs. In maize the most common potyviruses found in co-infections with MCMV are maize dwarf mosaic potyvirus (MDMV) A and/or B, and to a lesser extent wheat streak mosaic potyvirus. The symptoms

of CLN are much more severe than the additive symptoms of either MCMV or the potyvirus alone. CLN causes a severe systemic necrosis which culminates in death of the plant. In Kansas, crop losses due to CLN have been estimated to be between 50 and 90%, depending on the variety of corn and the year. If maize plants exhibit a rapid onset of necrosis followed by rapid plant death, it is likely that they are infected with both MCMV and a maize infecting potyvirus. The titer of MCMV in plants infected with both MCMV and a potyvirus is greater than five times higher than in plants infected with MCMV only.

Pathology and Histopathology

For field grown maize infected with MCMV, growth is stunted with the formation of short internodes. Leaf symptoms begin as chlorotic stripes running parallel to the veins, which later coalesce to produce elongated chlorotic blotches, finally resulting in leaf necrosis and epinasty. In severe infections of particularly susceptible lines, the extent of leaf necrosis can result in plant death. Male inflorescences are affected with hard panicles, short rachis and few spiklets. Fewer ears and ear malformation can also occur in severe infections. A general observation is that the younger the maize plant is when MCMV infects, the more severe the stunting and symptoms become. MCMV has been detected in all parts of an infected maize plant, including leaf, stem, roots, cob, husk, silk, kernel, seed, anther and sheath tissues.

Prevention and Control

The most effective control for MCMV will be the deployment of resistant varieties. A number of sources of resistance to MCMV have been identified and are being incorporated into commercial maize varieties throughout the Western Hemisphere. Alternatively, crop rotation with sorghum or another nonmaize crop has been shown to reduce the incidence of MCMV the following year. Soil fumigation apparently does not control MCMV.

CLN can be controlled by effectively controlling and eliminating the infection of either component virus. CLN has been controlled experimentally using a transgenic pathogen-derived resistance approach. Transgenic corn plants expressing the MDMV strain B capsid protein did not lead to CLN when inoculated with MDMV and MCMV.

Evolution

Based exclusively on the analysis of the amino acid sequence similarity of the various viral gene products, MCMV appears to be a composite virus composed of functional domains obtained from both *Tombusviruses* and *Sobemoviruses*. Additional domains of unknown phylogenetic origin are also present in the genome. The replicases of all known and sequenced positive-strand RNA viruses have been placed into three distinct phylogenetic supergroups. The MCMV replicase belongs in supergroup II along with the tombusviruses, carmoviruses, necroviruses, dianthoviruses, a subset of the luteoviruses, pestiviruses, hepatitis C virus, flaviviruses and the positive-strand RNA coliphages (notably Qβ) and the related yeast double-stranded RNA elements. It is generally assumed that the replicase from all of these viruses evolved from a common progenitor and was subsequently dispersed by modular evolution. The MCMV capsid protein fits into a rather large group of icosahedral-shaped viruses whose capsid protein(s) possess a conserved structural element termed the 'jelly-roll' conformation. It is suspected that this type of capsid protein has evolved only once. The capsid protein is most closely related to that of southern bean mosaic sobemovirus. The MCMV movement protein(s) have not been identified, however, significant similarity exists with a known movement protein from the *Carmoviruses*.

Future Perspectives

To date, the *Machlomovirus* genus is monotypic. It would not be surprising if additional machlomoviruses are identified in the near future. If new species are identified, it is likely that they would be found in monocot hosts from the temperate to subtropical climates in the Western Hemisphere. It is also anticipated that MCMV will be identified in maize in additional countries in the Western Hemisphere.

A full-length MCMV cDNA clone from which infectious RNA transcripts can be derived has been constructed. It is anticipated that a genetic study of the MCMV genome will be undertaken using this clone in the near future. It is expected that movement function and possibly vector transmission activities will be mapped to one or more of the unique ORFs.

See also: **Carmoviruses (*Tombusviridae*); Necroviruses (*Tombusviridae*); Sobemoviruses; Tombusviruses.**

Further Reading

Gordon DT, Bradfute OE, Gingery RE, Nault LR and Uyemoto JK (1984) Maize chlorotic mottle virus. In: *CMI/AAB Descriptions of Plant Viruses*, No. 284. Kew: Commonwealth Mycological Institute.

Lommel SA (1995) Genus *Machlomovirus*. In: Murphy FA, Fauquet CM, Bishop DHL *et al* (eds.) *Virus Taxonomy*. Sixth Report of the International Committee on Taxonomy of Viruses, p. 404. Wien: Springer.

Lommel SA, Kendall TL, Siu NF and Nutter RC (1991) Characterization of maize chlorotic mottle virus. *Phytopathology* 81: 819.

Nault LR, Styer WE, Coffey ME *et al* (1978) Transmission of maize chlorotic mottle virus by chrysomelid beetles. *Phytopathology* 68: 1071.

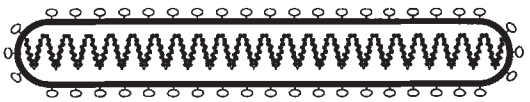
Nutter RC, Sheets K, Panganiban LC and Lommel SA (1989) The complete nucleotide sequence of the maize chlorotic mottle virus genome. *Nucl. Acids Res.* 17: 3163.

Uyemoto JK (1983) Biology and control of maize chlorotic mottle virus. *Plant Dis.* 67: 7.

Machupo Virus *see* **Lassa, Junin, Machupo and Guanarito Viruses**

Mammalian Hepadnaviruses *see* **Hepadnaviruses**

MARBURG AND EBOLA VIRUSES (*FILOVIRIDAE*)



Hans-Dieter Klenk, Werner Slenczka and **Heinz Feldmann**, Institut für Virologie, Philipps-Universität, Marburg, Germany

History

Marburg and Ebola viruses both cause severe hemorrhagic fevers. Marburg virus was first recognized in laboratory workers in Marburg, Germany, and Belgrade, Yugoslavia, in 1967. These workers had been exposed to tissues and blood from African green monkeys (*Cercopithecus aethiops*) imported from Uganda. There were 25 primary cases and six secondary cases in the outbreak. Seven of the primary cases died. Since then, sporadic, virologically confirmed Marburg disease cases have occurred in Zimbabwe, South Africa and Kenya. Ebola virus first emerged in two major disease outbreaks which occurred almost simultaneously in Zaire and Sudan in 1976. Over 500 cases were reported, with mortality rates of 88% in Zaire and 53% in Sudan. A single case was confirmed by virus isolation in Zaire in 1977, and in 1979 Ebola hemorrhagic fever occurred again in Sudan at the site that was involved in 1976. In 1994 the first case of Ebola virus disease occurred in western Africa, Cote d'Ivoire, when an ecologist was infected by examining a dead chimpanzee. Ebola virus re-emerged in Kikwit, Zaire, in 1995, with 316 cases and 245 deaths. From 1994 to 1997 three outbreaks of Ebola virus disease have been observed in Gabon. Ebola-Reston virus was first isolated from naturally infected nonhuman primates in 1989–1990, when *Cynomolgus* monkeys were imported from the Philippines into the USA, and later from monkeys at an export facility located in the Philippines. Further isolates have been made from exported Asian monkeys in 1992 in Italy and in 1996 in Texas, USA. While pathogenic for naturally and experimentally infected monkeys, Ebola-Reston virus may be less pathogenic for humans, having infected four animal caretakers without producing serious disease. At least three laboratory infections with Marburg and Ebola viruses (two fatal outcomes) occurred in Russia; a single nonfatal laboratory infection (Ebloa) occurred in the UK.

Taxonomy and Classification

Filoviruses are classified in the order *Mononegavirales*, a large group of viruses that have nonsegmented negative-stranded RNA as their genomes. The family *Filoviridae*, genus *Filovirus*, was created on the basis of unique morphologic, physicochemical, genetic and biological features of its members. Filoviruses are separated into two distinct species, Marburg and Ebola. The Marburg species consists of a single subtype Marburg including five strains. The Ebola

Lommel SA (1995) Genus *Machlomovirus*. In: Murphy FA, Fauquet CM, Bishop DHL *et al* (eds.) *Virus Taxonomy*. Sixth Report of the International Committee on Taxonomy of Viruses, p. 404. Wien: Springer.

Lommel SA, Kendall TL, Siu NF and Nutter RC (1991) Characterization of maize chlorotic mottle virus. *Phytopathology* 81: 819.

Nault LR, Styer WE, Coffey ME *et al* (1978) Transmission of maize chlorotic mottle virus by chrysomelid beetles. *Phytopathology* 68: 1071.

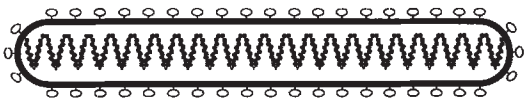
Nutter RC, Sheets K, Panganiban LC and Lommel SA (1989) The complete nucleotide sequence of the maize chlorotic mottle virus genome. *Nucl. Acids Res.* 17: 3163.

Uyemoto JK (1983) Biology and control of maize chlorotic mottle virus. *Plant Dis.* 67: 7.

Machupo Virus *see* **Lassa, Junin, Machupo and Guanarito Viruses**

Mammalian Hepadnaviruses *see* **Hepadnaviruses**

MARBURG AND EBOLA VIRUSES (*FILOVIRIDAE*)



Hans-Dieter Klenk, Werner Slenczka and **Heinz Feldmann**, Institut für Virologie, Philipps-Universität, Marburg, Germany

History

Marburg and Ebola viruses both cause severe hemorrhagic fevers. Marburg virus was first recognized in laboratory workers in Marburg, Germany, and Belgrade, Yugoslavia, in 1967. These workers had been exposed to tissues and blood from African green monkeys (*Cercopithecus aethiops*) imported from Uganda. There were 25 primary cases and six secondary cases in the outbreak. Seven of the primary cases died. Since then, sporadic, virologically confirmed Marburg disease cases have occurred in Zimbabwe, South Africa and Kenya. Ebola virus first emerged in two major disease outbreaks which occurred almost simultaneously in Zaire and Sudan in 1976. Over 500 cases were reported, with mortality rates of 88% in Zaire and 53% in Sudan. A single case was confirmed by virus isolation in Zaire in 1977, and in 1979 Ebola hemorrhagic fever occurred again in Sudan at the site that was involved in 1976. In 1994 the first case of Ebola virus disease occurred in western Africa, Cote d'Ivoire, when an ecologist was infected by examining a dead chimpanzee. Ebola virus re-emerged in Kikwit, Zaire, in 1995, with 316 cases and 245 deaths. From 1994 to 1997 three outbreaks of Ebola virus disease have been observed in Gabon. Ebola-Reston virus was first isolated from naturally infected nonhuman primates in 1989–1990, when *Cynomolgus* monkeys were imported from the Philippines into the USA, and later from monkeys at an export facility located in the Philippines. Further isolates have been made from exported Asian monkeys in 1992 in Italy and in 1996 in Texas, USA. While pathogenic for naturally and experimentally infected monkeys, Ebola-Reston virus may be less pathogenic for humans, having infected four animal caretakers without producing serious disease. At least three laboratory infections with Marburg and Ebola viruses (two fatal outcomes) occurred in Russia; a single nonfatal laboratory infection (Ebloa) occurred in the UK.

Taxonomy and Classification

Filoviruses are classified in the order *Mononegavirales*, a large group of viruses that have nonsegmented negative-stranded RNA as their genomes. The family *Filoviridae*, genus *Filovirus*, was created on the basis of unique morphologic, physicochemical, genetic and biological features of its members. Filoviruses are separated into two distinct species, Marburg and Ebola. The Marburg species consists of a single subtype Marburg including five strains. The Ebola

species is subdivided into four subtypes: Zaire, Sudan, Cote d'Ivoire and Reston. In terms of biohazard classification, filoviruses are classified as Biosafety Level 4 (BSL4) agents based on their high mortality rate, person-to-person transmission, potential aerosol infectivity, and absence of vaccines and chemotherapy. Maximum containment is required for all laboratory work with infectious material.

Properties of the Virion

By electron microscopy, filovirus particles are pleomorphic, appearing as long filamentous, sometimes branched forms, or as 'U'-shaped, '6'-shaped or circular forms. The particles vary greatly in length (up to 14 000 nm), but have a uniform diameter of about 80 nm. Virions purified by ratezonal gradient centrifugation are bacilliform in outline and show an average length associated with peak infectivity of approximately 665 nm for Marburg and 805 nm for Ebola virus. Except for the difference in length, filoviruses seem to be very similar in morphology. Virions contain a nucleocapsid consisting of a dark, central space (20 nm in diameter) surrounded by a helical capsid (50 nm in diameter) bearing cross-striations with a periodicity of approximately 5 nm. Within the nucleocapsid is an axial channel of 10–15 nm (**Fig. 1**). The nucleocapsid is composed of the genomic RNA and the large (L) protein, nucleoprotein and virion proteins 35 and 30. It is surrounded by a lipoprotein unit membrane envelope derived from the host cell plasma membrane. Spikes of approximately 7 nm length, spaced at approximately 10 nm intervals, are visible on the virion surface and are formed by the viral glycoprotein.

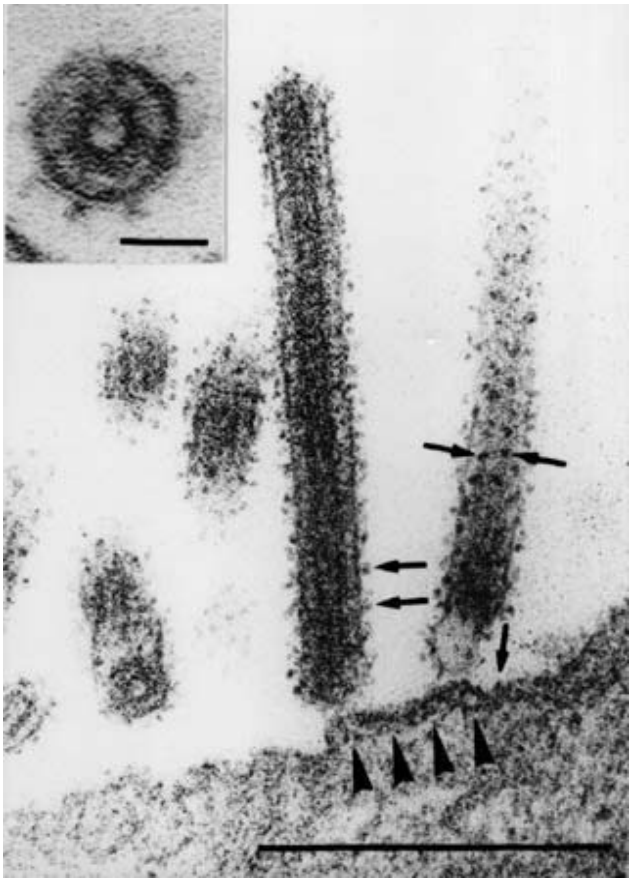


Figure 1 Marburg virus particles. An electron micrograph (ultrathin section) showing budding of Marburg virus particles from the plasma membrane of infected primary cultures of human endothelial cells. Particles consist of a nucleocapsid surrounded by a membrane in which spikes are inserted (arrows). The nucleocapsid contains a central channel (inset). The plasma membrane of infected cells is often thickened at locations where budding occurs (arrowheads). Bar = 0.5 μm; inset bar = 50 nm.

Physical Properties

Virus particles have a molecular weight of approximately $3–6 \times 10^8$ and a density in potassium tartrate of 1.14 g ml^{-1}. Uniform, bacilliform particles have a sedimentation coefficient of 1300–1400 S, whereas larger particles have a higher sedimentation coefficient. Virus infectivity is quite stable at room temperature. Inactivation can be performed by UV and gamma irradiation, 1% formalin, β-propiolactone, and brief exposure to phenolic disinfectants and lipid solvents, like deoxycholate and ether.

Properties of the Genome

The genome of filoviruses consists of a molecule of linear, nonsegmented, negative-stranded RNA which is noninfectious, not polyadenylated, and complementary to viral-specific messenger RNA. The genome amounts to 1.1% of the total virion weight and the sedimentation coefficient is 46 S (0.15 mol l^{-1} NaCl, pH 7.4). Filovirus genomes are approximately 19 kb in length and very rich in adenosine and uridine residues. Genomes show a linear gene arrangement in the order 3′ leader – nucleoprotein (NP) – viral structural protein (VP) 35 – VP40 – glycoprotein (GP) – VP30 – VP24 – polymerase (L) – 5′ trailer. All genes are flanked at their 3′ and 5′ ends by highly conserved transcriptional start (3′-CUnCnUnUAAUU-5′) and termination (3′-UaAUUCUUUUU-5′) signal sequences, respectively, which almost all contain the pentamer 3′-UAAUU-5′. Most genes are separated by intergenic sequences variable in length and nucleotide composition, but some genes overlap by the conserved pentanucleotide sequence. Subtype Zaire Ebola viruses show three overlaps that alternate with intergenic sequences (VP35/VP40, GP/VP30, VP24/

L), while the Marburg virus genome contains a single overlap at a different position (VP30/VP24). Extragenic sequences are present at the 3′ and 5′ end of filovirus genomes which are complementary at their very extremities. These sequences are comparable to those found in genomes of other nonsegmented negative-stranded RNA viruses and are known as leader sequences. However, neither (+) nor (−) leader RNAs have been detected in filovirus-infected cells.

Properties of Viral Proteins

Virions contain at least seven proteins with presumed identical functions for the different viruses. The electrophoretic mobility patterns of the structural proteins are characteristic for Marburg strains on the one hand and Ebola strains on the other. Four proteins are associated with the viral ribonucleoprotein complex (NP, L, VP30 and VP35), the single glycoprotein (GP) is inserted in the envelope, and the location of two proteins (VP40 and VP24) has not been determined exactly, but they seem to be membrane associated. The L protein is the largest protein and, like other L proteins of nonsegmented negative-stranded RNA viruses, represents the virion-associated RNA-dependent RNA polymerase. Its size, as calculated from the deduced amino acid sequence of the Marburg virus (Musoke strain) L gene, is 267 kDa. GP (Marburg virus 170 kDa; Ebola virus 160 kDa) is a type I transmembrane protein and inserted in the lipid membrane as a homotrimer, as shown directly for Marburg virus. It is reasonable to assume that GP is the mediator of virus entry into the cell. Functional sites for receptor recognition and binding, and perhaps for fusion, should be located on this protein. GP undergoes several post-translational modifications: glycosylation, acylation and proteolytic cleavage. The carbohydrate structures of this highly glycosylated protein account for >50% of its M_r. They include oligomannosidic and hybrid type *N*-glycans as well as bi-, tri- and tetra-antennary complex species, and high amounts of neutral mucin-type *O*-glycans. In contrast to Ebola viruses, sialylation of Marburg virus carbohydrate structures is cell line dependent. In case of Marburg virus GP, acylation occurs at cysteine residues located at the boundary between the membrane anchor and cytoplasmic domains. The Golgi-specific precursor (preGP) is proteolytically cleaved into the subunits GP1 and GP2 by a proprotein convertase, most likely furin. Mature GP (GP1/2) is a disulfide-linked complex of the cleavage subunits and is anchored in the membrane via the carboxy-terminal part of GP2. The major nucleoprotein (NP) (Marburg virus 96 kDa; Ebola virus 104 kDa) and VP30 (Marburg virus 28 kDa; Ebola virus 30 kDa), which may represent a minor nucleoprotein, seem to be intimately associated with the virion ribonucleoprotein complex. VP35 (Marburg virus 32 kDa; Ebola virus 35 kDa) is loosely associated with the ribonucleoprotein complex and seems to be a component of the transcriptase complex analogous to the P protein of paramyxoviruses and the NS (P) protein of rhabdoviruses. The functions of VP40 (Marburg virus 38 kDa; Ebola virus 40 kDa) and VP24 (Marburg virus 24 kDA; Ebola virus 24 kDa) are not known, but they are probably membrane components. VP40 is the most prominent viral structural protein and most likely represents the matrix protein of filoviruses. A nonstructural glycoprotein has recently been discovered with Ebola viruses. This protein, designated sGP, is the primary gene product of gene 4 and efficiently secreted from infected cells. Recently it was detected in high concentrations in the blood of acutely infected Ebola hemorrhagic fever patients. The function of sGP is unknown, but an interaction with the cellular and humoral host immune responses has been postulated. A similar protein is not found with Marburg viruses.

Replication

Cell entry seems to be mediated by GP as the only surface protein of virion particles. Studies on Marburg virus infections of hepatocytes have identified the asialoglycoprotein receptor as a receptor candidate. However, one has to postulate additional receptors as this protein is not expressed on many virus-susceptible cells. Whether the next step in virus entry involves a fusion process at the plasma membrane or fusion following endocytosis of virus particles is not known. The genetics of filoviruses are probably similar to those of *Paramyxoviridae* and *Rhabdoviridae*. Transcription and replication take place in the cytoplasm of infected cells. The 3′ leader of the genome probably provides the encapsidation site for the nucleoprotein as well as the entry site for the polymerase. Filovirus genomes are transcribed to yield mainly monocistronic subgenomic messenger RNA species which are complementary to viral genomic RNA. Transcription of the Ebola virus GP gene gives rise to at least two different messenger RNA species. The primary transcript encodes small (s) GP which is encoded by the amino-terminal open reading frame of gene 4. Full-length GP is expressed by transcriptional editing of a single nucleotide at a run of uridine residues that combines the sGP open reading frame with an overlapping additional reading frame in −1. The 5′ ends of the subgenomic RNAs

start precisely at the transcription start signal sequence, and the 3′ ends carry a poly(A) tail generated by the polymerase at a run of uridine residues located at the 5′ ends of all transcription termination signal sequences. Transcription efficiency might be influenced by gene order, formation of secondary structures at the 3′ ends of the genes, secondary structure formation within the intergenic sequences, overlapping genes, and presence of duplicated termination sites. Filovirus transcripts contain unusually long untranslated regions, especially at the 3′ ends. The 5′ end untranslated regions show a potential for a formation of stable hairpin structures, which might play a role in transcript stability and ribosome binding. Replication of the genome is mediated by the synthesis of a full-length complementary antigenome ((+) sense) which then serves as the template for the synthesis of progeny negative-stranded RNA molecules anticomplementary to the parental template RNA. The complementarity of the genome extremities suggests a single identical encapsidation site on the genome and antigenome and an identical entry site for the polymerase for both the transcription and the replication mode. The cytoplasm of virus-infected cells contains prominent inclusion bodies consisting of viral nucleocapsid. As infection proceeds, they grow and become highly structured. Budding of completed virus particles takes place at cell membrane sites which are altered by insertion of the viral glycoprotein and alignment of viral membrane-associated proteins as well as of preformed nucleocapsids.

Geographic Distribution

Filoviruses, with the exception of subtype Reston of Ebola viruses, appear to be indigenous to the tropical rain forest regions of Central Africa, as indicated by the geographic locations of known outbreaks and seroepidemiological studies. This is also suggested by the fact that almost all filovirus isolates from human patients are of African origin. This includes the European isolates of Marburg viruses that could be traced back to foci in Uganda, from where vervet monkeys were imported to Germany and the former Yugoslavia. The Ebola-Reston outbreak suggested for the first time the presence of a filovirus in Asia. Studies among captive macaques in the Philippines indicated that the source of Ebola-Reston virus might be wild nonhuman primates; thus, it appears that filoviruses are not confined to Africa.

Host Range and Virus Distribution

The natural reservoirs for human and nonhuman primate infections with filoviruses are unknown. Experimental hosts include monkeys, for which infection with Marburg and Ebola-Zaire virus are usually lethal, whereas some animals survive infections with Ebola-Sudan and Ebola-Reston viruses. Guinea pigs show febrile responses 4–10 days after inoculation with Marburg and Ebola viruses. However, none of these viruses kills guinea pigs consistently on primary inoculation. Ebola-Zaire virus is usually pathogenic for newborn mice after intracerebral and intraperitoneal inoculation. For growth in cell culture, primary monkey kidney cells and monkey kidney cell lines, usually Vero cells, are used. Filoviruses also grow in human endothelia maintained as primary cell cultures or as organ cultures.

Evolution

Molecular analyses of the genomes clearly demonstrated that filoviruses are the closest relatives to *Rhabdoviridae* and *Paramyxoviridae*. All nonsegmented negative-stranded RNA viruses share a similar genome organization, with conserved regions at both ends encoding the core and L proteins surrounding a variable part in the middle encoding the envelope proteins. Filovirus genomes are more complex than those of lyssaviruses and vesiculoviruses and align organizationally more closely to members of the genera *Paramyxovirus* and *Morbillivirus*. This relationship is confirmed on the amino acid level as demonstrated for the nucleoproteins and polymerases (L proteins). The Marburg species is genetically more homogenous. Comparative sequence analysis showed that two lineages coexist with the recent isolate from Kenya (Ravn strain, 1987) differing from the others by 21–23% at nucleotide level. Since this divergence is less than that separating Ebola subtypes (see below), the Ravn strain does not represent a distinct subtype. Significant differences within the Ebola species were first based on peptide and oligonucleotide mapping, which have since been confirmed by sequence comparison analysis of the glycoprotein genes. All four subtypes differ from one another by 37–41% and 34–43% at the nucleotide and amino acid level, respectively. Phylogenetic analysis of the glycoprotein open reading frame revealed a closer relationship of the three African subtypes compared with Reston, supporting the concept of an Asian origin of Reston viruses. Within individual subtypes of filoviruses the variation in nucleotide sequences has been shown to be $<7\%$ and even $<2\%$ among distinct subtype Zaire viruses. Recently it was reported that there is no genetic variability between strains isolated from different patients of single outbreaks in Gabon and Kikwit. All the data indicate a remarkable degree of stability over time for RNA viruses that are usually

thought to be extremely variable. Furthermore, it appears that filoviruses might have evolved into specific niches and may reflect a similar divergence in the natural hosts, assuming they have coevolved.

Serologic Relationships

Comparison of the two species of filoviruses, Marburg and Ebola, showed similarities between amino acid sequences of the structural proteins. This finding indicates that the structural proteins have maintained similar structures and functions. Despite this amino acid similarity, there is no indication that there is any significant serological (antigenic) crossreactivity between Ebola and Marburg viruses, but the different subtypes of the Ebola species share common epitopes. Neutralization tests for Marburg and Ebola viruses have not yet been shown sufficiently reliable to enable determination of taxonomic relationships.

Epidemiology

The reservoir of filoviruses remains a mystery. Many species have been discussed as possible natural hosts. However, no nonhuman vertebrate hosts or arthropod vectors have yet been identified. Epidemiological data, obtained in association with the 1967 Marburg outbreak and the 1994 Côte d'Ivoire case as well as the Ebola-Reston episodes, suggested monkeys as a potential reservoir of filoviruses. However, the high pathogenicity of filoviruses, especially of Marburg virus and subtypes Sudan and Zaire of Ebola virus, for nonhuman primates does not support such a concept. Similarities in biological properties to other viral hemorrhagic fever agents, such as 'Old World' arenaviruses, favor a chronic infection of an animal that regulates survival of filoviruses in nature. This is in line with recent data on experimentally infected fruit and insectivorous bats that demonstrated asymptomatic Ebola virus replication in these animals. Nosocomial transmission, mainly due to a lack of hygiene, is of major public health concern. Based on experiences of former episodes, isolation of patients and use of strict barrier nursing procedures (e.g. protective clothing, respirator) are sufficient to interrupt transmission.

Transmission and Tissue Tropism

The mode of primary infection with Marburg and Ebola viruses in any natural setting is not known. The physical contact route of infection is undoubtedly the most common means of transmission from humans to humans. Especially activities such as nursing and preparing bodies for burials are associated with an increased risk of becoming infected. One Marburg case was acquired by sexual contact more than 60 days after the original infection. Neonatal transmission has been reported for the 1976 outbreak in Zaire. Transmission by droplets and small-particle aerosols has been observed among experimentally infected and quarantined imported monkeys (Ebola-Reston virus, 1989–1990). This is confirmed by identification of filovirus particles in alveoli of naturally and experimentally infected monkeys. However, the contribution of aerosol transmission to the course of human outbreaks is still unknown.

Virus is usually recovered from acute-phase sera and has also been found in throat washes, urine, soft tissue effusates, semen and anterior eye fluid, even when the specimens were obtained late in convalescence. It has also been regularly isolated from autoptic material, such as spleen, lymph nodes, liver and kidney, but rarely from brain or other nervous tissues.

Pathogenicity

Marburg and Ebola viruses cause severe hemorrhagic fever in humans and laboratory primates. According to the evidence available to date, subtype Reston of Ebola virus causes hemorrhagic fever in monkeys, but appears to be apathogenic for humans. The subtype Zaire strains of Ebola virus apparently have the highest mortality in humans when compared with other subtypes or Marburg virus.

Clinical Features of Infection

Clinical symptoms are similar with Marburg and Ebola virus infections. Following incubation periods of 4–16 days, onset is sudden, marked by fever, chills, headache, anorexia and myalgia. These signs are soon followed by nausea, vomiting, sore throat, abdominal pain and diarrhea. When first examined, patients are usually overtly ill, dehydrated, apathetic and disoriented. Pharyngeal and conjunctival injections are usual. Most of the patients develop severe hemorrhagic manifestations, usually between days 5 and 7. Bleeding is often from multiple sites, with the gastrointestinal tract, lungs and gingiva the most commonly involved. Bleeding and oropharyngeal lesions usually herald a fatal outcome. Death occurs between days 7 and 16, usually from shock with or without severe blood loss.

Pathology, Histopathology and Pathogenesis

Marburg and Ebola viruses cause similar pathological changes in humans. The most striking lesions are

found in liver, spleen and kidney. These lesions are characterized by focal hepatic necrosis with little inflammatory response and by follicular necrosis of lymph nodes and spleen. In late stages of the disease, hemorrhage occurs in the gastrointestinal tract, pleural, pericardial and peritoneal spaces, and into the renal tubules with deposition of fibrin. Abnormalities in coagulation parameters include fibrin split products and prolonged prothrombin and partial thromboplastin times, suggesting that disseminated intravascular coagulation is a terminal event. There is usually also profound leukopenia in association with secondary bacteremia. Ebola-Reston virus causes similar pathological changes in monkeys, as described for human infections with Marburg and other subtype Ebola viruses. In Reston-infected animals it was clearly demonstrated that virus replication was extensive in fixed tissue macrophages, interstitial fibroblasts of many organs, circulating macrophages and monocytes, and less frequently in vascular endothelial cells, hepatocytes, adrenal cortical cells and renal tubular epithelium. Macrophages seem to be the first and preferred site of replication by filoviruses.

Clinical and biochemical findings support anatomical observations of extensive liver involvement, renal damage, changes in vascular permeability, and activation of the clotting cascade. Visceral organ necrosis is the consequence of virus replication in parenchymal cells. However, no single organ is sufficiently damaged to explain fatal outcome. Fluid distribution problems and platelet abnormalities indicate dysfunction of endothelial cells and platelets. Virus-induced release of humoral factors, such as cytokines, may increase endothelial permeability and may be a major factor in the shock syndrome regularly observed in severe and fatal cases.

Immune Response

Fatal filovirus infections usually end with high viremia and no evidence of an effective immune response. In monkeys infected with Ebola-Reston virus nonprotective antibodies have been observed shortly before death. Altogether, however, the data available today do not support an important role of neutralizing antibodies in virus clearance. Since circulating monocytes/macrophages are primary target cells in filovirus infections, and since the extensive disruption of the parafollicular regions in spleen and lymph nodes results in the destruction of the antigen-presenting dendritic cells, cellular immunity appears also to be affected during filoviral hemorrhagic fever. In addition to these cytolytic effects, there are several other mechanisms by which filoviruses may interfere with the immune system. Firstly, the high carbohydrate content of GP may suppress its immune reactivity. Secondly, the nonstructural sGP that is secreted from cells infected with Ebola virus may interfere with the virus-directed immune response. Thirdly, GP1 which is also secreted in large amounts from infected cells, may have similar effects. Fourthly, full-length GP displays a sequence motif homologous to an immunosuppressive domain observed in retroviral glycoproteins. Altogether, there is now a large body of evidence indicating that filoviruses induce immunosuppression in the infected host, which appears to be a major factor for the rapid spread and the severity of the disease.

Prevention and Control

Although neutralizing antibody titers in human convalescent sera can, if at all, only barely be detected in laboratory tests, there are anecdotal case reports suggesting the potential benefit of passive immunization against Ebola virus infection. Furthermore, recent reports from the 1995 outbreak in Zaire about effective treatment of acutely ill patients with whole blood transfusions from convalescent donors suggest that quantities of antibodies, predicted to be marginally effective in laboratory tests, may still be protective. There is experimental evidence that active immunization employing killed virus, recombinant expressed glycoprotein, and recombinant gene 4 (GP)-DNA (DNA vaccination) is partially successful in animals, suggesting that these may be feasible strategies to elicit protective immunity. At present, however, vaccines for human application are not available. A specific chemotherapeutic treatment is not available to date, but knowledge of the expected clinical course can anticipate medical complications, including disseminated intravascular coagulation, shock, encephalomyelitis, cerebral edema, kidney failure, superinfection, hypoxia and hypotension. Patients have to be isolated and clinical personal to be protected. Human interferon, human convalescent plasma and anticoagulation therapy has been used, but their success is still controversial.

Diagnosis

Because Marburg and Ebola viruses are highly virulent, special precautions need to be taken when samples are handled, and for some procedures biocontainment (BSL4) is necessary. For acute diagnosis polymerase chain reaction (PCR) and antigen-ELISA are suitable. For confirmation virus isolation

should be initiated in appropriate cell cultures (Vero E6 cells, MA 104 cells) from acute-phase serum or biopsy/autopsy materials (liver, spleen, lymph nodes, kidney, heart). During viremia particles can usually be visualized by electron microscopy. Serum antibody titers are determined by ELISA (IgM μ-capture, IgG) or indirect fluorescent-antibody immunofluorescence assay (IFA) (caution: prone to nonspecific positives). Serum from patients with suspected cases should be inactivated by gamma irradiation prior to serotesting. Neutralization tests are totally unreliable for filoviruses.

See also: **Emerging and re-emerging virus diseases.**

Further Reading

Feldmann H and Klenk H-D (1996) Filoviruses: Marburg and Ebola. *Adv. Virus Res.* 47: 1.

Feldmann H, Slenczka W and Klenk H-D (1996) Emerging and reemerging of filoviruses. *Arch. Virol. Suppl.* 11: 77.

Martini GA and Siegert R (eds) (1971) *Marburg Virus Disease.* New York: Springer.

Pattyn SR (ed.) (1978) *Ebola Virus Hemorrhagic Fever.* Amsterdam: Elsevier/North-Holland.

Peters CJ, Sanchez A, Feldmann H *et al* (1994) Filoviruses as emerging pathogens. *Semin. Virol.* 5: 147.

Peters CJ, Sanchez A, Rollin PE, Ksiazek TG and Murphy FA (1996) *Filoviridae*: Marburg and Ebola viruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology* 3rd edn, p. 1161. Philadelphia: Lippincott-Raven.

MAREK'S DISEASE VIRUS (*HERPESVIRIDAE*)

LN Payne, Institute for Animal Health, Compton, Newbury, Berkshire, UK

History

Marek's disease (MD) is a common lymphomatous and neuropathic disease of domestic fowl, and less commonly of turkeys and quail. It is named after the Hungarian pathologist, Jozsef Marek, who in 1907 first described the neuropathic form. In 1926, Pappenheimer and colleagues in the USA described many features of the disease and recognized visceral lymphomas as part of the pathology. Both the neural form (formerly termed 'neurolymphomatosis' or 'fowl paralysis') and the visceral lymphomatosis form (termed 'acute leukosis' or 'acute MD') were serious causes of economic loss to the poultry industry worldwide for many years (**Figs 1–3**).

Although MD was early considered to be an infectious disease, confusion with retrovirus-induced leukoses pathologically and in transmission experiments hindered understanding. However, experimental transmission of MD was established convincingly in the early 1960s, and in 1967 Churchill and Biggs in the UK and Nazerian, Solomon, Witter and Burmester in the USA established the cause as a herpesvirus. Two years later, MD virus (MDV) attenuated in tissue culture was shown to provide vaccinal protection against MD.

The nonpathogenic herpesvirus of turkeys (HVT) (turkey herpesvirus) was discovered in 1969 by Kawamura, King and Anderson in the USA, and in 1970 was shown to protect chickens against MD by Okazaki, Purchase and Burmester. Since then, HVT has become the most widely used vaccine against MD, and vaccination has greatly reduced mortality and economic loss from the disease. MD vaccinations were performed effectively until the late 1970s when evidence emerged from the USA of increased rates of

Figure 1 Paralysis of the legs in Marek's disease. (From Purchase (1985), in *Marek's Disease*, Payne, L.N. (ed), pp 17–42, Martinus Nijhoff Publishing, Boston, with permission.)

should be initiated in appropriate cell cultures (Vero E6 cells, MA 104 cells) from acute-phase serum or biopsy/autopsy materials (liver, spleen, lymph nodes, kidney, heart). During viremia particles can usually be visualized by electron microscopy. Serum antibody titers are determined by ELISA (IgM μ-capture, IgG) or indirect fluorescent-antibody immunofluorescence assay (IFA) (caution: prone to nonspecific positives). Serum from patients with suspected cases should be inactivated by gamma irradiation prior to serotesting. Neutralization tests are totally unreliable for filoviruses.

See also: **Emerging and re-emerging virus diseases.**

Further Reading

Feldmann H and Klenk H-D (1996) Filoviruses: Marburg and Ebola. *Adv. Virus Res.* 47: 1.

Feldmann H, Slenczka W and Klenk H-D (1996) Emerging and reemerging of filoviruses. *Arch. Virol. Suppl.* 11: 77.

Martini GA and Siegert R (eds) (1971) *Marburg Virus Disease.* New York: Springer.

Pattyn SR (ed.) (1978) *Ebola Virus Hemorrhagic Fever.* Amsterdam: Elsevier/North-Holland.

Peters CJ, Sanchez A, Feldmann H *et al* (1994) Filoviruses as emerging pathogens. *Semin. Virol.* 5: 147.

Peters CJ, Sanchez A, Rollin PE, Ksiazek TG and Murphy FA (1996) *Filoviridae*: Marburg and Ebola viruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology* 3rd edn, p. 1161. Philadelphia: Lippincott-Raven.

MAREK'S DISEASE VIRUS (*HERPESVIRIDAE*)

LN Payne, Institute for Animal Health, Compton, Newbury, Berkshire, UK

History

Marek's disease (MD) is a common lymphomatous and neuropathic disease of domestic fowl, and less commonly of turkeys and quail. It is named after the Hungarian pathologist, Jozsef Marek, who in 1907 first described the neuropathic form. In 1926, Pappenheimer and colleagues in the USA described many features of the disease and recognized visceral lymphomas as part of the pathology. Both the neural form (formerly termed 'neurolymphomatosis' or 'fowl paralysis') and the visceral lymphomatosis form (termed 'acute leukosis' or 'acute MD') were serious causes of economic loss to the poultry industry worldwide for many years (**Figs 1–3**).

Although MD was early considered to be an infectious disease, confusion with retrovirus-induced leukoses pathologically and in transmission experiments hindered understanding. However, experimental transmission of MD was established convincingly in the early 1960s, and in 1967 Churchill and Biggs in the UK and Nazerian, Solomon, Witter and Burmester in the USA established the cause as a herpesvirus. Two years later, MD virus (MDV) attenuated in tissue culture was shown to provide vaccinal protection against MD.

The nonpathogenic herpesvirus of turkeys (HVT) (turkey herpesvirus) was discovered in 1969 by Kawamura, King and Anderson in the USA, and in 1970 was shown to protect chickens against MD by Okazaki, Purchase and Burmester. Since then, HVT has become the most widely used vaccine against MD, and vaccination has greatly reduced mortality and economic loss from the disease. MD vaccinations were performed effectively until the late 1970s when evidence emerged from the USA of increased rates of

Figure 1 Paralysis of the legs in Marek's disease. (From Purchase (1985), in *Marek's Disease*, Payne, L.N. (ed), pp 17–42, Martinus Nijhoff Publishing, Boston, with permission.)

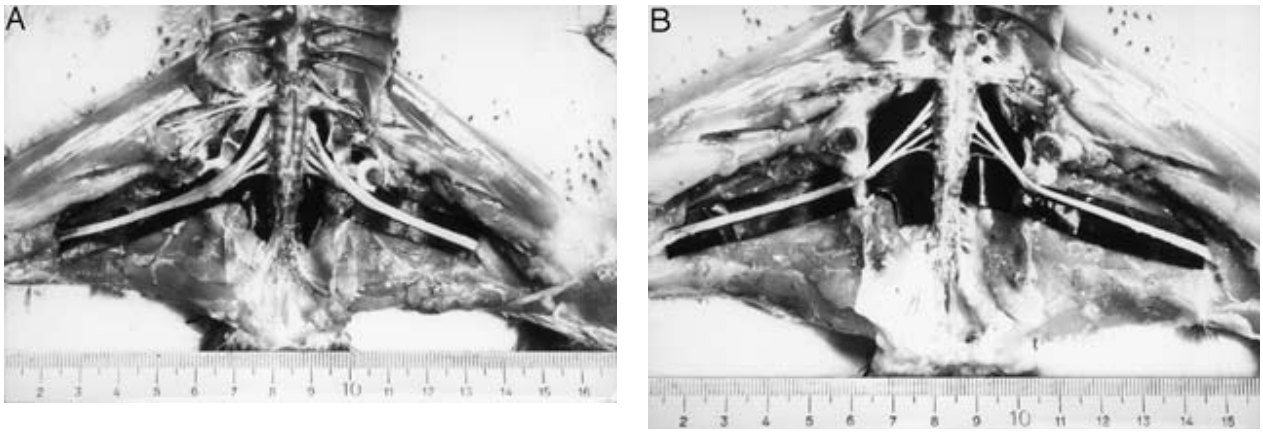


Figure 2 (**A**). Enlarged sciatic nerves in chicken with Marek's disease. (**B**). Normal chicken for comparison. (From Payne and Biggs (1967), *J. Natl. Cancer Inst.* 39, 281–302.)

condemnation from MD of vaccinated broiler chickens and vaccination failure in older birds. The so-called very virulent strains of MDV were isolated from affected flocks, against which HVT provided poor protection. Subsequently effective new vaccination strategies were developed but these have required further modification following the appearance of even more virulent strains of MDV.

Apart from its importance in veterinary medicine and to the poultry industry, MD has attracted considerable interest in biomedicine as a model of a lymphoma caused by a herpesvirus and preventable by vaccination.

Figure 3 Ovarian lymphoma (arrow) in chicken with Marek's disease. (From Payne *et al* (1976), *Int. Rev. Exp. Pathol.* 16, 59–154, with permission.)

Classification and Properties of the Virus

MDV and HVT are typical herpesviruses, in the family *Herpesviridae*, subfamily Alphaherpesvirinae, genus Marek's disease-like viruses. The nucleocapsid measures 85–100 nm and has 162 hollow capsomeres. Enveloped particles measure up to 400 nm. The nucleoid has a spherical to toroidal structure. Although originally placed with the gammaherpesviruses because of their lymphotropism, molecular studies show that MDV and HVT are more closely related to the alphaherpesviruses, such as herpes simplex virus.

Three serotypes of the virus are recognized: serotype 1, which comprises a variety of strains of MDV which vary from being highly to weakly oncogenic; serotype 2, which contains nononcogenic strains of MDV, although a virulent strain has been reported, and serotype 3, represented by HVT, which is also nononcogenic. These serotypes are distinguished in polyclonal or monoclonal antibody tests, polypeptide pattern and DNA analysis.

Serotype 1 isolates can also be classified pathotypically as mild (mMDV), virulent (vMDV), very virulent (vvMDV) and very virulent plus (vv + MDV).

Properties of the Genome

The DNAs of serotype 1 MDV and HVT are linear double-stranded molecules of approximately 180 and 167 kbp, respectively. Structurally the genomes consist, like herpes simplex virus, of a long unique region (U_L) and a short unique region (U_S) bounded by

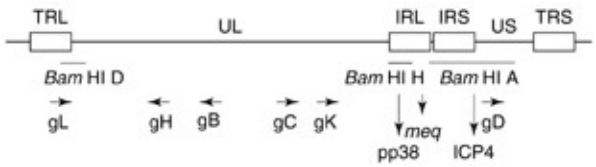


Figure 4 Schematic representation of the MDV genome. The 200 kbp sequence consists of a long unique sequence (UL) flanked by a terminal repeat (TRL) and an internal inverted repeat (IRL) and a short unique sequence (US) flanked by an internal (IRS) and an inverted terminal repeat region (TRS). Part of the BamHI linkage map and the positions of the MDV-specific proteins are shown. (From Venugopal and Payne (1995), *Avian Pathol.* 24, 597–609, with permission.)

inverted repeats (**Fig. 4**). Physical maps of restriction endonuclease fragments are available for MDV and HVT. There is little homology between the three serotypes under stringent hybridization conditions, although, under certain low-stringency conditions, the DNAs of serotype 1 MDV and HVT show about 70% homology. Some genes have been mapped, but those responsible for oncogenicity have not yet been identified. Candidate genes associated with oncogenicity include a 1.8 kb family, *meq* (which resembles the *jun/fos* family), *ICP4* and *pp38*.

Properties of Virus Proteins

More than 40 virus-specific polypeptides, many glycosylated, have been isolated from MDV- or HVT-infected cells. The A antigen detected by gel immunoprecipitation is a secreted cell-surface antigen of unknown function. Its gene is homologous to the *gC* gene of herpes simplex virus. The B antigen detected by immunoprecipitation stimulates virus-neutralizing antibodies and consists of three polypeptides. Its gene is homologous to the *gB* gene of herpes simplex virus. A C antigen has also been identified by immunoprecipation; its gene has not yet been identified.

Replication

Two main types of virus–cell interaction occur with MDV and HVT, similar to those described for other herpesviruses: (1) productive infections, which are cytolytic and in which virions are completely or partially formed; (2) nonproductive infections, which are noncytolytic and in which there is either no or only limited expression of the viral genome. Fully productive infection by MDV and HVT occurs *in vivo* in feather follicle epithelium from which enveloped infectious virus is released. RNA is transcribed from almost the whole viral genome. Semiproductive or restrictively productive infection occurs *in vivo* in lymphoid organs, epithelial cells and occasionally in tumors, and *in vitro* in cultured cells such as chick kidney cells or chick or duck embryo fibroblasts. Most of the virions produced are non-enveloped and noninfectious. Nonproductive infection by MDV is typified by lymphoma cells and by lymphoblastoid cell lines and transplantable lymphomas derived from them. Multiple (5–15) copies of the viral genome are present and are expressed by immortalization of the cells. Most of the viral genome is in the form of full-length closed circular DNA, but there is also evidence for some viral DNA integrated into the host chromosomes. A limited degree of latent MDV genome transcription has been demonstrated. HVT does not produce neoplastic transformation. A second type of nonproductive infection occurs in latently infected lymphocytes in birds infected with MDV or HVT, in which fewer than five viral genome copies are present.

Physical Properties

Cell-free preparations of MDV are stable at pH 7 but lose infectivity above and below this pH. Virus titer is stable at −65°C but titer is lost at −20°C. All infectivity is lost after 2 weeks at 4°C and after shorter periods at higher temperatures. Virus-infected feather materials retain infectivity at room temperature for many months. Cell-free MDV and HVT can be lyophilized in the presence of appropriate stabilizers. Cell-free MDV is sensitive to ether and formalin and is inactivated by common chemical disinfectants.

Viral Isolation and Propagation

Commonly favored for isolation of MDV or HVT in cell culture are viable whole blood cells, buffy coat cells, kidney cells or lymphoma cells. Almost all infectivity is lost from such tissues by treatments that disrupt cells. Cell-free MDV or HVT is present in feather follicle epithelium of infected chickens or turkeys, and viral preparations may be obtained by homogenization and sonication of skin with feather follicles or feather quill tips.

MDV or HVT can be detected in such materials by the following techniques.

1. Chick inoculation – in genetically susceptible chicks, pathogenic MDV induces microscopic lesions in nerves and other tissues from 2 weeks postinoculation and gross neural lesions and visceral lymphomas from 3 weeks. vvMDV can also cause an early mortality syndrome due to cytolytic infection. Nonpathogenic MDV and

HVT do not produce gross lesions, but may be detected by the presence of cell-associated viremia, precipitating or neutralizing antibodies, and antigen in feather tips.

2. Cell culture – cell-associated and cell-free MDV and HVT produce cytopathic plaques characteristic of herpesviruses within a few days when inoculated on to tissue culture monolayers of chick kidney cells, duck embryo fibroblasts, chick embryo fibroblasts and some other avian cells. Plaques vary in size and rate of development depending on the strain of virus, and degenerating cells contain type A intranuclear inclusion bodies. The virus in plaque cells is closely cell-associated, although some amounts of cell-free virus can be harvested from disrupted cells especially in medium containing stabilizers. Serial passage of pathogenic MDV in cell culture results in attenuation of the virus, a phenomenon used to produce vaccine strains.
3. Polymerase chain reaction assays have been developed which can discriminate between pathogenic and attenuated virus strains.
4. Embryonated eggs – MDV and HVT produce pocks on the chorioallantoic membrane of the chick embryo after yolk sac inoculation, but the technique is not of great practical value.

Distribution and Epizootiology

Under natural conditions, infection by MDV occurs mainly in domestic chickens and is ubiquitous among poultry populations throughout the world. Infection of turkeys and quail by MDV sometimes occurs and can cause clinical disease. In commercial chicken flocks, virtually all birds become infected, commonly within the first few weeks of life, and the virus persists throughout life. Whether or not infection leads to clinical disease is often unpredictable and depends on several factors, including the pathogenicity of the strain of MDV, the age at infection and the genetic constitution of the birds. Losses can be especially high in areas of intensive broiler rearing. Mixed infections can occur, and natural first infection by a nonpathogenic strain of MDV (of serotype 1 or 2) can provide immunity to subsequent infection by a virulent strain. HVT is widely distributed in turkey flocks. There is no evidence that MDV or HVT present any health hazard to humans.

The main source of MDV and HVT in the environment and the vehicle for virus spread is feather debris and dander from infected birds. Infectivity is also present in oral and nasal fluids. Contaminated poultry house dust and litter remain infective for many months. The darkling beetle (*Alphitobius diaperinus*) can carry MDV passively, but does not appear to be an important vehicle for spread of virus. The infections are readily transmitted by the airborne route. Egg transmission of MDV and HVT does not occur.

A number of stresses, including handling, change of housing, vaccination and debeaking, have been anecdotally associated with exacerbation of clinical MD in infected flocks, and experimental studies have shown that the stress induced by changes in social hierarchies of birds ('pecking orders') and genetically controlled responses to stress, as measured by corticosterone levels, can influence the incidence of disease.

Clinical Features

Four clinical forms of MD are recognized.

1. The 'classical form' refers to the neural form of the disease (formerly termed 'fowl paralysis' or 'range paralysis') in which up to a third of a flock may be affected with signs of paresis or paralysis, commonly of the legs and wings, occurring usually in birds of 2–12 months of age. Iritis sometimes occurs (**Fig. 1**).
2. The 'acute form' applies to the more virulent form of the disease in which lymphomatosis of various visceral organs is common, and where the mortality may reach 80% of a flock (**Fig. 3**). Birds may be affected as early as 6 weeks of age, and losses commonly occur between 3 and 6 months, and sometimes in adults. Neural involvement is usually also present. Lymphomatous involvement of the skin ('skin leukosis') and ocular involvement may also be evident. Visceral and skin lymphomas due to MD are important causes of carcase condemnation in poultry dressing plants.
3. 'Transient paralysis' is an uncommon acute encephalitic expression of infection by MDV, occurring in birds of 5–18 weeks of age. The sudden symptoms of locomotory disorders usually disappear within 24–48 h and mortality is low.
4. Birds may die from an 'acute mortality syndrome', before the onset of lymphomas. They show severe atrophy of the thymus and bursa of Fabricius, and degenerative changes in other tissues, as a result of a severe cytolytic infection.

Pathology and Pathogenesis

Enlarged peripheral nerves are present in almost all chickens with clinical signs of classical MD and in many with the acute form of the disease (**Fig. 2**).

Figure 5 Lymphoid cell infiltration in peripheral nerve of a chicken with Marek's disease. (From Payne (1972), in *Oncogenesis and Herpesviruses*, Biggs, P.M., de The, G., and Payne L.N. (eds), pp. 21–37, IARC Scientific Publications No. 2, International Agency for Research on Cancer, Lyon, with permission.)

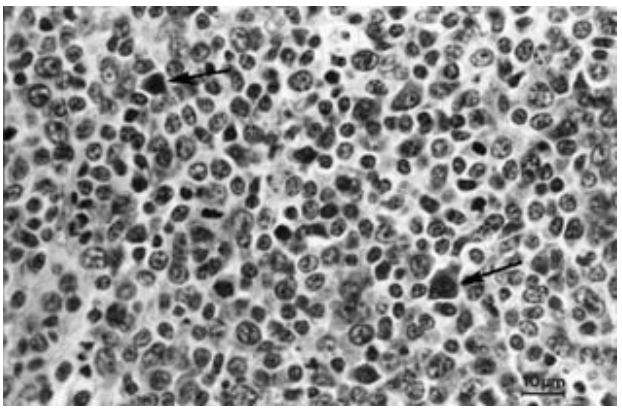


Figure 7 Ovarian lymphoma showing varying morphology of lymphoid cells, including so-called MD cells (degenerative lymphoblasts) (arrows). (From Payne (1985) in *Marek's Disease*, Payne, L.N. (ed), pp 43–75, Martinus Nijhoff Publishing, Boston, with permission.)

Affected nerves are usually two or three times their normal thickness, those commonly affected being the lumbosacral and brachial plexuses, the sciatic and brachial trunks and the celiac plexus. Two main pathological processes occur in the peripheral nerves: (1) a neoplastic lymphoproliferation (**Fig. 5**), similar to that which gives rise to lymphomas in other tissues (see later), and (2) a primary segmental cell-mediated demyelination (**Fig. 6**). Nerves with lymphoproliferation and demyelination have been termed A-type lesions, and those with demyelination, light inflammatory infiltration and interneuritic edema, B-type lesions. A mild form of the B-type lesion is termed C-type. Demyelination is associated with humoral and cell-mediated immune reactions to normal myelin.

Lymphomas in visceral organs occur in a minority of cases of classical MD, notably in the ovary (**Fig. 3**), and in various organs and tissues in acute MD, particularly the gonads, liver, spleen, proventriculus, kidneys, heart and skin. The lymphomas consist of a mixture of small and medium lymphocytes, lymphoblasts, reticulum cells and macrophages (**Fig. 7**). The majority of lymphoma cells are T cells and most of the other cells are B cells. Lymphoblastoid T cell lines (**Fig. 8**) and transplantable tumors can be developed from lymphomas. A minority of lymphoma cells and lymphoblastoid cells carry a membrane antigen,

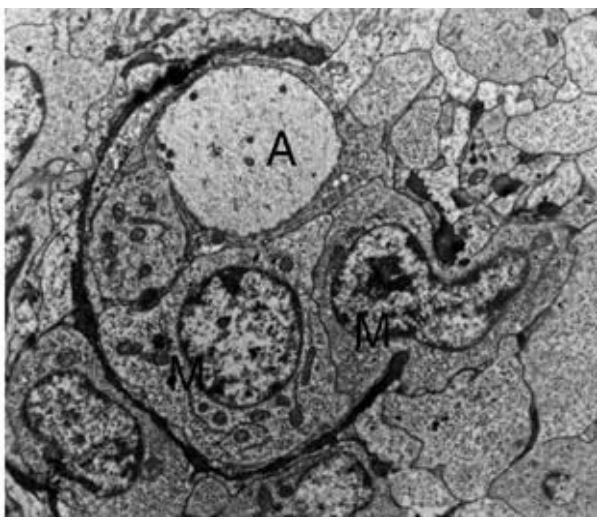


Figure 6 Invasion of a nerve fiber by macrophages (M) and demyelination of axon (A) in Marek's disease. (From Payne (1982), in *The Herpesviruses*, Vol. 1, Roizman, B (ed), pp. 347–431, Plenum Press, New York, with permission.)

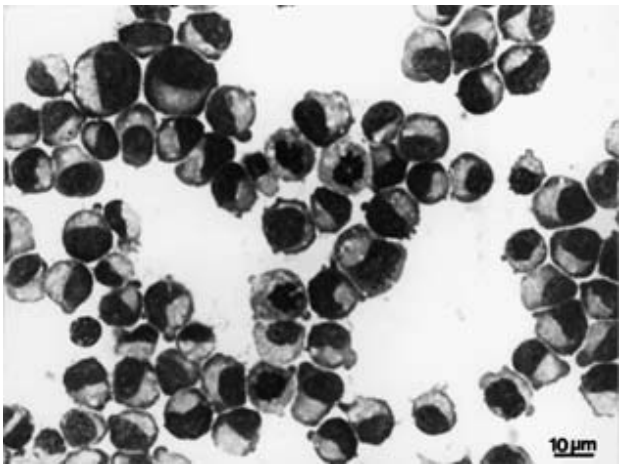


Figure 8 Lymphoblastoid cell line derived from a Marek's disease lymphoma. (From Frazier and Powell (1975), *Br. J. Cancer*, 31, 7–13, with permission.)

Marek's disease tumor-associated surface antigen (MATSA), once thought possibly to be a tumor antigen, but now considered to be a marker for an activated T cell.

Other pathological changes occurring in MD include lymphoid atrophy of the bursa of Fabricius and thymus, atherosclerosis and ocular changes. Lymphocytosis and, uncommonly, leukemia may occur. Lesions in the central nervous system are usually minimal in MD, although they are significant in the transient paralysis form.

The pathogenesis of MD is complex. Experimental studies indicate that, following infection, the virus produces an early restrictively productive and cytolytic infection mainly of B cells but also of some activated T cells. These events are seen as a phase of acute cytolytic infection particularly in the bursa, thymus and spleen occurring during the first week after infection, and there is an associated transient immunosuppression. At the end of this period, the infection switches to one of latent infection, particularly of activated T cells, associated with the development of immune responses. The regressive changes in the lymphoid organs tend to resolve and the normal architecture of the bursa, thymus and spleen is largely restored. At this time, activated T cells are believed to become targets for neoplastic transformation by MDV. CD4 + CD8−, CD4 − CD8+ and CD4 − CD8− T lymphocytes can be transformed, although lymphoblastoid tumor cell lines derived from MD lymphomas in chickens are almost exclusively CD4 + CD8− (helper) T cells. Lymphoblastoid cell lines of both T cell and B cell origin have been developed from MD lymphomas in turkeys. Development of lymphomas in chickens is associated with a second permanent period of immunosuppression and with atrophic changes in the bursa and thymus. During the first 2 weeks of infection, productive infection of feather follicle epithelium cells also becomes established with the appearance of intranuclear inclusion bodies, providing a source of cell-free infectious MDV.

Immune Responses

Birds infected with MDV develop both humoral and cell-mediated immune responses against a variety of MD-associated antigens, although the immunosuppression of MD may impair these responses. Antibodies against viral antigens can be detected in sera from infected birds by agar gel precipitation tests, immunofluorescence, virus neutralization, indirect hemagglutination, complement fixation and enzyme-linked immunosorbent assay. These antibodies are variously directed against internal antigens, membrane antigens and virus envelope. Precipitating antibodies appear 7–14 days after infection by MDV and are directed against the A (gC), B (gB) and C antigens in infected cells. Neutralizing antibodies, directed against gB antigen, peak between 6 and 12 days after infection, decline and then rise in titer, and correlate with survival. There is little evidence for significant humoral response against MD tumor cells. MDV-associated antibodies are passively transferred via the yolk to progeny chicks, and abrogate the severity of MDV infection during early life. Humoral responses are not essential for resistance to MD, since bursectomized birds may survive infection.

Cell-mediated immune responses occur against virus-associated antigens and possibly also against tumor-cell antigens believed to be present on lymphoblastoid line tumor cells. Original suggestions that MATSA was the antigen involved have not been confirmed.

Other immune responses detected include antibody-dependent cell-mediated cytotoxicity directed against virus-infected cells, and macrophages able to restrict MDV replication and MD lymphoblastoid cell line growth. Natural killer cells have also been shown to be cytotoxic to lymphoblastoid tumor cells.

Genetic Resistance

The existence of genetic resistance to MD among chickens has long been recognized, and susceptible and resistant lines can be developed by progeny testing, breeding from survivors or blood typing. Two distinct genetic loci that play a major role in controlling resistance have been identified. First, B-F region genes at the major histocompatibility (MHC)

locus, closely linked to B-G genes encoding B erythrocyte antigens, can influence resistance, the most notable being associated with the B^{21} allele. The resistant Cornell N line carries the B^{21} allele, in contrast to the susceptible P line which carries the B^{19} allele. B^{21}-associated resistance is absent at hatching but develops in early life, is accompanied by reduced numbers of infected T cells in comparison to susceptible birds, and may be due to a superior ability to reject infected or transformed T cells. A second type of resistance present at hatching, is shown by the Regional Poultry Laboratory line 6 birds and is associated with the lymphocyte antigen allele present at the non-MHC *Ly-4* locus. The resistance is associated with reduced numbers or susceptibility of target T cells for MDV infection or transformation.

Prevention and Control

MD is prevented mainly by vaccination, combined with good hygienic practices to minimize early exposure to MDV, and to some extent selection of genetically resistant stock.

Live virus vaccines have been used to control MD in commercial chickens since 1970. These are injected into chicks at hatching to provide early protection from pathogenic MDV. In recent years, an increasing number of chickens have been vaccinated *in ovo*, at 18 days of incubation. The minimum recommended dose of vaccine virus is 1000 plaque-forming units per chick. The vaccine viruses are nonpathogenic and establish a permanent infection that prevents lymphoma formation and clinical disease, although superinfection by pathogenic virus is not prevented.

Commercially available vaccines have been derived from all three serotypes, for use either alone or in combination.

Serotype 1 vaccines

Two types of commercial vaccine have been developed from this serotype.

1. Attenuated virulent serotype 1. The first commercial vaccine was of this type, developed by attenuation in cell culture of the virulent HPRS-16 strain of MDV. It is available only in a cell-associated form. Commercially its widespread use was soon superseded by the HVT (serotype 3) vaccine but it has retained some use for alternate-generation vaccination and also in combination with other serotype vaccines in localities with increased MD losses.
2. Attenuated mildly virulent serotype 1. This is exemplified by the CVI-988 (Rispens) strain of a cell-culture attenuated mildly virulent strain of MDV. It is also cell-associated, and has been widely used in The Netherlands and elsewhere. More recently it has been used in combination with HVT, and has shown protection against vv + MDV.

Serotype 2 vaccines

Serotype 2 viruses are naturally nonpathogenic strains of MDV which are widespread among poultry flocks. A representative of this serotype is the SB-1 strain which is protective, in a cell-associated form, against most strains of vMDV, but less so against vvMDV. In combination with HVT, SB-1 provides good protection against vvMDV.

Serotype 3 vaccines

The HVT represents serotype 3 and is typified by the FC-126 strain. It is widely used commercially in either a cell-associated form or a cell-free form and is highly effective against vMDV but less so when used alone against vvMDV.

Vaccine failure

A number of possible causes of vaccine failure have been recognized, the most important being challenge by MDV before vaccinal immunity has developed, delayed onset of vaccinal immunity due to interference by passively acquired antibodies, and the use of nonprotective strains of vaccine virus. Early exposure to MDV may be prevented by improved hygiene, and embryonal vaccination has been developed to provide earlier protection. Effects of passive antibodies have been counteracted by vaccinating alternate generations by different types of vaccine. Lack of protection by certain vaccines is seen when vvMDV is present: the use of bivalent vaccines, such as SB-1 plus HVT, is effective. However, even these are not fully protective against vv + MDV strains that have emerged.

Future Perspectives

Research on MD over the past 30 years has been remarkably successful in controlling one of the most serious worldwide diseases of poultry. Nevertheless, the emergence of the vv and vv+ strains of MDV during this period shows that new threats are likely to continue to appear and demand effective responses. Research continues on a wide front but most effort is currently devoted to molecular biological studies of MDV and HVT and with a view particularly to characterizing viral genes which encode antigens important in immunity and those responsible for viral virulence. This knowledge is being applied to the

development of recombinant vaccines needed to improve protection against disease. Interest is also focused on understanding the complex pathogenesis of MD, particularly the role of T cell subsets, and other cells, involved in transformation and immunity. Lastly, work on genetic resistance to MD continues, for the light it throws on the disease process, for understanding interactions between host genotype and vaccination, and because the availability of transgenic techniques in chickens means that development of genetically resistant chickens by this method offers an alternative to vaccination in disease control.

See also: **Diagnostic techniques: Detection of viral antigens, nucleic acids and specific antibodies, Isolation and identification by culture and microscopy; Epstein-Barr virus (*Herpesviridae*): General features, Molecular biology; Herpes simplex viruses (*Herpesviridae*): General features, Molecular biology; Immune response: Cell mediated immune response, General features; Vaccines and immune response.**

Further Reading

Calnek BW (1986) Marek's disease – a model for herpesvirus oncology. *CRC Crit. Rev. Microbiol.* 12: 193.

Calnek BW and Witter RL (1997) Marek's disease. In: Calnek BW (ed.) *Diseases of Poultry*, 10th edn, p. 369. Ames: Iowa State University Press.

Payne LN (ed.) (1985) *Marek's Disease. Scientific Basis and Methods of Control*, 359 pp. Boston: Martinus Nijhoff.

Silva RF, Cheng HH, Coussens PM, Lee LF and Velicer LF (eds) (1996) *Current Research on Marek's Disease*, Proceedings of the 5th International Symposium on Marek's Disease, 546 pp. Kennett Square: American Association of Avian Pathologists.

Mayaro Virus *see* **Chikungunga, O'yong Nyong and Mayaro Viruses**

MEASLES VIRUS (*PARAMYXOVIRIDAE*)

Sibylle Schneider-Schaulies and **Volker ter Meulen**, Institute for Virology and Immunobiology, University of Würzburg, Würzburg, Germany

History

Acute measles is an early childhood disease resulting from infection by the highly contagious measles virus (MV). With the isolation of the virus by Enders and Peebles in 1954 experimentation became possible and allowed significant progress with the development of tissue culture systems, the availability of monoclonal antibodies and molecular biological approaches. The virus is distributed worldwide, monotypic in nature and no significant differences between isolates from different locations have been found. MV persists in nature in populations large enough to support it, although it causes an acute infection in any individual only once in a lifetime. Thus measles is a highly successful virus, which has efficiently exploited its potential for spread, particularly as it has no animal reservoir. On rare occasions, MV may persist for years in an individual, but it is not shed as infectious virus. A single attack of measles confers lifelong immunity, even in the absence of re-exposure to the virus. Consequently, to remain endemic in a given community the virus has to infect the young who are still susceptible. This process is so efficient that the first known report of measles (in Egyptian hieroglyphs) failed to recognize the infectious nature of the illness, and described it as a normal part of child growth and development. In the prevaccine era in developed countries measles was predominantly seen in children aged 5–9 years. Infections and epidemics centered around elementary schools, and by the age of 20, approximately 99% of subjects had seroconverted. With the introduction of measles vaccine the age incidence and percentage of measles cases in different age cohorts has changed. In countries with an optimal vaccination regimen, measles has shifted to the teenage group whereas in areas with inefficient vaccine programs children up to 4 years of age reveal a high primary measles attack rate. In Third World countries measles has its greatest incidence in children

development of recombinant vaccines needed to improve protection against disease. Interest is also focused on understanding the complex pathogenesis of MD, particularly the role of T cell subsets, and other cells, involved in transformation and immunity. Lastly, work on genetic resistance to MD continues, for the light it throws on the disease process, for understanding interactions between host genotype and vaccination, and because the availability of transgenic techniques in chickens means that development of genetically resistant chickens by this method offers an alternative to vaccination in disease control.

See also: **Diagnostic techniques: Detection of viral antigens, nucleic acids and specific antibodies, Isolation and identification by culture and microscopy; Epstein-Barr virus (*Herpesviridae*): General features, Molecular biology; Herpes simplex viruses (*Herpesviridae*): General features, Molecular biology; Immune response: Cell mediated immune response, General features; Vaccines and immune response.**

Further Reading

Calnek BW (1986) Marek's disease – a model for herpesvirus oncology. *CRC Crit. Rev. Microbiol.* 12: 193.

Calnek BW and Witter RL (1997) Marek's disease. In: Calnek BW (ed.) *Diseases of Poultry*, 10th edn, p. 369. Ames: Iowa State University Press.

Payne LN (ed.) (1985) *Marek's Disease. Scientific Basis and Methods of Control*, 359 pp. Boston: Martinus Nijhoff.

Silva RF, Cheng HH, Coussens PM, Lee LF and Velicer LF (eds) (1996) *Current Research on Marek's Disease*, Proceedings of the 5th International Symposium on Marek's Disease, 546 pp. Kennett Square: American Association of Avian Pathologists.

Mayaro Virus *see* **Chikungunga, O'yong Nyong and Mayaro Viruses**

MEASLES VIRUS (*PARAMYXOVIRIDAE*)

Sibylle Schneider-Schaulies and **Volker ter Meulen**, Institute for Virology and Immunobiology, University of Würzburg, Würzburg, Germany

History

Acute measles is an early childhood disease resulting from infection by the highly contagious measles virus (MV). With the isolation of the virus by Enders and Peebles in 1954 experimentation became possible and allowed significant progress with the development of tissue culture systems, the availability of monoclonal antibodies and molecular biological approaches. The virus is distributed worldwide, monotypic in nature and no significant differences between isolates from different locations have been found. MV persists in nature in populations large enough to support it, although it causes an acute infection in any individual only once in a lifetime. Thus measles is a highly successful virus, which has efficiently exploited its potential for spread, particularly as it has no animal reservoir. On rare occasions, MV may persist for years in an individual, but it is not shed as infectious virus. A single attack of measles confers lifelong immunity, even in the absence of re-exposure to the virus. Consequently, to remain endemic in a given community the virus has to infect the young who are still susceptible. This process is so efficient that the first known report of measles (in Egyptian hieroglyphs) failed to recognize the infectious nature of the illness, and described it as a normal part of child growth and development. In the prevaccine era in developed countries measles was predominantly seen in children aged 5–9 years. Infections and epidemics centered around elementary schools, and by the age of 20, approximately 99% of subjects had seroconverted. With the introduction of measles vaccine the age incidence and percentage of measles cases in different age cohorts has changed. In countries with an optimal vaccination regimen, measles has shifted to the teenage group whereas in areas with inefficient vaccine programs children up to 4 years of age reveal a high primary measles attack rate. In Third World countries measles has its greatest incidence in children

Table 1 Morbilliviruses

Virus	*Natural host*	*Experimental infection*
Measles (MV)	Human	Primates, cotton rats, mouse, rat, ferret, hamster
Rinderpest (RPV)	Cattle, pig, goat, sheep	Rabbit
Peste des petits ruminants (PPRV)	Goat, sheep	Pig
Canine distemper (CDV)	Dog, fox, ferret, lion	Mouse, hamster, pig, cat
Phocine distemper (PDV)	Seal, sea-lion	Dog, mink
Dolphin (DMV)	Dolphin	Cattle, sheep, goat, dog
Porpoise (PMV)	Porpoise	Cattle, sheep, goat, dog
Horse[a]	Fruitbat, horse	?

[a] The virus was isolated recently in Australia from horses, fruitbats and two humans with fatal respiratory infections. Its natural host is not yet known, the human infection was accidental.

under 2 years of age. Here the disease is a serious problem with a high mortality (up to 10%) and its morbidity and mortality correlate with malnutrition.

Taxonomy and Classification

Measles virus (MV) is a member of the *Morbillivirus* genus in the subfamily *Paramyxovirinae* of the *Paramyxoviridae* family. It has a nonsegmented RNA genome of negative polarity and lacks a detectable virion-associated neuraminidase activity. MV is, therefore, grouped into a separate genus, the morbilliviruses, of which it is the type species. Other members of this group include: peste de petit ruminants which infects sheep and goats (PPRV), rinderpest virus which infects cattle, canine distemper virus (CDV) which infects dogs, phocine distemper virus (PDV) which infects seals and sea-lions, dolphin morbillivirus (DMV) and porpoise morbillivirus (PMV). More recently, a morbillivirus has been isolated in Australia which infects horses and accidentally humans (**Table 1**).

Molecular Biology

MV particles are highly pleomorphic with an average size of 120–250 nm and consist of a lipid envelope surrounding the viral ribonucleoprotein (RNP) complex which is composed of genomic RNA associated with proteins (**Fig. 1**). Two glycosylated viral transmembrane proteins project from the envelope surface: (1) the hemagglutinin protein (80 kDa) mediating the attachment of virions to cellular surface proteins including the MV receptor CD46, and (2) the fusion protein (only active after host-cell-mediated cleavage of a 60 kDa precursor protein (F_0) into an F_1 (40 kDa)/F_2 (22 kDa) heterodimer) mediating fusion between viral and host cell membrane at neutral pH. They interact with the matrix (M) protein which, in turn, links the envelope to the RNP core structure. The latter contains the viral genomic RNA, an about 16 kb nonsegmented RNA molecule of negative polarity, fully encapsidated by N (nucleocapsid), and the viral polymerase complex consisting of the P (phospho-) and the L (large) proteins. The L protein is a multifunctional RNP-specific RNA polymerase synthesizing replicative intermediates, progeny viral genomic RNAs and mRNAs which are also capped, methylated, edited and polyadenylated by this enzyme. The P protein is a phosphoprotein and, as cofactor, controls L protein activity during transcription and replication.

MV replication is confined to the cytoplasmic compartment. The viral polymerase complex initiates transcription at a promoter sequence located within a noncoding region at the 3′ end of the viral genome. MV-specific mRNAs are then synthesized along the viral genome with the N-specific mRNA being most abundant (followed by P, M, F, H and L). At the gene junctions, the polymerase first polyadenylates and cleaves the nascent transcript before continuing transcription of the distal gene. Alternatively, the polymerase leaves the template at the gene junctions to reinitiate at the 3′ promoter sequence. This is the reason why there is a gradient of frequency of the individual MV mRNAs in the infected cell, and this represents an efficient strategy of how the virus controls the accumulation of the corresponding translation products. The polymerase also synthesizes complete copies of the viral genome to yield the positive-stranded antigenome, that, in turn, serves as template for the synthesis of genomic RNAs of negative polarity. The replicating genome is immediately encapsidated by the viral N protein, with which the polymerase complex subsequently assembles to yield the RNP core structures. These are then

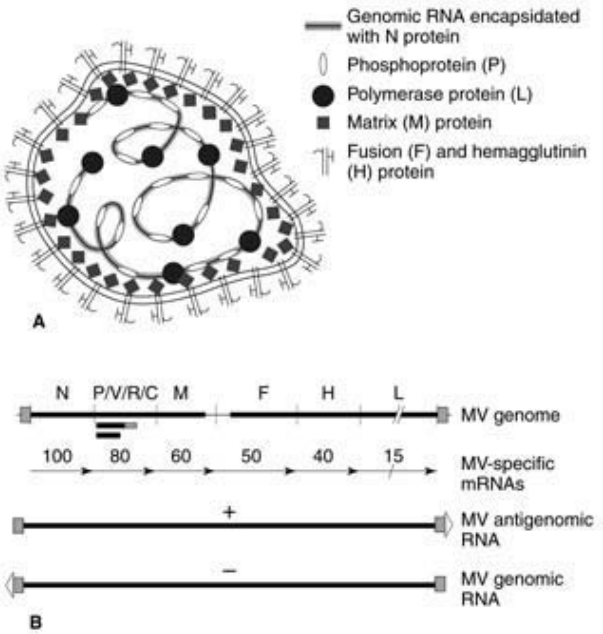


Figure 1 Measles virus, its genomic organization and transcription and replication. (**A**) The pleomorphic measles virus particle has two structural components: a host cell-derived lipid envelope bilayer in which the glycoprotein complex (containing fusion and hemagglutinin proteins) is inserted, and a ribonucleoprotein core complex consisting of the viral genomic RNA, tightly encapsidated with the nucleoprotein, and the viral polymerase complex (phospho- and large or polymerase proteins). These subunits are linked by the matrix (M) protein. (**B**) The polymerase complex initiates transcription at a promoter within the 3′ noncoding region (solid box) located upstream of the N-specific reading frame to sequentially transcribe the mRNAs for individual MV genes (arrows). The viral genes are interspersed by conserved intergenic regions where transcription either pauses (to be reinitiated at the 3′ promoter) or continues with the distal gene. Due to this phenomenon, MV specific mRNAs accumulate at different frequencies with the N-specific being most abundant (100%), the relative frequencies for the distal genes are also indicated above the arrows. For replication, the polymerase initiates within the 3′ noncoding sequence to synthesize positive-stranded replicative intermediates of the viral genome (antigenome). These, in turn, serve as templates for the synthesis of the negative-stranded genomic RNAs which, after encapsidation and association with the newly formed polymerase complexes are transported to the cell membrane to regions where the viral glycoproteins are inserted. Progenitor viral particles then bud from these locations.

transported to the cellular membrane at locations where the glycoproteins F and H are inserted, with a layer of M proteins beneath. Mature viral particles are then budding from the cell surface in order to find and attach to a new host cell. Receptor attachment mediated by the viral H protein triggers conformational changes within the F protein, thus enabling the F protein fusion domain to insert into the host cell membrane and initiating the fusion process. It is the activity of the glycoprotein complex that also mediates fusion between two adjacent cells thus enabling cell-associated spread of the virus. The fusion events occur at neutral pH and are responsible for the typical cytopathic effect of MV, the formation of giant cells, in tissue culture and *in vivo* (Warthin–Finkeldey cells in the respiratory epithelium).

The virus is dependent on the integrity of the envelope for infectivity and is inactivated by any procedure which disrupts this structure such as detergents or other lipid solvents. Particles are acid labile and inactivated below pH 4.5, although they remain infective in the range pH 5–9. The virus is also thermolabile. It may remain infective for two weeks at 4°C, but it is completely inactivated in 30 min at 56°C. At 37°C it has a half-life of 2 h. Virus can be stored for prolonged periods at −70°C and also freeze-dried.

Geographic and Seasonal Distribution

Measles occur worldwide and are endemic in regions with inadequate vaccine coverage. The efficiency of transmission is high and outbreaks have been reported in populations where as few as 3–7% of individuals are susceptible. There is generally no seasonal distribution of measles epidemics. The disease incidence in the northern hemisphere tends to rise in winter and spring when lowered relative humidity would favor respiratory transmission. In equatorial regions epidemics of measles can occur in the hot dry seasons. In large unimmunized populations, measles epidemics occur at regular intervals, and the number of susceptible individuals or the frequency of contact between infectious and susceptible individuals will lead to an increase in measles cases.

Host Range and Virus Propagation

Measles infections are confined to humans, although monkeys are also susceptible. Experimentally, only monkeys and cotton rats (*Sigmodon hispidus*) can be successfully infected intranasally. Rodents are generally nonsusceptible to inoculation by this route, and susceptibility to MV infection was not improved in both mice and rats genetically engineered to express the MV receptor CD46. Intracerebral infection with certain MV strains leads to the development of CNS diseases in rodents which have been extensively used to study the pathogenesis of MV-induced CNS-diseases in humans.

Both *in vivo* and in tissue culture, almost any cell type of human and primate origin supports MV

replication. During primary infection, MV reveals a pronounced tropism for peripheral blood mononuclear cells and is found in both lymphocytes and monocyte/macrophages. Apparently, in lymphocytes MV is replicated only efficiently after activation, whereas monocytes may not support productive replication at all. As a key determinant for viral tropism, the MV receptor consisting of a complex between CD46 and moesin is expressed on the surface of susceptible cells. Several isoforms of CD46, whose natural function is to protect cells from lysis by activated complement, exist, and all of them have been shown to support MV uptake. Expression of CD46 is not the only determinant of MV tropism. Stable expression of human CD46 does not confer susceptibility to MV-infection in many mouse cells and in CD46 transgenic animals. Rodent brain cells support MV replication both *in vivo* after experimental infection and in tissue culture, although they do not express CD46. Thus, additional or alternative receptors as well as intracellular factors that so far have not been characterized also essentially determine MV tropism.

Measles can be isolated directly from blood or from urine sediment cells using susceptible tissue cultures. These include primary human embryo kidney (HEK) or continuous cell lines derived from green monkey kidney cells (Vero or CV-1). Epstein–Barr virus-transformed B-lymphoblastoid cell lines (B95a, BJAB) are preferentially used for MV isolation as they reveal a high susceptibility to MV present in clinical specimens. Moreover, MV isolated in B95a cells differed in some biological properties from those adapted to Vero cells, suggesting that MV is subject to host cell-mediated selection. Cytopathic effects (CPE) usually develop between 48 h and 15 days, and consist either of a broad syncytium or of a stellate form.

Genetics and Evolution

The monotypic nature of MV in serological terms has masked the existence of a set of genotypes which accumulate mutations continuously. During the recent past, the molecular epidemiology of MV has been intensely studied to reveal whether MV strains differ in pathogenicity or to monitor the antigenic drift in wild-type MV strains. Sequence analyses on vaccine and wild-type MV strains as well as isolates from persistent MV central nervous system (CNS) infections (SSPE) have enabled the construction of evolutionary trees that clearly indicate their relationship. The sequence of the COOH-terminal 151 amino acids of the N protein of 46 strains of MV revealed an up to 7.2% divergence in the coding sequence and 10.6% divergence in the amino acid sequence between the most unrelated strains. MV strains group into several different genotypes some of which are extinct (i.e. have not been isolated recently) or are still cocirculating in the human population. The vaccine strains differ markedly from the wild-type isolates, but efficiently control MV infection with viruses of different genotypes. The CNS-derived isolates are similar to wild-type viruses. Based on these sequence similarities, it was possible to identify wild-type MVs that had circulated in a given population as infectious agents and found later in brain material from patients with subacute sclerosing panencephalitis (SSPE).

Serologic Relationships and Variability

Measles virus is monotypic in nature, i.e. only a single serotype of the virus has been described. Antigenic differences as observed with monoclonal antibodies between vaccine and wild-type viruses need further study as it is not clear how much this affects the ability of wild-type strains to replicate and be transmitted from persons with waning immunity generated by vaccination with a different genotype. MV isolates have been obtained from many different locations and from patients with different clinical conditions. Much effort has been invested in attempts to distinguish between these viruses and, in particular, to identify any strains which might be predisposed toward the production of encephalitis or SSPE. Conventional serological techniques have, so far, failed to demonstrate any significant differences. Thus infection by any one MV confers immunity to them all.

Epidemiology

The efficient spread of the virus is mediated by aerosol droplets and respiratory secretions which can remain infectious for several hours. Acquisition of the infection is via the upper respiratory tract, the nose and, possibly, the conjunctivae. Virus is also shed in the urine but this is unlikely to be an important means of transmission. The spread of measles has been used as a convenient example to illustrate the principles of epidemiology, and it has been calculated that any community of less than 500 000 is unlikely to have a high enough birth rate to supply the number of susceptible children required for the continuous maintenance of the virus in the population. In fact, there has been documentation of the complete elimination of measles from isolated groups which remained free of the disease until its reintroduction from outside, when susceptible individuals were once more at risk. There appears to be an interrelationship between continuous urban transmission and rural

outbreaks. Given the minimum population of 400 000–500 000 susceptible individuals to sustain measles transmission, rural transmission is likely to be initiated by cases from nearby cities. This observation underlines the importance of coverage not only at a country, but even at the village or community level.

Measles often leads to more serious disease in such communities experiencing the illness for the first time, because all age groups are susceptible to the infection. The WHO estimates that globally 45 million cases and 1.2 million deaths occur annually due to measles. In general, measles mortality is highest in children under 2 years of age and in adults. Death from uncomplicated measles is rare in the developed world, but the introduction of the virus to the Fiji Islands in 1875 resulted in an epidemic with a fatality rate of 20–25% and introduction into Greenland in 1951 produced an epidemic which infected 100% of the susceptible population and resulted in a death rate of 18 per 1000.

Natural immunity is known to last for at least 65 years even in the absence of re-exposure. In 1781 measles disappeared from the Faroe Islands following an epidemic and was not reintroduced until 1846. Individuals old enough to have experienced the disease 65 years previously were still protected. This unusual persistence of immunity has suggested that MV may normally persist inside the body and restimulates immunity from within.

Transmission and Tissue Tropism

MV is spread by droplets and first gains entry into the body through the upper respiratory tract or conjunctivae where it initially replicates. The first sign of infection is virus replication in the draining lymph nodes and destruction of lymphoid tissue which leads to a pronounced leukopenia. The virus then spreads to the rest of the reticuloendothelial system and respiratory tract through the blood (primary viremia). About five days after infection the virus overflows from the compartments in which it has hitherto been replicating, to infect the skin and viscera, kidney and bladder (secondary viremia). Although spread into the CNS does not usually occur, CNS involvement is quite frequent since about 50% of patients present with electroencephalogram (EEG) and cerebrospinal fluid (CSF) changes. Giant cells, lymphoid hyperplasia and inflammatory mononuclear cell infiltrates are observed in all infected tissues.

Pathogenicity

There is no evidence that circulating MV strains would be of different pathogenicity. This has been extensively analyzed when MV outbreaks occurred in populations with a high vaccine coverage in the late 1980s. Apparently, host rather than viral determinants are associated with MV pathogenicity such as immunocompetence. In addition, no particular MV strains could be associated with the developement of late CNS complications of acute measles. In West Africa, increased mortality after MV vaccination was due to the use of a high-titer instead of medium-titer vaccine strain.

Clinical Features and Infection

About 10 days after exposure the patient enters the prodromal phase which lasts from 2 to 4 days. The initial symptoms consist of fever, malaise, sneezing, rhinitis, congestion, conjunctivitis and cough. At the beginning of the prodromal stage, a transitory rash can sometimes develop which has an urticarial or macular appearance, but disappears prior to the onset of the typical exanthem. Once the exanthem has reached its height, the fever usually falls and the conjunctivitis as well as the respiratory symptomatology begin to subside. Antibody titers rise, virus shedding then decreases and the patient rapidly improves. Continuation of clinical symptoms of the respiratory tract or fever suggests complications (**Table 2**). Modified measles occur in partially immunized hosts such as infants with residual maternal antibodies or in the course of live vaccine failure. The illness is mild and follows the regular sequence of events seen in acute measles but is less symptomatic. Atypical measles may develop after incomplete measles vaccination prior to exposure to MV and present with high fever, headache, abdominal pain, myalgia, dry cough and a pleuritic chest pain. Unlike typical measles, the exanthem develops on the distal extremities and spreads centripetally with involvement of the palms and soles. Patients often develop pneumonia with a lobular or segmental appearance accompanied by pleural effusion. Recovery from the pulmonary symptomatology is rather slow, and pulmonary lesions can sometimes be still seen by radiography months after onset of the disease.

Complications of acute measles are relatively rare, and result mainly from opportunistic secondary infection of necrotic surfaces such as those in the respiratory tract. Bacteria and other viruses can invade to cause pneumonia or other complications such as otitis media and bronchitis. The most severe complications caused directly by measles virus are giant cell pneumonia and subacute measles encephalitis, both of which occur in the immunocompromised patient as well as acute measles encephalitis and SSPE in which no underlying susceptibility factor has been

Table 2 Established MV-associated diseases and their pathogenesis

Disease	*MV-infection*	*Pathogenesis*
Acute measles	Generalized	Acute, self-limiting infection
Modified measles	Generalized	Acute, less symptomatic infection in partially immunized hosts
Atypical measles	Generalized	Acute infection following exposure after incomplete vaccination
Measles pneumonia (giant cell pneumonia)	Generalized, lung	Interstitial pneumonia
Pneumonia, bronchitis, otitis media	Generalized	Opportunistic bacterial infections
Measles encephalitis (AME)	Generalized; virus undetectable in CNS	Virus-induced autoimmune disease
Subacute sclerosing panencephalitis (SSPE)	CNS	Persistent infection of brain cells
Measles inclusion body encephalitis (MIBE)	CNS	Persistent infection of brain cells in the immunocompromised host

identified. Unusual manifestations which may complicate acute measles are myocarditis, pericarditis, hepatitis, appendicitis, mesenteric lymphadenitis and ileocolitis. If measles infection occurs during pregnancy spontaneous abortions or stillbirth may occur. Congenital malformations have also been reported. Acute encephalitis during measles (AME) is observed at a frequency of about 1 per 1000–3000 cases, and is fatal in about 15% of cases. Encephalitis usually develops when exanthem is still present within a period of 8 days after the onset of measles. Long-term sequelae include selective brain damage with retardation, recurrent convulsive seizures, hemi- and paraplegia. SSPE is a rare, fatal, slowly progressing inflammatory disease of the brain occurring years after acute measles. For unknown reasons, boys are more likely to develop SSPE than girls and no unusual features of the acute measles have ever been demonstrated. The course of SSPE is variable and usually starts with intellectual deterioration or psychological disturbance. Neurological or motor dysfunctions may take the form of dyspraxia, generalized convulsions, aphasia, visual disturbances or mild, repetitive simultaneous myoclonic jerks. Viral invasion of the retina often leads to a chorioretinitis affecting the macular area followed by blindness. Finally the disease proceeds to progressive cerebral degeneration leading to coma and death. The illness usually lasts months to several years and is inevitably fatal.

Pathology and Histopathology

At the beginning of the rash giant cells are present in the sputum, nasopharyngeal secretions and urinary sediment cells. MV is present in blood and secretions, and the patient is highly infectious. During this period, giant cells containing inclusion bodies (Warthin–Finkeldey cells) are formed in lymphoid tissue and in the epithelial surfaces of the trachea and bronchi. Koplik's spots, the pathognomonic enanthem of measles, appear on the buccal and lower labial mucosa opposite the lower molars which begin to fade some two to four days after the onset of the prodromal phase as the rash develops. The distinctive maculopapular rash appears, about 10–12 days after exposure, behind the ears and on the forehead from where it spreads centrifugally to the feet within three days involving the face, neck, trunk, and upper and lower extremities. Once the entire body is covered the rash starts fading, sometimes accompanied by a fine desquamation. Histologically, the rash is characterized by vascular congestion, edema, epithelial necrosis and round cell infiltrates. MV-induced pneumonia is characterized by the formation of giant cells, squamous metaplasia of the bronchiolar epithelia and alveolar lung cell proliferation. Virus can easily be detected in lung biospsy material. To the contrary, MV is usually not found in the CNS of patients with measles encephalitis. Histopathological findings rather include demyelination, perivascular cuffing, gliosis, and the appearance of fat-laden macrophages near the blood vessel walls. CSF findings in measles encephalitis consist usually of mild pleocytosis and absence of measles antibodies. As SSPE develops on the basis of a persistent MV infection of the CNS, viral nucleic acids, proteins and intracellular nucleocapsid structures are detected in large amounts in neurons and glial cells. Characteristically, the expression of the viral envelope proteins is found severely impaired in SSPE brain material, and this is based on both transcriptional attenuation of and accumulation of mutations within the corresponding viral genes (**Fig. 2**). Due to these particular restrictions, budding of infectious viral particles does not occur, and the

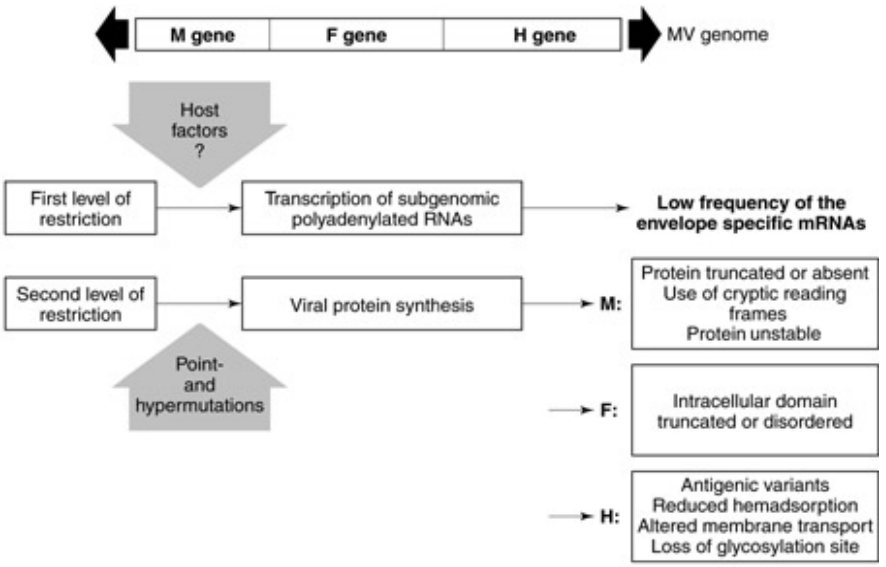


Figure 2 Measles virus gene expression is restricted in persistent brain infections. Whereas MV N and P proteins are easily detected within infected cells in brain material from patients with SSPE, the expression of the viral envelope proteins M, F and H is extremely low or undetectable. Viral gene expression is restricted both on the level of transcription and translation of the envelope-specific mRNAs. Host cell factors are presumably involved in downregulation of MV transcription, particularly that of the envelope protein genes which are distally located (see also **Fig. 1**). In addition, mutations are found within these reading frames which are due to the infidelity of the viral polymerase (point mutations) or are actively introduced by a host cell enzyme (as clustered transitions, called hypermutations). These mutations interfere with the production of functional envelope proteins.

virus is able to survive intracellularly in the presence of an efficient immune response. Neuropathologically, a diffuse encephalitis affecting both the white and the gray matter is seen, characterized by perivascular cuffing and diffuse lymphocytic infiltration. Glial cells may proliferate, and fibrous astrocytes, neurons and oligodendroglial cells contain intranuclear viral inclusion bodies. Giant cell formation or membrane changes consistent with virus maturation are absent. Characteristic EEG changes consist of periodic high-amplitude slow wave complexes (Radermecker) which are remarkably stereotyped in that their form remains identical in any given lead. They are bilateral, usually synchronous, symmetrical and consist of two or more delta waves which appear biphasic or polyphasic. Another pathognomonic finding is the state of hyperimmunization against measles virus as well as the prominent virus-specific gammaglobulin increase in CSF which is of restricted heterogeneity reflecting intrathecal Ig synthesis.

Immune Response

The rash clearly marks the onset of the immune response to infection as determined by the appearance of antiviral antibody and the infiltration of mononuclear cells into local areas of virus replication and the appearance of virus-specific T cells in the blood. Virus is then rapidly cleared from blood and tissue. Antibodies are induced to most viral proteins and can be measured by their biological effects. N-specific antibodies are most abundant and rapidly produced. They are the major antibodies detected in complement fixation tests; however, they are not protective. Virus-neutralizing antibodies are directed against the H protein, as they prevent the viral attachment to host cells. These antibodies are protective and can be measured by the inhibition of MV-induced agglutination of monkey erythrocytes (HI) and in neutralization tests. F-specific antibodies are not neutralizing but are important to prevent viral spread by cell fusion.

Activation of MV-specific cellular immunity is of greater importance for overcoming the acute infection than that of the humoral immune response. This is supported by the fact that children with agammaglobulinemia recover from acute measles normally whereas those with T cell deficiencies frequently have severe complications. Both MV-specific CD4+ and CD8+ T cells are present in the blood during acute measles, and lysis of MV-infected cells *in vitro* has been documented with CD8+ cytotoxic T lymphocytes and major histocompatibility (MHC) class II

restricted CD4+ T cells. Cytokine patterns observed in patients' plasma reveal that initially predominantly interleukin (IL)-2 and interferon γ (IFN-γ) are produced followed by high levels of IL-4. A delayed hypersensitivity reaction is implicated in the production of the rash, and thus the rash is not observed in patients with T cell deficiencies. After resolution of the rash, both CD4+ and CD8+ memory cells can be found in peripheral blood.

Concomitant with the induction of an efficient MV-specific immune response, immunity to other antigens is severely suppressed. This is characterized *in vivo* by a marked lymphopenia, an increased susceptibility to opportunistic infections and a transient loss of delayed-type hypersensitivity (DTH) reactions. Proliferation of lymphocytes isolated from measles patients is strongly impaired in response to recall antigen, allogen and mitogens. This transient general immune suppression is the major reason for the high morbidity/mortality rate associated with this disease particularly in Third World countries.

Prevention and Control

Currently, a live attenuated vaccine is used worldwide. The first attenuated vaccine strain was the Edmonston B strain produced by serial passage of the virus in human kidney cells, human amnion cells, chick chorioallantoic membrane and, finally, duck embryo cells. This vaccine was administered intramuscularly or subcutaneously 12–18 months after the disappearance of maternal antibodies. It was effective and achieved seroconversion in 95% of recipients, but side effects of mild measles were common (5–10%). The side effects and necessity to provide additional gammaglobulin led to the development of the further attenuated and less reactogenic Schwartz and Moraten (Enders) strains derived by further passage in the chick embryo at lowered temperature. Both vaccines produced a 95% seroconversion rate. In recent years, the Edmonston strain was further attenuated by passing it in human diploid cells. This vaccine, referred to as Edmonston Zagreb strain, has been shown to produce higher seroconversion rates than the Schwartz vaccine when administered at the same age. Similar observations have been made with the AIK-C vaccine, produced in Japan. Levels of antibodies induced are protective but generally lower than after measles and are still measurable up to 15 years after immunization in the absence of boosting infections.

In the prevaccine era, an estimated 4–5 million cases occurred annually in the USA and by the age of 15 years 95% of the population seroconverted. Following the rigorous implementation of the MV vaccination program, the case reports have fallen dramatically from 500 000 annually to 26 000 in 1978 and 1500 in 1983. From 1984 through 1988 only 3700 cases were registered. As a result of measles vaccination, the mortality and AME have also declined and the available experience indicates that SSPE can also be prevented by measles vaccination. However, in 1989 and 1990 a dramatic increase in acute measles cases was observed in industrialized countries with high vaccine coverage such as the USA indicating that lifelong immunity may not be induced by the application of live measles vaccine. As a result of these measles epidemics, the American Academy of Pediatrics (AAP) and the Immunization Practices Advisory Committee of the USA (ACIP) recommended a change from a one-dose to a two-dose schedule for measles vaccination which is expected to be optimal for measles control and eventual elimination. The first dose of measles vaccination should be administered at 15 months of age, as delayed primary measles vaccination (at 15 months of age or later) significantly reduces measles risk at later ages. A second dose is recommended at 4–6 years of age by ACIP, whereas the AAP recommends revaccination at the age of 11–12 years.

Future Perspectives

Since MV has no animal reservoir, it is an obvious target for a controlled campaign aimed at the eradication of the virus. In the USA and Canada, where vaccination of all children is required at or before commencing school, quite startling results have been achieved. Case reports have fallen by over 99% but eradication has not yet been achieved. In Germany and the UK, where vaccination is not mandatory, distrust of vaccination has led to a lower acceptance rate and less dramatic results have been achieved. In the Third World, where the consequences of MV infection are most severe, considerable effort has been expended with comparatively little return. Vaccination in these areas has so far failed to yield dramatic results. This is largely due to the epidemiology of measles in these areas. Interrupting transmission through vaccination would require high vaccination coverage rates early in life (>80%), and even with extremely high vaccination coverage rates outbreaks can be expected to occur. In order to achieve high population immunity in endemic areas, both the regimen of vaccine application and the generation of vaccines which are safe and efficient even in the presence of maternal antibodies require

further improvement. Progress made in the molecular biology of MV could lead to the development of genetically engineered measles vaccines which should be free of side effects and possibly suitable for the immunization of individuals currently at greatest risk, such as young infants or those suffering from chronic debilitating diseases.

See also: **Latency; Mumps virus (*Paramyxoviridae*); Parainfluenza viruses (*Paramyxoviridae*): Animal, Human; Pathogenesis: Animal viruses; Persistent viral infection.**

Further Reading

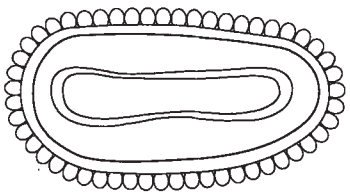
ter Meulen V and Billeter MA (eds) (1995) Measles virus. *Curr. Top Microbiol. Immunol.* 191.

Carter MJ and ter Meulen V (1995) Measles virus. In: Zuckerman AJ, Banatvala JE and Pattison JR (eds) *Principles and Practice of Clinical Virology*, 3rd edn, p. 337. Chichester, New York, Brisbane, Toronto, Singapore: Wiley.

Griffin DE and Bellini WJ (1996) Measles virus. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 1267. Philadelphia: Lippincott–Raven.

Mink Enteritis Virus *see* **Parvoviruses**

MOLLUSCUM CONTAGIOSUM VIRUS (*POXVIRIDAE*)



LC Archard and **BA Soteriou**, Division of Biomedical Sciences, Imperial College School of Medicine, London, UK

CD Porter, Institute for Cancer Research, London, UK

History

Molluscum contagiosum is a benign skin tumor of humans resulting from enhanced basal cell proliferation. The disease was first described by Bateman in 1814 and the characteristic intracytoplasmic inclusion bodies (molluscum bodies) were described independently in 1841 by Henderson and Paterson. The viral etiology of the disease was eventually established in 1905, when Juliusberg successfully transmitted the disease to one of three individuals inoculated with a filtered extract of molluscum contagiosum lesions. Molecular studies of the virus have been greatly hindered by the nonavailability of a means of propagation *in vitro*.

Taxonomy and Classification

Molluscum contagiosum virus (MCV) belongs to the *Poxviridae* family of DNA viruses and is a member of the subfamily *Chordopoxvirinae* (poxviruses of vertebrates). MCV is the sole member of the genus *Molluscipoxvirus* and is antigenically unrelated to any other genera of this subfamily, within which species are serologically related. Molecular studies have identified three subtypes of the virus (MCV I, II and III: **Fig. 1**) which show a degree of genome diversity greater than that shown amongst species of other poxvirus genera (e.g. the orthopoxviruses). The MCV subtypes are independent but closely related and cause clinically indistinguishable lesions.

Properties of the Virion

The MCV particle is approximately 100 × 200 × 300 nm in size and has the morphology typical of a poxvirus. The mature virion consists of inner and outer membranes enclosing a dumbbell-shaped core, with two lateral bodies underlying the membranes. The virion core contains the viral genome and virus-encoded enzymes necessary to establish the cytoplasmic site of replication in the newly infected cell, including those for viral gene transcription and mRNA modification. Virions purified from clinical lesions have similar physical and chemical properties to those of vaccinia virus with respect to sedimentation characteristics, DNA content and absence of RNA.

further improvement. Progress made in the molecular biology of MV could lead to the development of genetically engineered measles vaccines which should be free of side effects and possibly suitable for the immunization of individuals currently at greatest risk, such as young infants or those suffering from chronic debilitating diseases.

See also: **Latency; Mumps virus (*Paramyxoviridae*); Parainfluenza viruses (*Paramyxoviridae*): Animal, Human; Pathogenesis: Animal viruses; Persistent viral infection.**

Further Reading

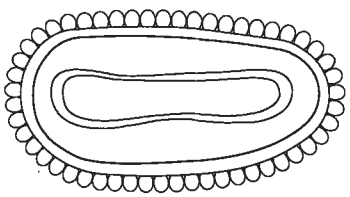
ter Meulen V and Billeter MA (eds) (1995) Measles virus. *Curr. Top Microbiol. Immunol.* 191.

Carter MJ and ter Meulen V (1995) Measles virus. In: Zuckerman AJ, Banatvala JE and Pattison JR (eds) *Principles and Practice of Clinical Virology*, 3rd edn, p. 337. Chichester, New York, Brisbane, Toronto, Singapore: Wiley.

Griffin DE and Bellini WJ (1996) Measles virus. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 1267. Philadelphia: Lippincott–Raven.

Mink Enteritis Virus *see* **Parvoviruses**

MOLLUSCUM CONTAGIOSUM VIRUS (*POXVIRIDAE*)



LC Archard and **BA Soteriou**, Division of Biomedical Sciences, Imperial College School of Medicine, London, UK

CD Porter, Institute for Cancer Research, London, UK

History

Molluscum contagiosum is a benign skin tumor of humans resulting from enhanced basal cell proliferation. The disease was first described by Bateman in 1814 and the characteristic intracytoplasmic inclusion bodies (molluscum bodies) were described independently in 1841 by Henderson and Paterson. The viral etiology of the disease was eventually established in 1905, when Juliusberg successfully transmitted the disease to one of three individuals inoculated with a filtered extract of molluscum contagiosum lesions. Molecular studies of the virus have been greatly hindered by the nonavailability of a means of propagation *in vitro*.

Taxonomy and Classification

Molluscum contagiosum virus (MCV) belongs to the *Poxviridae* family of DNA viruses and is a member of the subfamily *Chordopoxvirinae* (poxviruses of vertebrates). MCV is the sole member of the genus *Molluscipoxvirus* and is antigenically unrelated to any other genera of this subfamily, within which species are serologically related. Molecular studies have identified three subtypes of the virus (MCV I, II and III: **Fig. 1**) which show a degree of genome diversity greater than that shown amongst species of other poxvirus genera (e.g. the orthopoxviruses). The MCV subtypes are independent but closely related and cause clinically indistinguishable lesions.

Properties of the Virion

The MCV particle is approximately 100 × 200 × 300 nm in size and has the morphology typical of a poxvirus. The mature virion consists of inner and outer membranes enclosing a dumbbell-shaped core, with two lateral bodies underlying the membranes. The virion core contains the viral genome and virus-encoded enzymes necessary to establish the cytoplasmic site of replication in the newly infected cell, including those for viral gene transcription and mRNA modification. Virions purified from clinical lesions have similar physical and chemical properties to those of vaccinia virus with respect to sedimentation characteristics, DNA content and absence of RNA.

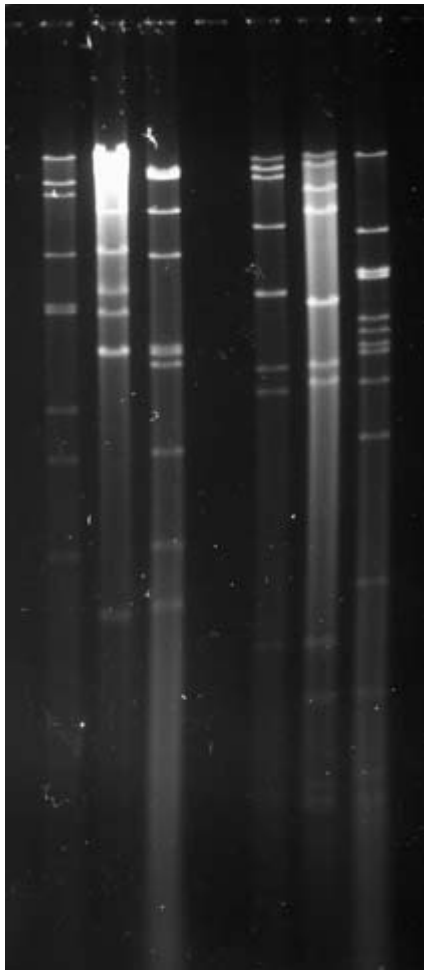


Figure 1 Restriction digests of DNA from MCV subtypes I–III. Digests with the restriction enzymes *Cla*I (left-hand panel) and *Bam*HI (right-hand panel) of DNA from MCV isolates of subtypes I, II and III (right to left).

Properties of the Viral Genome

The genome of MCV is a single, linear double-stranded DNA molecule. It is about 190 kb long, typical of poxvirus genomes which range from 130 kb (parapoxviruses) to 280 kb (avipoxviruses). In common with all poxvirus genomes and related to their mode of replication, the termini are covalently closed, the whole genome being a continuous strand of self-complementary DNA. The regions most easily denatured and so lowest in G + C content are those at the ends of the genome. The overall G + C content of MCV DNA is high (63%) and dissimilar to that of orthopoxviruses (34%). This base composition results from biased codon usage in MCV genes, with G or C occurring preferentially at the third codon position.

As with other poxvirus genomes, MCV DNA has terminal inverted repeats which vary in size from about 4 kb to 10 kb in particular isolates but are identical at each end of the genome. These inverted repeats contain numerous tandem repetitions, generally present as two major blocks: the length polymorphism arises from variation in the copy number of each particular tandem repeat sequence. These features are related to the mechanism of DNA replication.

Comparison of restriction endonuclease maps has defined three independent subtypes of MCV, designated MCV I, II and III, among over 250 isolates. Cross-hybridization shows that despite their distinguishable restriction maps, the subtypes are closely related at the nucleotide level and their genomes are essentially colinear. An exception to this colinearity is a 12 kb region unique to MCV I DNA and mapping around 150 kb from the left terminus; the significance of this is unknown as there are no biological differences apparent between the viral subtypes.

Partial nucleotide sequences from several MCV subtypes and the complete genomic sequence of one type have been analyzed extensively. This has demonstrated open reading frames whose predicted translation products are conserved both at the amino acid level and geographically between MCV and orthopoxviruses, confirming the principle of general colinearity of their genetic organization. There are, additionally, MCV genes whose putative products have no homologue in orthopoxviruses and, conversely, some orthopoxvirus genes are not represented in MCV. Specifically, analysis of the complete genomic sequence suggests that MCV encodes 182 proteins, of which 105 have homologues in orthopoxviruses: these include functions involved in genome replication and expression, suggesting that basic mechanisms are similar. The remaining 77 have no known homologues in other poxviruses and some may be involved in the evasion of host immune responses: examples include inhibitors of chemokine activities and of apoptosis (see below).

Properties of the Viral Proteins

More than 40 polypeptides are detectable in purified virions. Two major structural proteins, present in surface or subsurface virion layers but absent from virion cores, show size variation between independent isolates. An MCV homologue of the vaccinia virus major envelope antigen has a predicted 62% amino acid sequence similarity to the vaccinia protein, despite the marked difference in nucleotide composition of MCV and vaccinia DNA. However, amino acid identity in the N-terminal region preceding the apparent transmembrane domain is lower (26%) and

may explain the lack of antigenic crossreactivity of MCV and vaccinia.

Comprehensive analysis of the complete MCV genomic sequence reveals 182 open reading frames. Putative translation products of 105 of these show varying levels of homology with early or late vaccinia virus gene products, and some demonstrate conservation of geographical location between the genomes. As many vaccinia genes associated with virus genome replication and expression and some encoding structural proteins of the virion have counterparts in MCV, the implication is that functions required for the replication of a large DNA virus in the cytoplasm of infected cells are conserved between these antigenically unrelated poxviruses.

Of the 77 MCV genes with no obvious counterparts in other poxviruses, some have a degree of homology with cellular proteins, a number of which may be related to evasion of host immune responses (see below). Examples include a chemokine homologue with broad-spectrum chemokine antagonist activity and inhibitors of several pathways of apoptosis.

Despite the fact that the hypertrophic skin lesion is generated by increased proliferation of basal cells which do not support MCV replication (see below), no genes with homology to growth factors such as the epidermal growth factor (EGF)-like vaccinia virus growth factor have been found.

Replication and Transcriptional Control

MCV is typical of poxviruses in its genome structure and cytoplasmic site of replication and is expected to be functionally similar to the group as a whole in these respects. The open reading frames detected by MCV DNA sequence analysis show tight clustering of genes, transcribed from opposing strands in either direction, as seen in vaccinia virus. Transcriptional promoter and termination sequences are highly conserved by comparison with orthopoxviruses and, being AT-rich, are particularly obvious in relation to the GC-rich coding sequences of MCV DNA. For example, the 15 bp core promoter in the 5′ non-translated region of 29 known vaccinia early genes is similar in sequence and location in the corresponding MCV genes. Analogously, the TAAAT transcriptional initiator sequence of vaccinia late gene promoters is conserved in MCV homologues. Some orthopoxvirus genes contain both early and late promoter consensus sequences, and this remarkable combination of transcription signals is preserved in most of their MCV counterparts. Intermediate vaccinia promoters containing an AAANAA core sequence located 11–13 nucleotides upstream of a TAAA transcription initiator site are also conserved in the MCV gene homologues.

Transcription stops 20–50 bp downstream of the TTTTTNT termination sequence of vaccinia early genes and this sequence is present at the end of many predicted MCV early genes. Termination of transcription of late genes is poorly characterized but consensus early promotor sequences are often present, implying adjacent early genes.

Poxvirus DNA is replicated as a concatemer and resolution to genomic DNA in vaccinia is controlled by near terminal, late promoter-like sequences consisting of a TAAAT element separated by 7–9 nucleotides from a run of 5–7 consecutive T residues; this signal is conserved in MCV DNA.

These features suggest that genetic organization, control of gene expression and DNA replication in MCV is similar in many respects to vaccinia. A recombinant vaccinia virus containing an MCV type I DNA fragment, which includes the p43K gene encoding the major envelope antigen, has been used to demonstrate that MCV promoter elements are recognized by the transcriptional mechanisms of a serologically unrelated poxvirus.

Geographic and Seasonal Distribution

Molluscum contagiosum occurs worldwide and year round. Its incidence has been variously reported as 10% of children in Papua New Guinea in 1971, 4.5% of the entire population of a Fijian village in 1966 and 1.2% of outpatients in Scotland between 1956 and 1963. Preliminary data suggest that there is a different geographical distribution of the three MCV subtypes. The relative occurrence of MCV I to MCV II ranges from 1:1 in a small (eight patients) American study group, 2:1 for Australia, 3:1 for the UK, to 50:1 for Scotland. MCV III is relatively rare (approximately 1% of cases).

Host Range and Viral Propagation

MCV is one of two poxviruses regarded as being specific for the human host; the other is variola, the agent of smallpox. There have been occasional reports of molluscum contagiosum-like disease in other species, although these have not been substantiated by molecular analysis. MCV has been transmitted by inoculation to human subjects, but similar experiments with monkeys, apes, sheep, fowl, guinea pigs and mice were unsuccessful.

Attempts to grow the virus on the chick embryo chorioallantoic membrane, in human embryonic skin organ cultures and in primary and continuous cell cultures have been unsuccessful, although in the last

system limited transmission of a cytopathic effect (CPE) and induction of interferon were observed. Propagation of MCV in human amnion cells was reported, but attempts to repeat this were unsuccessful. The MCV-induced CPE lasted for 10 days, after which the cells returned to normal and could respond to fresh challenge. Virus attachment and release of virion cores into the cytoplasm occurred, but uncoating of the cores did not follow and the infection cycle aborted. Simultaneous infection with other poxviruses did not release MCV from this block. Limited replication in human foreskin grafted to mice has been reported.

As for the human papilloma viruses, the inability to culture MCV is thought to be due to the need for keratinocytes at a defined stage of differentiation, which is difficult to obtain or sustain *in vitro* but which is necessary for completion of the uncoating process. In this respect, MCV behaves as a host-dependent conditional lethal mutant.

Genetics, Evolution, Serological Relationships and Variability

Little is known of these aspects of MCV but they are expected to be similar to the poxvirus group as a whole. Specific antibody is generated during infection but MCV is serologically distinct from other poxviruses. Independent MCV isolates show variability in the size of the genomic inverted terminal repeats, as seen in orthopoxviruses. Nucleotide sequence analysis demonstrates a high degree of homology between the MCV subtypes, although their restriction endonuclease maps show greater variation than is seen between species of the orthopox, parapox or capripoxvirus genera. Phylogenetic analysis based on the sequence of the viral DNA polymerase, an enzyme essential to replication of a DNA virus in the cytoplasm of infected cells, suggests that MCV branched from a trunk common to the evolution of orthopoxviruses and parapoxviruses, after divergence of the avipoxviruses.

Epidemiology, Transmission and Tissue Tropism

The incidence of molluscum contagiosum varies in different populations. Infectivity is relatively low in a European environment, with a peak incidence of between 10 and 12 years of age. In contrast, the Fiji study showed that 25% of households harbored more than one case and the peak incidence was in children aged between 2 and 3 years.

Transmission of MCV is mechanical. Mature virus reaches the surface of the skin at the disease site by extrusion from the lesion core. Transmission follows close physical contact (hence *contagiosum*), presumably gaining access via a break in the skin. MCV replicates in the keratinocytes of the epidermis but not in the cells of the stratum basalis.

Molluscum contagiosum infection in adults is commonly transmitted by sexual contact. Molluscum contagiosum is prevalent in the sexually active and its incidence is rising commensurately with other sexually transmitted diseases, and in association with immunosuppression.

Pathogenicity

The incubation period varies from 1 week to several months. Infection is self-limiting and most individual lesions resolve spontaneously within a few months without treatment. However, the disease may persist as a result of autoinoculation, questioning the protective value of specific antibody. Complications may result from intradermal lesion rupture, giving rise to an inflammatory response. Inflammation is generally associated with clinical resolution of the lesion, suggesting that the virus-coded chemokine inhibitor may be functional in maintenance of persistent infection. For the majority of patients the disease is no more than a cosmetic problem, although persistence over long periods may imply impaired immunity. Widespread lesions are common in patients with atopic dermatitis.

Clinical Features of Infection

Molluscum contagiosum lesions consist of papules, 2–4 mm in diameter, with an umbilicated center, from which a white curdy core can be expressed. They may be solitary or clustered and can occur anywhere on the skin, although rarely on the palms and soles. Giant solitary lesions, with diameters of 15 mm or more, and intrafollicular lesions are rare variants. The lesions are often barely apparent and may go unnoticed by patients. However, eczema can develop around the lesions and they can become inflamed and secondarily infected prior to resolution. Molluscum contagiosum on the eyelids may be associated with conjunctivitis.

Pathology and Histopathology

In the molluscum contagiosum lesion, the epidermis grows down into the dermis and projects above the surface as a papule. The basement membrane remains intact. The basal cells are larger and more columnar than usual and intracytoplasmic inclusion bodies (Henderson–Paterson or molluscum bodies), the site of virus assembly, appear first in the cells of the

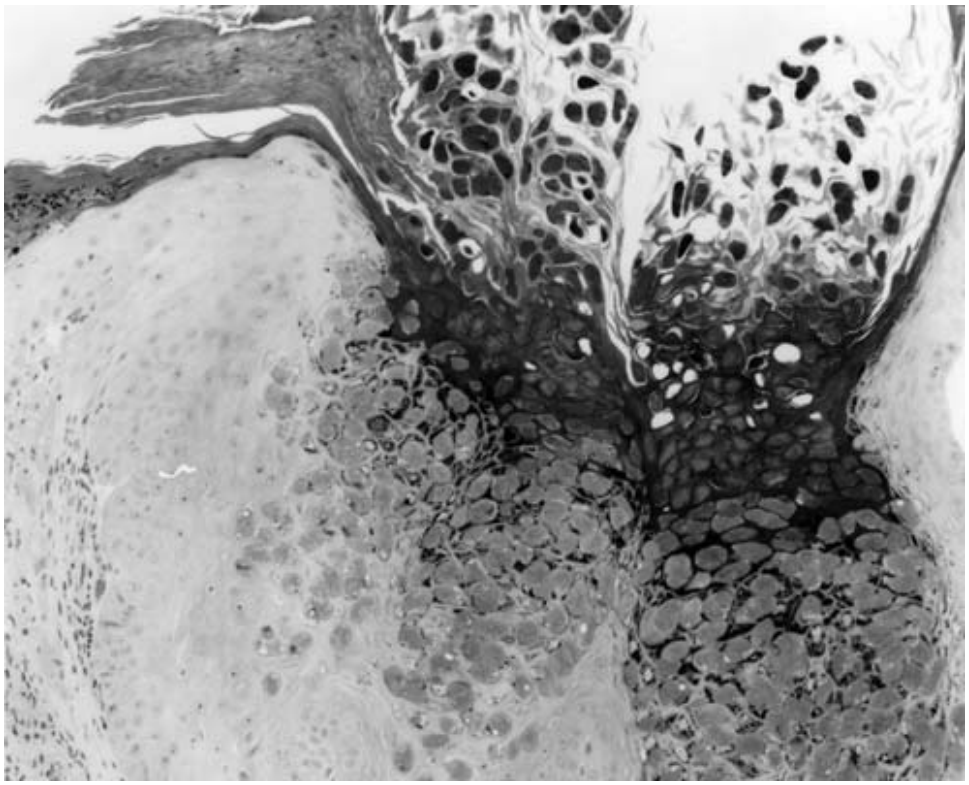


Figure 2 Molluscum contagiosum lesion. Skin section showing lobules of keratinocytes containing large intracytoplasmic inclusions (molluscum bodies). The stratum corneum has disintegrated in the center of the lesion, releasing the molluscum bodies and generating a central crater. Hematoxylin and eosin; ×100. (Reproduced with permission from Dr D Woodrow and R Barnett, Charing Cross Hospital, London.)

stratum spinosum. The molluscum bodies expand and fill the cytoplasm as the affected cells migrate through the stratum granulosum towards the surface (**Fig. 2**). Viral particles are present exclusively in the cytoplasm of infected cells and do not occur in basal cells or in uninfected cells adjacent to the lesion: the latter continue to develop normally. The virion colony is enclosed by a sac within each infected keratinocyte. In the stratum corneum, the degenerating keratinocytes with their inclusion bodies become enmeshed in the keratin network. The central core of the lesion is an expressible soft material consisting of husks of epidermal cells packed with the elementary bodies of the virus.

The molluscum contagiosum tumor results both from hypertrophy of epidermal cells, due to the presence of the virus-packed inclusion bodies, and from hyperplasia of basal cells, which show a twofold reduction in their division time. Infected cells migrate through the epidermis at a more uniform rate of 9–15 days to reach the stratum granulosum, compared with 9–30 days for uninfected cells. Viral DNA replication occurs within the first 4–5 days, and host transcription is inhibited. Some hyperplasia of the dermal fibroblasts subjacent to the lesion has been reported, although these cells, like the basal cells, do not contain virus. It has been proposed that MCV-infected keratinocytes may secrete a virus-encoded growth factor, analogous to vaccinia virus growth factor, which stimulates the proliferation of adjacent cells. Analysis of the MCV genomic sequence reveals no such coding capability (see above) and so this stimulation may be a secondary effect, mediated by a cellular product induced by infection of keratinocytes.

Immune Response

Lesions can persist in normal individuals for months or years without any sign of inflammation. Even when some lesions are removed, others may appear and the virus can be difficult to eradicate. There is a lack of T lymphocytes and natural killer cells in the base of the lesions and no inflammatory infiltrate in the early eruptive phase. While some orthopox genes encoding proteins expected to inhibit cellular immune responses (e.g. complement activation, interferon, tumor necrosis factor and interleukin 1 activities) are not represented, MCV encodes a truncated CC-type chemokine which lacks agonist activity but is a broad-spectrum chemokine antagonist. Both encode a

3β-hydroxysteroid dehydrogenase activity. Resolution may be accompanied by erythema, initially at the base of the lesion, and a predominantly mononuclear infiltrate has been taken to imply a cell-mediated immune response. In one study, two-thirds of patients had an antibody response, predominantly of the IgG class. The lack of response in the remainder may be due to the superficial site of infection and the outward migration of infected cells within the epidermis. Most patients retain virus-specific antibody after resolution. The involvement of immune responses in the resolution of molluscum contagiosum lesions is implicated by the observation that lesions may be numerous, widespread and long-lasting in the iatrogenically immunosuppressed, those with atopic dermatitis and those with acquired immunodeficiency syndrome (AIDS).

Prevention and Control

Treatment, when given, involves lesion disruption by expression of the core, or removal by curettage.

See also: **Genetics of animal viruses; Smallpox and monkeypox viruses (*Poxviridae*); Vaccinia virus (*Poxviridae*); Vectors: Animal viruses.**

Further Reading

Blake NW, Porter CD and Archard LC (1991) Characterisation of a molluscum contagiosum virus homologue of the vaccinia virus p37K major envelope antigen. *J. Virol.* 65: 3583.

Damon I, Murphy PM and Moss B (1998) Broad spectrum chemokine antagonist activity of a human poxvirus chemokine homolog. *Proc. Natl Acad. Sci. USA* 95: 6403.

Douglass NJ, Blake NW, Cream JJ *et al* (1996) Similarity in genome organisation between molluscum contagiosum virus (MCV) and vaccinia virus (VV): identification of MCV homologues of the VV genes for protein kinase 2, structural protein VP8, RNA polymerase 35 kDa subunit and 3β-hydroxysteroid dehydrogenase. *J. Gen. Virol.* 77: 3113.

Porter CD and Archard LC (1992) Characterisation by restriction mapping of three subtypes of molluscum contagiosum virus. *J. Med. Virol.* 38: 1.

Porter CD *et al* (1992) Molluscum contagiosum virus. In: Wright D and Archard L (eds) *Molecular Biology and Cell Biology Sexually Transmitted Diseases*, p. 233. London: Chapman & Hall.

Postlethwaite R (1970) Molluscum contagiosum: a review. *Arch. Environ. Health* **21**: 432.

Senkevich TG, Bugert JJ, Sisler JR *et al* (1996) Genome sequence of a human tumorigenic poxvirus: prediction of specific host response-evasion genes. *Science* 273: 813.

Senkevich TG, Koonin EV, Bugert JJ, Darai G and Moss B (1997) The genome of molluscum contagiosum virus: analysis and comparison with other poxviruses. *Virology* 233: 19.

Monkeypox Virus *see* **Smallpox and Monkeypox Viruses**

Morbilliviruses *see* **Rinderpest and Distemper Viruses (*Paramyxoviridae*)**

MOUSE MAMMARY TUMOR VIRUS (*RETROVIRIDAE*)

Jaquelin P Dudley, The Department of Microbiology and Institute for Cellular and Molecular Biology, The University of Texas at Austin, Austin, USA

History

The mouse mammary tumor virus (MMTV) first was reported in 1933 by the Jackson Memorial Laboratory and by Korteweg in 1934 as an extrachromosomal influence on the incidence of breast cancer in inbred mouse strains. Initial crosses between high-mammary-cancer-incidence mice and low-mammary-cancer-incidence mice revealed that progeny invariably had the mammary cancer incidence of the female

3β-hydroxysteroid dehydrogenase activity. Resolution may be accompanied by erythema, initially at the base of the lesion, and a predominantly mononuclear infiltrate has been taken to imply a cell-mediated immune response. In one study, two-thirds of patients had an antibody response, predominantly of the IgG class. The lack of response in the remainder may be due to the superficial site of infection and the outward migration of infected cells within the epidermis. Most patients retain virus-specific antibody after resolution. The involvement of immune responses in the resolution of molluscum contagiosum lesions is implicated by the observation that lesions may be numerous, widespread and long-lasting in the iatrogenically immunosuppressed, those with atopic dermatitis and those with acquired immunodeficiency syndrome (AIDS).

Prevention and Control

Treatment, when given, involves lesion disruption by expression of the core, or removal by curettage.

See also: **Genetics of animal viruses; Smallpox and monkeypox viruses (*Poxviridae*); Vaccinia virus (*Poxviridae*); Vectors: Animal viruses.**

Further Reading

Blake NW, Porter CD and Archard LC (1991) Characterisation of a molluscum contagiosum virus homologue of the vaccinia virus p37K major envelope antigen. *J. Virol.* 65: 3583.

Damon I, Murphy PM and Moss B (1998) Broad spectrum chemokine antagonist activity of a human poxvirus chemokine homolog. *Proc. Natl Acad. Sci. USA* 95: 6403.

Douglass NJ, Blake NW, Cream JJ *et al* (1996) Similarity in genome organisation between molluscum contagiosum virus (MCV) and vaccinia virus (VV): identification of MCV homologues of the VV genes for protein kinase 2, structural protein VP8, RNA polymerase 35 kDa subunit and 3β-hydroxysteroid dehydrogenase. *J. Gen. Virol.* 77: 3113.

Porter CD and Archard LC (1992) Characterisation by restriction mapping of three subtypes of molluscum contagiosum virus. *J. Med. Virol.* 38: 1.

Porter CD *et al* (1992) Molluscum contagiosum virus. In: Wright D and Archard L (eds) *Molecular Biology and Cell Biology Sexually Transmitted Diseases*, p. 233. London: Chapman & Hall.

Postlethwaite R (1970) Molluscum contagiosum: a review. *Arch. Environ. Health* **21**: 432.

Senkevich TG, Bugert JJ, Sisler JR *et al* (1996) Genome sequence of a human tumorigenic poxvirus: prediction of specific host response-evasion genes. *Science* 273: 813.

Senkevich TG, Koonin EV, Bugert JJ, Darai G and Moss B (1997) The genome of molluscum contagiosum virus: analysis and comparison with other poxviruses. *Virology* 233: 19.

Monkeypox Virus *see* **Smallpox and Monkeypox Viruses**

Morbilliviruses *see* **Rinderpest and Distemper Viruses (*Paramyxoviridae*)**

MOUSE MAMMARY TUMOR VIRUS (*RETROVIRIDAE*)

Jaquelin P Dudley, The Department of Microbiology and Institute for Cellular and Molecular Biology, The University of Texas at Austin, Austin, USA

History

The mouse mammary tumor virus (MMTV) first was reported in 1933 by the Jackson Memorial Laboratory and by Korteweg in 1934 as an extrachromosomal influence on the incidence of breast cancer in inbred mouse strains. Initial crosses between high-mammary-cancer-incidence mice and low-mammary-cancer-incidence mice revealed that progeny invariably had the mammary cancer incidence of the female

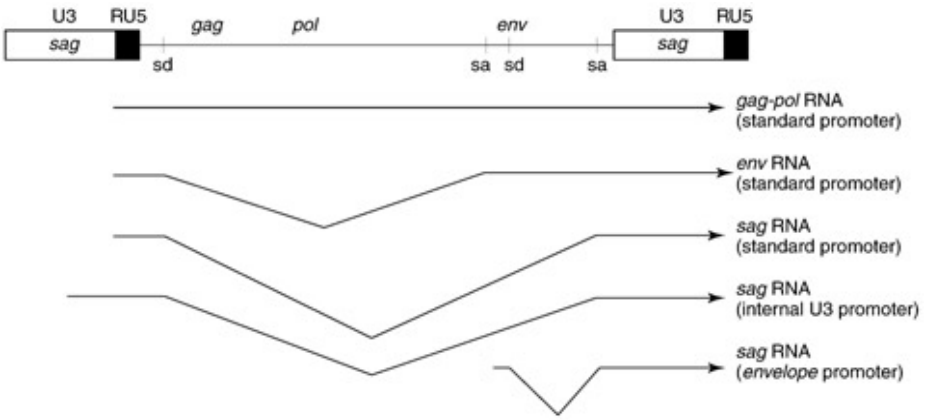


Figure 1 Proposed structure for spliced MMTV RNAs from promoters in the U3 and envelope regions. Arrows show the direction of transcription, and the V-shaped regions indicate sequences that are removed by splicing. All RNAs are shown with respect to a provirus containing the viral structural genes, *gag*, *pol* and *env*. LTRs are shown as boxes. RNAs initiating from the internal U3 promoter may contain additional splicing events upstream of the splice shown.

parent. Subsequently, Bittner showed that an extrachromosomal factor was transmitted through maternal milk, and this factor was later associated with viral particles, termed B-particles, that could cause breast cancer in susceptible animals.

Taxonomy and Classification

MMTV is the prototype species of the genus Gammaretrovirus-B within the family *Retroviridae*. Type B viruses (approximately 100 nm in diameter) are distinguished from other retroviruses by their characteristic morphology under the electron microscope, including an acentric electron-dense core. Milk-borne strains of MMTV often incorporate the name of the inbred mouse strain from which they were derived, e.g. C3H MMTV or MMTV (C3H). Endogenous MMTV proviruses found in the germline of most common inbred mouse strains are referred to as *Mtv* followed by an Arabic number, e.g. *Mtv-8*.

Properties of the Virion

The B-type particle contains a genomic RNA dimer that contains two copies of a single-stranded linear RNA encapsidated by NC protein as a helical ribonucleoprotein (RNP); reverse transcriptase (RT) is closely associated with this RNP. Surrounding the RNP is an icosahedral capsid (although this structure is not entirely clear) composed of CA protein, and this capsid is bound to the envelope by the matrix protein, MA. The detergent-sensitive viral envelope contains numerous spikes that are composed of a transmembrane (TM) protein and a surface (SU) protein.

Properties of the Genome

The viral RNA is bounded at either end by a short direct repeat (R) (15 bases) (**Fig. 1**). Unique regions are present at the 5′ (U5) and 3′ (U3) ends of the genome, and these regions comprise approximately 120 and 1200 bases, respectively. The primer-binding site (PBS), located just downstream of U5, is an exact complement of 18 nucleotides from the 3′ end of tRNA3Lys. Although the 'psi' site for RNA packaging has not been defined, this site is likely to include the region between U5 and the splice donor site for the envelope (*env*) mRNA. The PBS precedes the *gag* region that encodes the nonglycosylated proteins of the virion in the order NH_2-MA-p21-CA-NC-COOH, and this region is followed by the genes for protease (PR) and RT. At the 3′ end of the genome, the envelope proteins are encoded in the order NH_2-SU-TM-COOH just prior to the U3 and R regions. Unlike most other retroviruses, which contain multiple stop codons within the long terminal repeat (LTR), MMTV has an open reading frame (simply known as *orf* or *sag*) within the U3 region. The *sag* gene encodes a protein that has been conserved in all known strains of endogenous and milk-borne MMTVs. The Sag protein plays a critical role in transmission of milk-borne virus from the gut of newborn mice to the mammary gland (see below).

Virus Replication

As with other retroviruses, little is known about the processes that govern the entry and exit of MMTV from infected host cells. However, it is clear that the surface glycoprotein gp52 (SU) mediates the attachment of the virus to the host-encoded viral receptor

(also known as MTVR). Monoclonal antibodies against gp52 will block viral entry. Following entry and partial uncoating due to adsorptive endocytosis, the virally encoded reverse transcriptase produces a double-stranded (ds) DNA copy (also known as the provirus) of the viral RNA. The viral RT synthesizes a minus-strand DNA primed by lysyl-tRNA to form an RNA–DNA hybrid. Using the virally encoded ribonuclease H, the majority of the virion RNA is degraded, and the plus-stranded DNA is completed to form the provirus that, in contrast to the template RNA, contains a long terminal repeat composed of U3, R and U5 regions (**Fig. 1**).

The provirus then enters the nucleus subsequent to nuclear membrane breakdown during mitosis, and viral DNA integrates using the MMTV-encoded integrase (IN) protein, one of the products of the *pol* gene. The IN protein introduces an asymmetric cut 2 bp from the linear ends of the provirus as well as an asymmetric break exactly 6 bp apart on opposite DNA strands of the host cell chromosome. Although there appear to be no discrete sites for proviral integration, like other retroviruses, it is possible that integration occurs preferentially in regions that have an open chromatin structure, e.g. transcriptionally active sites. Following joining events, the repair of the virus–cell junction generates a direct repeat of cellular DNA that, in turn, flanks the viral LTRs. This structure resembles the transposable elements of bacteria, yeast and *Drosophila*.

The integrated provirus contains all the signals necessary for recognition by RNA polymerase II, and many of these signals are present in the U3 region of the LTR. Generally, transcription from the standard promoter is initiated in the 5′ LTR starting at the U3/R junction and terminating at the R/U5 junction in the 3′ LTR (**Fig. 1**). Because of the terminal redundancy, the polyadenylation signals are present in both LTRs, but the sequence in the 5′ LTR is ignored because of the multipartite nature of the poly(A) signal. Termination appears to be reasonably inefficient, and a number of MMTV transcripts probably terminate in the adjacent cellular DNA. Transcripts are capped by cellular enzymes in the nucleus. A portion of this RNA leaves the nucleus in the unspliced form, whereas the remainder is spliced in at least two alternative ways to generate the *envelope* mRNA and, in some cases, the *superantigen* or *sag* mRNA.

Full-length transcripts (8.7 kb) are translated into Gag, Gag-Pro and Gag-Pro-Pol precursor proteins. Since *pro* (the viral *protease* gene) and *pol* (the *polymerase* gene) are out-of-frame with respect to *gag* and each other, two independent ribosomal frameshifts are required to obtain Gag-Pro and then Gag-Pro-Pol. Because of the ribosomal frameshifting events, the viral PR and RT are made at a fraction of the amounts of Gag synthesized. The viral envelope mRNA is translated into a precursor protein on membrane-bound polyribosomes. The protein precursor is modified by glycosylation in both the endoplasmic reticulum and the Golgi, and protein cleavage to SU and TM also occurs in the latter compartment. The *sag* mRNA (approximately 1.4 kb) appears to be translated into a type II transmembrane protein of 36 kDa; this protein is glycosylated and reportedly cleaved to generate a C-terminal 18 kDa fragments. Sag is associated with major histocompatibility complex (MHC) type II protein. Unlike the other virally encoded proteins, Sag is not believed to be a structural component of virions (see below).

Recent evidence suggests that *sag* mRNA can be initiated from at least two other MMTV promoters (**Fig. 1**). One of these promoters is located within the U3 region of the LTR, resulting in RNA initiation approximately 500 bp upstream of the U3/R junction. This RNA can be processed to form both singly and doubly spliced RNAs containing only the *sag* open reading frame. In addition, a promoter within the envelope region gives a spliced RNA with a different splice donor, but the same splice acceptor as that originally described for spliced *sag* RNAs from the standard promoter. The use of the standard promoter, the internal U3 promoter or the *envelope* promoter for synthesis of Sag protein appears to be specific for different strains of MMTV. The amount of spliced *sag* mRNA produced from any of these promoters appears to be very low compared to the amount of *gag-pol* or *env* mRNAs produced.

The precursors for Gag, Gag-Pro, and Gag-Pro-Pol aggregate within the cell cytoplasm into procapsids (approximately 70 nm in diameter) that are referred to as intracytoplasmic A particles. This process is distinct from the maturation of C-type particles which assemble the Gag precursors at the cell surface membrane concomitant with the budding process. Presumably the precursor proteins are folded so that nucleocapsid and RT proteins are sequestered inside the particle to interact with a dimer of viral RNA. The viral PR, which is present in a fraction of the Gag precursors, is apparently responsible for the cleavage events that produce the mature virion proteins MA, p21 (function unknown), CA and NC. The procapsid very likely initiates budding through viral glycoprotein-modified cellular membranes following interaction between the MA protein and the cytoplasmic tail of the TM protein. RT appears to be inactive in newly forming virions since enzyme activation requires several cleavage events by PR to form the infectious mature B-particles.

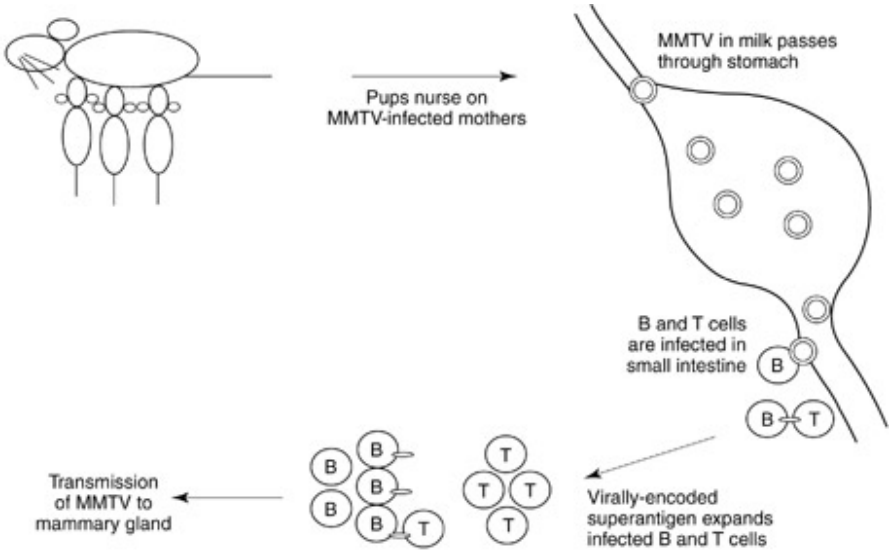


Figure 2 Life cycle of milk-borne MMTV. Newborn pups ingest virus-infected milk from their mothers, and virus particles pass through the stomach to encounter lymphoid cells associated with the gut epithelial cells. B cells become infected and express Sag at the cell surface in conjunction with MHC class II protein. The Sag–MHC complex is recognized by specific T cell subsets that respond by the release of cytokines. These cytokines stimulate the proliferation of bystander B and T cells that may be the target for additional MMTV integrations. Cytokines also amplify the number of previously infected cells. The mechanism for transfer of MMTV infection by lymphoid cells to the mammary gland is unclear.

Transmission and Tissue Tropism

MMTV is transmitted horizontally through maternal milk (called exogenous or milk-borne virus) or vertically through the germline (endogenous viruses). The exogenous viruses are responsible primarily for the high mammary cancer incidence of mouse strains such as RIII and C3H. However, some strains, such as GR, carry an endogenous virus (in this case, *Mtv-2*) that is also transmitted through milk.

Our current understanding of MMTV transmission through milk is shown in **Fig. 2**. MMTV particles ingested from mother's milk survive passage through the stomach during the first few weeks of life prior to maturation of the intestinal tract. These particles enter the small intestine where they probably cross the intestinal epithelial layer via the specialized M cells; under this layer, MMTV virions encounter and infect B cells. Following integration and transcription of the infectious provirus, the Sag protein is presented at the B-cell surface in association with MHC class II protein. Sag is recognized by a certain subset of T cells that are stimulated to release cytokines. Such cytokines trigger the proliferation of additional B and T cells, leading to the amplification of infected cells. Thus, MMTV develops a reservoir of infected lymphoid cells through typical viral replication events that require dividing cells to establish an integrated provirus as well as through division of previously infected cells. Both T and B cells are required for MMTV transmission since knockout or transgenic animals lacking either lymphoid subset cannot be infected by the milk-borne route. This lymphoid cell reservoir is apparently required to preserve MMTV infectivity prior to the onset of puberty in mice when a source of dividing mammary gland cells is available. Recent data also suggest that Sag-mediated stimulation of lymphoid cells is required to improve the efficiency of viral transfer within the mammary gland. MMTV production is increased during lactation when glucocorticoid hormones elevate transcription from the hormone-responsive element (HRE) in the LTR U3 region (**Fig. 3**). Increased expression of virions during lactation ensures that large amounts of particles will be produced at a time when newborn offspring can be infected.

Horizontal transmission of MMTV by seminal or salivary fluid has been reported. It is possible that the low infectivity of most body fluids (other than milk) is attributable to the presence of virally infected lymphoid cells and the absence of large amounts of infectious MMTV particles.

Genetics

MMTV causes mammary adenocarcinomas by infection of the mammary epithelium with either exogenous or endogenous virus. The genetic factors that influence mammary tumor incidence in mice include

(1) the presence or absence of milk-borne virus, (2) the presence of an infectious endogenous MMTV, (3) cellular factors that determine genetic resistance and susceptibility to the virus and (4) host factors that may influence the immune response (including the Sag response) or hormonal levels in the animals. The presence of milk-borne virus has been shown in all high-mammary-cancer-incidence strains by foster-nursing experiments, as originally described by Bittner. However, shortly thereafter, it was discovered that high-mammary-cancer incidence strains (such as C3H) could be converted permanently to a lower mammary tumor frequency by foster-nursing them on low-mammary-cancer-incidence strains (e.g. BALB/c). Such strains, known as C3Hf, have a mammary tumor incidence between 38 and 47% (average latency 600 days) in multiparous animals whereas C3H strains containing milk-borne MMTV have a mammary tumor incidence of 88–95% (average latency 300 days) in breeding females. Mammary tumors appearing in C3Hf mice are the result of expression of a replication-competent endogenous provirus known as *Mtv-1*. Strains, such as BALB/c and C57BL, which have a mammary tumor incidence of 1% or less and long tumor latencies, lack a replication-competent MMTV.

A number of cellular and host factors also influence the incidence of mammary tumors in different mouse strains. Most of these factors have not been defined, but it is likely that many of them affect the ability of MMTV to replicate. For example, the resistance of C57BL mice to MMTV infection appears to be the result of failure to express the MHC class II I-E molecule, one of two available class II molecules in the mouse. Although many MMTV Sag proteins apparently interact with class II I-A, this interaction is less efficient than the Sag/class II I-E complex in the stimulation of lymphoid cells. Loss of class II molecules is not the only defense against MMTV infection. The presence of endogenous MMTV proviruses that express Sag proteins results in the deletion of Sag-reactive T cells in the thymus during the period when the immune system selects against self-reactive T cells. Because the Sag proteins from different MMTV strains react with different T-cell subsets, endogenous MMTVs provide protection against milk-borne infection by MMTVs that encode Sags with the same T cell receptor (TCR) reactivity. For example, endogenous *Mtv-7* expresses a Sag protein that reacts with and causes deletion of Vβ6+ T cells; therefore, the milk-borne MMTV (SW) strain that encodes a TCR Vβ6-reactive Sag cannot infect mouse strains that carry *Mtv-7* because these mice lack the T cell subset required for efficient MMTV transmission.

Resistance to exogenous MMTV infection is usually a recessive characteristic, and this has been used to determine if various mouse strains have the same or different resistance genes. For example, C57BL and I strain mice are resistant to infection by C3H MMTV, yet F_1 hybrids of these strains have a high incidence of mammary cancers when infected by C3H virus. This result suggests that I strain mice ($H\text{-}2^j$) encode a functional MHC class I-E molecule that can complement the defect in C57BL mice and overcome resistance to MMTV infection. Resistance of I strain mice to MMTV infection has been linked to a strong immunological response to the virus. This idea is supported by experiments that indicate that the H-2 histocompatibility locus, particularly the D end that excludes the involvement of class II molecules, influences the susceptibility of different mouse strains to MMTV infection. Similar experiments have shown that NH mice (also resistant to C3H MMTV infection) have an independent resistance factor, perhaps related to hormonal changes that lead to early reproductive difficulties in this strain.

Traditional types of genetic experiments with MMTV have been difficult for two reasons. First, since MMTV does not form plaques or foci *in vitro*, cloning of viral stocks has not been possible. Second, molecular cloning of an intact MMTV provirus (easily obtained for most retroviruses) has been difficult for some MMTV strains because of selection against a specific region of *gag* called the 'poison' sequence. However, this difficulty has been overcome by combining the 5′ end of an endogenous virus (e.g. *Mtv-1*) with the 3′ end of an exogenous virus (e.g. C3H MMTV). Such hybrid proviruses have been transfected into tissue culture cells and shown to produce infectious virions that retain oncogenicity for the mammary gland *in vivo*.

Pathogenicity

MMTV induces primarily type A and B mammary adenocarcinomas or adenoacanthomas which often are preceded by preneoplastic lesions known as hyperplastic alveolar nodules (HANs). The appearance of HANs has been used as a means of quantifying MMTV virion preparations. The mammary tumors that arise may be hormone-independent (C3H tumors) or hormone-dependent (GR or RIII tumors). Many hormone-dependent or plaque (P)-type tumors progress to hormone-independent tumors.

Insertional mutagenesis has been presumed to be involved in MMTV-induced tumors because of the relatively long period of tumor latency and the apparent absence of a virally encoded oncogene.

Table 1 Common integration sites found in MMTV-induced mammary tumors

Locus	*Gene family*	*Mouse chromosome*	*Integration frequency*
Wnt1 (Int-1)	Wingless	15	80% in C3H; 70% in BR6; 30% in GR
Fgf3 (Int-2)	Fibroblast growth factor	7	65% in BR6; 5% in C3H; 20% in GR
Notch4 (Int-3)	Notch	17	20% in Czech II; 8% in BR6
Wnt3 (Int-4)	Wingless	11	10% in GR
Cyp19 (Int-5/Int-H/aromatase)	Cytochrome P450; aromatase	9	3 chemically induced BALB/c hyperplasias
Fgf4 (Hstf-1/Fgfk)	Fibroblast growth factor	7	10% in BR6
Fgf8	Fibroblast growth factor	19	80% in *Wnt-1* transgenic
Int-6		15	6% in *Fgf3* transgenic
Wnt10b	Wingless	15	23% of *Fgf3* transgenic

Nusse and Varmus first identified a common region of MMTV integration in 80% of C3H mammary tumors originally known as *int-1*, but renamed *Wnt-1* to signify its homology with the *Drosophila* gene *wingless*. However, current data have implicated at least nine different loci (termed *int* or integration site genes) in MMTV-induced mammary tumors (summarized in **Table 1**). Most of these integration sites share the following characteristics.

1. The majority of MMTV integrations are outside the *int* coding regions, and the MMTV promoter is rarely used to initiate *int* gene transcription. Thus, an unmodified Int protein is produced.
2. The MMTV provirus apparently activates *int* gene transcription over considerable distances (some in excess of 15 kb).
3. A definite orientation specificity of the provirus with respect to the *int* gene has been observed.
4. *int* gene transcription is not detectable in normal adult mammary gland.
5. Transcription of *int* genes appears to be regulated developmentally.
6. All *int* genes appear to be conserved evolutionarily, and a number of these genes encode growth factors or truncated growth factor receptors.

Such observations are all consistent with the transcriptional activation of conserved genes that are normally silent in the mammary gland; these genes are activated by their proximity to enhancer sequences present in the MMTV LTR. Recent evidence suggests that more than one of the *int* genes may be activated by MMTV proviral insertion in the same tumor cell; different Int proteins in mammary cells apparently cooperate during the generation and progression of tumors.

MMTV also induces T-cell lymphomas, presumably as a byproduct of replication in T cells. Like MMTV-induced mammary tumors, these lymphomas contain clonally acquired MMTV proviruses. However, unlike virally induced mammary tumors, MMTV proviruses from T-cell lymphomas invariably contain a large and specific deletion of 350–500 bp that removes several negative regulatory elements (NREs) as well as the 3′ one-third of the *sag* gene (**Fig. 3**). Many of these LTR deletions are accompanied by a duplication of U3 sequences that presumably act as a T cell-specific enhancer. Substitution of such modified LTRs into a mammotropic molecular clone causes the resulting virus to induce exclusively T cell lymphomas. However, current data do not indicate whether the alteration of *cis*-acting or *trans*-acting LTR sequences or both contribute to T cell tumor induction.

Several other tumor types, including kidney and pituitary tumors, have been shown to contain acquired MMTV proviruses. Most, if not all, of the proviruses found in these tumors also have LTR deletions, suggesting that there is a strong selection against an intact U3 region during MMTV replication in nonmammary cells.

Transcriptional Regulation

The tissue distribution of MMTV expression is linked tightly to viral transcriptional control. Many of the control elements located within the LTR have been mapped by mutation studies or by DNA-binding studies with nuclear factors (NF) (**Fig. 3**). As for many other promoters recognized by RNA polymerase II, the standard MMTV promoter contains a TATA element that is located approximately 30 bp upstream of the transcription initiation site (+1). Just

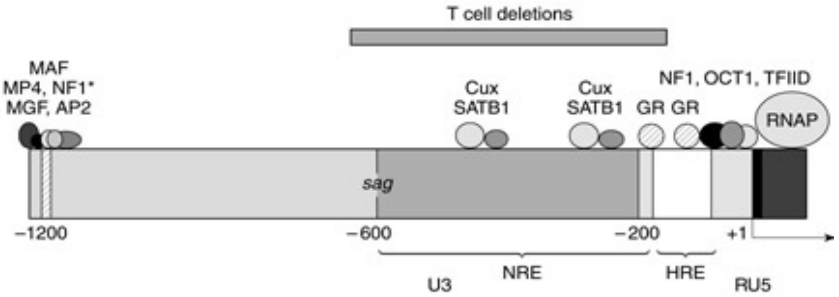


Figure 3 Location of transcription regulatory sequences within the MMTV LTR. DNA binding factors are shown as circles or ovals. In some cases, the exact number and position of the DNA-binding sites is unknown. The mammary gland enhancer is shown near the 5′ end of the LTR. The maximal deletion observed in acquired MMTV proviruses from T cell tumors is shown above the LTR; the deletion always includes the Cux and SATB1 sites shown within the NRE and, in some cases, a portion of the HRE. There appear to be multiple binding sites for OCT-1 upstream of the TATA box. Numbers below the LTR refer to the distance (in nucleotides) from the transcriptional start site of the standard promoter (+1). Circles are not intended to indicate the relative size of the DNA-binding proteins or their interactions with each other. Abbreviations: NRE, negative regulatory element; HRE, hormone response element; SATB1, special AT-rich binding protein 1; NF1, nuclear factor 1; NF1*, a member of the NF1 family; TFIID, transcription factor IID; GR, glucocorticoid receptor; OCT1, octamer-binding protein 1; RNAP, RNA polymerase II. This list of binding factors is not intended to be comprehensive; other tissue-specific and constitutive factors that bind to the MMTV LTR have been reported.

5′ to this element (−60 to −38) is a region containing 10 bp direct repeats that are mutation-sensitive and bind the nuclear factor OCT-1 (OTF-1). A third mutation-sensitive region (located at approximately −70) serves as a binding site for NF-1.

The MMTV HRE is one of the best characterized transcriptional control elements, and it has served as a model system for the study of hormonally regulated genes. Early experiments showed that MMTV proteins and virion production were increased 10- to 50-fold in the presence of glucocorticoid hormones, and this hormonal effect was demonstrable within 15 min of hormone addition, suggesting that glucocorticoids act to stimulate transcription initiation. Progesterone and androgens have also been shown to elevate MMTV-specific RNA levels. Hybrid constructs containing a minimal MMTV HRE linked to a reporter gene have shown that the hormonal inducibility of MMTV is conferred in *cis,* consistent with the presence of at least four binding sites for steroid receptors located approximately between −80 and −190. Each of these binding sites contains the consensus sequence TGTTCT, yet DNase I footprinting experiments indicate that different steroid receptors have unique DNA contacts. There appears to be cooperativity among receptor-binding sites so that the effects of binding are not strictly additive.

Although most of the receptor-binding studies have used naked DNA, some experiments have suggested that hormone receptors allow NF-1 accessibility to MMTV DNA–nucleosome complexes since NF-1 does not bind to nucleosome complexes in the absence of hormone-bound receptor. Mutations within the OCT-1 and NF-1 sites appear to affect both basal and glucocorticoid-stimulated transcriptional levels, but NF-1 may not be crucial for the response to progesterone.

The MMTV specifies several enhancer elements, and the HRE behaves as a conditional enhancer since it can function upstream or downstream of a promoter in either orientation. More recently, enhancer elements that affect mammary gland-specific transcription have been identified at the extreme 5′ end of the U3 spanning the region from −1094 to −858. These elements contain binding sites for multiple nuclear factors, including MP4, MAF, MGF (Stat5), AP-2, and a member of the NF-1/CTF transcription factor family. Synthesis of some factors is inducible by prolactin, epidermal growth factor, or tumor necrosis factor α (TNFα). Current data suggest that these factors cooperate with the HRE to provide maximal stimulation of the MMTV promoter during lactation when milk-borne transmission occurs.

Several negative elements have been reported upstream of the HRE or between the proximal and distal NRE. At least two of these NREs are deleted in MMTV proviruses integrated in T cell lymphomas, suggesting that these NREs suppress transcription in T cells. These NREs are bound by at least two homeodomain proteins called special AT-rich binding protein 1 (SATB1) and Cux/CDP. Intriguingly, the highest levels of SATB1 are expressed in T cells, but not in mature B cells or lactating mammary gland, and mutations in one SATB1 binding site elevate transcription from the MMTV promoter in transient

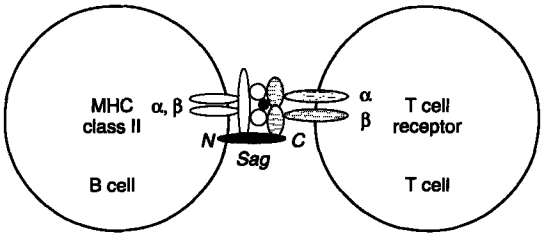


Figure 4 Interaction of MMTV Sag with the T cell receptor (TCR). The α and β chains of the TCR on CD4+ T cells combine to recognize a peptide (foreign or self) (small black circle) residing in the binding pocket of the α and β chains of the MHC class II molecule on MMTV-infected B cells. The C-terminal end of Sag interacts with the variable portion of the TCR β chain, although the TCR α chain has been reported to affect Sag binding. Accessory molecules such as CD4 probably affect the stability of Sag–TCR interaction.

transfection assays and in lymphoid tissues of transgenic mice. These data suggest that MMTV transcription is highest in lactating mammary gland because the mammary gland enhancer is active and because transcriptional suppression is abolished due to the absence of Cux and SATB1.

Immune Response

Recently, endogenous MMTVs have been found to cosegregate in mouse-breeding experiments with several genes that encode minor-lymphocyte-stimulating (Mls) antigens or Sag proteins. Unlike conventional antigens where a peptide associates with the groove formed by the MHc class II α and β chains, the central portion of Sag binds to the class II molecule (**Fig. 4**). Both endogenous and exogenous MMTVs encode Sag. Since Sag is a type II transmembrane protein, the C-terminal portion is available to interact with the TCR on a subset of CD4+ or CD4 + CD8+ cells. Sag reacts with the variable portion of the β chain of the TCR instead of the junction of the α and β chains, and therefore, Sag recognizes entire classes of T cells (up to 30% of the entire T cell repertoire). This is in sharp contrast to conventional antigens that recognize the TCR on less than 1 in 10^4 T cells. Sag stimulation leads to the rapid deletion of reactive cells if Sag is expressed in the thymus, whereas if expression occurs in nonthymic cells, but is low or absent in thymus, deletion of reactive cells appears to be slower.

Sag is a relatively conserved protein, except at the C-terminus where interaction occurs with the TCR. The polymorphism of the C-terminus has been shown to correlate with the reactivity to specific TCR β chains; for example, C3H MMTV Sag reacts with Vβ14 and 15 chains, whereas *Mtv-3* Sag reacts with Vβ3 and 5 chains. Molecular switching experiments have shown that the C-terminal half of Sag is sufficient to specify TCR reactivity, and recent experiments indicate that virtually any mutation (including two or three amino acid substitutions) within the 30–40 polymorphic C-terminal amino acids is sufficient to abolish Sag reactivity and function. Because T cells mediate MMTV infection of the mammary gland, deletion of certain T cell subsets by endogenous MMTV Sags provides immunity against specific milk-borne MMTVs or potentially other pathogens that require the same T cell subsets for infection.

Future Perspectives

MMTV has proven to be an invaluable model for studies of: the molecular mechanism of hormone action, the tissue-specificity of transcriptional control, the immune response, and mechanisms of oncogenesis. There is every reason to believe that MMTV will continue to provide insights into many biological mysteries.

See also: **Immune response: Cell mediated immune response, General features; Retroviral Oncogenes; Transformation: Animal viruses.**

Further Reading

Coffin JM (1996) Retroviridae: the viruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fundamental Virology*, 3rd edn, p. 763. Philadelphia: Lippincott–Raven.

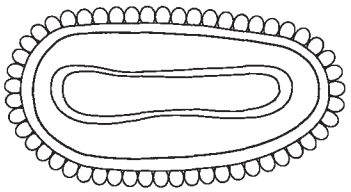
Golovkina TV, Dudley JP, Jaffe AB and Ross SR (1995) Mouse mammary tumor viruses with functional superantigen are selected during *in vivo* infection. *Proc. Natl. Acad. Sci. USA* 92: 4828.

Huber BT, Hsu PN and Sutkowski N (1996) Virus-encoded superantigens. *Microbiol. Rev.* 60: 473.

Liu J, Bramblett D, Zhu Q *et al* (1997) The matrix attachment region-binding protein SATB1 participates in negative regulation of tissue-specific gene expression. *Mol. Cell. Biol.* 17: 5275.

van Leeuwen F and Nusse R (1995) Oncogene activation and oncogene cooperation in MMTV-induced mouse mammary cancer. *Semin. Cancer Biol.* 6: 127.

MOUSEPOX AND RABBITPOX VIRUSES (*POXVIRIDAE*)



Frank Fenner, John Curtin School of Medical Research, Australian National University, Canberra, Australia

Ectromelia (mousepox) virus and rabbitpox virus share two features; they are both orthopoxviruses and both are known only as infections of laboratory animals, the mouse and rabbit respectively.

Ectromelia Virus

History

Ectromelia virus was discovered in 1930 by Marchal, as a spontaneous infection of laboratory mice at the National Institute of Medical Research in London. It was called infectious ectromelia because of the frequent occurrence of amputation of a foot in animals that had recovered from infection. Soon after, Barnard showed by UV microscopy that it had oval virions about the same size as those of vaccinia virus. The only other experiments done with the virus at that time involved studies of experimental epidemics by Topley and his colleagues.

In 1946 Burnet, in Melbourne, showed that ectromelia virus was serologically related to vaccinia virus. During experimental epidemics carried out in Burnet's laboratory, Fenner found that in animals that did not die of acute hepatitis there was a rash, and he named the disease mousepox. Subsequent studies led to the development of a model to explain the spread of virus around the body in generalized virus infections with rash.

In laboratories in Europe and the USA the virus was regarded as a major menace to colonies of laboratory mice, and stringent steps were taken to prevent its entry to the USA. Only after extensive outbreaks in several cities of that country in 1979 were studies of the virus undertaken in the USA, in high-security laboratories.

Classification

Ectromelia is a species within the genus *Orthopoxvirus*, as evidenced by the morphology of the virion, crossprotection tests and restriction endonuclease mapping. Most strains of ectromelia virus recovered from naturally infected mouse colonies appear to be of high virulence. However, a substrain of Marchal's original strain was attenuated by serial passage on the chorioallantoic membrane.

Geographic Range and Seasonal Distribution

Ectromelia virus has been spread around the world inadvertently by scientists working with laboratory mice, and has been repeatedly reported from laboratories in several countries of Europe and from Japan and China. Mousepox has never been enzootic for prolonged periods in mouse colonies in the USA, but accidental importations sometimes occurred with mice or mouse tumor material from European laboratories, with devastating consequences.

There is only one report of the occurrence of ectromelia virus in wild animals, an unconfirmed report of its presence in several species of wild rodents in Germany.

Host Range and Virus Propagation

Ectromelia virus produces disease in *Mus musculus* and several other species of mice. The rabbit, guinea pig and rat can be infected by intradermal or intranasal inoculation, with the production of small skin lesions or an inapparent infection. Ectromelia virus will grow on the chorioallantoic membrane of the developing chick embryo.

Epidemiology

In laboratory mice, ectromelia virus is infectious by all routes of inoculation; it always produces a generalized infection but there are local lesions in the lungs after intranasal inoculation and in the peritoneal cavity after intraperitoneal injection. The response of mice is strongly conditioned by mouse genotype (see below).

The usual source of natural infection is via minor abrasions of the skin, which may occur from contaminated bedding or during manipulations by animal handlers. Infection may also occur by the respiratory route, but probably only between mice in close proximity to each other. A primary lesion usually develops at the site of infection. Since mice are readily infected by inoculation, virus-contaminated mouse serum, ascites fluid or mouse cells, tumors or tissues constitute a risk to laboratory colonies previously free of infection.

Enzootic mousepox

Mousepox was enzootic in many mouse breeding establishments in Europe and Japan until the 1960s. A variety of mechanisms probably operated to maintain the virus, without so disrupting the mouse breeding program as to make control mandatory. One important factor was probably the high level of genetic resistance and trivial symptomatology exhibited by many mouse genotypes. Another may have been maternal antibody. Another possible mechanism for maintaining enzootic infection is chronic, clinically inapparent infection, which sometimes occurs after oral administration, when some mice show infection of Peyer's patches, excretion of virus in the feces and lesions in tail skin.

Susceptibility of different strains of mice

Analysis of spontaneous epizootics and deliberate experiments in the 1980s showed that C56BL/6 and AKR mice were highly resistant to mousepox and BALB/c, DBA and CH3 mice were highly susceptible. Although there was no clinical evidence of infection in C57BL/6 mice except for the swelling of the inoculated foot, serial transmission between such mice was observed for at least six generations. Strain differences are best demonstrated after footpad inoculation or in natural epizootics, as C57BL mice are relatively susceptible by intranasal, intracerebral or intraperitoneal infection.

Experimental epidemiology

In the early 1920s the British bacteriologist, Topley, embarked upon a long-term study of experimental epizootics in mice housed in specially designed cages. Studies of long-continued epizootics of ectromelia (1.75 and 3.25 years) in herds of mice maintained by the regular addition of normal mice suffered from the fact that the only indication of infection was death. In similar experiments carried out after the demonstration of the nature of the virus and the pathogenesis of the disease, Fenner, working at the Hall Institute in Melbourne, constructed life tables for mice exposed to a virulent and an attenuated strain of virus. In addition, closed epizootics were used to study several parameters of naturally spreading orthopoxvirus infection, such as immunization with vaccinia virus and the effects of old age. More recently, Anderson and May have used the results of the long-term experiments by Greenwood and Fenner in an analysis of the population biology of infectious diseases.

Pathogenesis

Fenner used mousepox as a model system for the study of generalized viral infections. Mice were infected in the footpad, and after an incubation period of some 7 days (as found in natural infections) a local ('primary') lesion developed at the inoculation site. A few days later some mice died, with no other visible skin lesions but with acute necrosis of the liver and spleen, and in those which survived a rash developed which went through macular and pustular stages before it scabbed.

By titrating organs of infected mice at daily intervals after inoculation, he showed that during the incubation period the virus passed through the mouse body in a stepwise fashion: infection, multiplication and liberation, usually accompanied by cell necrosis, first in the skin, and then the regional and possibly the deeper lymph nodes until it reached the bloodstream (primary viremia). Working in Canberra during the 1960s, Mims showed that during the primary viremia, virus was ingested by the phagocytic littoral cells of the liver and spleen. After a day or so, much larger amounts of virus were liberated into the circulation and during this secondary viremia focal infection of the skin and other organs occurred. There was again an interval during which the virus multiplied to high titer before visible changes were produced, so that an interval of 2 or 3 days usually elapsed between the appearance of the primary lesion and the secondary rash. Some animals died before skin lesions appeared, but titration experiments and histological examination showed that early skin lesions were present.

Clinical Features of Infection

Early workers described two forms of the disease, a rapidly fatal form in which apparently healthy mice died within a few hours of the first signs of illness, and showed extensive necrosis of the liver and spleen at autopsy, and a chronic form characterized by ulcerating lesions of the feet, tail and snout. Fenner found that in natural infections most mice developed a primary lesion (**Fig. 1A,B**), usually on the snout, feet or belly. Subsequently, virus multiplied to high titer in the liver and spleen. Some mice died at this stage, but if they survived they almost invariably developed a generalized rash (**Fig. 1C**).

Age affects the response of genetically susceptible mice. Both virulent and attenuated strains produced higher mortalities in suckling mice and in mice about a year old than in the 8-week-old mice.

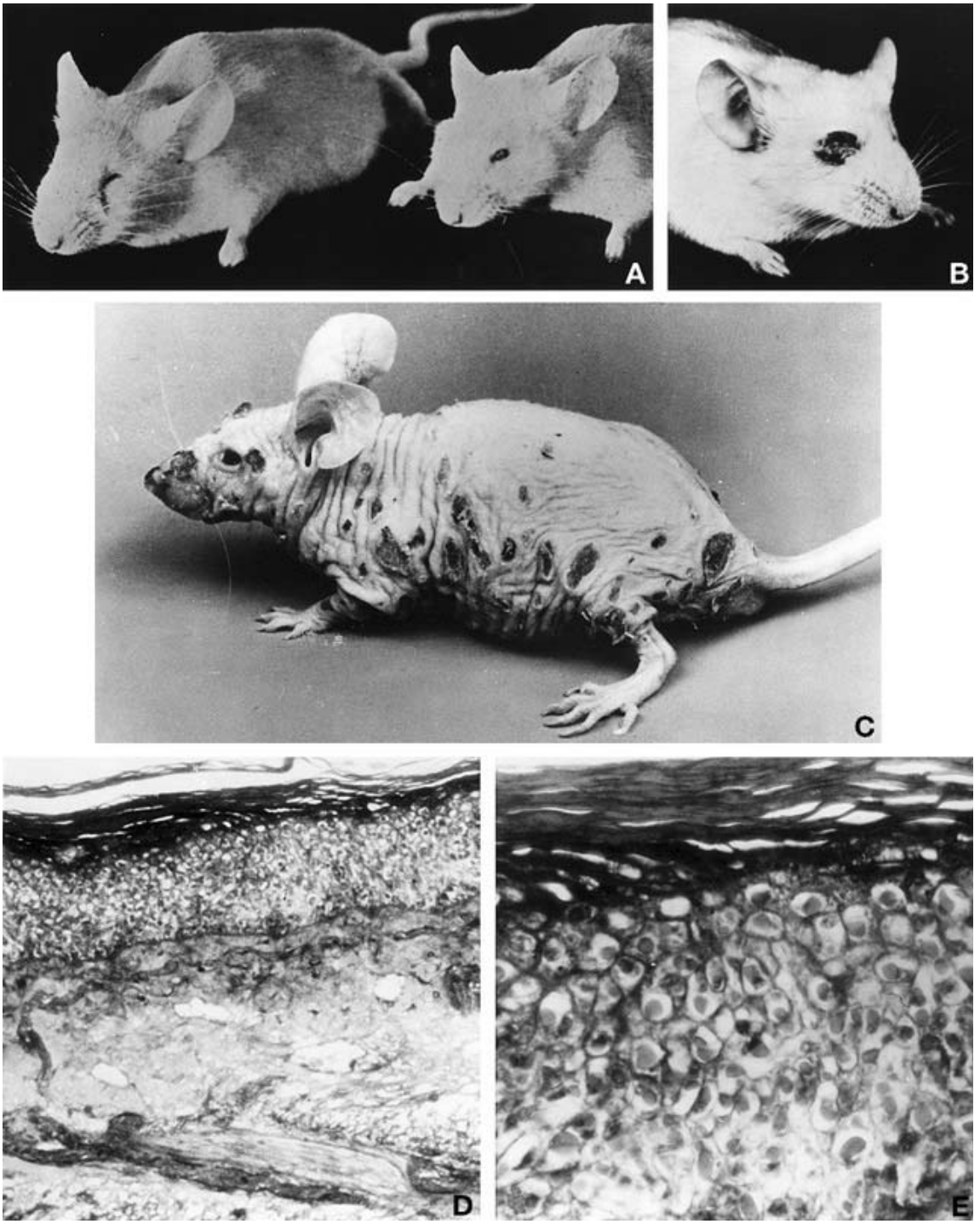


Figure 1 (**A**) An early primary lesion of mousepox beneath the left eyebrow of an outbred susceptible mouse exposed to infection with ectromelia virus 7 days earlier. Comparison with the normal mouse on the right shows the swelling of the face due to edema around the primary lesion. (From Fenner F (1948) *J. Pathol. Bacteriol.* 40: 529. Copyright John Wiley & Sons Limited. Reproduced with permission.) (**B**) Advanced primary lesion of mousepox, first seen 8 days after exposure to contact infection and photographed 5 days later. (From Fenner F (1948) *Br. J. Exp. Pathol.* 29: 29. Reproduced with permission of the publisher, Blackwell Science Ltd.) (**C**) The rash of mousepox as it appears 14 days after infection, in a naturally infected genetically hairless mouse (not athymic). Similar lesions occur beneath the hair of other strains of susceptible mice and can be clearly demonstrated by epilation. (Courtesy of the Zentralinstitut für Versuchtiere, Hannover, Germany.) (**D**) Low power and (**E**) high power view of a section of the skin of the foot of a mouse injected with ectromelia virus in the footpad 6 days earlier. Mann's strain. Almost every epithelial cell contains an eosinophilic A-type inclusion body. With most strains of ectromelia virus, these A-type inclusion bodies contain large numbers of mature virions.

Pathology and Histopathology

The pathological changes in naturally occurring mousepox in susceptible mice are quite characteristic. Additional lesions in the peritoneal cavity or lung occur after intraperitoneal or intranasal inoculation respectively; these are important because mousepox sometimes occurs after unwitting passage of ectromelia virus by these routes.

Intracytoplasmic inclusion bodies

Ectromelia virus produces two types of intracytoplasmic inclusion body in infected cells, A-type and B-type. The latter occur in all poxvirus infections and are the sites of viral multiplication; more characteristic of mousepox are the prominent acidophilic inclusion bodies (A-type), which are always found in infected epithelial cells but rarely in liver cells (**Fig. 1D,E**).

Skin lesions

The earliest primary lesions that could be recognized macroscopically were the seat of advanced histological changes, for viral multiplication had then been in progress for several days. There was no macroscopic breach of the skin surface, but the dermis and subcutaneous tissue were edematous and there was widespread lymphocytic infiltration of the dermis. Inclusion bodies could be seen in the epidermal cells at the summit of the lesion. Necrosis of these epidermal cells was followed by ulceration of the surface. The exudate formed a scab, beneath which healing occurred. Histologically the changes of the rash were similar to those in the primary lesion.

Lesions of the liver

The liver and spleen were invariably invaded during the incubation period and virus multiplied to high titer there. The liver remained macroscopically normal, until within 24 h of death, when it appeared enlarged and studded with minute white foci. The necrotic process extended rapidly and at the time of death the liver was enlarged, with many large semiconfluent necrotic foci. In animals which survived, the liver usually returned to its normal macroscopic appearance, but occasionally numerous white foci occurred.

Histologically, little change was apparent until macroscopic changes had appeared, although with fluorescent antibody staining it could be shown that infection always occurred first in the littoral cells of the hepatic ducts, from which it spread to contiguous parenchymal cells (**Fig. 2**). Numerous scattered foci of necrosis then appeared throughout the liver parenchyma, and in fatal cases these rapidly extended until they became semiconfluent. The portal tracts showed slight infiltration with lymphoid cells, but A-type inclusion bodies were rarely found in infected hepatic parenchymal cells. Liver regeneration commenced early and was active, especially in nonfatal cases, and fibrosis did not occur.

Lesions of the spleen

The spleen showed macroscopic changes at least a day earlier than the liver, and higher titers of virus were found in the spleen. Virus reached the spleen in infected lymphocytes, which initiated infection in the substance of the follicles. While infected follicles were destroyed by the spreading infection, neighbouring follicles showed the proliferative response characteristic of antibody production.

In surviving mice, lesions of the spleen varied from small, raised plaques about a millimeter in diameter to strands of fibrous tissue which after severe attacks almost completely replaced the normal splenic tissue. These changes constitute reliable autopsy evidence that a mouse has recovered from an attack of mousepox.

Lesions of other organs

The regional lymph nodes draining the site of the primary lesion were enlarged from the time that the primary lesions could be detected, and they usually showed localized areas of necrosis, with pyknotic nuclear debris in a featureless background. In fatal cases the gut was often engorged and the lymphoid follicles enlarged. Small necrotic foci with typical inclusion bodies occurred in the intestines in most acutely fatal cases of mousepox. Occasionally, especially in very young mice, there were hemorrhagic foci in the kidneys.

Lesions after intraperitoneal inoculation

There is no primary skin lesion, but in acutely fatal cases the necrosis of the liver and spleen resembles that found after natural infection. In addition there is usually some increase in intraperitoneal fluid and a considerable amount of pleural fluid, and the pancreas is often grossly edematous. In animals that survive the acute infection there is a great excess of peritoneal and pleural fluid, the peritoneal surfaces of the liver and spleen are covered with a white exudate, the walls of the gut are thickened and rigid, and there is often fat necrosis in the intraperitoneal fat. Extensive adhesions between the abdominal viscera develop later.

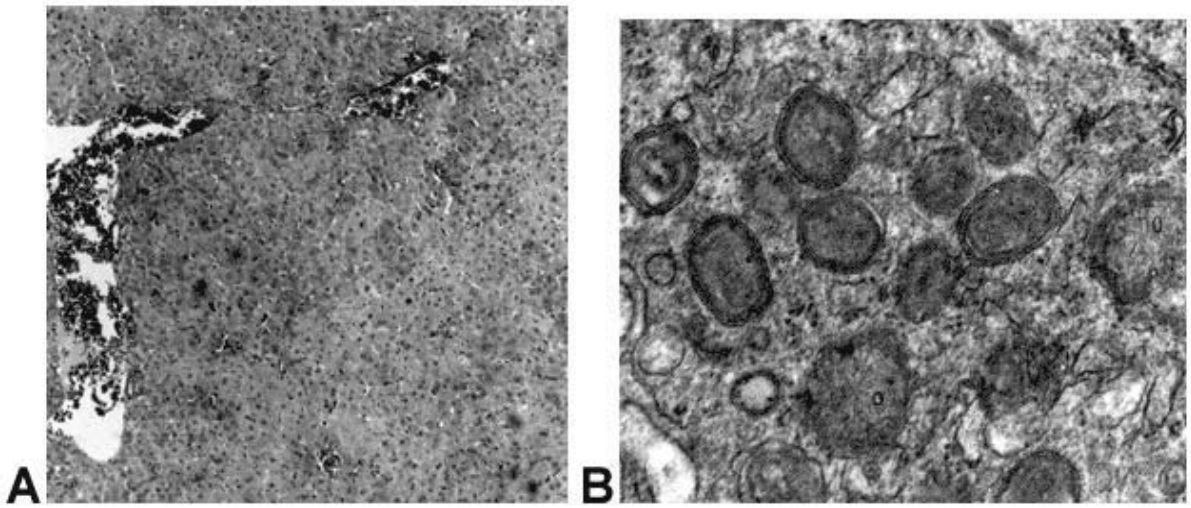


Figure 2 (**A**) Section of the liver of a mouse sacrificed when moribund, 7 days after the injection of a large dose of ectromelia virus in the footpad. There is extensive irregular necrosis of the hepatic cells, with little inflammatory reaction; no A-type inclusion bodies are found. (**B**) Electron micrograph of the same liver; there are numerous virions in the cytoplasm of most hepatic cells. (Courtesy of D. O. Irving, John Curtin School of Medical Research.)

Lesions after intranasal inoculation

When small doses of virus are inoculated intranasally there is usually little change in the lungs except patchy congestion; the changes in the liver and spleen are those characteristic of naturally acquired mousepox. With larger doses of virus, congestion of the lungs is more pronounced and consolidation may occur, and, when very large doses are given, death occurs with patchy or complete consolidation of the lungs and little change in the liver and spleen. The apparent pneumotropism is due to the fact that the local reaction which occurs after the intranasal inoculation of very large doses of virus kills the animal before there is time for the characteristic changes in the liver and spleen to occur.

Immune Response

Active immunity

Two weeks after infection, mice are solidly immune to reinfection by footpad inoculation of the virus. This immunity declines slowly but even a year after recovery multiplication of the virus after footpad inoculation is confined to the local skin lesion.

Passive immunity

Newborn mice received some antibody via the placenta but much more in the milk during the first 7 days after birth. Maternal antibody declines to undetectable levels by the seventh week after birth, but before this it confers protection against death but not against infection with moderate doses of ectromelia virus.

Serologic changes

Sera from mice which have recovered from mousepox contain antibodies that can be recognized by a variety of serologic tests. Hemagglutination-inhibition tests were long used for serologic surveys of mouse sera in the USA, but during outbreaks in 1979–1980 it was found that they occasionally gave both false-positive and false-negative results. ELISA is now preferred for large-scale screening tests.

Cell-mediated immunity

Understanding of the role of cell-mediated immunity in mousepox has been greatly expanded by the investigations of Blanden and his colleagues in Canberra. The full story is complicated and involves different classes of T cells and the H-2 antigens. Briefly, mechanisms controlling viral growth in the liver and spleen are operating 4–6 days after infection. Virus-specific cytotoxic T cells are detectable 4 days after infection and reach peak levels in the spleen 1–2 days later, while delayed hypersensitivity is detectable by the footpad test 5–6 days after infection. In contrast, significant neutralizing antibody is not detectable in the circulation until the eighth day.

Mice pretreated with antithymocyte serum die from otherwise sublethal doses of virus due to uncontrolled viral growth in target organs. These mice have impaired cell-mediated responses but normal neutralizing antibody responses, elevated interferon

levels in the spleen, and unchanged innate resistance in target organs.

Very large doses of interferon or immune serum transferred to preinfected recipients are relatively ineffective against the established infection in target organs, but immune spleen cells harvested 6 days after donor immunization transfer specific and highly efficient antiviral mechanisms which rapidly eliminate infection from the target organs, although neither antibody nor interferon is detectable in the recipients. The active cells in the immune population are cytotoxic T cells, which retard viral spread by lysing infected cells before the assembly of progeny virions. This T-cell activity attracts blood monocytes, which contribute to the elimination of infection.

Laboratory Diagnosis

Diagnosis of a suspicious case

Mousepox can be diagnosed by the microscopic or electron microscopic examination of the tissues of suspected cases, the diagnostic features being the distinctive eosinophilic cytoplasmic inclusion bodies and the poxvirus particles, respectively. Virus can be recovered in mouse embryo cell cultures. Inoculation of genetically susceptible nonimmune mice will produce the symptoms and signs described earlier, whereas vaccinated mice of the same strains should prove resistant.

Screening tests for mouse stocks

The consequences of the reported discovery of mousepox in a mouse colony are so serious that it is unwise to rely on any one serologic test for diagnosis. ELISA should be used as the basic screening test, and any positive results should be confirmed by another serologic test.

Prevention and Control

Most well-managed laboratory colonies of mice are now free of mousepox, and the aim is to keep them free of the disease while still importing mice (for genetic purposes) or mouse organs or tissues (as sources of arboviruses, for example, or tumors). Quarantine and regulation of the importation and distribution of ectromelia virus or materials infected with it is mandatory in the USA; however, such regulations offer no protection against unsuspected sources of infection, and various procedures involving serologic testing and vaccination have been proposed.

Future Perspectives

Mousepox is now very rare as a natural infection in laboratory colonies of mice and it is likely that strict controls on its use will continue to restrict its study in all except a few laboratories. However, workers in Australia, the USA and the UK are now using it as a model for studies of such problems as immunocontraception and the role of the many homologues of mammalian immunomodulatory genes that are found in all poxviruses. Laboratory workers importing mice or mouse products will need to be aware of the risk of introducing the disease and of methods for minimizing its spread if it is accidentally introduced.

Rabbitpox Virus

History and Classification

Rabbitpox is a laboratory artifact, due to the infection of laboratory rabbits with vaccinia virus, usually with 'neurovaccinia' variants; hence this account of rabbitpox virus will omit reference to those aspects that are covered in the entry on vaccinia virus.

The name 'rabbitpox' was originally given to devastating outbreaks of a generalized disease, likened to smallpox in humans, in a colony of laboratory rabbits at the Rockefeller Institute of Medical Research in New York in 1932–1934. Other scientists had been working with neurovaccinia virus in rabbits in an adjacent room before the outbreak. The virus recovered from the outbreak, which was called rabbitpox virus, was shown to be very similar to neurovaccinia virus in its biological properties, and subsequently the restriction map of the Utrecht strain (see below) was found to be almost identical with that of vaccinia virus.

Another outbreak occurred in The Netherlands in 1941. It began among rabbits, bought from a dealer, a few days after they were introduced into the laboratory colony, and spread amongst the stock rabbits. The disease was usually lethal, death occurring before there was time for the development of a rash. The virus that caused this outbreak, designated rabbitpox-Utrecht, caused similar highly lethal epizootics when it 'escaped' in the Institut Pasteur in Paris in 1947; other outbreaks have been described in laboratory rabbits in the USA in the 1960s.

Epidemiology

In all outbreaks, spread appeared to occur by the respiratory route, and experiments confirmed that infection occurred readily by this route. Rabbits infected by contact were not infectious for other

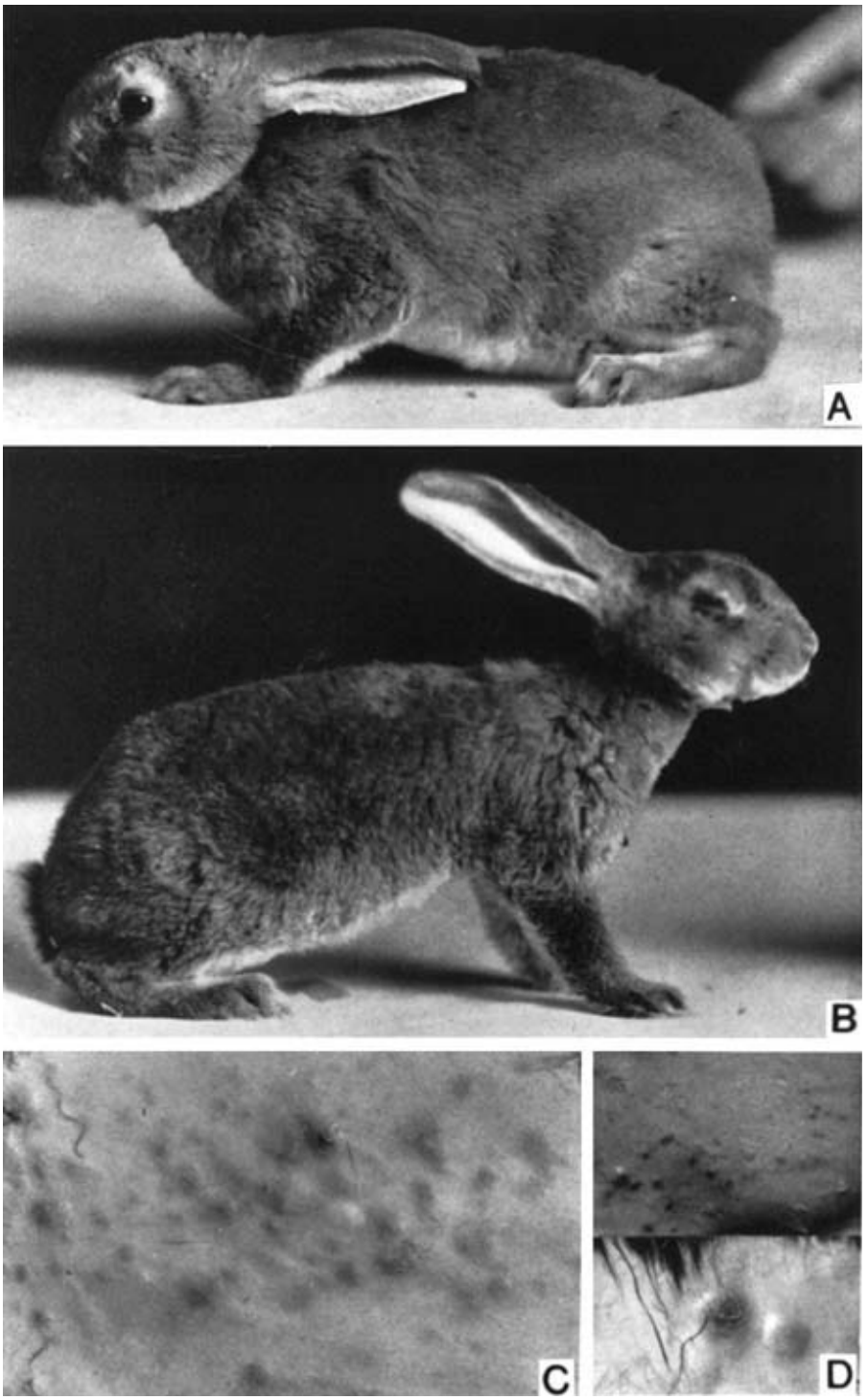


Figure 3 Rabbitpox. (**A**) and (**B**) Littermates with different types of disease. The course of infection was mild in the rabbit shown in the upper panel and external lesions were limited in the skin; the animal in the lower panel was seriously ill and its posture is a manifestation of acute respiratory distress resulting from extensive mouth lesions. (**C**) Cutaneous lesions on the trunk. The coat in this area was loose and easily plucked by hand. Papules varied in size, up to one centimeter in diameter, even among papules of the same age. (**D**) Skin of a pregnant doe self-plucked for nest fur, showing both dry crusted pustules and others in earlier stages of development. (From Greene, HSN (1934) *J. Exp. Med.* 60: 427, with permission.)

rabbits until the second day of illness, which was usually 5 days after infection. Actual contact was not necessary: transmission could occur across the width of a room, and air sampling revealed the presence of rabbitpox virus in the air of rooms housing infected rabbits.

Genetics

Since 1960 the Utrecht strain of rabbitpox virus has been used for genetic studies on poxviruses, as it was found to give rise to white pock mutants on the chorioallantoic membrane and host range mutants in a pig kidney cell line, both of which entail deletions and transpositions of DNA. Recombination experiments with the white pock mutants were used to construct the first crude 'genetic map' produced for an animal virus.

Pathogenesis

A good deal of experimental work has been carried out on the pathogenesis of rabbitpox as an animal model of smallpox, with results that were largely confirmatory of those obtained with mousepox. In rabbits infected by the intranasal instillation of a small dose of virus, by aerosol, or after intradermal inoculation or contact infection, there was a stepwise spread of virus through the organs, although the incubation period was shorter than in mousepox and there seemed to be little delay at the regional lymph nodes. Viremia was leukocyte-associated.

Clinical Features of Infection

Rabbitpox caused an acute generalized disease in which a rash appeared in animals that survived long enough, presenting as pocks on the skin and mucous membranes (**Fig. 3**). Rabbits dying of hyperacute infection showed no obvious skin lesions, the so-called 'pockless' rabbitpox. Such infections are analogous to acutely lethal cases of mousepox, and perhaps to early hemorrhagic-type smallpox, in which death occurs before there is time for pustular skin lesions to develop.

Pathology and Histopathology

The most distinctive lesions are the pocks on the skin and mucous membranes, and occasionally small areas of focal necrosis are found in the internal organs (liver, spleen, lung, testes, ovaries, uterus, adrenals and lymph nodes). In the so-called pockless form, a few pocks may occur around the mouth and they may be visible on the shaved skin. The most prominent gross lesions are pleuritis, focal necrosis of the liver, enlarged spleen, and edema and hemorrhage of the testes.

Rabbitpox virus, being a strain of vaccinia virus, produces B-type inclusions (Guarnieri bodies) in infected cells, but not the prominent A-type inclusions found in cells infected with ectromelia virus.

Immune Response

Rabbits that have recovered from rabbitpox are immune to infection with vaccinia virus, but in very severe infections rabbits die before there is time for an effective immune response. The importance of enveloped virions in the pathogenesis and immunology of orthopoxvirus infections was first demonstrated in experiments with rabbitpox virus. Passive immunization with sera that did not contain antibody to the viral envelope failed to protect rabbits against challenge infection, even though the neutralization titer of the ineffective antiserum (produced by immunization with inactivated vaccinia virus) was much higher, as judged by conventional neutralization tests. This work helped to explain the failure of inactivated vaccinia to provide protection against infection with orthopoxviruses.

Prevention and Control

Since rabbitpox appears to be a laboratory artifact, owing to the introduction of strains of vaccinia virus that can spread from one rabbit to another in rabbit colonies, prophylaxis appears to be a matter of preventing such events. They were rare even when vaccinia and especially neurovaccinia viruses were extensively used in animal experiments, several decades ago, and it is unlikely that further episodes will occur now that most research with vaccinia virus utilizes cultured cells rather than intact animals. Nevertheless, laboratory managers who use rabbits should be aware of the possibility that some strains of vaccinia virus can spread naturally from one rabbit to another. If such an outbreak did occur again, the appropriate method of control would be slaughter of all animals in the colony and disinfection of animal quarters.

See also: **Potyviruses (*Potyviridae*); Poxviruses (*Poxviridae*): Capripoxviruses, Leporipoviruses and suipoxviruses; Vaccinia virus (*Poxviridae*).**

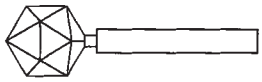
Further Reading

Fenner F (1982) Mousepox. In: Foster HL, Small JD and Fox JG (eds) *The Mouse in Biomedical Research*, p. 209. New York: Academic Press.

Fenner F and Buller RML (1996) Mousepox. In: Nathanson N (ed.) *Viral Pathogenesis*, p. 535. Philadelphia: Lippincott-Raven.

Fenner F, Wittek R and Dumbell KR (1989) *The Orthopoxviruses*. San Diego: Academic Press.

MU-LIKE PHAGES (*MYOVIRIDAE*)



Michael S DuBow, McGill University, Montreal, Quebec, Canada

Introduction

Since the pioneering work of Barbara McClintock with the mobile genetic elements of maize, DNA transposition has been shown to be a ubiquitous process in all well-studied life forms. Transposable elements were originally thought to be capable only of intragenomic DNA transposition. However, since DNA transposition can lead to the duplication of genetic information (whether it is directly from DNA→DNA, or RNA→DNA→RNA), it was not surprising that this mechanism could be used for the propagation and amplification of a virus genome. It was Larry Taylor, in 1963, who first reported the discovery of a virus which uses DNA transposition as a lifestyle. He named this temperate coliphage Mu (for *Mu*tator) because he found that its prophage was capable of randomly inserting in the bacterial chromosome, including within a gene, and thus providing Mu lysogens with a much higher frequency of auxotrophic mutants. This phage was initially called Mu-1 because it was assumed that other transposable mutator phages would be subsequently found. However, only one other bacteriophage that uses DNA transposition as a lifestyle, called D108, has been characterized from *Escherichia coli*. This phage was initially studied as a generalized transducing phage different from P1, and only later shown to be related to phage Mu. In contrast, transposable phages have been found to be quite common within the genus *Pseudomonas*, and can be readily isolated from lysogens of bacteria of this genus. Several other reports have also identified transposable phages in Gram-negative bacteria, though no transposable phages have yet been found in either Gram-positive bacteria or archaebacteria. It is significant to note that DNA transposition as part of the life cycle is used by the retroviruses present in eucaryotes from yeast (Ty elements) to man [human immuno-deficiency virus (HIV)] for the integration of their reverse-transcribed genomes. This entry will summarize what is known of transposable phages and will highlight both their similarities and differences at the molecular level.

Transposable Phage Life Cycle

The three best-studied transposable phages include the coliphages Mu and D108, as well as the *Pseudomonas* transposable phage D3112. These phages all share several important properties, in addition to the fact that their genomes act as mobile genetic elements:

1. The phage genomes are all composed of double-stranded DNA, approximately 37 kb in length.
2. Chromosomal DNA is covalently linked to both ends of the mature phage DNA in the capsid. The right end of the phage genome contains approximately 1000–3000 bp of DNA, while the left end contains approximately 50–150 bp in Mu and D108, and 30–33 bp of DNA in D3112.
3. All of these phages are capable of a low level of generalized transduction.
4. These phages cause 5 bp duplications at the target site of integration during DNA transposition.
5. The lytic cycle takes approximately 60 min at 37°C. However, the burst size of Mu and D108 (50–100 phages per cell) is higher than that of D3112 (10–50 phages per cell).
6. These transposable phages are all temperate, and between 5 and 10% of the infected cells will ultimately go on to form lysogens.
7. The genetic maps and gene organization of the phages are strikingly similar (**Fig. 1**). The arrangement of the repressor genes, and early and late operons, may indicate evolutionary relatedness. However, the phages are heteroimmune and their virion proteins do not cross react in Western blots. Mu and D108, however, are highly related (approximately 90% at the DNA level) and their virion proteins are immunologically related.

The life cycle of a typical transposable bacteriophage is outlined in **Figure 2**, but the discussion below will focus on coliphages Mu and D108. These two phages recognize and attach to different lipopolysaccharide components on the cell surface of their respective hosts. In the case of Mu, after phage attachment the DNA is injected into the cell along with the product of the *N* gene, which binds to the extremities of the injected DNA (phage plus host) where it acts to noncovalently circularize and partially supercoil the infecting DNA. The DNA is then inserted into a random location within the host genome (either on the chromosome or on a plasmid) in a conservative reaction with both infecting strands being integrated. Nonetheless, this is a true DNA

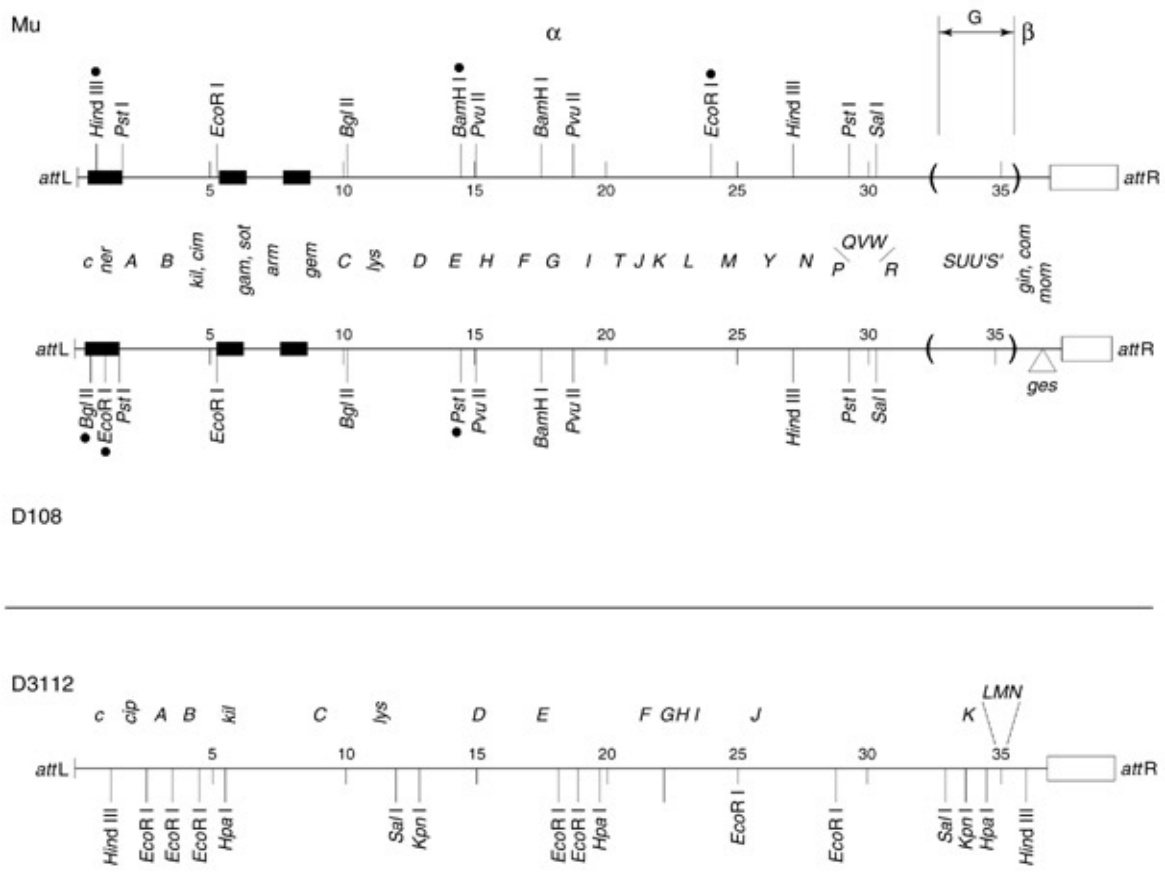


Figure 1 Physical and genetic maps of coliphages Mu and D108, and *Pseudomonas* phage D3112. The location of genes and restriction endonuclease cleavage sites are indicated. The Mu and D108 genomes are divided into three segments, α, the G-loop and β. The regions of nonhomology between Mu and D108 are indicated by thickened lines, plus the 520 bp insertion, containing the ges gene, located near the D108 right end. Restriction site polymorphisms between Mu and D108 are indicated by a • next to the enzyme site.

transposition event in that 5 bp at the target sequence are duplicated and directly flank the inserted DNA. It is important to note that the flanking host DNA of the infecting genome is not integrated along with the phage DNA. If the lysogenic response is chosen, the product of the *c* gene (the repressor) is expressed, and this DNA-binding regulatory protein regulates its own synthesis and represses the expression of the early (lytic cycle) phage operon. Alternatively, if the lytic cycle is chosen, or if a lysogen is induced, the early operon is expressed. This gives rise to the synthesis of the transposase protein (*A* gene product) which is unstable and used *in vivo* stoichiometrically. Transposase, along with other phage- and bacterial-encoded proteins, catalyzes the duplicative transposition of the phage genome to other random bacterial genomic locations. Towards the end of the lytic cycle, the *ner* gene product represses the expression of the early operon, and the late operon is expressed through the positive action of the *C* gene product. The late operons encode functions required for capsid construction and genome maturation. The integrated and dispersed viral genomes are then matured from the host chromosome starting near the left end (*att*L). Double-stranded cleavages are made in the adjacent host DNA, and the viral genomes then begin to be encapsidated. Because these genomes are too small to fill up a phage prohead, adjacent chromosomal DNA from the right end of the phage genomes is also encapsidated prior to the second DNA cleavage and maturation of the phage DNA and subsequent addition of the virion tails. The bacteria then lyse and liberate the progeny phages, to begin the cycle anew.

The transposable phage genomes are always inserted within host DNA (as are all DNA copies of mobile genetic elements). Thus, even in the virus capsid, the viral genome is 'inserted' in chromosomal DNA. In the newly infected cell, phage DNA can therefore transpose from its 'old' genomic location to

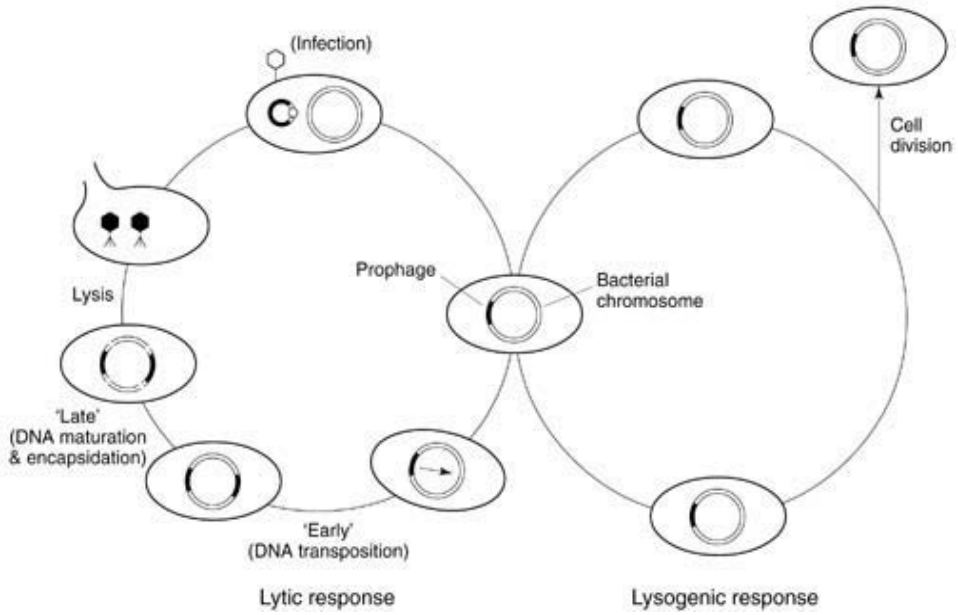


Figure 2 Outline of the life cycle of a transposable phage. The phage genome is represented by a solid line, while the bacterial DNA is represented by a double line. The Mu phage N protein is represented by an open circle joining the infecting DNA. Only two phage genomes are shown during the lytic cycle.

a new chromosomal site. It should be stressed that the transposable bacteriophages form a prophage irrespective of whether or not they choose the lytic or the lysogenic pathway for propagation. Moreover, during the lytic cycle these phages are amplifying their genomes through transposition to form an increasing number of prophages within their host's DNA. This mode of genome propagation presents interesting problems for phage DNA maturation and encapsidation, as these phages use headfull packaging and the chromosomal DNA behaves as a large discontinuous concatemer substrate for DNA maturation.

Lytic/Lysogenic Decision and Regulation of Early Gene Expression

The left end regulatory regions of Mu, D108 and D3112 contain the elements necessary for control of gene expression and the decision between lytic growth or lysogeny. A glance at the regulatory region (**Fig. 3**) of these three transposable phages reveals a distinct organizational resemblance and, perhaps, evolutionary relatedness. The following discussion will again concentrate on the transposable coliphages Mu and D108. At an indeterminate time after infection, the early operon is expressed and the phage transposes, in a conservative reaction, into host DNA. It is unclear at this time whether the choice between lytic or lysogenic growth is made prior to, during, or after the first integration of the infecting phage DNA. Phages Mu and D108 have a promoter, called P_c, which is the major promoter for expression of the repressor gene (*c*). The repressor genes of the phages are transcribed from right to left on the genetic maps and the repressor proteins are nonhomologous and incapable of binding to each other's operator sites or conferring immunity to superinfection by the other phage. In the lysogenic state, repressor binds to two operator sites in phage D108, and three operator sites in phage Mu. These operators repress the expression of the early operon (from promoter P_e), yet somehow allow continued expression of repressor for the maintenance of lysogeny.

During lytic growth (including the induction of a lysogen), the early genes are expressed from promoter P_e. This results in the production of proteins from the *ner* gene and the *A* (transposase) and *B* genes. The Ner protein binds to its operator and acts as a repressor to turn off expression of the repressor operon and commit the phage to the lytic cycle. The Ner protein is also capable of turning down expression of the early operon to allow a level of early gene expression sufficient to amplify, via DNA transposition, the viral genome. This is necessary because the transposase protein of Mu is used stoichiometrically and is unstable *in vivo*, thus requiring its continued expression. *In vitro*, Mu DNA transposition reactions containing elevated levels of Mu transposase protein predominantly result in 'suicide' intramolecular transposition products. Consequently, transposase

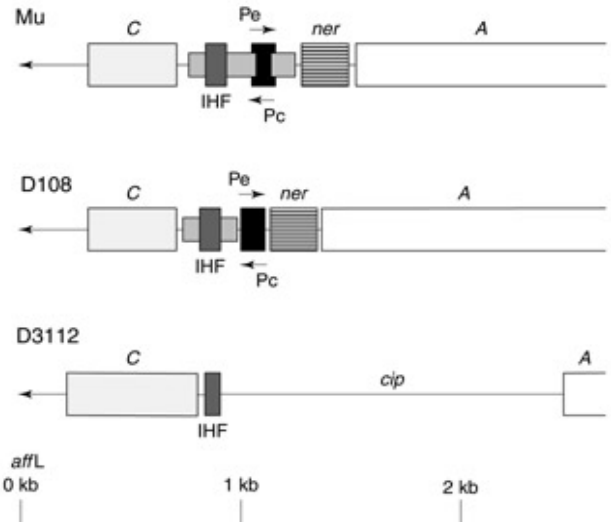


Figure 3 Maps of the Mu, D108 and D3112 left end regulatory regions. The genes and sites are drawn approximately to scale. The repressor (c) genes (▩), *ner* genes (■), and the 5′ ends of the A genes (□) are shown. The binding sites for IHF (▩), Ner (■) and repressor (▨) are also delineated. The arrows for Pe and Pc represent the 5′ ends of the mRNA for the early and repressor promoters, respectively. The scale at the bottom shows the distance (in kb) from the left end (*att*L) of the phage genomes.

gene expression must be adequately regulated to ensure a productive lytic cycle. It is the binding of the bacterial protein IHF that allows RNA polymerase to initiate transcription from the early promoter even in the presence of the Ner repressor. The mechanisms governing gene regulation and the lytic/lysogenic decision in phages Mu and D108 are not as well studied as in phage λ. Nonetheless, the outline of these mechanisms is now being elucidated, as is the fact that DNA supercoiling and local DNA conformation play a role in the relative expression of the overlapping, yet divergent, P_e and P_c promoters.

Transposable *Pseudomonas* phage D3112 bears striking similarities, though not homologies, to transposable coliphages Mu and D108. Phage D3112 also encodes a repressor protein in its left end which can act to confer immunity to D3112 superinfection *in vivo*. This repressor gene is transcribed from right to left on the genetic map of phage D3112. Moreover, D3112 contains an IHF site, which is capable of being bound by *E. coli* IHF *in vitro*, in a location on the phage DNA which is similar to that of Mu and D108 (**Fig. 3**). However, the transposase gene of phage D3112 does not begin until well after 2 kb from the left end. A *ner*-like function, called *cip*, has been postulated to exist in phage D3112 and to be located between the repressor and transposase genes. However, the region containing the *cip* gene is well over 1.5 kb in length and several open reading frames, on both strands, have been found, though none of these protein products bind to D3112 DNA *in vitro* when overexpressed in crude protein extracts. Thus, D3112 may regulate its early and repressor genes in a manner different from that of the coliphages Mu and D108.

Mechanism of DNA Transposition

The high frequency of DNA transposition undergone by Mu during the lytic cycle (up to 100 events per cell per one hour lytic cycle) has made it highly amenable to biochemical and genetic analysis. It was Mu DNA transposition that was the first system to be available for study *in vitro*. Analysis of this system has demonstrated that the requirements for DNA transposition are the Mu *A* (and *B*) gene products, *E. coli* histone-like protein HU, Mg^{2+}, ATP, a supercoiled 'donor' plasmid containing the left and right ends of Mu in the proper orientation, and a target DNA molecule. The DNA sequences at the Mu left and right ends contain three transposase-binding sites (termed L1–L3 and R1–R3, respectively), as well as an enhancer-like element located between base pairs 890 and 1000 from the Mu left end. This element, termed the IAS (internal activating sequence), helps to bring the two ends of Mu DNA together in a synaptic complex catalyzed by the Mu transposase and *E. coli* Hu proteins. Mu transposase protein, the N terminus of which contains homology to the DNA-binding domain of the Mu repressor, is capable of binding not only to the ends of Mu DNA (using a second DNA-binding domain), but also to the 01 and 02 repressor operators (in effect, the IAS). When the ends are

brought together, a nick is placed at the extreme 3′ ends of Mu DNA in a nucleoprotein complex containing transposase tightly bound to the L1, R1 and R2 sites. Mg^{2+} ions are required for the transposase-induced single-strand cleavage. The strand-transfer reaction utilizes the nucleoprotein complex, plus the 3′-hydroxyl groups at the Mu ends acting as the attacking nucleophiles, to nick and ligate to the target DNA in a 5 bp 5′-staggered fashion. Following DNA replication, primed by the free 3′ ends of the target sequence, Mu DNA is replicatively copied using the bacterial DNA replication machinery. Filling in the gaps leaves the characteristic 5 bp directly repeated duplication of the target sequence. This mechanism of DNA transposition can also account for all of the DNA rearrangements (e.g. co-integrate formation, deletions, translocations, inversions, etc.) that Mu DNA is capable of catalyzing *in vivo*. In addition, the mechanism allows a conservative transposition of Mu DNA to occur. In this case, after the initial ligation to the target DNA site, a second set of nick-ligation reactions at the Mu termini, plus filling in of the target sequence gaps, will lead to both DNA strands being inserted at the new location.

Phages D108 and D3112 also cause 5 bp duplications at the site of insertion. D108 and Mu are over 90% homologous at the DNA level, yet are non-homologous in their left and regulatory region (*c*, *ner* and the 5′ end of the *A* gene). However, their three transposase-binding sites are very well conserved, though the IAS is not. *In vivo*, Mu and D108 can catalyze the transposition of each other's DNA, although, owing to the nonhomology of their repressor binding sites and IAS, to a lower extent than the homologous DNA. The N-terminal portion of the putative transposase protein of D3112 shares 40% amino acid homology with that of phage Mu. However, the D3112 left end DNA sequences have no similarity to that of phages Mu and D108.

The initial infecting Mu DNA transposition reaction may proceed by a variation of the duplicative transposition reaction outlined above. However, the infecting Mu DNA is only partially supercoiled due to the noncovalent attachment of the terminal DNA sequences. Moreover, the initial reaction is a conservative reaction in which both strands of the infecting molecule are integrated without significant DNA replication occurring.

Late Gene Expression and DNA Maturation/Encapsidation

Late in the lytic cycle, the Ner protein is responsible for the reduction of early gene expression. Conversely, the product of the *C* gene is a positive activator of late gene transcription in phage Mu which allows the σ^{70} form of RNA polymerase to transcribe the late genes from several promoters scattered throughout the rightmost two-thirds of the Mu genome. These genes encode the morphogenetic functions of the virus as well as genes for host range determination (the G-loop) and a DNA modification function (see below).

Mu DNA maturation begins by the recognition of a sequence, called *pac*, which is located between nucleotides 32 and 54 from the Mu left end and nestled between the first two transposase-binding sites (L1 and L2). This sequence is identical in Mu and D108, and these phages can package each other's genomes *in vivo* and *in vitro*. A double-stranded cleavage is then made in the adjacent host DNA between 50 and 150 nucleotides from the left end of Mu and D108. There is preference for this double-stranded cleavage to occur in 10 bp intervals, indicative of a process that occurs once every helical turn of DNA. The enzyme(s) responsible for this process (called 'pacase'), as well as the nature of the reaction that causes these cleavages, is not currently known. The prohead is then filled with DNA with the left end entering first and, as the Mu genome is too small to totally fill the prohead, the terminal cleavage for Mu DNA maturation is made in the adjacent host DNA between 1 and 3 kb from the right end. Recently, an *in vitro* reaction to study this packaging mechanism has been developed and the template requirement was determined to be circular DNA, in this case an entire Mu prophage inserted within the plasmid pSC101, while the poorest substrate was mature Mu DNA. The packaging reaction requires Mg^{2+} ions, a crude extract prepared from Mu-infected cells late in the lytic cycle, spermidine and ATP. It was also shown that the presence of transposase can inhibit the reaction, suggesting that the depletion of active transposase protein is necessary to allow the Mu prophages to be competent for DNA packaging. The cells are finally lysed, and the progeny phage released, by the action of the *lys* gene product.

DNA Modification Function Encoded by Mu and D108

Phages Mu and D108 encode an unusual DNA modification function from the *mom* or *mod* genes, respectively. The consensus recognition sequence, 5′ C/G A C/G Py 3′ is the recognition site for this modification function. The modified base is the adenine residue, and it is modified to the unusual nucleotide α-*N*-(9-β-D-2′-deoxyribofuranosylpurin-6-yl)glycinamide. This unusual modification of DNA is not required for Mu lytic or lysogenic

growth, though it does act to protect the phage DNA from several bacterial restriction endonucleases.

The *mom* gene has an unusual mode of regulation of its gene expression. Its promoter is positively regulated both by the C gene product of Mu, as well as the host Dam methylase. In addition, the *mom* gene consists of two open reading frames which overlap, one of which comprises the *com* gene. The *com* gene encodes a 62-amino acid zinc finger protein which binds to *mom* mRNA and acts to destabilize a translational inhibitor stem loop structure to expose the Shine-Dalgarno sequence for *mom* translation initiation. The net effect is for *mom* function to be expressed late in the lytic cycle. However, owing to the Dam regulation of the *mom* promoter, *mom* is more highly expressed after prophage induction than after infection. This is most likely due to the *mut*H gene product of *E. coli* acting as a repressor of *mom* transcription by binding to unmethylated (or hemimethylated) dam (5′ GATC 3′) sites in the *mom* promoter.

The Invertible G-Loop

Phages Mu and D108, but not the transposable phages of *Pseudomonas*, contain an invertible region located near their right end. This 3015 bp region was initially identified as a heteroduplex bubble in electron micrographs of denatured and renatured Mu DNA. It has since been found that the invertible G segment of Mu and D108 plays a role in the adsorption specificity and host range of these two phages. The tail fiber proteins of Mu and D108 are partially encoded by these regions such that, in one orientation, the expression of the S and U genes leads to phages that can adsorb to *E. coli* K12 (for example). In the other orientation, the G-loop will encode the two genes S′ and U′, which yields phages which are incapable of adsorption to *E. coli* K12, but can adsorb to other Gram-negative bacteria such as *Citrobacter freundii*. The N-terminal portion of the S and S′ genes are identical and encoded by the adjacent sequences to the left of the invertible G-loop. Transcription proceeds into the G-loop (from left to right) and, in one orientation, gives rise to an mRNA that contains the constant N-terminal region of the S gene, plus the C-terminal region of the S gene and the entire U gene. In the other orientation, the constant region of the S gene is now transcribed with the carboxyl portion of the S′ gene and then the U′ gene. Thus, the S and S′ genes share their N termini, but differ in their C-terminal sequences, yielding phages which recognize different lipopolysaccharide components on the exterior of the cell.

The inversion of the G segment is catalyzed by a gene just to the right of the G-loop and before the *mom*/*com* genes and *att*R. The product of this gene, called *gin* (for *G inversion*), catalyzes the reaction through 34 bp inverted repeat segments that flank the G-loop. This reaction is stimulated by a thermostable host protein, called Fis, which binds to a site within the *gin* gene and which acts as an enhancer for efficient catalysis of G-loop inversion. Since G-loop inversion is a relatively slow and inefficient process *in vivo*, the majority of phages that arise after infection generally contain their G-loop in the initial orientation that occurred upon infection. However, phages prepared from induction of lysogens will have had adequate time during growth of the culture to arrange their G-loops in one or the other orientation, and will thus give rise to an approximately equal mixture of phages with their G-loops in one, or the other, orientation.

Gin is a member of, and related to, other DNA invertases. For example, the C-loop of bacteriophage P1 fulfills a similar function to the G-loop in determining the phage's host range, and its inversion is catalyzed by a protein homologous to Gin. Moreover, Gin can catalyze the inversion of the C-loop of P1, in spite of the fact that the C-loop of P1 contains 600 bp inverted repeats flanking the invertible segment. Gin is also highly homologous to the *Salmonella* flagellum invertase (*hin*) and contains a striking degree of homology with the resolvase protein of Tn*3* (TnpR).

Transposable Phages from the Genus *Pseudomonas*

Transposable phages have been found to be quite common in the genus *Pseudomonas*. Much of this work has been the result of extensive research by Dr Victor Krylov and his co-workers at the Institute for Genetics and Selection of Industrial Micro-organisms in Moscow, Russia. They have isolated a wide variety of transposable phages, including phage D3112, and have studied a great deal of their biology. All of the transposable phages of *Pseudomonas* have been found to be inducible by DNA-damaging agents, which is not the case for bacteriophage Mu. The DNAs of many of these phages have been isolated and characterized by restriction enzyme and electron microscopic heteroduplex analyses. This has allowed the transposable phages to be sorted into four main evolutionarily related groups. These groups are named according to their best characterized member. The phages of the B3 group appear to be less related to the other three groups. For example, there is more extensive host DNA present in their left ends (50–

100 bp) than in the phages of the other groups such as D3112 (30–33 bp). Western blot analysis using Mu virion antiserum has revealed that the virion proteins of phage D3112 are non-homologous to those of Mu or D108. Phage D3112, however, does contain repressor and transposase genes that are organized in a similar manner to phages Mu and D108, as well as an IHF site suggestive of the presence of an IHF-like protein in *Pseudomonas aeruginosa*.

The extensive physical characterization of these phage genomes, as well as the construction of viable hybrid phages by homologous recombination, has revealed the presence of regions that are conserved among the various transposable *Pseudomonas* phages. These regions are interspersed with areas of nonhomology, and suggest that the phages have evolved by a process of modular evolution. It is unclear at this time why these phages should be so common within the genus *Pseudomonas*, yet only two such transposable phages have been found in *E. coli*. It is possible that the transposable phages may be a consequence, or source, of the unusual metabolic and genomic plasticity observed within the genus *Pseudomonas*.

Transposable Phages of Other Bacteria

Reports of transposable phages from other bacterial species have been rare. Nonetheless, phage VcA1, from *Vibrio cholera*, has been shown to be a Mu-like transposable phage. It is interesting to note that its genome is approximately 37 000 bp in length. However, very little is known of its genetics or mechanism of transposition. This phage can create co-integrates of plasmids into the *V. cholera* genome and suggests that transposition of the genome progresses through a co-integrate intermediate, as seen for the other transposable phages. Phage VcA1 appears to also randomly insert within the *V. cholera* chromosome, and lysogens contain an increased frequency (0.6–2%) of auxotrophic mutations, a result similar to that observed in coliphage Mu and D108 lysogens.

A second phage, called Psi (ψ), was shown to be a mutagenic temperate phage of *Agrobacterium tumefaciens*. This phage also contains a double-stranded DNA genome of approximately 38 kb in length, and lysogens of this temperate phage contain various chromosomal mutations. However, these mutants were found to be leaky and to revert at a detectable frequency. Further work has shown that phage ψ DNA forms a covalently closed and supercoiled molecule after infection, and integrates into the chromosome at a unique site to form a prophage. This suggests that ψ is much more like phage λ in the behavior of its DNA. The increased frequency of mutations observed in lysogens may be due to some product expressed in the lysogen which acts as a mutator-like enzyme.

Lastly, a coliphage, called B278, has also been observed to act as a mutator-like phage. However, very little information is available concerning this 87 kb double-stranded DNA phage and the behavior of its genome.

The Use of Transposable Phages as Genetic Tools

The properties of transposable phages that enable integration into random locations in host DNA, and that catalyze a wide variety of DNA rearrangements, have allowed molecular biologists to prepare a number of derivatives of these transposable phages for *in vivo* genetic engineering. One important characteristic of these phages is to act as insertional mutagens and thus allow the creation of stable recessive mutations. The use of cloned antibiotic resistance markers within the phage genomes allows the facile cloning and characterization of the gene in which the prophage is inserted. In addition, a number of mini-Mu derivatives have been prepared which contain the *cis*-acting sequences required for transposition (and, in many cases, the transposase gene). These mini-Mu derivatives can contain reporter genes cloned near their right end and allow the determination of the expression of the gene in which they are inserted through the use of gene fusions. Upon infection, or induction of a thermosensitive helper prophage, one can create virtually any genetic combination that one wishes *in vivo*. Moreover, various plasmid replicons have been cloned within the ends of mini-Mus to allow these mini derivatives to be used as cloning vehicles such that the adjacent DNA is packaged by a helper phage and, when introduced to a new cell by infection, will circularize to form a plasmid derivative containing the gene to be studied. A wide variety of host range mutants of Mu have allowed it to be grown in many Gram-negative bacterial species, and recent research is directed at the preparation of D3112 derivatives to allow all of the benefits of Mu to be used in the medically and industrially important genus *Pseudomonas*.

Thus, it can be seen that transposable phages can provide model paradigms for the study of gene expression, DNA inversion and DNA transposition. It is important to note in this regard that the mechanism of HIV-1 DNA integration appears to be identical to that used by Mu, and that this transposition reaction mechanism may thus be conserved from bacteria to humans. The use of Mu and other transposable phages as *in vivo* gene manipula-

tion vehicles has greatly aided the studies of their host bacteria at the molecular level. It is perhaps not a great leap to predict that these kinds of benefits will some day be extrapolated to the genomes of all well-studied organisms.

See also: **Human immunodeficiency viruses (*Retroviridae*): Molecular biology; Retrotransposons of fungi.**

Further Reading

Harshey RM (1998) Phage Mu. In: Calendar R (ed.) *The Bacteriophages*, p. 193. New York: Plenum Press.

Pato ML (1989) Bacteriophage Mu. In: Berg DE and Howe MM (eds) *Mobile DNA*, p. 23. Washington, D.C.: American Society for Microbiology Press.

Strzelecka TE, Clone GM, Gronenborn AM. The solution structure of the Mu Ner protein reveals a helix-turn-helix DNA recognition molif. *Structure* 3: 1087–95.

Stern B, Molven A, Kamp D Conserved sequence patterns in phages Mu and lambda DNA. *Biochimica et Biophysica Acta* 1264: 115–20.

Symonds N *et al.* (eds) (1987) *Phage Mu.* Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Toussaint A and Resibois A (1983) Phage Mu: transposition as a life-style. In: Shapiro JA (ed.) *Mobile Genetic Elements*, p. 105. London: Academic Press.

[This article is reproduced from the 1st edn (1994).]

MUMPS VIRUS (*PARAMYXOVIRIDAE*)

Bertus K Rima, The Queen's University of Belfast, Belfast, Northern Ireland

History

The primary clinical manifestation in mumps is swelling of the salivary glands because of parotitis. This symptom is so characteristic that the disease was recognized very early as different from other childhood illnesses which give rise to skin rashes. Hippocrates described mumps as a separate entity in the fifth century BC. He also noted swelling of the testes (orchitis) as a common complication of mumps. Infection in the central nervous system (CNS) and meninges in some cases of mumps was first noted by Hamilton in 1790. In 1934, Johnson and Goodpasture demonstrated the filterable nature of the causative agent and Koch's postulates were fulfilled by infection of human volunteers with virus propagated in the salivary glands of monkeys.

Taxonomy and Classification

Mumps virus (MuV) is sensitive to ether and other membrane-destroying reagents and has hemagglutinating and neuraminidase activity (HN). It contains a nonsegmented negative-stranded RNA genome. MuV is now classified in the family *Paramyxoviridae*, subfamily *Paramyxovirinae* and placed in the genus *Rubulavirus* with Newcastle disease virus and several other mammalian viruses on the basis of genetic characteristics. Paramyxoviruses consist of an inner ribonucleoprotein (RNP) core surrounded by a lipid bilayer membrane from which spikes protrude. The latter are involved in the hemagglutination and neuraminidase activities as well as the hemolysis of erythrocytes of different origins. The latter reflects the ability of the virus to fuse with infected cells. Fusion is required for the entry of the RNP cores into cells.

Properties of the Virion

The MuV virion appears to be roughly spherical with diameters ranging from 100 to 300 nm when grown in cultured cells or eggs, but bizarre rod-shaped and other pleomorphic particles have been observed. Electron microscopy shows that MuV has the typical paramyxovirus structure with a lipid bilayer membrane surrounding an internal RNP complex, the nucleocapsid. This contains the RNA genome covered with nucleocapsid protein (N) as well as a phosphoprotein (P) and the large (L) protein. The RNP is surrounded by a lipid bilayer membrane derived from the host cell in which the matrix protein of the virus is embedded. This protein interacts with the internal core N protein and the viral glycoproteins. Spikes (10–15 nm in length) protrude from the membrane and these contain the viral glycoproteins (HN and F) in homo- or hetero-oligomeric complexes the composition of which has not yet been elucidated. The internal nucleocapsid displays the herring-bone structure characteristic of *Paramyxoviridae* and is approxi-

tion vehicles has greatly aided the studies of their host bacteria at the molecular level. It is perhaps not a great leap to predict that these kinds of benefits will some day be extrapolated to the genomes of all well-studied organisms.

See also: **Human immunodeficiency viruses (*Retroviridae*): Molecular biology; Retrotransposons of fungi.**

Further Reading

Harshey RM (1998) Phage Mu. In: Calendar R (ed.) *The Bacteriophages*, p. 193. New York: Plenum Press.

Pato ML (1989) Bacteriophage Mu. In: Berg DE and Howe MM (eds) *Mobile DNA*, p. 23. Washington, D.C.: American Society for Microbiology Press.

Strzelecka TE, Clone GM, Gronenborn AM. The solution structure of the Mu Ner protein reveals a helix-turn-helix DNA recognition molif. *Structure* 3: 1087–95.

Stern B, Molven A, Kamp D Conserved sequence patterns in phages Mu and lambda DNA. *Biochimica et Biophysica Acta* 1264: 115–20.

Symonds N *et al.* (eds) (1987) *Phage Mu.* Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Toussaint A and Resibois A (1983) Phage Mu: transposition as a life-style. In: Shapiro JA (ed.) *Mobile Genetic Elements*, p. 105. London: Academic Press.

[This article is reproduced from the 1st edn (1994).]

MUMPS VIRUS (*PARAMYXOVIRIDAE*)

Bertus K Rima, The Queen's University of Belfast, Belfast, Northern Ireland

History

The primary clinical manifestation in mumps is swelling of the salivary glands because of parotitis. This symptom is so characteristic that the disease was recognized very early as different from other childhood illnesses which give rise to skin rashes. Hippocrates described mumps as a separate entity in the fifth century BC. He also noted swelling of the testes (orchitis) as a common complication of mumps. Infection in the central nervous system (CNS) and meninges in some cases of mumps was first noted by Hamilton in 1790. In 1934, Johnson and Goodpasture demonstrated the filterable nature of the causative agent and Koch's postulates were fulfilled by infection of human volunteers with virus propagated in the salivary glands of monkeys.

Taxonomy and Classification

Mumps virus (MuV) is sensitive to ether and other membrane-destroying reagents and has hemagglutinating and neuraminidase activity (HN). It contains a nonsegmented negative-stranded RNA genome. MuV is now classified in the family *Paramyxoviridae*, subfamily *Paramyxovirinae* and placed in the genus *Rubulavirus* with Newcastle disease virus and several other mammalian viruses on the basis of genetic characteristics. Paramyxoviruses consist of an inner ribonucleoprotein (RNP) core surrounded by a lipid bilayer membrane from which spikes protrude. The latter are involved in the hemagglutination and neuraminidase activities as well as the hemolysis of erythrocytes of different origins. The latter reflects the ability of the virus to fuse with infected cells. Fusion is required for the entry of the RNP cores into cells.

Properties of the Virion

The MuV virion appears to be roughly spherical with diameters ranging from 100 to 300 nm when grown in cultured cells or eggs, but bizarre rod-shaped and other pleomorphic particles have been observed. Electron microscopy shows that MuV has the typical paramyxovirus structure with a lipid bilayer membrane surrounding an internal RNP complex, the nucleocapsid. This contains the RNA genome covered with nucleocapsid protein (N) as well as a phosphoprotein (P) and the large (L) protein. The RNP is surrounded by a lipid bilayer membrane derived from the host cell in which the matrix protein of the virus is embedded. This protein interacts with the internal core N protein and the viral glycoproteins. Spikes (10–15 nm in length) protrude from the membrane and these contain the viral glycoproteins (HN and F) in homo- or hetero-oligomeric complexes the composition of which has not yet been elucidated. The internal nucleocapsid displays the herring-bone structure characteristic of *Paramyxoviridae* and is approxi-

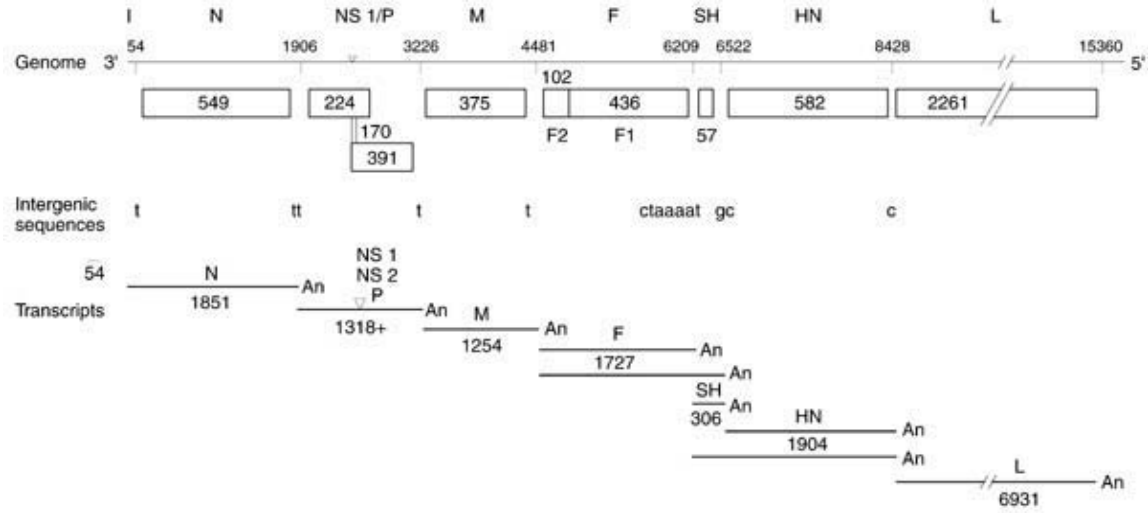


Figure 1 Gene order and transcription of mumps virus genome. ▽, editing site; An, poly(A) tail.

mately 1 μm in length with a diameter of 17 nm and an internal central core of 5 nm. Some of the pleomorphic particles have been reported to contain more than one RNP structure. The biological significance of such polyploid particles has not been investigated.

Properties of the Genome

MuV (as all other paramyxoviruses) has a single nonsegmented negative-stranded RNA genome. The nucleotide sequence of the entire genome of 15 384 nucleotides is now known. The order of the genes of MuV and transcription of the genome is depicted in **Fig. 1**, and is similar to that of other paramyxoviruses. **Figure 1** also shows the number of amino acid residues in each protein and the lengths of each messenger RNA.

Properties of the Proteins

The presence of six structural proteins, i.e. the nucleocapsid (N) protein, the phosphoprotein (P), the matrix protein (M) and the large protein (L) as well as two glycosylated membrane-spanning proteins, the hemagglutinin-neuraminidase (HN) and fusion (F) protein, has been demonstrated in mumps virions. Furthermore, the virus induces the synthesis of at least two and possibly more nonstructural proteins from transcripts of the NS1/P gene. The properties of the proteins of MuV are summarized in **Table 1**. It should be emphasized that this assignment is largely based on analogy of the MuV proteins with those of other paramyxoviruses, as gene identifications have not been carried out directly for all genes. However, the similarities to other paramyxoviruses are so striking that this assignment is beyond dispute. Recently, the presence of SH protein has been demonstrated in MuV-infected cells using antisera to peptides derived from the deduced amino acid sequence. It is not clear as yet whether the protein is incorporated into virions. At least one strain (Enders) expresses the SH gene as a tandem readthrough transcript with the F gene in tissue culture. It is unlikely that the SH protein is translated from such an F-SH bicistronic mRNA and growth of this strain in tissue culture may not require the expression of this protein.

Physical Properties

The MuV virion is very susceptible to heat and treatment with UV light. The UV target size is one genome equivalent of RNA. The virus is also inactivated by 0.2% formalin and the presence of the lipid bilayer leads to sensitivity to ether and chloroform. Treatment with 1.5 M guanidine hydrochloride leads to selective inactivation of neuraminidase and not hemagglutinating activity of the virus. This indicates that separate domains of the HN molecule are responsible for these two functions.

Replication

MuV is capable of infecting a variety of cells in culture even though *in vivo* it may be more restricted. The attachment of MuV is mediated primarily through the interaction of the HN protein with a sialoglycoconjugate receptor, but the exact nature of the MuV receptor is not known. The cooperative

binding of a number of HN molecules to cellular receptors probably leads to invagination of the host's membrane and this may allow fusion protein in its proteolytically cleaved activated state to fuse the membrane of the virus and the host cell. Whether viropexis is another mechanism of entry of MuV is not known.

After introduction of the RNP into the cell, primary transcription of the negative-stranded genome occurs mediated probably by the L and P proteins of the virion. This leads to the synthesis of positive-strand monocistronic mRNAs encoding the proteins of MuV (see **Fig. 1**). The presence of RNA-dependent RNA polymerase has been demonstrated in MuV virions but the role of the various viral proteins in this has not been assessed directly. Most of what follows is analogous to other paramyxoviruses. The first gene encoding the N protein is preceded by a leader region. No reports have appeared on the presence or absence of encapsidated or unencapsidated leader transcripts in infected cells or on the question of whether the leader region is transcribed in tandem with the first gene. The transcription complex recognizes the 3′ end of the genome and transcribes the genes in the order given in **Fig. 1**, stopping at each of the intergenic regions and synthesizing the polyadenylate tails of the various mRNAs by presumed repeated transcription (stuttering) of a poly(U) stretch in the genome.

The sequence signals surrounding the stop and start sites of transcription have the following (positive-strand) consensus:

$$\binom{A_3}{T_4}T\binom{A_3A_5}{T_4T_2}A\binom{G_6}{T_1}AAAAAA(A)\text{-intergenic-}$$

$$A\binom{A_5}{G_2}G\binom{C_5C_5}{R_2A_2}NN\binom{A_4A_6}{G_3G_1}$$

The intergenic sequences in the genome are short (1–7 nucleotides; **Fig. 1**). Occasionally, the transcription stop and start signal sequences are ignored and tandem readthrough transcripts of two or more genes are generated. At any intergenic sequence there is a finite chance that the RNA polymerase complex will leave the template. This gives rise to a transcription gradient in which the 3′ proximal gene (N) is most frequently transcribed and the 5′ proximal L gene very infrequently. The gradient has not been quantified in MuV-infected cells.

Cotranscriptional editing has been shown to be responsible for the generation of mRNAs encoding the P and NS2 proteins of MuV. Insertion of one or up to five extra G residues occurs at a site in the NS1 gene with a sequence similar to the polyadenylation signal. This leads to the formation of mRNAs that encode the NS2 or P protein or NS1, NS2 or P proteins with an extra glycine residue respectively. Recognition of transcription signals appears to be dependent on host as well as viral factors. Viral strains adapted to eggs give rise only to large tandem readthrough RNA transcripts when they are used to infect mammalian cells in culture, but monocistronic mRNAs are generated in chicken embryo fibroblasts.

The mRNAs of MuV are polyadenylated but the 5′ structure (presence and methylation state of cap) has not been determined. The mRNAs are translated by the cell's protein-synthesizing machinery and there is no indication of preferential translation of viral mRNAs or shut-off of translation of host mRNAs in MuV-infected cells. Indications in the literature of temporal translational control and control of transcription and translation of host mRNAs by the presence of viral glycoproteins in the cell membrane have not been confirmed.

The roles of various viral proteins and their post-translational modifications are indicated in **Table 1**. The processing pathway for the HN protein differs from that for the F protein. The HN protein forms oligomers during transport through the Golgi apparatus, and carbohydrate side chains are modified slowly in the *trans*-Golgi cisternae, whereas the fusion protein is maturing rapidly with respect to its glycosylation. The proteolytic cleavage of the F_0 precursor into the F_1 and F_2 activated complex has already occurred in the *trans*-Golgi cisternae.

Replication of the genome probably starts with the same promoter as is used for transcription, but all the intergenic sequence signals are ignored during this process. One long positive-stranded RNA molecule is formed which is encapsidated by the N protein immediately after the start of synthesis. The concentration of the N protein may regulate the balance between transcription and replication. The positive-strand in the RNP then forms the template for further negative-strand synthesis again with concomitant encapsidation by N protein. The presence of small leader transcripts from the positive-strand has not been reported.

There are a number of outstanding issues with respect to the replication of MuV which are not yet clear, some of which are specific to the virus. The roles of the 'V'-like NS1 and the NS2 nonstructural proteins in the processes of replication, transcription, translation and assembly have not been elucidated. Neither is it clear what role the SH protein plays. Studies on the assembly of the RNP and the localization of the P and L proteins as well as the

transport of the whole complex to the plasma membrane also remain to be carried out for MuV. Particularly, since the host cell cytoskeleton appears to play a role in the replication of other paramyxoviruses, it is important to investigate this aspect of MuV replication.

Budding probably takes place as a consequence of the interaction of the matrix protein with the RNP (most probably the C-terminal part of the N protein) and the cytoplasmic domains of the viral glycoproteins which accumulate in patches on the cell membrane. RNPs containing the genome or the antigenome strands have been found in mumps virions, but whether the budding process is selective in favoring inclusion of genomic, negative strand RNPs into virions is not yet clear. The budding process itself and its regulation have not been studied in great detail for MuV.

Both the F and the HN protein appear to play a role in fusion of virus-infected cells and syncytium formation. This is indicated by the observations that firstly, fusing (syncytium forming strains) as well as nonfusing strains contain cleaved activated F protein, secondly, levels of neuraminidase are higher in nonfusing strains and thirdly, proteolytic cleavage of the HN protein can activate fusion.

Geographic and Seasonal Distribution

The virus has a worldwide distribution and requires a minimum population for it to be able to circulate by continuous infection. The minimum population size is assumed to be similar to that for measles virus (approximately 250 000) but no systematic study has been undertaken to ascertain this for mumps virus. Before successful control of mumps by vaccination had been achieved, outbreaks of mumps were more often observed in the winter and spring than in the summer, at least in the temperate northern hemisphere, but this seasonal pattern was not observed in the tropics.

Evolution

From the fact that human populations have only become dense enough to sustain MuV about 4000 years ago, it has been suggested that the virus must have evolved from another animal pathogen. However, a closely related primate or other animal pathogen has not yet been identified.

Host Range and Viral Propagation

Humans are the only known host for the virus but dogs can be infected and show parotid swelling, though they do not pass on the virus. The virus can infect a variety of animals experimentally, including monkeys, cats, dogs, ferrets and a number of rodent species such as rabbits, suckling rats and mice, hamsters and guinea pigs. Its adaptation to growth in 8-day-old embryonated eggs allowed the biological activities of the virus to be studied before the advent of tissue culture. The virus infects a wide range of cells in culture and causes a distinct cytopathic effect in most cell cultures with either rounding of and

Table 1 Properties of the proteins of MuV

Protein	*Size in SDS-PAGE[a] (kDa)*	*Function*
Nucleocapsid (N)	68–73	Phosphorylated structural protein of nucleocapsid, protects genome from RNases, possible role in regulation of transcription and replication, S antigen
Phospho- (P)	45–47	Phosphorylated protein associated with the nucleocapsid; possible role in transport of N protein, role in RNA synthesis
Large (L)	>200	Protein with RNA polymerase activity associated with the nucleocapsid; possibly protein kinase, role in capping, methylation and polyadenylation?
Matrix (M)	39–42	Hydrophobic protein associated with inner side of membrane, role in budding by interactions with the N, HN and F proteins
Fusion (F)	65–74	Acylated, glycosylated protein F_2–F_1 heterodimer activated by proteolytic cleavage, hemolysis antigen. Fusion of membrane of virus with host cells may also involve HN
Hemagglutinin–neuraminidase (HN)	74–80	Acylated and glycosylated protein with hemagglutinin and neuraminidase activity; role in fusion and attachment, major V antigen
Small hydrophobic (SH)	6	Membrane protein, detected in infected cells but not in virions so far
Nonstructural NS1 (V)	23–28	Phosphorylated protein role unknown, Cys-rich (metal-binding?) domain
Nonstructural NS2	17–19	Phosphorylated protein role, unknown, artifact of misediting?

[a]Abbreviations: SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; kDa, mass of the protein in kilodaltons.

detachment of the cells from the substratum or widespread syncytium formation. This cytopathic effect varies from strain to strain as fusing and nonfusing variants have been described. The virus also readily establishes persistent infection in tissue culture systems, a property shared with many of the other viruses in the family *Paramyxoviridae*. Only low titers of virus are produced in such infections.

Serologic Relationships and Variability

MuV is a monotypic virus. Tests with human sera indicate the presence of only a single serotype. The virus crossreacts serologically to a low extent with human parainfluenza virus type 2 and simian virus 5. At the more detailed level of single epitopes of MuV, variability between strains has been demonstrated using monoclonal antibodies, with the HN and N proteins displaying the greatest variability. Recent sequence comparisons have demonstrated the existence of more than six genotypes of MuV.

Genetics

Temperature-sensitive or any other conditional lethal mutants of MuV have not been reported. Recombination has not been described in any nonsegmented negative-strand RNA virus. Although host range mutants have not been isolated, the adaptation of the virus to growth in eggs or in chicken embryo cells requires a number of blind passages, and strains so adapted do not readily grow or form plaques in mammalian cells in culture. Neutralizing monoclonal antibody escape mutants of the HN protein are the only type of MuV mutants described.

Epidemiology

Outbreaks of mumps show annual periodicities but the length of the cycle may vary as a result of poorly understood factors from 2 to 7 years. Traditionally, the virus caused severe problems when troops were assembled for war and it was observed that male recruits from rural populations were affected more often. It is, therefore, assumed that children in isolated, rural and island populations are exposed to the virus later in life than those in densely populated urban conglomerates. The infection gives rise to lifelong immunity from disease, but it is not clear whether a single dose of vaccine achieves the same. The existence of a number of different genotypes has allowed the development of molecular epidemiology for mumps. Specific genotypes appear to dominate in Japan and China, respectively. In some European epidemics two genotypes have been found to cocirculate. Changes in nucleotide sequences of viral isolates during an epidemic have not been studied but differences between viral isolates from a given geographical area over a timespan of several years indicate that the virus gradually accumulates nonexpressed and to a lesser extent expressed mutations. However, strains isolated more than 40 years apart are still recognized easily as belonging to a specific genotype.

Transmission and Tissue Tropism

The virus grows in the upper respiratory tract and the salivary glands and is transmitted in salivary droplets. Patients are infectious from 3 days before until approximately 4 days after the onset of clinical symptoms. Mumps can also cause viruria but this is not considered important in transmission. Transmission occurs only in the acute phase, and longer term mumps infections are known not to produce infection in susceptible contacts. From the attack rate in virgin populations, it can be concluded that the virus is highly contagious ($R = 5$) but not as contagious as, for example, measles ($R = 16$–450) and chickenpox viruses. The infection is systemic and the virus can multiply in a wide variety of tissues in the human host. The tropism for the pancreas, particularly the β cells, has been implicated as a potential link between MuV and juvenile onset diabetes mellitus, but direct evidence for a link is missing (**Table 2**).

Clinical Features of the Infection

The incubation period of infection with MuV is 14–21 days, but occasionally has been estimated to extend much longer (>50 days). The most common clinical feature is parotitis. However, as much as one-third of infections are subclinical. Other complications such as orchitis in males are frequent (**Table 2**). Contrary to popular belief, orchitis has not been linked to an increase in male sterility. Mastitis occurs in females with the same frequency and the incidence of both these clinical features increases with the age at which MuV infection is first contracted. MuV was the most common cause of viral meningitis and encephalitis in the US before vaccination controlled the virus. The encephalitis is usually benign although minor neurological changes, learning and concentration impairments and sudden deafness are well documented sequelae in a number of patients. More rare complications are oophoritis, thyroiditis, pancreatitis, otitis, retinitis, conjunctivitis and keratitis (**Table 2**).

Pathology and Histopathology

Upon infection, the virus replicates primarily in the nasal mucosa and the epithelial layer of the upper

respiratory tract and penetrates the draining lymph nodes after which a transient viremia occurs. From this, various target organs such as the salivary glands, the kidney, pancreas and the CNS are infected. Infection in the salivary glands produces the most predominant clinical feature of the virus. Viral replication leads to tissue damage and the subsequent immune response leads to inflammation and swelling of the gland. Dissemination to the kidneys can lead to prolonged infection of this organ and viruria. Virus can be isolated from throat swabs, salivary fluid and urine. Involvement of the CNS may be as high as 50% of cases and parotitis is not required for this to take place. MuV can be readily isolated from the cerebrospinal fluid (CSF) in cases of meningoencephalitis.

Pathogenicity

Strains of different pathogenicity for animals have been described. Similar differences have not been described for human infection, but varying levels of meningitis have been shown to be associated with some MuV strains (see below). The ability of different strains to infect the CNS of suckling hamsters after intraperitoneal injection has been correlated with their ability to fuse cells in culture. Some strains do not spread to the CNS and the ability to infect neurons after CNS penetration also varies. MuV strains appear to establish persistent infections readily in the CNS of suckling hamsters and mice. Some neutralizing monoclonal antibody escape mutants show alterations in neuropathogenicity in a rodent model.

Immune Response

Whether the humoral or cellular immune response (CMI) is the most important in clearance of MuV from the infected host is unknown. Both play a role but neither seems required for successful control of

Table 2 Clinical features, complications and prognosis of mumps virus infection in various organs

Organ	*Clinical feature*	*Frequency*	*Pathology*	*Prognosis*
Salivary gland	Parotitis (bilateral or unilateral)	95% of symptomatic cases	Blockage of duct of Stensen	Swelling disappears usually in 3–4 days
Submaxillary and sublingual salivary glands	Parotitis (bilateral or unilateral)	Rare	—	Swelling subsides in 3–4 days
Testes	Orchitis (mostly unilateral)	25% of males	—	Good; no lasting effect on sperm production
Breast	Mastitis	15% of females	—	Good; cause of virus in breast milk
Ovaries	Oophoritis	Rare	—	Good
Meninges	Meningitis fever/ vomiting	Mononuclear pleocytosis in CSF in 40–60% of cases	Ependymal epithelium destroyed by virus	Benign and self-limiting: ataxia some permanent damage possible
Brain	Encephalitis	2% of cases <1% = fatal	Virus spreads by neuronal pathways	Poor
Kidney	—	—	—	Good/cause of viruria
Pancreas	Pancreatis with nausea and vomiting	50% of cases	Could be due to interferon responses rather than specific tissue infection	Good/no established link to IDDM
Middle ear	Deafness	Very rare <3%	Cochlear infection	Permanent deafness is very rare
Heart	Myocarditis	Very rare	Fibroelastosis	Altered ECG; can cause myocardial infarct
Blood	Immunosuppression	All cases	Infection in macrophages and lymphocytes	Viremia resolves
Fetus	Abortion in first trimester	Frequent	Virus is widespread in tissues of aborted fetus	In live births CMI response in the absence of humoral response

IDDM, insulin-dependent diabetes mellitus; ECG, electrocardiogram; CMI, cellular immune response.

the infection by the host. Eleven days after infection, the humoral immune response is well established and the presence of neutralizing viral antibodies probably terminates viremia. Similarly, the appearance of IgA in the salivary fluid terminates excretion of infectious MuV via saliva. The time of first appearance of cytotoxic T lymphocytes reactive with MuV is unclear, but these have been demonstrated in blood of patients with the natural disease as well as in vaccinees. The magnitude and effectiveness of this response may be related to the genetic (HLA) background of the host. A complication in the development of the CMI response is the tropism of the virus for T and B cells. Reduction in CMI responses to antigens previously recognized has been observed, but the mechanism is unclear. It is less severe and of shorter duration than the immunosuppression associated with measles. The virus grows well in activated but not in resting T lymphocytes and infection of the CNS during MuV infection is thought to occur via transfer of infected monocytes into the choroid plexus. Perinatal exposure of the fetus to MuV by the placenta of the infected mother does not appear to lead to infection of the fetal tissues, but can give rise to anomalous immune responses to MuV in the newborn child. In these, CMI but no humoral responses are detectable. One neutralizing B-cell epitope has been defined in the HN gene of MuV but no other B- or T-cell epitopes have been delineated, as yet.

Prevention and Control

Adaptation of the virus to growth in eggs and chicken embryo cells allowed an early development of life attenuated vaccines for mumps. In 1946, Enders observed that adaptation of MuV to chicken cells was associated with the loss of virulence for monkeys. Killed virus preparations have been used for human vaccination but these do not lead to life-long protective immunity. In general, the disappointing appearance of atypical cases of measles after vaccination with killed virus has led to a preference for live attenuated viruses for the control of paramyxoviruses. Mumps vaccination has substantially reduced the incidence of mumps worldwide. In the USA, the incidence of mumps after licensing of the vaccine in 1967 dropped from 76 per 100 000 to 2 per 100 000 population. There are now a number of live attenuated vaccine strains such as the Jeryl Lynn, Rubini and others available for MuV. These are usually administered in a trivalent vaccine, which contains live attenuated strains of measles and rubella virus as well as MuV (MMR). Recently, the Urabe strain has been removed from MMR vaccines since reports in several countries indicated a higher incidence of meningitis associated with the use of this strain of MuV. Most developed countries have now gone over to a two-dose schedule for MMR vaccination.

MuV and Inclusion Body Myositis

MuV has been implicated in the past in inclusion body myositis. However, recent research using *in situ* hybridization, polymerase chain reaction (PCR) and immunocytochemistry has been unable to link MuV to the paramyxovirus-like nucleocapsids observed in muscle cells of patients with this disease. In contrast, myocarditis in patients with fibroelastosis has been associated with the presence of mumps viral RNA demonstrated with reverse transcriptase-PCR. The link to arthritis is also unclear (**Table 2**).

Future Perspectives

The generation of a cDNA clone which represents the full length of the MuV genome and rescue of virus from such a clone will allow a further definition of structure and function relationships and the role that various proteins play in attenuation of the virus. The generation of vectors that express individual viral proteins will allow dissection of the humoral and cellular immune responses and, hopefully, a better description of the role that both have in virus clearance. It is paramount that the immune status of vaccinees and their susceptibility to reinfection and the occurrence of sequelae is monitored after the introduction of large-scale vaccination. Factors involved in the neurovirulence of the virus remain to be determined.

See also: **Epidemiology of viral diseases; Genetics of animal viruses; Immune response: Cell mediated immune response; General features; Measles virus (*Paramyxoviridae*); Nervous system viruses; Newcastle disease virus (*Paramyxoviridae*); Parainfluenza viruses (*Paramyxoviridae*): Animal, Human; Persistent viral infection; Sendai virus (*Paramyxoviridae*); Vaccines and immune response.**

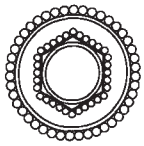
Further Reading

Feldmann HA (1989) Mumps. In: Evans AS (ed.) *Viral Infections of Humans*, p. 471. New York: Plenum Medical Book Company.

Kingsbury DW (ed.) (1991) *The Paramyxoviruses*, p. 1. New York: Plenum Press.

Wolinsky JS (1996) Mumps virus. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, p. 1273. Philadelphia: Lippincott–Raven.

MURINE LEUKEMIA VIRUSES (*RETROVIRIDAE*)



Hung Fan, Department of Molecular Biology and Biochemistry, University of California, Irvine, California, USA

History

Murine leukemia viruses (MuLVs) were originally identified by breeding of high-leukemia-incidence mouse strains in the 1920s and 1930s by Furth. Attempts to passage a cell-free filterable leukemogenic agent were ultimately successful, leading to independent MuLV isolates by Gross, Friend, Moloney, Rauscher and others. Different MuLVs induce distinct leukemias: Gross and Moloney MuLVs induce T lymphoma, while Friend and Rauscher MuLVs induce erythroleukemia. C57 Black 6 mice were found to develop T lymphoma with high incidence after X-irradiation, and Kaplan isolated another MuLV associated with the leukemogenesis, RadLV. Beginning in the 1970s, MuLVs have been among the most intensively studied retroviruses. Much of basic retroviral replication was worked out for MuLVs, and they have also been important subjects for the study of retroviral leukemogenesis and carcinogenesis in general.

During the early tumorigenesis experiments, passage of MuLV or MuLV-induced tumor cells occasionally gave rise to variants with increased pathogenicity *in vivo*. In some cases, solid tumors instead of leukemias were induced. Further studies indicated that these variants were classical acute transforming retroviruses that carry transduced cellular oncogenes. These viruses and their corresponding oncogenes include Moloney murine sarcoma virus (M-MSV, the *mos* oncogene), Harvey MSV (H-*ras*), Kirsten MSV (K-*ras*), FBJ MSV (*fos*) and Abelson MuLV (*abl*). More recently isolated acute transforming murine retroviruses include AKT8 (*akt*) and myeloproliferative sarcoma virus (*mpl*). In addition, the original Friend MuLV stocks contained an acute transforming retrovirus, spleen focus-forming virus (SFFV). The oncogene of SFFV is a deleted form of recombinant envelope glycoprotein (see below). Since these acute transforming viruses are replication defective, they are generally propagated by co-infection with a 'helper virus', a replication-competent MuLV that supplies the viral proteins necessary for replication.

Classification, Structure and Replication

MuLVs are members of the family *Retroviridae*, genus *Gammaretrovirus*. The structure and genetic organization of MuLVs are typical of all simple retroviruses. The virions are enveloped particles of 120–150 nm, with a classical C-type morphology. The viral envelope surrounds a spherical capsid (core) that contains the RNA genome. Virions also contain three virus-encoded enzymes (reverse transcriptase (RT), integrase (IN) and protease (PR)), as well as cellular transfer RNA, one of which (usually $tRNA^{Pro}$) is a primer for viral DNA synthesis. The RNA genome is a dimer of two identical positive-stranded RNAs of *c.* 8.2 kb. As with all simple retroviruses, MuLV genomes contain three genes: 5′-*gag-pol-env*-3′. The *gag* gene encodes the capsid proteins: a matrix protein (MA or p15, *c.* 15 kDa), a capsid protein (CA or p30, *c.* 30 kDa), a nucleocapsid protein (NC or p10, *c.* 10 kDa) and a type-specific protein of 12 kDa (pp12) whose function is unknown. The order within the *gag* gene is 5′-MA-pp12-CA-NC-3′. The *pol* gene encodes the viral enzymes in the order 5′-PR-RT-IN-3′. The *env* gene encodes two envelope proteins (from 5′ to 3′): a glycosylated surface protein (SU or gp70, *c.* 70 kD) and a transmembrane protein (TM or p15E, *c.* 15 kD).

MuLVs also encode a nonstructural form of glycosylated *gag* polyprotein ($gPr80^{gag}$) that is exported to the cell surface and shed into the medium. The fact that glycosylated Gag is conserved among all MuLVs as well as the closely related feline leukemia virus suggests that it plays a role in virus replication. While glycosylated Gag mutant viruses replicate well *in vitro*, glycosylated Gag is required for efficient *in vivo* replication. For instance, if glycosylated Gag-negative MuLVs are infected into animals, there is a strong *in vivo* selection for back-reversion to the glycosylated Gag-positive genotype. The glycosylated Gag is also a major pathogenic determinant for a neurotropic MuLV (see below). A portion of glycosylated Gag protein (the C-terminal half) is shed from infected cells, and the remainder (the N-terminal half) is embedded in the plasma membrane of the infected cell.

MuLV replication is typical of all retroviruses, with the following steps:

- Binding of viral particles to cell surface receptors. This involves physical interaction of the envelope

SU (gp70) protein with a cellular protein (the receptor – see below).
- Fusion of the viral and cellular plasma membrane, resulting in internalization of the viral core into the cytoplasm.
- Activation of reverse transcriptase to synthesize double-stranded viral DNA using the viral RNA genome as template.
- Transport of the viral DNA (in the form of a complex with some of the viral core proteins – the preintegration complex) to the nucleus. In order for the preintegration complex to enter the nucleus, the infected cell must past through mitosis, during which the nuclear envelope breaks down.
- Integration of viral DNA at random chromosomal sites to yield the provirus form. This process is mediated by the viral integrase (IN) protein.
- Transcription of integrated proviral DNA by cellular RNA polymerase II to give genomic RNA.
- Transport of viral mRNA (unspliced and spliced) to the cytoplasm and translation to give viral proteins. The initial viral translation products are polyproteins that contain all of the protein domains for each of the genes.
- Packaging at the plasma membrane of genomic viral RNA with viral proteins to form virus particles that bud from the cell surface. Infection by MuLVs is not lytic, and results in stably infected producer cells.
- Maturation of the released virus particles by cleavage of the polyproteins by the viral protease (PR). This maturation is essential for infectivity; immature virions are not infectious.

As a result of reverse transcription, the resulting viral DNA is longer than its template RNA, containing long terminal repeats (LTRs) at either end. LTRs can be divided into three regions, U3-R-U5, according to their origins in the viral RNA. The U3 region contains promoter and enhancer sequences necessary for viral transcription, as well as the signals for RNA cleavage/polyadenylation. The organization of a viral LTR and its relationship to virion RNA is shown in **Fig. 1**.

The virus specifies all of the enzymes necessary for viral DNA synthesis (RT), integration (IN) and proteolytic maturation (PR). These processes can be carried out *in vitro* using purified MuLV particles or enzymes.

As with all retroviruses, translation of MuLV Gag and Pol proteins is from unspliced RNA. Initiation is at the N-terminus of the *gag* gene, giving rise to either a Gag polyprotein (processed to the Gag proteins) or a Gag-Pol polyprotein (processed to Pol proteins). The balance between *gag* and *pol* translation (20:1) is

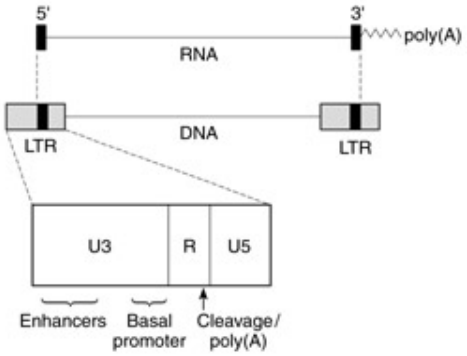


Figure 1 MuLV DNA organization. The relationship of reverse transcribed MuLV DNA to its template viral RNA is shown. The dark boxes on the RNA indicate direct repeats (R regions) at either end of the viral RNA. The LTRs on either end of the viral DNA are shown. The subdivision of the LTR into the U3, R and U5 regions is shown, as is the location of enhancer, basal promoter and cleavage/polyadenylation signals. When integrated proviral DNA is transcribed, initiation begins at the U3–R junction in the upstream (5′) LTR, and terminates at the R–U5 boundary in the downstream (3′) LTR.

controlled by suppression of an amber termination codon (by a cellular glutaminyl tRNA) between the *gag* and *pol* genes. This mechanism is different from most other retroviruses that generate Gag-Pol fusion proteins by translational frameshifting. Ribosomes also initiate on unspliced mRNA at a CUG codon slightly upstream from the AUG initiation site for the Gag and Gag-Pol polyprotein preprecursors; this yields the primary translation product that is processed independently to glycosylated Gag polyprotein. Env polyprotein is translated from a singly spliced mRNA.

Early classification of MuLVs was based on serology. Group-specific antigens were shared by all MuLVs, and were later found to be predominantly on Gag proteins (hence the name Gag, for group-specific antigens). Type-specific antigens subdivided the MuLVs into the Friend–Moloney–Rauscher (FMR) group and the Gross virus group. The main proteins carrying type specificity are the Env proteins, as well as ppl2^{gag} protein. Current classification of MuLVs is based on host range (see below).

Distribution of MuLVs and Endogenous Viruses

MuLVs are widely distributed in domestic and feral mice. While the original isolations were from laboratory mouse strains, related MuLVs have been isolated from wild mice. The wild mouse MuLVs have been important because they have shown new receptor specificities and pathologies (see below).

All mouse strains carry genetic information for MuLV-related viruses (endogenous viruses) in their chromosomes. Endogenous viruses represent rare germline infections, which result in mendelian transmission of the integrated proviruses to all progeny. Most endogenous MuLV proviruses are replication-defective, although some inbred mouse strains (e.g. AKR) carry and spontaneously activate replication-competent endogenous viruses. Treatment of cells from other strains by DNA-demethylating agents such as halogenated pyrimidines (e.g. bromodeoxyuridine (BrdU) or 5-AzaC) can activate endogenous viruses as well. There are approximately 50–80 endogenous MuLV proviruses in most mouse strains. In evolutionary terms, they were recently acquired – other subspecies of mice have significantly different patterns of endogenous viruses, although distantly related endogenous elements (usually defective) can be found in all mammals, including humans. Moreover, different inbred strains of laboratory mice have endogenous MuLV proviruses at different chromosomal loci. This extensive polymorphism can be exploited for mouse genetic mapping. In some cases, the integration of an endogenous MuLV virus into a cellular gene has caused a genetic mutation (e.g. the *dilute* and *hairless* mutations). The endogenous MuLVs represent germline infection by three different MuLVs, based on differences in their *env* regions (see below): xenotropic, polytropic and modified polytropic.

Host Range

The host range of an MuLV is controlled by two systems. The first system is the interaction of envelope glycoprotein (SU) with the cell surface receptor. On the basis of cell surface receptor specificities, five different classes of MuLVs have been identified so far: ecotropic, xenotropic, amphotropic, polytropic (or MCF recombinant) and 10AA1. These groups can be distinguished on the basis of interference assays: cells infected with one MuLV are resistant to superinfection by another MuLV of the same class, owing to blockage of the receptor by SU protein of the first virus. The species specificity of the MuLVs is also useful in separating the different classes. Ecotropic MuLVs (most exogenously infecting MuLVs) infect mouse-derived cells, but not cells from other species. The ecotropic MuLV receptor is a membrane transporter of basic (cationic) amino acids (mCAT-1). Xenotropic viruses (generally endogenous viruses) infect nonmouse cells but not mouse cells, while amphotropic viruses infect both mouse and nonmouse cells. The amphotropic MuLV receptor is a membrane transporter of inorganic phosphate (PIT-1). MCF recombinant viruses are *env* recombinants that arise during *in vivo* passage in mice of ecotropic viruses; they result from recombination with endogenous polytropic (or modified polytropic) MuLV proviruses. MCF MuLVs also infect both mouse and nonmouse cells (but by a different receptor from that of amphotropic MuLVs). 10A1 MuLV has dual specificity: it can infect cells via the amphotropic receptor (PIT-1), or by a related phosphate transporter (PIT-2).

The second MuLV host range system is Fv-1 restriction or N/B tropism. This is controlled by the mouse Fv-1 locus. Mice of the Fv-$1^{n/n}$ genotype (prototypic strain NIH) are susceptible to infection by N-tropic viruses (e.g. AKR MuLV) and relatively resistant to B-tropic MuLVs (e.g. RadLV). Similarly Fv-$1^{b/b}$ mice are resistant to infection by N-tropic viruses, and susceptible to B-tropic viruses. Fv-1 resistance is dominant, i.e. Fv-$1^{n/b}$ mice are resistant to infection by both N-tropic and B-tropic viruses. However, forced passage of viruses in restrictive cells can result in development of NB-tropic viruses that can infect cells of any Fv-1 genotype. Many exogenous MuLVs (e.g. Moloney, Rauscher, Friend) are NB-tropic. At the cellular level, Fv-1 restriction is after viral penetration, but before viral DNA integration, although the exact mechanism by which this occurs remains to be determined. N/B tropism and cell receptor specificity are independent host range systems. For instance, M-MuLV is an NB-tropic ecotropic virus, and 1504A MuLV is an N-tropic amphotropic virus. The Fv-1 gene has been molecularly cloned; it encodes a Gag protein of a distantly related retrovirus.

Leukemogenesis by MuLV

MuLVs induce leukemias in mice with latencies ranging from 2 to 18 months, depending on the strain of virus and strain of mouse. Neonatal infection is by far the most efficient means of leukemogenesis, whereas infection of adults is not leukemogenic for most viruses. Prior to development of leukemia, the predominant sites of infection are hematopoietic tissues (bone marrow, spleen, thymus), although the tissue specificity of the enhancers in the LTR can modulate this. In addition, infection in the intestines has also been reported for M-MuLV, although it is possible that this represents mucosal lymphoid cells. MuLVs can presumably infect a number of other cell types (at least as far as integration of proviral DNA), as they can act as helper viruses for acute transforming murine retroviruses that induce fibrosarcomas, osteosarcomas and carcinomas. Several characteristic events occur during MuLV leukemogenesis. The most

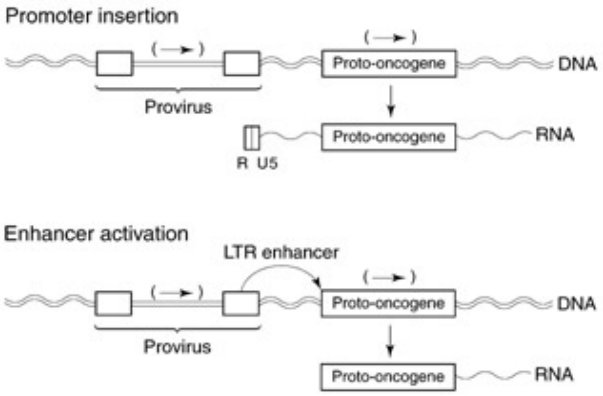


Figure 2 Proto-oncogene activation during retroviral leukemogenesis. The promoter insertion mechanism is illustrated at the top of the diagram. In this case, transcription initiates in the downstream LTR of an inserted provirus and proceeds into proto-oncogene-coding sequences. The result is overexpression of the proto-oncogene (as a hybrid transcript) under the control of the retroviral LTR. The enhancer activation mechanism is illustrated at the bottom of the diagram. In this case, the LTR enhancer activates transcription from the proto-oncogene via its own promoter.

well-understood event is LTR activation of proto-oncogenes (first elucidated by Hayward for avian leukosis virus; **Fig. 2**). Although retroviral DNA integration is generally random with respect to chromosomal location, when independent MuLV-induced tumors are examined, common sites of proviral insertion are detected. These sites reflect activation of a cellular proto-oncogene in the tumor by the viral LTR (either through its enhancer sequences or by 'promoter insertion'). This activation supplies an oncogenic stimulus to the cell, resulting in outgrowth of a monoclonal tumor. Many proto-oncogenes have been found to be activated by different MuLVs, as summarized in **Table 1**.

Oncogenesis is a multistep process, and LTR activation of proto-oncogenes probably supplies only one step in the process. Several experiments support this conclusion. First, sequential LTR activation of additional proto-oncogenes has been associated with *in vitro* tumor progression of M-MuLV-induced tumor lines. Second, even for acute murine retroviruses such as Abelson MuLV and Friend SFFV (which carry their own oncogenes), other events, such as LTR activation of cellular proto-oncogenes by the helper virus or by the transforming virus, or inactivation of a tumor suppressor gene (p53), also appear to be involved. Third, mice transgenic for the *pim*-1 proto-oncogene (activated in many M-MuLV-induced tumors) do not rapidly develop leukemia. However, infection of these mice with M-MuLV results in extremely rapid induction of T lymphomas, resulting from LTR activation of a second proto-oncogene (c-*myc* or n-*myc*). Also, crossbreeding of mice transgenic for *pim*-1 and for c-*myc* results in high tumor incidence in doubly transgenic progeny.

A feature of MuLV leukemogenesis is that the specificity of the disease is largely controlled by the specificity of the LTR enhancers. Chimeras between

Table 1 Common proviral insertion sites or activated proto-oncogenes in MuLV-induced tumors

Virus	*Disease*	*Common insertion site/activated proto-oncogene*
Moloney MuLV	T lymphoma	
	in mice	c-*myc*, *pim*-1, *pvt*-1/*mis*-1/*mlvi*-1, *lck*, *pim*-2[a], n-*myc*[a], *bmi*-1[a], *frat*-1[a], *pal*-1/*gfi*-1[a]
	in rats	c-*myc*, *pvt*-1/*mis*-1/*mlvi*-1, *mlvi*-2, *mlvi*-3, *mlvi*-4, *dsi*-1, *lck*, *tpl*-1/*ets*-1[a], *tpl*-2[a], *gfi*-1/*pal*-1[a], *gfi*-2/*IL*-9R
	Myeloid leukemia	c-*myc*, *mml*-1
AKR MuLV/Gross virus; SL3-3 MuLV	T lymphoma	c-*myc*, *gin*-1, n-*ras*
Friend MuLV	Erythroleukemia	*fli*-1, *fre*-2
	Myeloid leukemia	*fis*-1, *fim*-1, *evi*-1/*fim*-3, c-*fms*/*fim*-2
Endogenous MuLV (AKXD recombinant inbred mice)	Myeloid leukemia	*evi*-1/*fim*-3, *evi*-2, *meis*-1
	B lymphoma	*evi*-3
Abelson MuLV (contains v-*abl* oncogene)	B lymphoma	*ahi*-1, *ahi*-2 (M-MuLV helper inserted)
Friend SSFV (SFFV gp52 is an oncogene)	Erythroleukemia	*Spi*-1[b], p53[b,c]

[a]Insertions associated with tumor progression or that collaborate with other proto-oncogene activations.
[b]Insertions by the SFFV provirus, not the F-MuLV helper.
[c]Insertion at *p53* inactivates its function.

Moloney MuLV (T lymphoma) and Friend MuLV (erythroleukemia) have been generated by molecular cloning; substitution of Friend enhancers into the M-MuLV LTR converts M-MuLV from T lymphomagenic to erythroleukemogenic, and vice versa. This can be explained by LTR activation of proto-oncogenes (**Fig. 2**): for LTR activation to occur, the enhancers must be active in that particular differentiated cell type.

The long latency of MuLV-induced disease is only partially due to the time needed for the low probability insertion of a provirus near a proto-oncogene in an incipient tumor cell. Other experiments have indicated virus-driven preleukemic events. Tumor transplantation in AKR spontaneous leukemia and Moloney MuLV-induced leukemia has identified 'potentially leukemic cells' (PLCs) that precede the development of leukemia. Moreover, the PLCs are first evident in the bone marrow and spleen, but not in the thymus, where the tumors eventually develop. Other virus-induced changes observed in AKR and M-MuLV-induced leukemia include thymic atrophy and mild splenomegaly. The preleukemic splenomegaly represents hematopoietic hyperplasia of multiple lineages that may result from a defect in bone marrow hematopoiesis.

Another characteristic event in MuLV leukemogenesis is the appearance *in vivo* of MCF recombinant viruses. As described above, these are *env* gene recombinants between the infecting (or endogenously activated) MuLV and endogenous MuLV proviruses, and they are characteristically found in leukemic animals. All MCF recombinants contain chimeric *env* genes. In addition, some also show changes in the LTR, which increase the strength of the promoters/enhancers. Several lines of evidence indicate that MCFs are important for leukemogenesis. First, injection of MCF recombinants can acelerate leukemogenesis in AKR mice. Second, mice carrying the $Rmcf^R$ genetic allele are resistant to MCF recombinant formation, and they are also relatively resistant to leukemogenesis by Friend MuLV. Third, an enhancer variant of M-MuLV (Mo+PyF101, containing heterologous polyoma virus enhancers inserted into the LTR) does not generate MCF recombinants *in vivo* under conditions where it is poorly leukemogenic. Finally, in spontaneous leukemia of AKR mice (caused by endogenous ecotropic Akv MuLV), the proviruses in tumor cells are generally MCF recombinants, and many tumors are not infected with the original Akv MuLV.

The predominant evidence is that MCF recombinants act late in leukemogenesis, i.e. that they are the 'proximal leukemogens'. For instance, as mentioned above, in T lymphomas of AKR mice, the proviruses responsible for activating proto-oncogenes are MCF recombinants. It has also been shown that MCF envelope glycoprotein can bind to hematopoietic growth factor receptors such as the erythropoietin receptor. Infection with MCF viruses can render cells independent from growth factors such as erythropoietin (epo) and perhaps interleukin 2 (T cell growth factor). Thus MCF glycoprotein may provide infected hematopoietic cells with an autocrine signal for growth, leading to abnormal proliferation. Epo receptor binding and induction of epo-independent growth of erythroid precursors is even more dramatic for the gp55 oncogene form encoded by Friend SSFV.

Despite the clear role for MCF recombinants in efficient leukemogenesis by MuLVs in mice, they are not absolutely required for disease. This is evident since: (1) mice genetically resistant to MCF formation ($Rmcf^R$) still develop F-MuLV-induced leukemia (although with a longer latency); (2) mice infected with Mo+PyF101 M-MuLV can develop leukemia; and (3) M-MuLV can induce T lymphoma in rats, which cannot form MCFs because they do not harbor endogenous polytropic MuLV proviruses.

Neuropathic MuLVs

Certain MuLV strains (e.g. the CasBrE MuLV of wild mice, the *ts*1 mutant of M-MuLV and certain strains of Friend MuLV) cause neurological symptoms. This is generally evident as hind limb paralysis, or occasionally hemiplegia. Spongiform lesions in the brain or spinal cord accompany the neuropathology, and they are reminiscent of the spongiform lesions associated with the dementia of the acquired immune deficiency syndrome (AIDS) in humans. Studies with genetic recombinants indicate that the nature of the envelope glycoprotein (SU) determines whether the virus is neuropathic. In addition, the severity of disease is influenced by the LTRs, presumably reflecting the relative infection efficiency of neural tissue. Also for one particularly neuropathic MuLV (a recombinant between Friend and CasBrE MuLVs), the glycosylated Gag protein is an important determinant in neuropathogenicity.

The mechanism by which neuropathic MuLVs cause disease is not understood. While efficient infection of the CNS is necessary for eventual development of neurological symptoms, it is not sufficient, as some areas of the brain or central nervous system show substantial infection but no degeneration. Most of the neuropathic MuLVs infect microglial cells in the brain, but it is less clear if they infect neuronal cells. Brain capillary endothelial cells may also be infected.

Immunodeficiency (MAIDS)

An MuLV stock originally isolated by Duplan induces immunodeficiency in infected mice. Mice infected with this stock show hyperproliferation of B lymphocytes and ultimately develop T cell anergy and immunodeficiency (both B and T lymphoid). This has been referred to as murine acquired immune deficiency syndrome (MAIDS), and it has been studied as a model for human AIDS. Further experiments led to isolation of a defective MuLV genome (MAIDS or LPBM5 virus) that makes a truncated Gag polyprotein that is responsible for induction of the immunodeficiency. It appears that MAIDS virus causes a B-lymphoproliferative disease that secondarily leads to immunodeficiency. It has also been proposed that MAIDS virus encodes a 'superantigen' that leads to polyclonal proliferation of B lymphocytes and ultimately to exhaustion of lymphoid precursors, although this model is currently less favored.

MuLV-based Retroviral Vectors

Retroviral vectors are currently of great interest for introduction of genes into eucaryotic cells, and many retroviral vectors are based on MuLV. The principles of generating a retroviral vector are shown in **Fig. 3**. The gene of interest is inserted into a deleted form of an MuLV genome by molecular cloning. The viral sequences in the vector include those *cis*-acting sequences necessary for reverse transcription, integration and transcription of vector RNA (the LTRs and adjacent sequences), as well as signals that are responsible for packaging of vector RNAs into retroviral particles. The target gene may be inserted in a vector such that it is transcribed from the LTR, or it may be inserted under control of an internal promoter. Frequently, a second gene that allows for selection of cells expressing the retroviral vector (e.g. bacterial neomycin phosphotransferase, selectable in mammalian cells by resistance to the antibiotic G418) is also included. The cloned retroviral vector is then transfected into 'packaging cells'. Packaging cells express all of the retroviral proteins (Gag, Pol and Env) but do not release virus particles carrying viral RNA themselves, owing to the lack of packaging signals on the mRNAs for the retroviral proteins. The transfected packaging cells will release viral particles containing RNA transcribed from the transfected vector DNA. These particles can infect cells, reverse transcribe and integrate the vector DNA, yielding cells stably harboring integrated vector DNA. Packaging lines based on amphotropic MuLV allow for

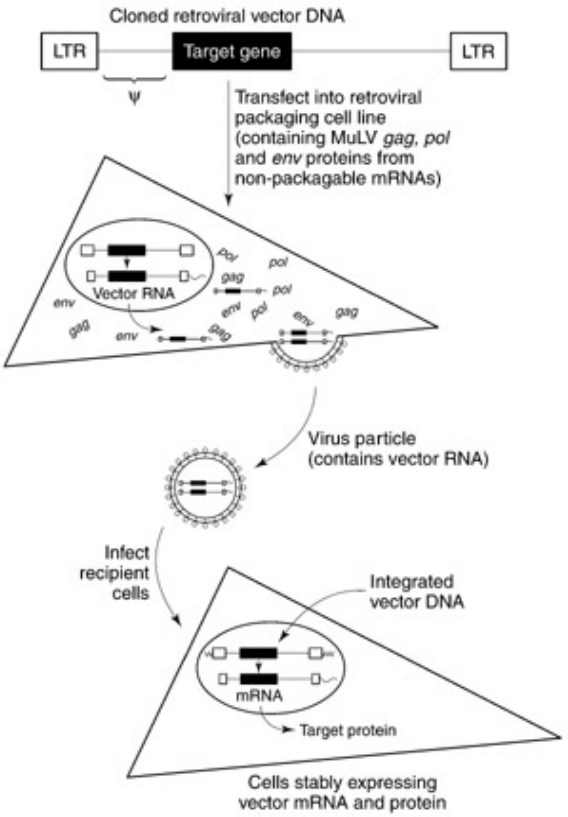


Figure 3 MuLV-based retroviral vectors. Organization of cloned retroviral vector DNA is shown at the top of the diagram. The target gene is inserted into a deleted version of an MuLV provirus; the critical viral sequences are the LTRs and the sequences required for packaging of RNA into retroviral particles (Psi). Upon transfection of the vector DNA into a retroviral packaging cell line, transcribed vector RNA will be packaged into virus particles and released. These virus particles are then infected into recipient cells, in which reverse transcription and integration of the vector DNA takes place. Transcription from the vector DNA into RNA followed by translation leads to stable expression of the target protein. Expression from retroviral vector DNA is highly efficient, much more so than from DNA introduced into cells by transfection.

generation of retroviral vectors that can infect a wide variety of cells, including human cells.

Retroviral vectors offer several advantages: infection is not lytic to cells, the transduced DNA is stably integrated into host cell chromosomal DNA, and expression from DNA integrated by retroviral infection is much more efficient than from transfected DNA. Retroviral vectors have been of particular interest for gene therapy experiments (e.g. correction of genetic defects such as severe combined immunodeficiency). Current technical problems include maintaining vector expression over prolonged periods of time in whole animals or people, infection of stem

cells and achieving expression in specific differentiated cell types.

Recent modifications to MuLV-based vectors include the use of vesicular stomatitis virus (VSV) envelope glycoprotein (G protein) to pseudotype the vector particles in place of MuLV Env protein. The advantage is that VSV-G pseudotyped particles can be concentrated to higher titers by ultracentrifugation under conditions where MuLV Env protein would be lost from the virions. Packaging systems have also been developed that allow efficient MuLV-based vector production after transient transfections. These have the advantage that vectors expressing genes that are toxic to stably transfected cells can be produced. Another area of vector development involves modification of the MuLV Env protein by addition of binding domains from other proteins. The goal of this appoach is to generate vectors that specifically bind to and infect certain cell types. Some success has been achieved by this approach (e.g. targeting vectors to cells that express the erythropoietin receptor), although the vector titers are still very low.

See also: **Vectors: Animal viruses; Retroviruses – type D (*Retroviridae*).**

Further Reading

Coffin JM (1996) *Retroviradae* and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Virology* 3rd edn, p. 763. Philadelphia: Lippincott-Raven.

Coffin JM, Hughes SH and Varmus HE (eds) (1997) *Retroviruses*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Fan H (1994) Retroviruses and their role in cancer. In: JA Levy (ed.) *The Retroviradae*, vol. 3, p. 313. New York: Plenum Press.

Fan H (1997) Leukemogenesis by Moloney murine leukemia virus: a multistep process. *Trends Microbiol.* 5: 74.

Gross L (1983) *Oncogenic Viruses* 3rd edn. Oxford: Pergamon Press.

Murine Parvoviruses *see* **Parvoviruses**

Murray Valley Encephalitis *see* **Encephalitis Viruses**

Myxoma Virus *see* **Poxviruses**

N

NECROVIRUSES (*TOMBUSVIRIDAE*)

Frank Meulewaeter, Plant Genetic Systems NV, Gent, Belgium

History and Classification

The *Necrovirus* genus belongs to the family *Tombusviridae* and contains all viruses that are closely related to tobacco necrosis virus (TNV). Tobacco necrosis was first described in 1935 by Smith and Bald as a viral disease of tobacco seedlings in greenhouses. On mechanical inoculation of a wide range of plants, the virus produced necrotic lesions in the inoculated leaves but did not cause systemic infection. In the 1960s and early 1970s, several new isolates of TNV were characterized. Based on the level of immunological crossreactivity between their coat proteins, the TNV isolates were divided into two major groups: the TNV-A and -D serotypes. Some of the TNV isolates are associated with small viruses. These satellite viruses are dependent for their propagation on the presence of TNV as a helper virus. In 1980, the sequence of the RNA genome of STNV-1 (a satellite virus of TNV-A) was the first full-length sequence of a plant viral RNA reported. It took another 10 years before the first sequence of a TNV isolate was published. At this moment several TNV isolates have been sequenced and it has become apparent that at least two groups of TNV isolates can be distinguished that are sufficiently different to be considered as separate species: TNV-A and TNV-D. In the mean time, two new viruses, olive latent virus 1 (OLV-1) and leek white stripe virus (LWSV), appeared sufficiently related to TNV to be included in the *Necrovirus* genus.

Symptoms, Distribution and Transmission

TNV is naturally present in the roots of a great variety of plants. Worldwide, around 20 different isolates have been described. Isolates are differentiated on the basis of host range and the symptoms that are induced on mechanically inoculated leaves. Typically, the size and color of the lesions on primary leaves of French bean are characteristic for specific isolates. These may vary from pinpoint lesions to lesions that spread along the veins and eventually kill the inoculated leaf. TNV does not induce systemic infection in most test plants, except for *Nicotiana benthamiana* in which several TNV isolates cause systemic mottling and stunting.

In general, TNV virulence is not associated with systemic disease symptoms and plant growth is not affected. Exceptions are the Augusta diseases of tulip, stipple streak of bean, and necrosis disease of cucumber and soybean. LWSV causes winter whitening of leek, which is characterized by a white striping of leaf blades and has spread epidemically in France since 1987. Latent systemic infections by TNV have been detected in woody crop plants, like apple and pear. Similarly, OLV-1 was isolated from symptomless olive trees.

Several strains of TNV have also been found in soils and in rivers and irrigation water. The virus particles present in the drainage water of infected plant roots are believed to be the virus source that establishes new plant infections. This is achieved through association with the zoospores of its natural vector, the chytrid soil fungus *Olpidium brassicae*. The virus particles attach on the outside of the zoospores in a strain specific reaction. Upon penetration of the fungus in a plant root, the TNV particles are concomitantly transmitted and the plant gets infected. The virus determinant for vector specificity probably lies within the coat protein. This specificity was exploited to achieve transient gene expression in plant roots. Purified TNV coat protein was used to encapsidate plasmid DNA containing a *cat* gene under control of a CaMV 35S promoter. Transfer of the nucleoprotein complex by *Olpidium brassicae* into wheat roots resulted in transient expression of the *cat* gene in the root cells. The epidemiology of LWSV suggests that its vector is also soil-borne.

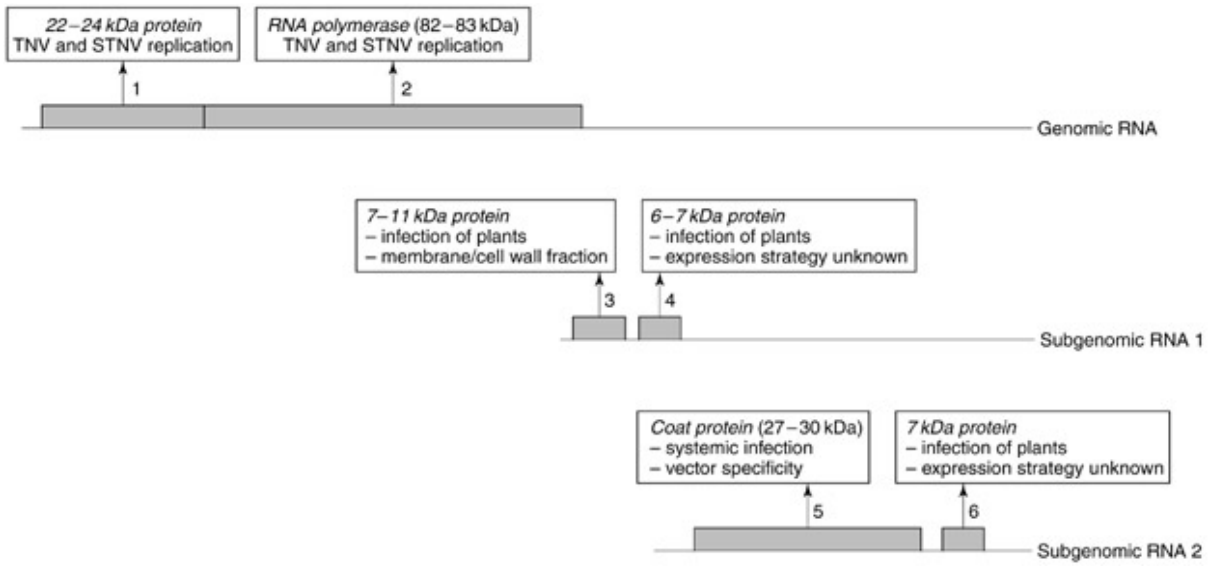


Figure 1 Genome organization and expression of the necroviruses. The locations of the major ORFs of TNV-A are shown on the RNAs from which they are presumably translated. The function of the encoded proteins as determined for TNV-A/D is given in the boxes. In TNV-D, the equivalent of ORF 6 overlaps with the 3′ end of ORF 2, whereas it is absent in the other necroviruses.

Properties of Virions

TNV and STNV form icosahedral viral particles with diameters of 28 and 17 nm, respectively, and triangulation numbers of 3 and 1, respectively. The three-dimensional structure of the STNV particle has been determined at 0.3 nm resolution. It consists of one genomic RNA molecule packaged by 60 STNV coat protein molecules. The TNV particles consist of one genomic RNA encapsidated in 180 copies of TNV coat protein and have also been crystallized. Both TNV and STNV are very stable viruses. The thermal inactivation point of most strains is about 90°C. Exceptions are a German TNV isolate that loses infectivity at 50–60°C, and TNV-B, which is very unstable on freezing and purification.

Genome Organization and Homologies

The necroviruses contain one single-stranded genomic RNA of a length between 3660 and 3760 nucleotides (nt). The genomic RNA is of positive polarity and has an organization that is similar to that of the carmoviruses. The TNV RNA has a 5′ terminal di- or triphosphate and lacks a 3′ terminal poly(A) tail or tRNA-like structure. The full-length RNA sequence has been determined for TNV-A, -D and -D^H and for LWSV and OLV-1. Infective cDNA clones are available for the three TNV isolates.

The necrovirus genome contains five or six major open reading frames (ORFs) (**Fig. 1**). No large ORFs are present on the complementary minus strand. The 5′ ORF encodes a protein of 22–24 kDa. It ends with a UAG stop codon. Suppression of this stop codon would result in the synthesis of an 82 kDa protein. This 82 kDa protein has amino acid sequence motifs characteristic for viral RNA polymerases and is thus the putative polymerase. Typically, this protein does not contain RNA helicase or capping motifs. The putative polymerase of OLV-1 shows high homology with that of TNV-A, whereas that of LWSV is more closely related to the TNV-D polymerase.

The second largest ORF is located near the 3′ end of the genome and encodes the viral coat protein. The size of the coat protein varies between 28 and 30 kDa. The necrovirus coat protein is very similar to the coat protein of southern bean mosaic virus. By analogy, it probably consists of an amino-terminal arm that interacts with the RNA and a C-terminal shell domain that forms the outer surface of the virus particle. The coat proteins of the TNV isolates are more closely related to each other than to that of the other necroviruses.

All necroviruses contain two small ORFs between the polymerase and the coat protein genes. These ORFs encode proteins of 6–11 kDa. The level of homology between these proteins shows a similar hierarchy as for the polymerase proteins. Both TNV-A and -D contain a sixth ORF that is not found in LWSV and OLV-1. This ORF encodes a 7 kDa protein. In TNV-D it overlaps with the 3′ end of the polymerase ORF, whereas in TNV-A it is located

downstream of the coat protein coding region. These two proteins show a low level of homology.

Gene Function

The coat protein gene was identified on the basis of the size and amino acid composition of the encoded protein, and by immunoprecipitation of its *in vitro* translation product with coat protein antiserum. TNV mutants that have internal deletions in the coat protein gene replicate efficiently in protoplasts. For TNV-D, the coat protein deletion mutant induced the same symptoms as wild-type virus in inoculated leaves. However, it did not cause a systemic infection in *N. benthamiana*. This indicates that the coat protein or intact virus particles are required for efficient long-distance movement. Cell-to-cell movement, however, does not require the coat protein.

Deletion of the central portion of the readthrough region of the 82 kDa protein ORF abolished TNV-D replication. This is compatible with the idea that the 82 kDa protein is the viral RNA polymerase. However, expression of the 82 kDa protein is probably not sufficient for RNA replication as a mutant that has the internal amber stop codon replaced by a tyrosine codon failed to replicate in protoplasts. This suggests that both the 22 kDa protein and the 82 kDa readthrough protein are required for RNA replication. Expression of the corresponding TNV-A proteins from a chimeric transgene was sufficient to support the replication of the satellite virus in tobacco. Moreover, mutation of the three small ORFs from TNV-D did not affect replication in protoplasts. This shows that the 22/23 kDa and 82 kDa proteins are the only TNV proteins that are required for TNV and STNV RNA replication.

The mutants in the three small TNV-D ORFs failed to show symptoms in plants. This suggests that these small proteins are involved in cell-to-cell movement in the plant. This is in agreement with the observation that in TNV-D infected plants one of these proteins could be detected in the combined cell wall and cell membrane fraction.

Genome Expression of TNV

In vivo protein labeling of TNV-infected leaves showed the induction of specific proteins, several of which correspond in size to TNV-encoded proteins. However, the coat protein and the TNV-D P7a are the only viral-encoded proteins that have directly been shown to accumulate in TNV-infected plants. By analogy with related viruses, it is most likely that the genomic RNA serves as mRNA for the 23 kDa protein and its readthrough protein. In a wheat germ translation system, however, the major translation products of the TNV-A genomic RNA are the 23 kDa protein and the coat protein. No 82 kDa protein synthesis by suppression of the amber stop codon could be observed. Expression of this protein in infected plant cells would thus require factors that are not present in the wheat germ system.

Coat protein synthesis from the genomic RNA in the wheat germ system is most likely the result of internal initiation of translation. However, studies in tobacco protoplasts did not reveal internal initiation by TNV sequences. *In vivo,* the coat protein is most likely synthesized from a subgenomic RNA. Necroviruses produce two subgenomic RNAs that are 3′-coterminal with the genomic RNA. For TNV-A, the shortest subgenomic RNA has a length of 1224 nt and is the mRNA for the coat protein. The longer subgenomic RNA of TNV-A is 1501 nt long and the first AUG codon is the start codon of the first small ORF. This subgenomic RNA is thus probably the mRNA for the 8 kDa protein. Moreover, *in vitro* translation experiments suggested that the subgenomic RNA might also direct the synthesis of the 6 kDa protein from the downstream ORF. The mechanism by which this could occur is, however, unclear. If and how the third small ORF of TNV is expressed is unclear as no subgenomic RNA is found that has this ORF at its 5′ end.

An important feature of the TNV genomic RNA is the absence of a 5′ cap and a 3′ poly(A) tail. Similarly, there are no indications that the subgenomic RNAs are capped. Both the cap and the poly(A) tail are crucial for the translation of most cellular mRNAs. However, synthesis of TNV coat protein from subgenomic RNA 2 is very efficient in infected tobacco protoplasts, with one coat protein molecule made per mRNA every 10–12 s. This efficient cap-independent translation requires sequences from both the subgenomic RNA leader and trailer. The trailer element contains an 18 nt sequence, which is absolutely conserved in related viruses and is required for cap-independent translation of barley yellow dwarf virus. This suggests that both viruses might share a similar mechanism for cap-independent translation.

Replication Strategy

Relatively little is known about the replication strategy of necroviruses. The only proteins that are absolutely required for TNV RNA replication are the 22/23 kDa and 82 kDa proteins. TNV can accumulate to very high levels in infected cells. In tobacco protoplasts the genomic RNA of TNV-A accumulates up to 10% of total RNA within 36 h after inoculation. TNV accumulation is reduced by the presence of

STNV. This reduction is proportional to the efficiency of STNV replication. This indicates that the STNV RNA competes with the TNV RNA for a limiting amount of replicase. The accumulation of the genomic RNA is linear in time up to 24 h after inoculation. In contrast, accumulation of the subgenomic RNAs slows down about 10 h after inoculation. Infected cells also accumulate three RNAs that are complementary to the genomic RNA. These RNAs correspond in size to the genomic and the two subgenomic RNAs and are also isolated from infected plants as part of three double-stranded RNAs. The full-length minus-strand RNA is probably the substrate for the synthesis of both the genomic and the subgenomic RNAs. This however requires experimental verification. The *cis*-acting sequences that are required for TNV replication have not been systematically mapped. Deletion mutants revealed that the 3′ sequences that are required for TNV replication are contained within the last 367 or 342 nt of TNV-D or TNV-A, respectively.

Satellite Viruses

TNV is commonly associated with viral icosahedral particles of about 17 nm diameter. These satellite virus particles consist of a single genomic RNA packaged by 60 coat protein subunits of 22 kDa. Three STNV serotypes have been described: STNV-1, STNV-2, and STNV-C. The earlier described STNV-4 is identical to STNV-2. The coat protein is the only protein encoded by the STNV RNA. This RNA has a length of 1220–1240 nt and lacks both a 5′ cap structure or Vpg and a 3′ poly(A) tail. The STNV RNAs are dependent for their replication on the presence of specific TNV isolates. For instance, STNV-1 and -2 RNA replication is supported by TNV-A, whereas the STNV-C RNA is replicated in the presence of TNV-D and not of TNV-A. Expression of both the 23 and 82 kDa protein of TNV-A is sufficient to support the replication of STNV-2 in tobacco protoplasts. In plants, however, STNV might depend on other TNV proteins for specific functions, like cell-to-cell transport.

The RNAs of STNV-1 and -2 show very similar organizations. The 5′ untranslated region is short (29 or 32 nucleotides). The extreme 5′ end can fold into a potential hairpin structure that is similar to the structure predicted for the 5′ end of the TNV-A RNA. The coat protein coding region is about 600 nt long and is followed by a 620 nt long trailer sequence. The trailer sequence shows a sequence similarity of 64% between STNV-1 and -2, which is higher than for the coat protein coding region (55%). The trailer can fold into phylogenetically conserved secondary structures. A first stem–loop structure is located just downstream of the coding region and is followed by three pseudoknots, one small hairpin, and a long stem–loop structure that includes the 3′ end of the RNA.

The coat protein coding region can be deleted without affecting replication in protoplasts. In contrast, both the leader and trailer sequences are important for replication of the STNV RNA. The 3′-terminal hairpin of STNV-2 is required for replication in protoplasts whereas the three pseudoknots and hairpin II have a quantitative effect on RNA accumulation. The only STNV 5′sequence that is required for an efficient replication is the 5′ terminal stem–loop structure. As a similar stem–loop structure is found at the 5′ end of the TNV-A RNA this might be one of the elements that specify the replication of both RNAs by the same viral RNA polymerase. At the 3′ end, there is no obvious primary or secondary structure similarity between the STNV-2 and the TNV-A RNAs that might explain their recognition by the TNV-A replicase.

STNV has been a model system for studying cap-independent translation in plants for more than 25 years. Translation of the STNV RNA is not increased by adding a cap to the 5′ end of the RNA and requires low levels of initiation factors for optimal translation. Deletion analysis of both the STNV-1 and -2 RNAs revealed a translational enhancer domain (TED) in the first 120 nt of the trailer, including hairpin I and part of the first pseudoknot. TED enhances translation of the STNV RNA in a wheat germ translation system more than an order of magnitude. Deletion of TED renders STNV translation cap-dependent. When fused to a heterologous mRNA, TED stimulates cap-independent translation both in a wheat germ translation system and in tobacco protoplasts. This stimulation is largely independent of the position of TED within the mRNA but is strictly dependent on the primary sequence of TED. Furthermore, the 5′ 38 nucleotides of the STNV-2 RNA cooperate with TED to further increase cap-independent translation both *in vivo* and *in vitro*. Two features of the STNV leader and TED could explain the dependence of TED for full activity on STNV 5′ sequences. First, the STNV leader contains two conserved regions of complementarity with TED that would allow base pairing between the leader and TED. Secondly, both the leader and TED show complementarity with the 3′ end of the 18S ribosomal RNA. Mutational analysis of STNV sequences complementary between leader, TED, and the 18S ribosomal RNA showed the importance of the primary sequence for the cooperative interaction but did not reveal a role for the complementarity.

The structures of the STNV-C leader and trailer are substantially different from those of STNV-1 and -2. The leader is 101 nt long and contains two AUG codons, the second of which is in frame with the coat protein initiation codon. This raises the question of how translation of the coat protein ORF is initiated. The STNV-C trailer is predicted to form a complex secondary structure, consisting of four pseudoknots and eight hairpins, that is clearly different from that of the STNV-1 and -2 trailers. As for STNV-2, the 3′-terminal structures of STNV-C are required for replication of the RNA.

Future Perspectives

Although the necroviruses are of limited economic importance, they have been studied for a long time as model systems for satellite virus replication and cap-independent translation. These will also be some of the most interesting aspects of the necrovirus research in the future. The availability of full-length infective cDNA clones of TNV-A and -D, as well as of all known STNV strains now provides the opportunity to investigate at the molecular level what elements of both the helper and satellite viruses determine the specificity of satellite activation. Furthermore, translation studies of both the STNV and TNV RNAs might help to unravel the mechanism of cap-independent translation in plants. Finally, the replication strategy of the necroviruses and the function of the various TNV-encoded proteins in the viral life cycle will require more detailed research.

See also: **Carmoviruses (*Tombusviridae*); Luteovirus; Satellite RNAs and Satellite viruses; Sobemoviruses.**

Further Reading

Fraenkel-Conrat H (1988) Tobacco necrosis, satellite tobacco necrosis, and related viruses. In: Koenig R (ed.) *The Plant Viruses* Part III, *Polyhedral Viruses with Monopartite RNA Genomes*, p. 147. New York: Plenum Press.

Uyemoto JK (1981) Tobacco necrosis and satellite viruses. In: Kurstak E (ed.) *Handbook of Plant Viruses Infections – Comparative Diagnosis*, p. 123. Amsterdam: Elsevier/North-Holland.

NEPOVIRUSES (*COMOVIRIDAE*)

MA Mayo and **AT Jones**, Scottish Crop Research Institute, Dundee, UK

History

The name 'NEPO virus' was proposed in 1963 by Cadman for a group of NEmatode transmitted viruses with POlyhedral particles. The convenience of the grouping and the euphony of the name led to its rapid acceptance, and nepoviruses were one of the first 16 groups of plant viruses to be recognized as taxa and assigned names by the International Committee on Taxonomy of Viruses (ICTV). Originally the group comprised ArMV, CLRV, RpRSV, SLRSV (now considered a tentative nepovirus), TRSV and TBRV (see **Table 1** for acronyms) but now it has grown to contain 29 definitive viruses (see later). However, not all current members are transmitted by nematodes and some are transmitted in association with pollen.

Taxonomy and Classification

The Sixth Report of ICTV lists 29 definitive members and eight tentative members of the genus *Nepovirus* (**Table 1**) in the family *Comoviridae*. The principal characters used at present to assign a virus to this genus are: isometric particles about 28 nm in diameter; a single coat protein of approximate molecular weight 53 000–60 000; and a bipartite genome with RNA-1 of about 8 kb and RNA-2 between 4 and 7 kb.

The genus can be subdivided in various ways. The simplest is by the size of RNA-2 so that, for example, subgroup I would comprise viruses with RNA-2 of <5 kb (i.e. a difference of >3 kb between RNA-1 and RNA-2) and subgroup II would comprise the remainder. More elaborate schemes have been proposed and both serological properties and geographical distribution have been used as discriminatory characters (**Table 1**). The genus is classified in the family *Comoviridae*, along with the genera *Comovirus* and *Fabavirus*. The largest genome RNA molecules of viruses in this family are similar in size and, at least for nepoviruses and comoviruses, the genomes are organized similarly, in that each RNA encodes a polyprotein which is cleaved by proteases to yield the

The structures of the STNV-C leader and trailer are substantially different from those of STNV-1 and -2. The leader is 101 nt long and contains two AUG codons, the second of which is in frame with the coat protein initiation codon. This raises the question of how translation of the coat protein ORF is initiated. The STNV-C trailer is predicted to form a complex secondary structure, consisting of four pseudoknots and eight hairpins, that is clearly different from that of the STNV-1 and -2 trailers. As for STNV-2, the 3′-terminal structures of STNV-C are required for replication of the RNA.

Future Perspectives

Although the necroviruses are of limited economic importance, they have been studied for a long time as model systems for satellite virus replication and cap-independent translation. These will also be some of the most interesting aspects of the necrovirus research in the future. The availability of full-length infective cDNA clones of TNV-A and -D, as well as of all known STNV strains now provides the opportunity to investigate at the molecular level what elements of both the helper and satellite viruses determine the specificity of satellite activation. Furthermore, translation studies of both the STNV and TNV RNAs might help to unravel the mechanism of cap-independent translation in plants. Finally, the replication strategy of the necroviruses and the function of the various TNV-encoded proteins in the viral life cycle will require more detailed research.

See also: **Carmoviruses (*Tombusviridae*); Luteovirus; Satellite RNAs and Satellite viruses; Sobemoviruses.**

Further Reading

Fraenkel-Conrat H (1988) Tobacco necrosis, satellite tobacco necrosis, and related viruses. In: Koenig R (ed.) *The Plant Viruses* Part III, *Polyhedral Viruses with Monopartite RNA Genomes*, p. 147. New York: Plenum Press.

Uyemoto JK (1981) Tobacco necrosis and satellite viruses. In: Kurstak E (ed.) *Handbook of Plant Viruses Infections – Comparative Diagnosis*, p. 123. Amsterdam: Elsevier/North-Holland.

NEPOVIRUSES (*COMOVIRIDAE*)

MA Mayo and **AT Jones**, Scottish Crop Research Institute, Dundee, UK

History

The name 'NEPO virus' was proposed in 1963 by Cadman for a group of NEmatode transmitted viruses with POlyhedral particles. The convenience of the grouping and the euphony of the name led to its rapid acceptance, and nepoviruses were one of the first 16 groups of plant viruses to be recognized as taxa and assigned names by the International Committee on Taxonomy of Viruses (ICTV). Originally the group comprised ArMV, CLRV, RpRSV, SLRSV (now considered a tentative nepovirus), TRSV and TBRV (see **Table 1** for acronyms) but now it has grown to contain 29 definitive viruses (see later). However, not all current members are transmitted by nematodes and some are transmitted in association with pollen.

Taxonomy and Classification

The Sixth Report of ICTV lists 29 definitive members and eight tentative members of the genus *Nepovirus* (**Table 1**) in the family *Comoviridae*. The principal characters used at present to assign a virus to this genus are: isometric particles about 28 nm in diameter; a single coat protein of approximate molecular weight 53 000–60 000; and a bipartite genome with RNA-1 of about 8 kb and RNA-2 between 4 and 7 kb.

The genus can be subdivided in various ways. The simplest is by the size of RNA-2 so that, for example, subgroup I would comprise viruses with RNA-2 of <5 kb (i.e. a difference of >3 kb between RNA-1 and RNA-2) and subgroup II would comprise the remainder. More elaborate schemes have been proposed and both serological properties and geographical distribution have been used as discriminatory characters (**Table 1**). The genus is classified in the family *Comoviridae*, along with the genera *Comovirus* and *Fabavirus*. The largest genome RNA molecules of viruses in this family are similar in size and, at least for nepoviruses and comoviruses, the genomes are organized similarly, in that each RNA encodes a polyprotein which is cleaved by proteases to yield the

Table 1 Some properties of definitive and tentative nepoviruses

Virus	*Main vector*	*Coat protein mol. wt**	*RNA-1 size†*	*Database accession numbers*	*RNA-2 size†*	*Database accession numbers*	*Seed transmission*	*Possible origin*
Definitive nepoviruses‡								
Arabis mosaic[a] (ArMV)	*Xiphinema diversicaudatum*	54 000	2.8	X81815	1.3	D10086 X55460	+	Europe
Arracha virus A	—	53 000	(2.5)		(1.4)		+	S. America
Artichoke Aegian ringspot[e]	—	54 000	2.4		1.4			Mediterranean
Artichoke Italian latent	*Longidorus apulus; L. fasciatus*	55 000	(2.4)	X87254	1.7			Mediterranean
Artichoke yellow ringspot	—	53 000	(2.3)		1.9		+	Mediterranean
Blueberry leaf mottle[b] (BLMV)	—	54 000	(2.4)	U20622	(2.2)	U20621	+	N. America
Cassava American latent	—	57 000	7400		4200			S. America
Cassava green mottle	—	53 000	2.9		2.3		+	Australasia
Cherry leaf roll (CLRV)	—	54 000	2.8	S84124 Z34265	2.3	S84125 S84126 S63537	+	Europe
Chicory yellow mottle (ChMV)	—	54 000	(2.4)		(2.1)		+	Mediterranean
Cocoa necrosis[c]	—	60 000	(2.4)		(1.4)			Africa
Crimson clover latent	—	52 000	(2.2)		(1.6)		+	Europe
Cycas necrotic stunt	—	65 000	(2.5)		(1.5)		+	Asia
Dogwood mosaic	—	54 000	(2.9)		(1.4)			N. America
Grapevine chrome mosaic[c] (GCMV)	—	57 000	7212	X15346	4441	X15163		Europe
Grapevine fanleaf[a] (GFLV)	*X. index; X. italiae*	56 000	7342	D00915	3774	X16907	+	Europe
Hibiscus latent ringspot	—	54 000	(2.5)		(2.2)			Africa
Lucerne Australian latent	—	55 000	2.8		2.5		+	Australasia
Mulberry ringspot	*L. martini*	—	2.8		1.5		+	Asia
Myrobalan latent ringspot	—	53 000	2.8		2.0			Mediterranean
Olive latent ringspot	*X. americanum* s.l.	56 000	(2.3)		(1.5)			Mediterranean
Peach rosette mosaic	*L. diadecturus*	57 000	(2.5)	A016626	(2.2)		+	N. America
Potato black ringspot[d]	—	59 000	(2.5)		(1.5)			S. America
Potato U	—	58 000	—		—		+	S. America

Table 1 Continued

Virus	*Main vector*	*Coat protein mol. wt**	*RNA-1 size†*	*Database accession numbers*	*RNA-2 size†*	*Database accession numbers*	*Seed transmission*	*Possible origin*
Raspberry ringspot[e] (RpRSV)	*L. elongatus; L. macrosoma*	57 000	2.8		3928	S46011	+	Europe
Tobacco ringspot[d] (TRSV)	*X. americanum* s.l.	57 000	2.8	U50869	1.3	L09205	+	N. America
Tomato black ring (TBRV)[c]	*L. elongatus; L. attenuatus*	57 000	7356	D00322	4463	X04062 X80831	+	Europe
Tomato ringspot (ToRSV)	*X. americanum* s.l.	58 000	2.8	L19655	7273	D12477	+	N. America
Tentative nepoviruses								
Arracacha B	—	26 000 22 000 27 500	(2.5)		(1.3)		+	S. America
Artichoke vein banding	—	24 500 22 000 24 000	(2.1) (2.0)		(1.5)		?	Mediterranean
Cherry rasp leaf	*X. americanum* s.l.	22 500			(1.5)		+	N. America
Lucerne Australian symptomless	—	40 000 26 000	(2.5)		(1.4)		+	Australasia
Rubus Chinese seed-borne[f]	—	47 000 25 000	—		1.4		+	China
Satsuma dwarf (SDV)	—	42 000 21 000	(1.9)	G1304230	(1.9)		+	Asia
Strawberry latent ringspot[f] (SLRSV)	*X. diversicaudatum* *X. coxi*	44 000 29 000	2.9		1.4	X75165 X77466	+	Europe
Tomato top necrosis	—	—	—		—			N. America

*Estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.
†Values <3 are molecular weight × 10^6, values >3700 are numbers of nucleotides; values in brackets were determined by electrophoresis in non-denaturing conditions and are probably underestimates at least for RNA-1.
‡Similar letters (a to f) indicate viruses known to be serologically related.

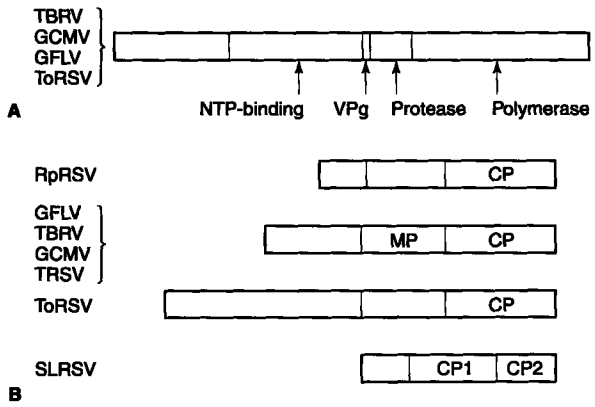


Figure 1 Diagrams illustrating the arrangement of some nepovirus proteins in the polyprotein translation products of RNA-1 (**A**) or RNA-2 (**B**). CP, coat protein(s); MP, putative movement proteins.

mature virus proteins. Comoviruses and fabaviruses differ from definitive nepoviruses in having two particle proteins of approximate molecular weights 43 000 and 25 000, but so do some tentative nepoviruses (**Table 1**). Recently comparisons among nucleotide sequences have prompted the suggestion that the tentative nepovirus SDV should be classified in a genus distinct from any of the three current genera. The distinction between the current genera is mainly in the type of vector; comoviruses are transmitted by beetles, fabaviruses by aphids, and several nepoviruses by nematodes. However, nematode transmissibility is not a requirement for membership of the genus because it has been demonstrated for only one-third of the viruses listed in **Table 1** that they are transmitted by nematodes.

Virion Properties

Particles of definitive nepoviruses are 26–30 nm in diameter and appear to have hexagonal outlines in the electron microscope. Some particles are penetrated by negative stain. Typically, particles of nepoviruses are of three types, named according to their relative rates of sedimentation. Top component (T) particles sediment at about 50S and contain no RNA, middle component (M) particles sediment at 86–128S and contain 27–40% RNA and bottom component (B) particles sediment at about 115–134S and contain 42–46% RNA. With some nepoviruses, T particles are less stable than the nucleoprotein M and B particles and do not survive some purification procedures. Freezing can cause a loss of nucleic acid from nucleoprotein particles of some nepoviruses and this can result in the appearance of T particles.

Nepovirus particles contain two sizes of RNA molecule (**Table 1**). M particles contain the smaller species (RNA-2) of 3.8–7 kb, B particles contain either a single 8 kb molecule (RNA-1) or, with nepoviruses that have RNA-2 molecules of <4 kb, two molecules of RNA-2. Because of their different RNA contents, T, M and B particles can be resolved by sedimentation to equilibrium in CsCl.

The coat proteins of definitive nepoviruses consist of molecules with molecular weights of 53 000–60 000 (**Table 1**). Calculation suggests that nepovirus particles have 60 of these protein molecules, presumably arranged in a $T = 1$ structure. Some tentative members of the genus have two or three types of protein molecule in their coat protein which have a combined monomer molecular weight of 60 000–70 000 (**Table 1**).

Genome Properties

Organization

Nepovirus RNA molecules are 3′-polyadenylated and are linked at their 5′ ends to a small protein (VPg). The sequences for the VPg of GFLV, ToRSV, GCMV and TBRV comprise 24–28 amino acids with a putative consensus sequence of:

$$E/D\text{-}x_{(1-2)}\text{-}Y\text{-}x_{(2)}\text{-}R\text{-}N\text{-}x_{(4-5)}\text{-}R.$$

Protease treatment, which degrades the VPg, either abolishes (e.g. TRSV, TBRV) or diminishes (e.g. RpRSV) the infectivity of nepovirus RNA. Translation of nepovirus RNA *in vitro* yields large polypeptides which correspond in size to the approximate coding capacity of each RNA species. Smaller polypeptides arise by proteolysis of each polyprotein but this process is very inefficient *in vitro*. Nucleotide sequences are known for the RNA of several nepoviruses and each contains one large open reading frame. The 3′ noncoding regions of the RNA-1 and RNA-2 of each nepovirus are virtually identical but only about ten of the 5′-most nucleotides of the 5′ noncoding regions are the same in the two RNA species. The polyprotein encoded by RNA-2 contains the coat protein at the C-terminal end and one or two other proteins, one of which may have a transport function. The polyprotein encoded by RNA-1 contains putative polymerase, protease and helicase domains. **Figure 1** shows the proposed genetic map of the polyproteins of TBRV, GFLV, GCMV, ToRSV (RNA-1 and RNA-2) and RpRSV and SLRSV (RNA-2 only). Cleavage at particular dipeptide sites is characteristic of polyprotein genomes cleaved by

Table 2 Dipeptides cleaved during proteolytic processing of nepovirus polyproteins

Cleaved dipeptide	*Virus*
R-G	GFLV, ArMV
R-A	GCMV
C-A	GFLV, RpRSV, TRSV
C-S	GFLV
G-E	GFLV
K-A	TBRV
K-S	TBRV
Q-S	CLRV
Q-G	ToRSV
N-S	BLMV

virus-coded proteases. The cleavage sites so far identified in nepovirus polyproteins are shown in **Table 2.**

Genetics

The two genome RNA species of nepoviruses are analogues of linkage groups. By inoculating plants with heterologous pairs of RNA from related strains and characterizing the resulting pseudo-recombinant isolates, functions have been assigned to each RNA. For RpRSV, RNA-1 determines systemic symptoms in *Chenopodium quinoa,* infection of Lloyd George raspberry, systemic invasion of *Phaseolus vulgaris* and the type of symptom induced in *C. quinoa*; RNA-2 determines the serotype of the particles, transmission by nematodes and chloroplast symptoms in *Petunia hybrida.* TBRV RNA-1 can replicate in inoculated protoplasts in the absence of RNA-2 and therefore presumably encodes the enzymes necessary for RNA replication and for the protease activity needed to release these enzymes from the polyprotein precursor. Progeny RNA molecules from such an experiment are infective and, because VPg is essential for TBRV RNA to be infective, it was inferred that RNA-1 also encodes the VPg. Sequencing located the VPg in the RNA-1 polyprotein (**Fig. 1**). With RpRSV and CLRV, there is evidence that at least one phenotypic character is controlled by more than one determinant (epistasis) and that some determinants have more than one phenotypic effect (pleiotropy).

Sequence Comparisons

Comparisons among the amino acid sequences of corresponding proteins of those nepoviruses for which sequence is known show few marked similarities. Most information is available for coat proteins. Among six coat proteins compared, the percentage identity was greater than about 24% only between viruses known to be serologically related (GFLV and ArMV; TBRV and GCMV). For other proteins in comparisons among TBRV, GCMV, GFLV and ToRSV, only the putative polymerase (81% for TBRV/GCMV; 45–53% among other pairs) and NTP-binding proteins (67% for TBRV/GCMV; 30–40% among other pairs) showed any significant similarities. A consensus cysteine protease motif is discernible in multiple sequence alignments of the putative protease sequences, and a consensus sequence has been proposed for nepovirus VPg (see above). But the percentage identity among either proteases or VPgs is not much different from that between unrelated proteins.

Satellites

Several nepoviruses have strains that support the multiplication of satellite RNA molecules. Some satellites (e.g. those of TBRV, SLRSV, GFLV, ChYMV and ArMV) are mRNA larger than 0.7 kb and encode proteins with molecular weights of 40 000–50 000. Others (e.g. those of TRSV, CYMV and ArMV) are about 0.3 kb and do not encode protein. These smaller satellites undergo self-cleavage during replication.

Geographic Range

Most nepoviruses are distributed throughout Europe or North America but a few appear much more restricted geographically. By contrast, GFLV occurs worldwide due to its widespread dissemination in planting stock.

Host Range and Virus Propagation

Most nepoviruses have wide natural and experimental host ranges that include woody and herbaceous plants. A notable exception is GFLV which is confined to grapevine in nature. Diseases of several important crop plants are induced by nepovirus infection and the viruses are prevalent in perennial species. Hosts include both monocotyledonous and dicotyledonous angiosperm plants and also some gymnosperms. The commonest experimental hosts used for the propagation of nepoviruses are *C. quinoa, Cucumis sativus, Nicotiana clevelandii, Pe. hybrida* and *Ph. vulgaris.*

Particles of many nepoviruses are stable and relatively easy to purify by clarification with butan-1-ol, alone or mixed with chloroform, followed by differential centrifugation and/or precipitation from solutions of polyethylene glycol. Particles of other nepoviruses are much less stable and may reach only

low concentrations in herbaceous hosts. Yields are usually 0.5–5 mg from 100 g of infected leaf.

Transmission

Some nepoviruses are transmitted by nematodes (**Table 1**) which are soil-inhabiting dorylaimid species in the genera *Longidorus* and *Xiphinema*. The nematodes acquire virus by feeding for a few hours on roots of infected plants. They can remain viruliferous thereafter for up to 9 weeks (*Longidorus* spp.) or 11 months (*Xiphinema* spp.) but the virus is lost when the nematodes molt. There is no evidence that nepoviruses multiply in their vectors. Nepovirus particles have been detected in the stylet lumen and attached to the guiding sheath of *Longidorus* spp. (RpRSV, TBRV) or the cuticular lining of the esophagus of *Xiphinema* spp. (ArMV, GFLV, TRSV, SLRSV). Experimentally, nepoviruses are readily transmissible by mechanical inoculation, although woody species can be difficult to infect and can yield poor inocula unless additives such as nicotine solution are used. A characteristic of most nepoviruses is that a high proportion (often >50%) of the seeds from an infected plant will germinate to give an infected plant (**Table 1**). Infection in seeds can come from either gamete although pollen from infected plants may not compete effectively with healthy pollen for fertilization of the ovule. Pollen transmission leading to infection of the mother plant appears to be a feature of the natural spread of CLRV and probably of BLMV.

Pathogenicity: Effects on Host Plants

Grapevine fanleaf is the most widespread and economically significant nepovirus worldwide due to the widespread dissemination of infected plants and of its nematode vector in planting stocks. Yield losses can be 50%. Many nepoviruses induce ringspots in certain hosts; chlorotic or necrotic rings on inoculated leaves and concentric rings or line patterns on the first leaves to be infected systemically. Leaves that are infected later may show no symptoms although they contain the virus ('recovery' phase). However, in many species, both naturally and experimentally inoculated, infection is symptomless; the rarity with which infected wild plants show symptoms indicates a close adaptation to these hosts, and suggests that nepoviruses are primarily pathogens of wild plants. All known nepoviruses infect *Chenopodium* species and many nepoviruses induce necrotic or chlorotic local lesions, making them useful assay plants.

Epidemiology

For nematode-transmitted nepoviruses, diseased plants occur in patches and spread from such infection foci is slow, reflecting the restricted mobility of the vector. Plants germinated from infected seeds are important sources from which nonviruliferous nematodes can acquire virus. Nepoviruses can be disseminated in seeds from infected plants over long distances to provide sources of inoculum into previously nepovirus-free areas for acquisition and transmission to crop plants by the relatively immobile vectors. Seed transmission is also important for the long-term survival of nepoviruses in the soil. An exception is GFLV which, in nature, does not infect common weed species but survives in long-lived pieces of grapevine root.

Many crop hosts of nepoviruses (such as grapevines and fruit trees) are vegetatively propagated, and so the propagation and movement of virus-infected plant stocks has been an important method of long-distance dispersal of these viruses (e.g. GFLV).

Cytopathology

Cells infected with some nepoviruses often contain cytoplasmic inclusion bodies which are readily detectable, some even by light microscopy. They contain membranous vesicles, endoplasmic reticulum and ribosomes, and are formed at a relatively early stage of infection; the bodies resemble those found in cells infected with cowpea mosaic comovirus. Another major cytological effect is the formation of tubules which contain virus particles in linear arrays. Such tubules are sometimes found in plasmodesmata between cells. Electron microscope observations suggest that nepovirus replication occurs in the cytoplasm, probably in or near the inclusion bodies.

Prevention and Control

Nepovirus infection is controlled by (1) establishing healthy stocks of plant material and distributing material certified to be virus-free, (2) planting resistant cultivars when available, (3) fumigating infested soil with nematicidal chemicals and (4) good weed control to remove possible sources of virus for re-infection.

See also: **Pathogenesis: Plant viruses; Plant virus disease – economic aspects.**

Further Reading

Brunt AA, Crabtree K, Dallwitz MJ, Gibbs AJ and Watson

L (1996) Nepoviruses: *Comoviridae*. In: *Viruses of Plants*, p. 37. Wallingford: CAB International.

Goldbach R, Martelli GP and Milne RG (1995) Genus *Nepovirus*. In: Murphy FA, Fauquet CM, Bishop DHL *et al* (eds) *Virus Taxonomy –The Classification and Nomenclature of Viruses:* Sixth Report of the International Committee on Taxonomy of Viruses, p. 341. Vienna: Springer-Verlag.

Martelli GP and Taylor CE (1989) Distribution of viruses and their nematode vectors. *Adv. Dis. Vector Res.* 6: 151.

Mayo MA and Robinson DJ (1996) Nepoviruses: molecular biology and replication. In: Harrison BD and Murant AF (eds) *The Plant Viruses*, vol. 5, p. 139. New York, London: Plenum Press.

Mayo MA, Robertson WM, Legorboru FJ and Brierley KM (1994) Molecular approaches to an understanding of the transmission of plant viruses by nematodes. In: Lamberti F, De Giorgi C, Bird DMcK (eds) *Advances in Molecular Nematology*, p. 277. New York and London: Plenum Press.

Mayo MA, Berns K, Fritsch C *et al* (1995) Satellites. In: Murphy FA, Fauquet CM, Bishop DHL *et al* (eds) *Virus Taxonomy – The Classification and Nomenclature of Viruses:* Sixth Report of the International Committee on Taxonomy of Viruses, p. 487. Vienna: Springer-Verlag.

NERVOUS SYSTEM VIRUSES

Richard T Johnson, The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

Introduction

Most viral infections of the nervous system represent serious and potentially life-threatening complications of systemic viral infections. With the possible exception of rabies, viruses are not neurotropic in the literal sense of having a specific affinity for the nervous system. Some viruses frequently invade the nervous system yet seldom cause serious disease; for example, mumps virus may cause meningitis but even during uncomplicated mumps parotitis cerebrospinal fluid changes in over 50% of patients indicate probable nervous system infection. Other viruses, such as herpes simplex virus, rarely infect the central nervous system; but when they do, they often cause fatal disease. Thus both mumps and herpes simplex viruses are regarded as neurotropic; mumps is highly neuroinvasive but has limited neurovirulence; herpes simplex has low neuroinvasiveness but is highly neurovirulent. Such variations are dependent on the structural and functional determinates of nervous system invasion, on the particular neural cells which specific viruses infect, on the effect of this infection on the host cells, and on the immune response or immunopathologic responses to the infection. Several terms need definition:

- *Neurotropic:* able to infect neural cells;
- *Neuronotropic:* able to infect neurons in contrast to other nervous system cells;
- *Neuroinvasive:* able to enter the nervous system;
- *Neurovirulent:* able to cause neurologic disease.

Anatomic Considerations

Both structural and functional features of the central nervous system present a unique milieu for viral replication. The blood–brain barrier and the compact structure of the brain and cord pose formidable barriers to the entry or dissemination of viruses within the nervous system. Furthermore, neurons are unique cells with high metabolic rates, intense membrane specialization, and no regenerative capacity. The same barriers that exclude viruses also limit access of immunocompetent cells and antibodies, and the nervous system lacks an intrinsic lymphatic system and has a paucity of phagocytic cells. Thus, the barriers that inhibit virus invasion also deter viral clearance. Therefore, many persistent infections involve the central nervous system.

The blood–brain barrier was originally conceptualized from the observation that dyes, such as trypan blue, stain all tissues except the brain and spinal cord after injection into the systemic circulation. The barrier at the cerebral capillary level consists of tight junctions between the capillary endothelial cells (beyond which most dyes do not pass), a dense basement membrane around the cells and tightly opposed astrocytic footplates. In the choroid plexus, the blood–cerebrospinal fluid barrier is structurally different. The capillaries of the plexus are fenestrated,

L (1996) Nepoviruses: *Comoviridae*. In: *Viruses of Plants*, p. 37. Wallingford: CAB International.

Goldbach R, Martelli GP and Milne RG (1995) Genus *Nepovirus*. In: Murphy FA, Fauquet CM, Bishop DHL *et al* (eds) *Virus Taxonomy –The Classification and Nomenclature of Viruses:* Sixth Report of the International Committee on Taxonomy of Viruses, p. 341. Vienna: Springer-Verlag.

Martelli GP and Taylor CE (1989) Distribution of viruses and their nematode vectors. *Adv. Dis. Vector Res.* 6: 151.

Mayo MA and Robinson DJ (1996) Nepoviruses: molecular biology and replication. In: Harrison BD and Murant AF (eds) *The Plant Viruses*, vol. 5, p. 139. New York, London: Plenum Press.

Mayo MA, Robertson WM, Legorboru FJ and Brierley KM (1994) Molecular approaches to an understanding of the transmission of plant viruses by nematodes. In: Lamberti F, De Giorgi C, Bird DMcK (eds) *Advances in Molecular Nematology*, p. 277. New York and London: Plenum Press.

Mayo MA, Berns K, Fritsch C *et al* (1995) Satellites. In: Murphy FA, Fauquet CM, Bishop DHL *et al* (eds) *Virus Taxonomy – The Classification and Nomenclature of Viruses:* Sixth Report of the International Committee on Taxonomy of Viruses, p. 487. Vienna: Springer-Verlag.

NERVOUS SYSTEM VIRUSES

Richard T Johnson, The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

Introduction

Most viral infections of the nervous system represent serious and potentially life-threatening complications of systemic viral infections. With the possible exception of rabies, viruses are not neurotropic in the literal sense of having a specific affinity for the nervous system. Some viruses frequently invade the nervous system yet seldom cause serious disease; for example, mumps virus may cause meningitis but even during uncomplicated mumps parotitis cerebrospinal fluid changes in over 50% of patients indicate probable nervous system infection. Other viruses, such as herpes simplex virus, rarely infect the central nervous system; but when they do, they often cause fatal disease. Thus both mumps and herpes simplex viruses are regarded as neurotropic; mumps is highly neuroinvasive but has limited neurovirulence; herpes simplex has low neuroinvasiveness but is highly neurovirulent. Such variations are dependent on the structural and functional determinates of nervous system invasion, on the particular neural cells which specific viruses infect, on the effect of this infection on the host cells, and on the immune response or immunopathologic responses to the infection. Several terms need definition:

- *Neurotropic:* able to infect neural cells;
- *Neuronotropic:* able to infect neurons in contrast to other nervous system cells;
- *Neuroinvasive:* able to enter the nervous system;
- *Neurovirulent:* able to cause neurologic disease.

Anatomic Considerations

Both structural and functional features of the central nervous system present a unique milieu for viral replication. The blood–brain barrier and the compact structure of the brain and cord pose formidable barriers to the entry or dissemination of viruses within the nervous system. Furthermore, neurons are unique cells with high metabolic rates, intense membrane specialization, and no regenerative capacity. The same barriers that exclude viruses also limit access of immunocompetent cells and antibodies, and the nervous system lacks an intrinsic lymphatic system and has a paucity of phagocytic cells. Thus, the barriers that inhibit virus invasion also deter viral clearance. Therefore, many persistent infections involve the central nervous system.

The blood–brain barrier was originally conceptualized from the observation that dyes, such as trypan blue, stain all tissues except the brain and spinal cord after injection into the systemic circulation. The barrier at the cerebral capillary level consists of tight junctions between the capillary endothelial cells (beyond which most dyes do not pass), a dense basement membrane around the cells and tightly opposed astrocytic footplates. In the choroid plexus, the blood–cerebrospinal fluid barrier is structurally different. The capillaries of the plexus are fenestrated,

lack a basement membrane and are surrounded by a loose stroma. Dyes and particles readily pass into the choroid plexus but are prevented from entering the cerebrospinal fluid by tight junctions located at the apices of the secretory epithelial cells of the choroid plexus. Tight junctions between the arachnoid cell over the surface of the brain complete the barrier.

There is no comparable barrier between the brain and the spinal fluid. The ependymal cells are not joined by tight junctions and, therefore, there is a free exchange between the extracellular space of the brain and the cerebrospinal fluid. The bulk flow is centrifugal, however, and solutions in the cerebrospinal fluid have limited entry into central nervous system tissue in normal circumstances. Furthermore, the intracellular gap between neural cells measures only 10–15 nm, less than the diameter of even the smallest virus, so that free movement of virus particles or inflammatory cells within the extracellular space is relatively restricted.

Neurons have specialized membranes for the transmission and receipt of specific messages; they also have axonal extensions to carry signals to and from distant neuronal populations, motor endplates, and sensory endings. In humans these cytoplasmic extensions may exceed a meter in length. These features are important in viral infections, since different subpopulations of neurons have different receptors usurped by viruses to permit entry into cells. Furthermore, viruses in some cases can be carried by axoplasmic transport systems either into the nervous system or within the nervous system where axonal processes link functionally related neurons. The high metabolic rates and lack of regenerative capacity may be important in chronic noncytopathic infections. A generalized infection that deranged cellular metabolism or caused shortened cell survival might present as a neurological disease and might have pathological findings restricted to the nervous system. Lack of neuronal replacement also assures that latent infection of neurons confers lifelong persistence in the host.

Antibodies found in the normal central nervous system are derived entirely from the serum. Antibody levels of immunoglobulin G (IgG) are approximately 0.4% of the serum levels. Since diffusion of macromolecules across the barrier is largely size dependent, immunoglobulin M (IgM) is present in even lower levels. Complement is largely excluded. When inflammation disrupts the blood–brain barrier, antibody molecules leak into the nervous system along with other serum proteins. When a mononuclear inflammatory response is mounted against infection, T lymphocytes usually enter the nervous system first followed by macrophages and B lymphocytes. These B cells from the peripheral circulation move into the perivascular space and can generate immunoglobulins intrathecally.

Pathways of the Central Nervous System Invasion

Viruses have been shown to enter the nervous system both along nerves and from the blood. The first experimental studies of viral invasion employed rabies, herpes simplex or polioviruses, all of which under experimental circumstances can penetrate the nervous system along peripheral nerves. The precise mechanism of neural spread remained a mystery for many years, since it was thought that the axoplasm slowly oozed in an anterograde direction. It was proposed that virus might move in perivascular lymphatics, by ascending infection of the supportive cells within the peripheral nerve, or even by replication in axons; a speculation that is now untenable because of the observed lack of ribosomes or protein synthesis within axons. In the 1960s active anterograde and retrograde axon transport systems were found. Viruses or other particles can be taken up in vesicles at the nerve terminals and transported to the cell body of the sensory or motor neuron (**Fig. 1**). This neural route of entry is important in primary viral infections such as rabies and possibly poliomyelitis. Retrograde transport also moves herpes simplex and varicella-zoster viruses from mucous membranes or skin into sensory ganglia at the time of primary infection. Subsequently, anterograde transport carries the reactivated virus from the ganglia to the periphery during exacerbations. Anterograde transport of herpes simplex virus by nerves innervating the dura from the trigeminal ganglia may explain the unique temporal lobe localization of herpes simplex virus encephalitis.

The olfactory spread of virus is a variation of neural spread. In the olfactory mucosa neural fibers provide a unique pathway; the apical processes of receptor cells extend beyond the free surface of the epithelium as olfactory rods and the central processes synapse in the olfactory blub. These are the only nerve cells with processes that link the central nervous system and the ambient environment. Indeed, some colloidal particles placed on the olfactory mucosa can be found in the olfactory bulbs within 1 h. Experimental studies show that viruses can enter through this route, and this may occur in some aerosol infections in humans such as laboratory accidents or rabies virus infections in bat-infested caves. Also the olfactory pathway has been postulated as a possible route of herpes simplex virus entry into the nervous system as an alternate explanation for the orbital-

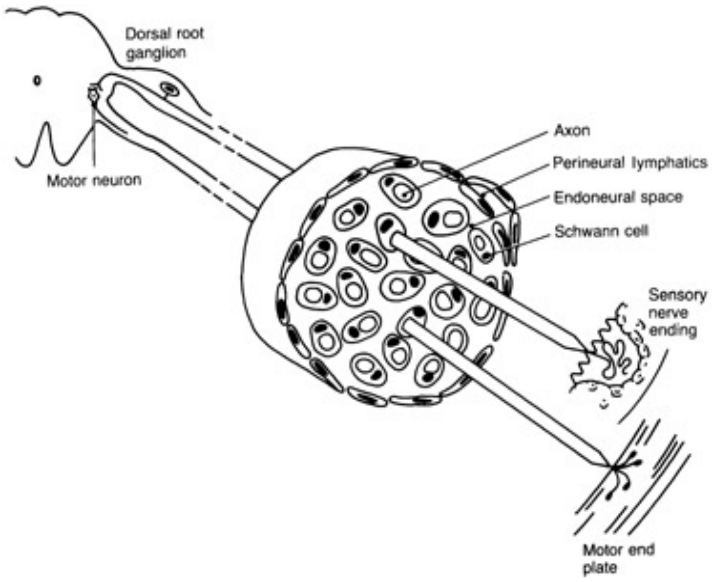


Figure 1 Schematic diagram of possible routes of neural spread of viruses to the nervous system. (Reproduced with permission from Johnson RT (1982) *Viral Infections of the Nervous System,* New York, Raven Press.)

frontal and medial temporal lobe localization of herpetic encephalitis. Nevertheless, despite the apparent ease of spread along this route, it appears to be a rare route of natural infection.

In most experimental and natural infections, viruses invade the brain from the blood. Historically, the blood–brain barrier was believed to be impervious to viruses. This belief was based in part on the fact that viruses experimentally inoculated directly into brain cause disease after a brief inoculation period, whereas the incubation period after intravenous inoculation is longer and comparable to that following cutaneous or peritoneal inoculation. The reason for this delay in infection is that virus in the blood is rapidly removed by the reticuloendothelial system; therefore, intravenous inoculation is, in fact, an inoculation primarily of the Kupffer cells of the liver and other reticuloendothelial cells. Therefore, virus must establish a nidus of peripheral replication that can effectively seed a viremia of sufficient magnitude and duration to allow invasion across the blood–brain barrier (**Fig. 2**). Thus, some viruses grow in lymphatics and seed into the blood directly via the thoracic duct, others grow in vascular epithelial cells, and others replicate in highly vascular tissue such as muscle.

A persistent viremia can be achieved by several mechanisms. Rate of clearance is dependent upon particle size; small particles such as togaviruses and flaviviruses can maintain high titer plasma viremias with sufficient rapid replication in peripheral tissue. Other viruses adsorb to red blood cells and thus evade clearance. Many large viruses such as measles and herpesviruses infect white blood cells thus evading clearance and replicating at the same time.

Some viruses enter the nervous system across the capillary endothelium and others across the choroid plexus. Some viruses infect the capillary endothelial cells and simply grow into the brain while others are able to transit across endothelial cells despite their paucity of endopinocytotic vesicles. Entry in infected leukocytes is a theoretical possibility but leukocyte traffic into the nervous system is limited, although trauma or inflammation due to other causes may predispose the nervous system to infection with viruses that infect white blood cells. Although there are areas of increased blood–brain barrier permeability, no viral infection has been shown to selectively infect these areas. Other viruses, such as mumps virus, grow in choroid plexus epithelium and seed into cerebrospinal fluid. Thus, the clearance of particles by the reticuloendothelial system, barriers of non-susceptible extraneural cells, production of interferon and other nonspecific inhibitors, and the physical barriers of the nervous system itself probably explain why viral infections of the brain are rare, even though systemic infections with the potential pathogens are very common.

Infection of Neural Cells

Once a virus has penetrated into the nervous system it must contact a susceptible cell and spread through the compact neuropil which is a theoretical problem. The fact that some viruses can be neutralized by extra-

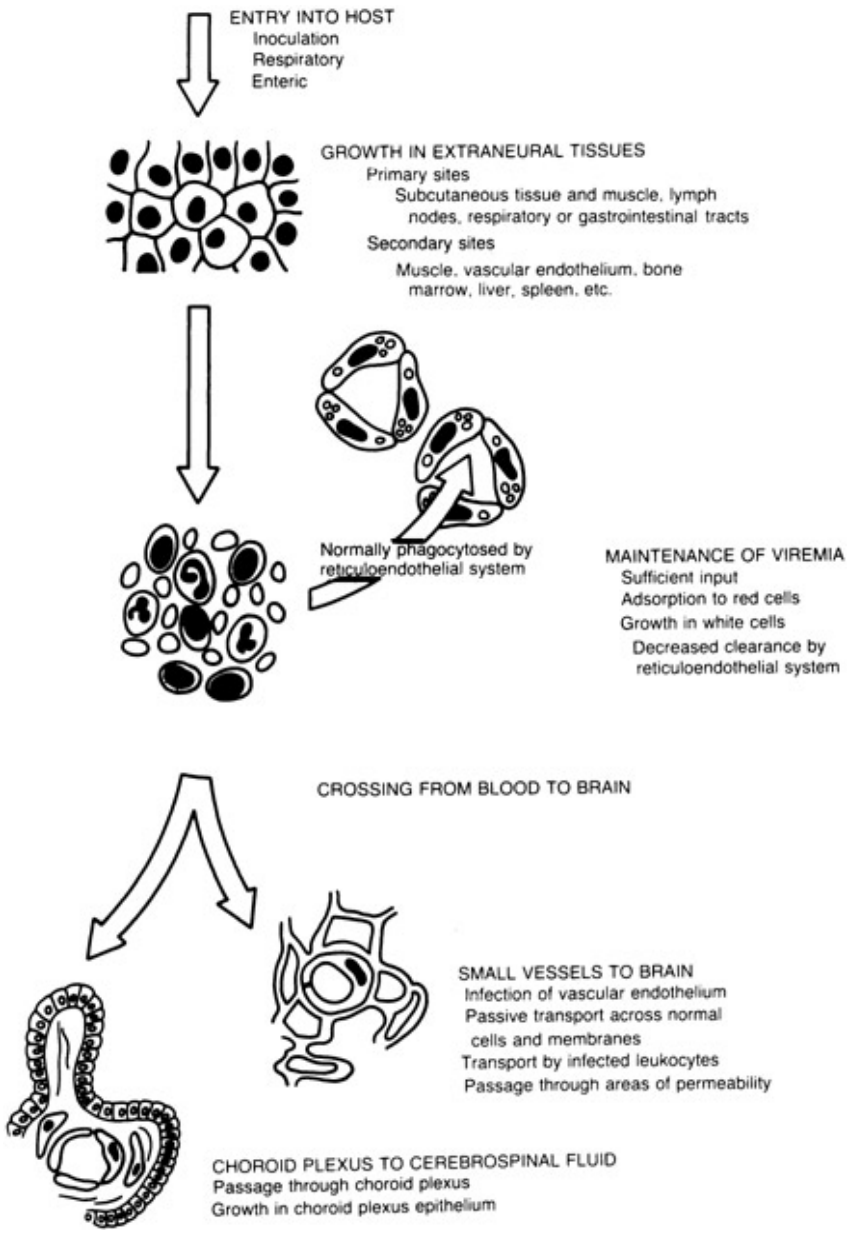


Figure 2 Schematic diagram of steps in the hematogenous spread of viruses to the nervous system. (Reproduced with permission from Johnson RT (1982) *Viral Infections of the Nervous System,* New York; Raven Press.)

cellular antibody even after central nervous system invasion shows that viruses such as togavirus and flaviviruses do spread in extracellular space, but this is not true of larger viruses. Conversely, the compact neuropil may facilitate the contiguous cell-to-cell spread of viruses. For example, in subacute sclerosing panencephalitis, a chronic brain infection of humans with measles virus, extracellular, enveloped virus is never seen, and there are enormous titers of extracellular antibody. Apparently the fusion protein allows measles virus to move from cell to cell through the brain.

Cell-to-cell spread may also involve axoplasmic transport causing infection of functionally linked cells; for example, in poliovirus infections the virus is rapidly spread through the motor system. Some viruses infect only neuronal populations such as rabies, polioviruses, and the arthropod-borne viruses, and some only infect selective neuron populations. Other viruses, such as herpes simplex virus, appear to infect neurons and glial populations with little selectivity.

Infection limited to vascular endothelial cells is found with rickettsial infections but is not recognized

in any viral infection of the nervous system. Infection limited to choroid plexus and meningeal cells appears to occur with those viruses that cause benign meningitis. In experimental studies with a number of viruses, widespread lytic infection of ependymal cells can lead to closure or stenosis of the aqueduct of Sylvius and resultant hydrocephalus. Similar aqueductal stenosis and hydrocephalus has been described in children after mumps virus meningitis.

The selective infection of oligodendrocytes has been recognized in nature in the disease progressive multifocal leukoencephalopathy caused in humans by the JC virus and in monkeys by SV-40 virus. In the course of immunosuppression, now seen most frequently with acquired immunodeficiency syndrome (AIDS), a selective lytic infection of oligodendrocytes causes multifocal areas of demyelination in the brain.

With changes in age, the specificity of infection and vulnerability of neural cells may change. For example, bluetongue virus infection of fetal sheep destroys the precursors of neurons and glia of the subependymal plate which leads to hydranencephaly or porencephaly depending on the age of fetal development, whereas the virus fails to infect the mature postmigratory cells in the late gestational or postnatal animal. Similarly, the external granular cells of the cerebellum in fetal or newborn animals are selectively infected by parvoviruses, and destruction of these mitotic cells leads to the granuloprival cerebellar degeneration seen in both natural and experimental animal infections.

Mechanisms of Cell Damage

Viral infection can have diverse effects on the host cell.

- Virus products may disrupt the integrity of the plasma membrane of the cell causing cell lysis.
- Infection with many viruses activates programmed cell death or apoptosis. This process of cell elimination is important in embryonic development, normal cell turnover, immunocyte clone deletion and other homeostatic processes but is particularly important in the nervous system, where massive cell elimination takes place during sculpting of the brain and cord and tight inhibition of apoptosis of mature neurons is required since they have no replicative capacity. This activation of death suppressor genes has explained the age-dependent effects of some neurotropic viruses.
- Virus genes or gene products may accelerate or inhibit host cell growth rate or can transform the cell so that it lacks contact inhibition.
- Infection can alter the antigenic composition of the cytoplasmic membrane making the cell the target of cytotoxic T cells and autoimmune cell lysis. This is the process that causes acute meningoencephalitis and death in mature mice infected with lymphocytic choriomeningitis virus.

Noncytopathic infections of neural cells also occur and lead to persistent infection with no disease or disorders without obvious histological changes. For example, neuroblastoma cells in culture infected with noncytopathic viruses such as rabies can show normal morphology, growth rates and protein synthesis, but reduced synthesis of specific neurotransmitters or receptors. These have been termed 'luxury functions', although *in vivo* the ability of neurons to synthesize transmitters or receptors would hardly be considered a luxury. Analogous noncytopathic infection has been demonstrated in mice congenitally infected with lymphocytic choriomeningitis virus. Congenitally infected mice are usually 'runts', but studies have shown selective infection of cells of the anterior pituitary that normally generate growth hormone. The animals are actually pituitary dwarfs responsive to growth hormone therapy.

Indirect cell damage can occur in viral infections that lead to sensitization of the host to neural antigens. This has been demonstrated in rats infected with coronaviruses, where the infection of neural cells leads to a cell-mediated autoimmune response to myelin proteins and to subsequent demyelination that can continue in the absence of ongoing infection. In postmeasles encephalomyelitis of humans, autoimmune demyelination appears to occur in the absence of infection of neural cells. Infection of lymphoid tissue leads to disruption of normal immune regulation, and about 1 per 1000 persons develop a symptomatic autoimmune reaction to myelin basic protein.

Other indirect mechanisms of neural cell damage have been postulated to explain the diverse neurological diseases seen in the course of human immunodeficiency virus (HIV) infection. The virus does not appear to cause infection of neurons, although neuronal processes and function are altered. The primary cells that are infected are macrophages and the microglia of the brain derived from macrophage populations. Possibly viral proteins produced by these cells or cytokines released by these cells interfere with neuronal function or are toxic to neurons or glial cells. For example, tumor necrosis factor, a lymphokine often elevated in brains of neurological-affected AIDS patients, has been shown *in vitro* to induce demyelination.

Clinical Features

Acute infections

The varied clinical features of viral infections of the nervous system can be explained in large part by the factors discussed above. Thus, a virus may infect only meningeal or ependymal cells, and cause a clinical syndrome known as viral meningitis or acute aseptic meningitis. This clinical syndrome is characterized by fever, headache and nuchal rigidity secondary to meningeal irritation but without clinical signs suggesting parenchymal disease. The commonest causes of acute viral meningitis are enteroviruses and mumps virus.

Encephalitis is a clinical syndrome in which, in addition to fever, headache and stiff neck, there is paralysis, seizures or other evidence of parenchymal disease of the brain. The commonest causes of severe encephalitis in man are herpes simplex virus and the arthropod-borne viruses. The former infects neurons and glia and causes diffuse necrotizing encephalitis in the newborn, but focal encephalitis in the immune child or adult presumably because diffuse virus spread is contained by immune responses. Focal signs, hallucinations, behavioral abnormalities and aphasia are more common with herpes encephalitis because of the localization to temporal lobes. Arboviruses have a propensity to infect neurons, and some flaviviruses tend to infect basal ganglia and brainstem neurons causing movement disorders and sudden respiratory failure. If signs of spinal cord involvement accompany encephalitis the term encephalomyelitis often is used. However, the term encephalomyelitis is also used to distinguish an acute postinfectious demyelinating disease of assumed autoimmune origin from acute viral encephalitis. Postinfectious encephalomyelitis (or acute disseminated encephalomyelitis) usually occurs 3–14 days after exanthems (measles, varicella or rubella) or respiratory infections (mumps, influenza and others) and clinically is characterized by the abrupt onset of fever, obtundation, seizures and multifocal neurological signs.

The clinical syndromes of rabies and poliomyelitis are the most distinctive of viral infections because of the selective infection of specific populations of neurons. Polioviruses selectively infect motor neurons which leads to flaccid paralysis. Rabies virus infects limbic system neurons with a relative sparing of cortical neurons early in disease leading to behavioral abnormalities. Rabies virus infections represent a diabolical adaptation of virus to animal host, causing the animal to remain alert but to lose timidity and develop aggressive behavior to transmit the virus. The advantage of this selectivity is evident considering that strains of rabies that cause the so-called 'dumb' or passive rabies are seldom transmitted in nature.

Slow infections

Slow infections are characterized by long incubation periods of months to years followed by an afebrile progressive disease. The term was originally coined in veterinary medicine to describe several transmissible diseases in sheep. The prototype slow infections are scrapie, a chronic noninflammatory spongiform encephalopathy due to a transmissible agent in which no nucleic acid has been identified (a prion), and visna, a chronic inflammatory encephalomyelitis caused by a lentivirus.

The first slow infections identified in humans was kuru, a progressive ataxia of a tribal group in New Guinea where the agent, resembling the agent of scrapie, was transmitted by ritual cannibalism. Creutzfeldt–Jakob disease, a subacute dementia with myoclonus, is a worldwide disease due to prion agents. In both of these human spongiform encephalopathies the clinical disease progresses irrevocably to death in about six months, but without fever or other clinical or histological findings to suggest infection.

In 10–15% of cases of Creutzfeldt–Jakob disease transmission is genetic resulting from insertions or point mutations in the gene coding for the prion protein. Some of these mutations result in phenotypes resembling sporadic Creutzfeldt–Jakob disease except that onset may be at an earlier age and progression to death may be more prolonged; in other mutations the phenotype is distinctive as in Gertmann–Straussler disease with prominent cerebellar ataxia or familial fatal insomnia where sleep disturbances dominate the early phase of disease. In rare cases, transmission has been iatrogenic with corneal and dural grafts, with neurosurgical procedures and with the injection of pituitary hormones extracted from human cadavers. Recent evidence suggests some cases are transmitted from beef contaminated with bovine spongiform encephalopathy. In the vast majority of cases no abnormality of the prion gene is found, and no common exposure is detected; in 85% of cases the mode of transmission is unknown.

Dementia, a chronic deterioration of cognitive function, is also caused by several conventional viruses. Subacute sclerosing panencephalitis is a chronic dementing illness caused by measles virus. One per million otherwise healthy children develop this chronic illness at an average of seven or eight years after uncomplicated measles. Dementia evolves slowly, associated with myoclonic movements and high levels of measles antibody in serum and cerebrospinal fluid. Children usually die a year or

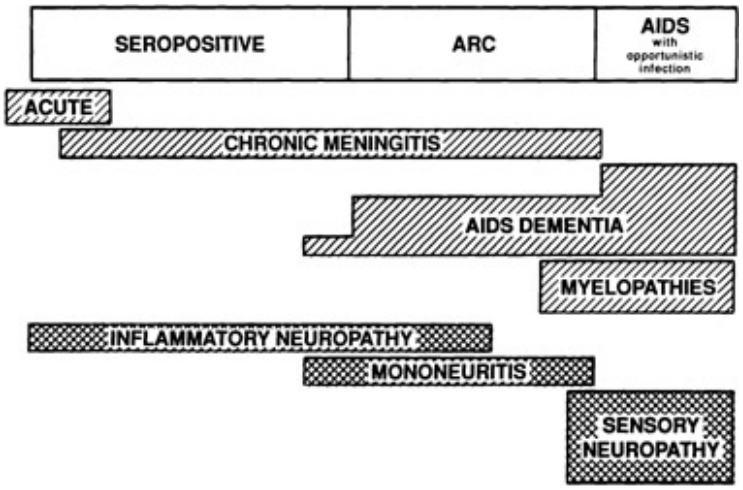


Figure 3 Schematic diagram of human immunodeficiency virus-related neurological diseases. Disease affects central (diagonal lines) and peripheral (cross-hatched lines) nervous systems, evolve at different stages of infection (asymptomatic seropositive, AIDS-related complex [ARC] and full-blown AIDS) and occur at different frequencies (indicated by vertical width). (Reproduced with permission from Johnson RT, McArthur JC and Narayan O (1988) *FASEB J* 2: 2970.)

less after the onset of clinical signs, but survival may vary from six weeks to six years. The disease is due to a subacute, slowly progressive diffuse infection of neurons and glial cells.

Progressive multifocal leukoencephalopathy due to JC virus also evolves as a slow viral infection with a selective infection of oligodendrocytes and a progressive demyelinating disease. Multifocal neurological signs evolve including cognitive dysfunction, paralysis, blindness and disorders of speech. Fever is absent and the cerebrospinal fluid shows no evidence of inflammation. Disease progresses relentlessly to death over a period of months.

Clinical symptoms associated with HIV are very diverse (**Fig. 3**). This virus now represents the commonest viral infection of the nervous system. From prospective studies of cerebrospinal fluid changes, it appears that the majority of people infected with the virus have early invasion of the nervous system; that is, the virus is highly neuroinvasive. However, early during this infection, neurological disease is rare. A presumed autoimmune disorder occasionally causes a demyelinating peripheral neuropathy (Guillain–Barré syndrome); acute meningitis is occasionally seen at the time of seroconversion or during early asymptomatic infection. An asymptomatic pleocytosis is often found. At this stage therefore, the virus is not highly neurovirulent. However, after the onset of immunodeficiency the infection is neurovirulent; 50% of AIDS patients develop progressive dementia with cerebral involvement, myelopathies or painful sensory neuropathies. The pathogenesis of these complications is unknown, but they are thought to be due to some viral protein or lymphokine incited by the virus because of the relative paucity of the virus in the nervous system and its localization to the microglial and macrophage populations.

Tropical spastic paraparesis complicating human T cell leukemia virus, type 1, infections is a recently recognized slow nervous system infection. Less than 2% of the patients infected with this virus develop either acute T cell leukemia or neurological disease. Since many of those infected are infected by breast milk and the onset of tropical spastic paraparesis is usually in the fourth or fifth decade of life, the incubation period is extraordinarily long. A subacute disease develops with progressive paralysis of the lower extremities associated with impotence, incontinence and sensory symptoms, but usually minimal sensory findings. Disease progresses slowly until the patients are wheelchair bound, but the disorder remains primarily at the level of the thoracic spinal cord. The involvement of the upper extremities is minimal with hyperreflexia but usually good function, and there is usually little, if any, indication of cerebral involvement. Early pathology in the spinal cord has shown vasculitis. In late stages hyalinization of vessels with necrosis and demyelination of spinal cord are found, and findings are most prominent in the thoracic cord. Whether virus replicates in any cells other than T lymphocytes is still uncertain. These observations lead to the questions of why less than 1 in 100 who are infected develop disease; why the incubation period is as long as 40 years; why the disease localizes to the thoracic spinal cord; and why over years the disease becomes relatively quiescent despite the fact that there are ongoing high levels of

intrathecal antibody synthesis suggesting that there is still antigenic stimulation by virus within the nervous system.

Identification of viruses or virus-like agents (prions) in a variety of chronic neurological diseases has led to speculation of a viral etiology for multiple sclerosis, Parkinson's disease, amyotrophic lateral sclerosis, Alzheimer's disease, schizophrenia and other illnesses. Experimental evidence for viruses in these chronic diseases is still tenuous.

See also: **Apoptosis and virus infection; Autoimmunity; Encephalitis viruses (*Flaviviridae*): Encephalitis viruses and related viruses causing hemorrhagic disease, Tick-borne encephalitis and Wesselsbron viruses; Enteroviruses (*Picornaviridae*): Animal and related viruses, Human enteroviruses (serotypes 68–71); Herpes simplex viruses (*Herpesviridae*): General features, Molecular biology; Human immunodeficiency viruses (*Retroviridae*): Molecular biology, Anti-retroviral agents, General features; Human T-cell leukemia viruses (*Retroviridae*): HTLV-1, HTLV-2; JC and BK viruses (*Papovaviridae*); Latency; Lymphocytic choriomeningitis virus (*Arenaviridae*): General features, Molecular biology; Measles virus (*Paramyxoviridae*); Mumps virus (*Paramyxoviridae*); Orbiviruses and coltiviruses (*Reoviridae*): General features, Molecular biology; Parvoviruses (*Parvoviridae*): Cats, dogs and mink, Molecular biology, Rodents, pigs, cattle and waterfowl, General features; Pathogenesis: Animal viruses, Plant viruses; Persistent viral infection; Polioviruses (*Picornaviridae*): General features, Molecular biology; Prions: Human and Animal, Yeast and Fungi; Rabies virus (*Rhabdoviridae*); Varicella-Zoster virus (*Herpesviridae*): General features, Molecular biology; Viral receptors; Virus–host cell interactions; Visna-Maedi viruses (*Retroviridae*).**

Further Reading

Glass JD and Johnson RT (1996) Human immunodeficiency virus and the brain. *Ann. Rev. Neurosci.* 19: 1.

Johnson RT (1998) *Viral Infections of the Nervous System*, 2nd edn. Philadelphia: Lippincott-Raven.

Levin MC and Jacobson S (1997) HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP): a chronic progressive neurological disease associated with immunologically mediated damage to the central nervous system. *J Neurovirol* 3: 126.

Prusiner SB (1997) Prion diseases and the BSE crisis. *Science* 278: 245.

Takahashi K, Wesselingh SL, Griffin DE *et al* (1996) Localization of HIV-1 in human brain using PCR/*in situ* hybridization and immunocytochemistry. *Ann. Neurol.* 39: 705.

NEWCASTLE DISEASE VIRUS (*PARAMYXOVIRIDAE*)

Peter T Emmerson, Department of Biochemistry and Genetics, Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne, UK

History

Newcastle disease was the name given by Doyle to a highly contagious viral infection of poultry, also known as fowl pest, which was first reported on a farm near Newcastle upon Tyne, UK, in 1926. Shortly after the reported disease at Newcastle, two further outbreaks occurred in the UK, one in Somerset and the other in Staffordshire. At about the same time, a disease with similar symptoms was observed in Java (in the city now known as Jakarta), Indonesia, and shortly thereafter in other regions of southeast Asia, notably around seaports of the Indian Ocean. Subsequent crossimmunity tests showed that the viruses isolated in southeast Asia and Newcastle upon Tyne were indistinguishable. The causative agent of the disease in Newcastle upon Tyne was identified as a virus which was distinct from fowl plague (avian influenza virus), although the symptoms bore some resemblance. It is thought likely that the virus was transported to the port of Newcastle upon Tyne by ship from southeast Asia. Whatever its origin, the new disease emerged or was first recognized in 1926 and rapidly spread throughout the world.

Newcastle disease was first recognized in various countries as a highly pathogenic disease with up to 100% mortality. In California, a relatively mild respiratory disease was first observed in the mid-

intrathecal antibody synthesis suggesting that there is still antigenic stimulation by virus within the nervous system.

Identification of viruses or virus-like agents (prions) in a variety of chronic neurological diseases has led to speculation of a viral etiology for multiple sclerosis, Parkinson's disease, amyotrophic lateral sclerosis, Alzheimer's disease, schizophrenia and other illnesses. Experimental evidence for viruses in these chronic diseases is still tenuous.

See also: **Apoptosis and virus infection; Autoimmunity; Encephalitis viruses (*Flaviviridae*): Encephalitis viruses and related viruses causing hemorrhagic disease, Tick-borne encephalitis and Wesselsbron viruses; Enteroviruses (*Picornaviridae*): Animal and related viruses, Human enteroviruses (serotypes 68–71); Herpes simplex viruses (*Herpesviridae*): General features, Molecular biology; Human immunodeficiency viruses (*Retroviridae*): Molecular biology, Anti-retroviral agents, General features; Human T-cell leukemia viruses (*Retroviridae*): HTLV-1, HTLV-2; JC and BK viruses (*Papovaviridae*); Latency; Lymphocytic choriomeningitis virus (*Arenaviridae*): General features, Molecular biology; Measles virus (*Paramyxoviridae*); Mumps virus (*Paramyxoviridae*); Orbiviruses and coltiviruses (*Reoviridae*): General features, Molecular biology; Parvoviruses (*Parvoviridae*): Cats, dogs and mink, Molecular biology, Rodents, pigs, cattle and waterfowl, General features; Pathogenesis: Animal viruses, Plant viruses; Persistent viral infection; Polioviruses (*Picornaviridae*): General features, Molecular biology; Prions: Human and Animal, Yeast and Fungi; Rabies virus (*Rhabdoviridae*); Varicella-Zoster virus (*Herpesviridae*): General features, Molecular biology; Viral receptors; Virus–host cell interactions; Visna-Maedi viruses (*Retroviridae*).**

Further Reading

Glass JD and Johnson RT (1996) Human immunodeficiency virus and the brain. *Ann. Rev. Neurosci.* 19: 1.

Johnson RT (1998) *Viral Infections of the Nervous System*, 2nd edn. Philadelphia: Lippincott-Raven.

Levin MC and Jacobson S (1997) HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP): a chronic progressive neurological disease associated with immunologically mediated damage to the central nervous system. *J Neurovirol* 3: 126.

Prusiner SB (1997) Prion diseases and the BSE crisis. *Science* 278: 245.

Takahashi K, Wesselingh SL, Griffin DE *et al* (1996) Localization of HIV-1 in human brain using PCR/*in situ* hybridization and immunocytochemistry. *Ann. Neurol.* 39: 705.

NEWCASTLE DISEASE VIRUS (*PARAMYXOVIRIDAE*)

Peter T Emmerson, Department of Biochemistry and Genetics, Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne, UK

History

Newcastle disease was the name given by Doyle to a highly contagious viral infection of poultry, also known as fowl pest, which was first reported on a farm near Newcastle upon Tyne, UK, in 1926. Shortly after the reported disease at Newcastle, two further outbreaks occurred in the UK, one in Somerset and the other in Staffordshire. At about the same time, a disease with similar symptoms was observed in Java (in the city now known as Jakarta), Indonesia, and shortly thereafter in other regions of southeast Asia, notably around seaports of the Indian Ocean. Subsequent crossimmunity tests showed that the viruses isolated in southeast Asia and Newcastle upon Tyne were indistinguishable. The causative agent of the disease in Newcastle upon Tyne was identified as a virus which was distinct from fowl plague (avian influenza virus), although the symptoms bore some resemblance. It is thought likely that the virus was transported to the port of Newcastle upon Tyne by ship from southeast Asia. Whatever its origin, the new disease emerged or was first recognized in 1926 and rapidly spread throughout the world.

Newcastle disease was first recognized in various countries as a highly pathogenic disease with up to 100% mortality. In California, a relatively mild respiratory disease was first observed in the mid-

1930s. This disease, with a mortality usually less than 15%, was first called pneumoencephalitis but was later shown to be caused by a virus which was indistinguishable immunologically from Newcastle disease virus (NDV). The observation that NDV was not always highly pathological was important and was followed by numerous reports of other strains with low virulence. Such isolates were used later as live vaccines.

Taxonomy and Classification

Newcastle disease virus is a member of the genus *Rubulavirus* of the family *Paramyxoviridae* in the order *Mononegavirales*. This order consists of viruses possessing a genome of nonsegmented, single-stranded RNA of negative sense (i.e. complementary to the mRNA sense). The order *Mononegavirales* contains four separate families of viruses: the *Paramyxoviridae*, the *Rhabdoviridae*, the *Filoviridae* and the recently assigned *Bornaviridae*. The family *Paramyxoviridae* is divided into two subfamilies, the *Paramyxovirinae* and the *Pneumovirinae*. The *Paramyxovirinae* subfamily is divided further into three genera: *Paramyxovirus* (type species human parainfluenza virus 1), *Morbillivirus* (type species measles virus) and *Rubulavirus* (type species mumps virus). Prior to 1995, NDV was classified as a member of the *Paramyxovirus* genus.

Other members of the *Rubulavirus* genus include human parainfluenza viruses 2, 4a and 4b, mumps virus, simian parainfluenza viruses 5 and 41 (SV5 and SV41), porcine rubulavirus and the avian paramyxoviruses 2 to 9 (where NDV is also known as avian paramyxovirus 1). The genus *Morbillivirus* includes measles virus, canine distemper virus and rinderpest virus. The genus *Paramyxovirus* includes Sendai virus and human parainfluenza viruses type 1 and 3. The genus *Pneumovirus* includes human respiratory syncytial virus, bovine respiratory syncytial virus, pneumonia virus of mice and turkey rhinotracheitis virus.

Properties of the Virion

NDV is a pleomorphic, membrane-enveloped virus of roughly spherical shape ranging in diameter from 150 to 400 nm. It contains a helical nucleocapsid structure 1000 nm long and 17–18 nm in diameter, with a central hole of 5 nm. The main protein subunit of the nucleocapsid is the nucleocapsid protein (NP). Two other proteins, the phosphoprotein (P) and the large (L) protein are also associated with the nucleocapsid. The envelope is a lipid bilayer derived from the host cell plasma membrane and has embedded in it, and protruding from it, the spike glycoproteins hemagglutinin-neuraminidase (HN) and fusion (F). The matrix protein (M) is interposed between the nucleocapsid and the viral envelope (**Fig. 1**).

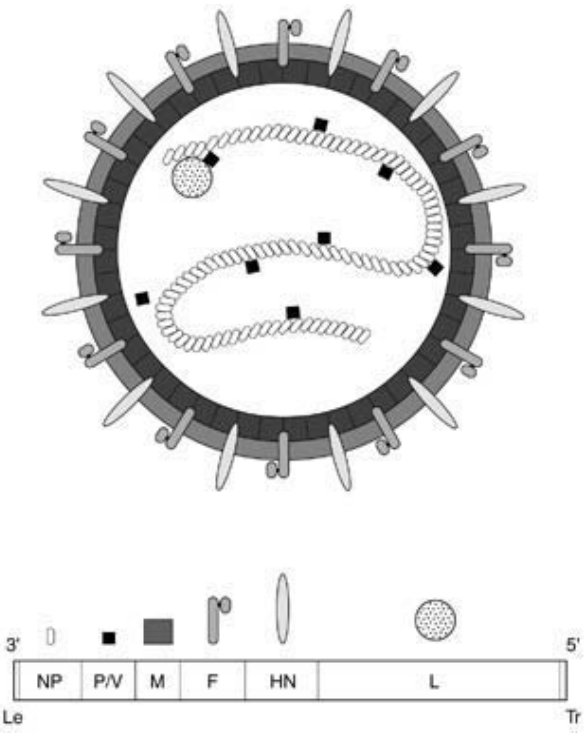


Figure 1 Newcastle disease virus.

Properties of the Genome

The genome of NDV is a linear, single strand of RNA of 15 186 nucleotides and of negative polarity. This strand must be transcribed into mRNA by the virion-associated RNA-dependent RNA polymerase before translation can take place. The genomic RNA itself is not infectious. On the genomic RNA, the genes are arranged in the order: 3′-NP-P-M-F-HN-L. There is a leader sequence at the 3′ end, upstream of the NP gene, and a trailer sequence at the 5′ end, downstream of the L gene.

The leader sequence (Le in **Fig. 1**) is 55 nucleotides long, and the first eight nucleotides of the leader sequence are identical in NDV, Sendai, measles and human parainfluenza 3 viruses.

The trailer sequence (Tr in **Fig. 1**) is 56 nucleotides long in strains Ulster and D26, and 114 nucleotides long in strain Beaudette C, based on the apparent poly(A) signal at the end of the L gene. Use of these differently located poly(A) signals would change the length of the corresponding mRNA but not affect the length of the L gene coding sequence or the total length of the genome. The nine nucleotides at the 5′

terminus of the trailer sequences of NDV and human parainfluenza virus type 3 are identical.

The genome displays inverse complementarity at its termini, with 11 out of 12 nucleotides at the 3′ terminus of the genome identical to those at the 3′ terminus of the positive-sense antigenome. This is to be expected as the 3′ end of the genome and the 3′ end of the antigenome both act as promoters to which the RNA-dependent RNA polymerase must bind to initiate transcription and replication.

Properties of Virus Proteins

The nucleocapsid protein NP, M_r 53 162 in strain Ulster, binds tightly to the genomic and antigenomic RNAs, producing helical structures. RNA that is not bound to NP cannot be transcribed or replicated. By analogy to studies with related viruses, the concentration of free (unassembled) NP within the infected cell is thought to control the relative rates of transcription and replication.

The phosphoprotein (P), M_r 42 241 (strain Ulster), is subject to phosphorylation. Together with the L protein it forms the viral polymerase (P-L). The P gene is transcribed to give two different mRNAs. One is a faithful copy of the P gene and leads to normal P protein production. The other has an extra guanosine residue inserted during transcription, which leads to the production of another protein, designated V, which has a cysteine-rich zinc binding domain. The letter 'vee' stems from the use of the roman numeral V to designate a fifth protein in SV5-infected cells.

The matrix protein (M), M_r 39 605 (strain Beaudette C), interacts with both the viral membrane and the nucleocapsid and is involved in viral assembly.

The fusion protein (F), one of two integral membrane glycoproteins, is first produced as an inactive precursor F_0 (M_r 59 042 before glycosylation in strain Beaudette C) which is cleaved post-translationally to give two disulfide-linked F_1 and F_2 polypeptides in the active protein. The active protein permits penetration of NDV into host cells by fusion of the viral envelope with the host cell plasma membrane.

The hemagglutinin-neuraminidase protein (HN), M_r 63 149 before glycosylation in strain Beaudette C, the other integral membrane glycoprotein, is responsible for the attachment of the virus to sialic acid-containing receptors on the cell surface. It also mediates cleavage of sialic acid residues from the surface of virions and infected cells and may be responsible for prevention of self-aggregation of viral particles during budding. In some avirulent NDV strains a larger inactive precursor HN_0 protein is cleaved to give the biologically active HN.

The large (L) protein, M_r 248 822 (strain Beaudette C), is required, together with the P protein, for viral RNA synthesis. The L protein is present in only about 30 copies per nucleocapsid. It is also probably involved in methylation, capping, phosphorylation and polyadenylation.

Replication

The replication of NDV, as with all viruses with negative-strand RNA genomes, is complicated by the absence in the mammalian host cells of the machinery to replicate RNA. The negative-sense RNA genome cannot be translated directly into protein but must first be transcribed into a positive-strand (mRNA) copy. Since the virus cannot therefore synthesize the required RNA-dependent RNA polymerase on entry, these proteins (L and P) must enter the cell with the genome on infection. Replication is considered together with transcription in the next section.

Transcription

Transcription and replication take place in the cytoplasm. Immediately after penetration, the virus initiates transcription by the P-L protein complex (an RNA-dependent RNA polymerase) using the RNA in the nucleocapsid as template. The transcriptive complex isolated from NDV-infected cells contains the viral nucleocapsid composed of a single strand of genomic RNA completely covered with NP protein, together with L and P proteins.

Viral RNA synthesis must be regulated such that it produces monocistronic mRNAs during transcription but full-length positive-strand complementary copies of the genomic RNA to act as templates for full-length negative-strand RNA synthesis during replication. Replication differs from transcription in that it requires *de novo* protein synthesis.

The 3′ leader contains the viral promoter for synthesis of the positive-sense RNAs (mRNA and antigenome). The P-L polymerase presumably binds to the promoter and transcribes the genome sequentially, beginning with the leader, and stopping and restarting at the gene-end and gene-start transcription signals in the RNA template. This process, primary transcription, produces mRNAs in amounts which decrease as the polymerase moves along the genome, due to attenuation at each intergenic control sequence. The first of the mRNAs to be translated produces NP.

For transcription to occur in this way, the polymerase must restart at the beginning of the NP gene after terminating at the end of the leader sequence. This is thought to be the critical step in the switch

between transcription to give individual mRNAs or replication to give full-length (+) antigenome. In order to give full-length antigenomic RNA the P-L polymerase must ignore the stop and start signals at the gene junctions. This is thought to be accomplished by binding to the nascent (+) RNA of newly synthesized NP which permits read-through of the stop signals by the polymerase, giving full-length (+) antigenomic RNA entirely coated with NP.

Replication of the virus then requires that the full-length (+) antigenome be used as template to produce full-length (−) strands for packaging. This requires binding of the polymerase to the promoter at the 3′ end of the (+) strand followed by complete synthesis of a complementary (−) strand. In this direction, there are no stop and start signals at the gene junctions.

Efficient replication of some paramyxovirus genomes requires that the genome length be an exact multiple of six. This 'rule of six' holds for the NDV genome length of 15 186 nucleotides and is thought to be the result of the binding of each NP monomer to exactly six nucleotides of RNA in the production of the helical nucleocapsid structures. This suggests that initiation of (−) strand synthesis only occurs if the last six nucleotides at the 3′ end of the antigenomic (+) strand, which constitute an essential part of the promoter, are precisely covered by one NP monomer.

Translation

The amounts of NDV proteins produced in infected cells suggests that transcription is polar, sequential and discontinuous. Thus, NP mRNA is the most abundant, while L mRNA is the least abundant, and this is reflected in the levels of protein synthesized.

Post-translational Processing

The newly synthesized NP, P and L proteins rapidly associate with the cytoskeletal framework and assemble into the nucleocapsid together with the genomic RNA.

The newly synthesized HN and F_0 proteins are inserted into the membrane of the rough endoplasmic reticulum, transported to and through the Golgi apparatus and inserted into the plasma membrane. Glycosylation of the HN and F proteins is initiated in the rough endoplasmic reticulum and further processed in the Golgi apparatus.

Assembly

The components of the virus are assembled at the host cell plasma membrane and mature virus is released by budding. Assembly probably involves binding of the M protein to the NP of the nucleocapsids and also to the HN and F proteins embedded in the cell membrane.

Geographic and Seasonal Distribution

NDV is very widely distributed, almost worldwide. Because of the wide variation in the severity of the disease caused by different strains it is likely that many outbreaks go unreported, particularly those involving strains of low pathogenicity. Vaccination by mesogenic and lentogenic strains is very widespread and this is likely to mask the presence of virulent strains of the virus. The geographic distribution of the disease can change rapidly. For example, a pigeon-adapted variant of NDV, apparently originating in Iraq in 1974, spread from there to Europe, reaching the UK in 1983, where limited spread to domestic poultry occurred in 1984.

Newcastle disease is a serious problem throughout Asia and the commercial poultry industry is totally dependent on control by vaccines. The disease is thought to be endemic throughout southeast Asia. It is a major problem in much of Africa, where traditional poultry raising at the village level is common and where effective vaccination is difficult to achieve. Velogenic virus has been recorded in the USA and many countries in South America. Canada and Chile were free of the disease in 1985 (the last survey) but vaccination was practised. Only two countries in Europe, Luxembourg and Norway, have never recorded outbreaks of NDV. NDV is present in certain parts of the former USSR and vaccination is practised. In 1985 no velogenic strains were reported in the former USSR. In Australia and New Zealand, avirulent strains are present in the poultry population, as indicated by the production of antibodies, but no disease is evident and vaccination is not routinely carried out.

An outbreak of Newcastle disease in poultry in Northern Ireland in 1997, which resulted in the death or compulsory slaughter of more than 900 000 birds, led to the order by the UK government of 15 million emergency vaccinations.

Newcastle disease has been reported in both temperate and tropical regions and it occurs in all seasons.

Host Range and Virus Propagation

Approximately 240 of the 8000 known species of birds have been shown to be susceptible to NDV and it is likely that virtually all species are susceptible. Chickens seem to be the most susceptible, while ducks and geese appear to be resistant even to those viruses that

are most pathogenic for chickens. Ducks and geese are often infected with the virus but rarely show clinical signs. Canaries, parrots and cranes have also been reported to be fairly resistant. Such birds, when infected, may shed velogenic virus for several weeks without showing any signs of disease. NDV causes mild conjunctivitis in humans.

NDV replicates at the site of entry, usually the conjunctiva, or the respiratory or alimentary tracts. It spreads rapidly to neighboring cells and by the second day there is considerable inflammatory cell infiltration. By the fifth day, the inflammatory reaction decreases and by the eighth day viremia is apparent. Within 48 hours of infection, high titers of virus are present in the kidney, lungs, spleen and bursa and quite high titers are present in the duodenum, trachea, pancreas and brain. Mesogenic and lentogenic strains may not reach the brain.

NDV can be readily grown in embryonated hens' eggs and in several tissue culture systems, notably chick embryo fibroblasts.

Genetics

The pathogenic and nonpathogenic strains of NDV presumably arise from each other by mutation. Sequence analysis of the HN and F genes of various strains has shown that susceptibility to cleavage of the precursor F_0 and HN_0 glycoproteins to their active form is determined by the specific sequences at or near the cleavage site. Mutations at these sites have major effects on the virulence of the strains.

Evolution

Sequence analysis of the genomes of various NDV strains and other paramyxoviruses is beginning to permit the construction of phylogenetic trees. For example, analysis of the L gene of various paramyxoviruses indicates a close relationship between NDV and parainfluenza virus type 2. Further sequencing of other viruses will eventually give a detailed account of the evolutionary relationships. Similarly, a start has been made at comparing gene sequences within NDV. Analysis of the HN sequences of 13 NDV strains permits the construction of a preliminary evolutionary tree, which, for example, places the mesogenic Beaudette C strain on the same 'branch' as the avirulent B1 strain.

Serologic Relationships and Variability

Some 30 years after the initial isolation of NDV in Newcastle upon Tyne, a paramyxovirus serologically distinct from NDV was isolated in Yucaipa, California, which caused serious outbreaks of disease in turkeys. A third paramyxovirus, serologically different from both types, was isolated from turkeys in Canada in 1967 and the USA in 1968. In the following decade there was a large increase in the number of isolations of serologically distinct paramyxoviruses throughout the world. It was suggested that the nomenclature should be based on the serological tests and that groups of viruses should be termed PMV-1 for NDV, PMV-2 for the Yucaipa type, PMV-3 for the North American turkey isolate, and so on. Furthermore, it was proposed that the names should also include the species of the bird from which the virus was first isolated, the geographical location, a reference number (if any) and the year of isolation. For example, a paramyxovirus isolated from domestic ducks in New York in 1978 is given the serotype PMV-9/duck/New York/22/78.

Antigenic variation has been studied by the use of monoclonal antibodies. On this basis, NDV (PMV-1) isolates can be differentiated and grouped into at least eight groups (A–H) which appear to correlate with shared biological and epizootiological properties.

Epidemiology

There have been at least three panzootics. The first began in 1926 and spread to most countries before it eventually subsided in the late 1950s as the result of widespread vaccination. The second panzootic emerged in the late 1960s in the Middle East and spread rapidly to all continents and most countries by 1973. The third panzootic appears to have emerged in the late 1970s in the Middle East and is thought to have been spread across Europe and then to many other countries by racing pigeons, both by contact during races and by international trade in the birds.

Transmission and Tissue Tropism

The virus is transmitted by inhalation and ingestion. Indeed, live vaccines can be administered by both methods, either as an aerosol spray or in the drinking water.

Pathogenicity

NDV strains differ widely in pathogenicity, depending largely on the host cell-specific cleavability and activation of the HN and F glycoproteins (discussed above). Whereas velogenic strains such as Herts 33 kill virtually 100% of an infected poultry flock, avirulent strains such as Hitchner B1 kill an extremely low percentage, if any.

Clinical Features of Infection

The first clinical sign in the bird is loss of appetite, usually about the fourth or fifth day after infection. Respiration is increased and is characterized by long, gasping inhalation through a half-opened beak. In many cases there is diarrhea and a thick mucus discharge from the nostrils and the mouth. The bird's temperature rises gradually until about the sixth or seventh day and falls quickly to below normal just before death.

Pathology and Histopathology

There is considerable strain dependence in the pathology of NDV infection. Birds infected with velogenic and mesogenic virus show gross lesions to the respiratory system. Changes to the trachea can vary from excess catarrhal exudate to severe hemorrhage. Less frequently, the lungs may be enlarged and congested. Also, swelling of the face tissue and eyelids is associated with edema, which may extend throughout the connective tissue of the neck and around the trachea.

Immune Response

The HN and F surface glycoproteins are important in the humoral response to NDV infection. On infection of a susceptible cell, the HN protein binds to a receptor containing neuraminic acid. Penetration into the cell is mediated by fusion of the viral envelope with the lipid bilayer of the cell, which requires the F protein. Monoclonal antibodies raised against the HN and F proteins have been used to map the antigenic sites within these proteins. Furthermore, the HN and F coding sequences when cloned into fowlpox or Marek's disease virus vectors protect chickens against a potentially lethal infection with NDV.

Prevention and Control

Full protection against Newcastle disease requires a combination of hygienic precautions and vaccination. Until recently, the only vaccines available against NDV were either live strains of low virulence or inactivated strains. Among live avirulent strains, Hitchner B1 and La Sota are used extensively worldwide as primary vaccines. Vaccines can be administered by a variety of convenient methods including addition to the drinking water and the spraying of aerosols. Where such mass treatment is not necessary, vaccines may be administered in a more controlled fashion by individual application to the eye or nostril, or by beak dipping. In Europe and North America, secondary vaccines are usually inactivated in oil emulsions and administered by injection. In many countries where virulent NDV is enzootic, live mesogenic secondary vaccines may be used.

Recently, recombinant DNA techniques have been used to construct genetically engineered bivalent viral vaccines. Inoculation of chickens with fowlpox virus containing the NDV HN or F genes protects them against both fowlpox and Newcastle disease. Similarly, vaccination with Marek's disease virus (herpes disease of turkeys) containing the NDV HN or F genes protects them against both Marek's disease and Newcastle disease. The first recombinant viral vaccine to be approved by the US Department of Agriculture was a recombinant fowlpox virus carrying the NDV HN and F genes.

The worldwide poultry industry is of great economic importance and the large-scale, intensive rearing of poultry greatly increases the vulnerability to infection. Some idea of the magnitude of the problem is given by the report that in the former USSR, where some large premises house up to two million adult birds, more than three billion birds are vaccinated against NDV each year.

Newcastle disease vaccine is probably the most widely used viral vaccine in the world.

Future Perspectives

The use of recombinant DNA technology can be expected to lead to the production of safer, more effective vaccines, as they can be more readily tailor-made to requirements and subsequently modified as required. Genetic engineering also offers the opportunity to construct multivalent vaccines in which the genes for several different antigens can be inserted into the same vector. In addition to the present recombinant vaccines available, which consist of the NDV HN or F gene sequences inserted into attenuated fowlpox or Marek's disease virus vectors, it should be possible to include the coding sequences for the surface glycoproteins of other poultry pathogens such as infectious bronchitis virus, infectious bursal disease virus, infectious laryngotracheitis virus, and so on.

See also: **Epidemiology of viral diseases; Measles virus (*Paramyxoviridae*); Parainfluenza viruses (*Paramyxoviridae*): Animal, Human; Sendai virus (*Paramyxoviridae*); Vaccines and immune response; Vesicular stomatitus viruses (*Rhabdoviridae*).**

Further Reading

Alexander DJ (ed.) (1988) *Newcastle Disease*. Boston: Kluwer Academic.

Collins PL, Chanock RM and McIntosh K (1996) Parainfluenza viruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology* 3rd edn, p. 1205. Philadelphia: Lippincott-Raven.

Doyle TM (1927) A hitherto unrecorded disease of fowls due to a filter-passing virus. *J. Comp. Ther. Pathol.* 40: 144.

Lamb RA and Kolakofsky D (1996) *Paramyxoviridae:* The viruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology* 3rd edn, p. 1177. Philadelphia: Lippincott-Raven.

Nagai Y, Hamaguchi M and Toyoda T (1989) Molecular biology of Newcastle disease virus. In: Pandy R (ed.) *Progress in Veterinary Microbiology and Microbiology*, Vol. 5, pp. 16–64. Karger: Basel.

NODAVIRUSES (*NODAVIRIDAE*)

L Andrew Ball, Department of Microbiology University of Alabama at Birmingham, Birmingham, Alabama, USA

Introduction

Nodaviruses are small, nonenveloped, isometric, riboviruses with bipartite, positive-sense RNA genomes. Within the *Nodaviridae* there are two genera: the alpha nodaviruses which predominantly infect insects, and the beta nodaviruses which infect fish. Under experimental conditions, some family members can also replicate in mammalian, plant or yeast cells. The two genomic RNAs (called RNA 1 which contains about 3100 nucleotides, and RNA 2 which contains about 1400 nucleotides) are both required for productive infection. These RNAs are copackaged in 29–32 nm diameter virions that contain a single major coat protein of approximately 40 kDa. The small genome size of these viruses, their unique genome strategy, abundant replication, ease of purification, and accessibility to high resolution structural analysis make the nodaviruses attractive systems for studies of RNA replication, gene expression, virion assembly and three-dimensional structure.

Members of the Family

The alpha nodaviruses include seven recognized members: nodamura virus (NOV, the type species of the genus), black beetle virus (BBV), flock house virus (FHV), boolarra virus (BoV), Manawatu virus (MwV), *Lymantria ninayi* virus (Greenwood) (LNV_G), and New Zealand virus (NZV) which is also known as *Drosophila* line 1 virus (DLV). These viruses were isolated from mosquitoes (NOV), New Zealand black beetles (BBV), two species of grass grubs (FHV, BoV and MwV) and gypsy moths (LNV_G).

The beta nodaviruses were discovered only within the last few years, but distinct viral species have now been isolated from several marine fish: striped jack nervous necrosis virus (SJNNV, the type species of the genus), barfin flounder nervous necrosis virus (BFNNV), *Dicentrarchus labrax* (sea bass) encephalitis virus (DlEV), Japanese flounder nervous necrosis virus (JFNNV), Lates calcarifer (barramundi) encephalitis virus (LcEV), redspotted grouper nervous necrosis virus (RGNNV), and tiger puffer nervous necrosis virus (TPNNV). Typically, these viruses were isolated from immature fish larvae and hatchlings in commercial fish hatcheries where they can cause significant economic losses. Capsid protein sequences show that the two genera are distantly related, but the viruses within each genus share antigenic determinants although they all show distinct reactivities. NOV is more distantly related to the other alpha nodaviruses than are the other six. No serological relationship has been found to any virus outside the *Nodaviridae*.

Host Range

All the alpha nodaviruses were originally isolated from insects, and some of them can be transmitted experimentally to several different insect species, including mosquitoes, bees, ticks, beetles and moths. In the laboratory, larvae of the wax moth (*Galleria mellonella*) provide convenient hosts. SJNNV and the other beta nodaviruses were isolated from fish larvae,

Further Reading

Alexander DJ (ed.) (1988) *Newcastle Disease*. Boston: Kluwer Academic.

Collins PL, Chanock RM and McIntosh K (1996) Parainfluenza viruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology* 3rd edn, p. 1205. Philadelphia: Lippincott-Raven.

Doyle TM (1927) A hitherto unrecorded disease of fowls due to a filter-passing virus. *J. Comp. Ther. Pathol.* 40: 144.

Lamb RA and Kolakofsky D (1996) *Paramyxoviridae:* The viruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology* 3rd edn, p. 1177. Philadelphia: Lippincott-Raven.

Nagai Y, Hamaguchi M and Toyoda T (1989) Molecular biology of Newcastle disease virus. In: Pandy R (ed.) *Progress in Veterinary Microbiology and Microbiology*, Vol. 5, pp. 16–64. Karger: Basel.

NODAVIRUSES (*NODAVIRIDAE*)

L Andrew Ball, Department of Microbiology University of Alabama at Birmingham, Birmingham, Alabama, USA

Introduction

Nodaviruses are small, nonenveloped, isometric, riboviruses with bipartite, positive-sense RNA genomes. Within the *Nodaviridae* there are two genera: the alpha nodaviruses which predominantly infect insects, and the beta nodaviruses which infect fish. Under experimental conditions, some family members can also replicate in mammalian, plant or yeast cells. The two genomic RNAs (called RNA 1 which contains about 3100 nucleotides, and RNA 2 which contains about 1400 nucleotides) are both required for productive infection. These RNAs are copackaged in 29–32 nm diameter virions that contain a single major coat protein of approximately 40 kDa. The small genome size of these viruses, their unique genome strategy, abundant replication, ease of purification, and accessibility to high resolution structural analysis make the nodaviruses attractive systems for studies of RNA replication, gene expression, virion assembly and three-dimensional structure.

Members of the Family

The alpha nodaviruses include seven recognized members: nodamura virus (NOV, the type species of the genus), black beetle virus (BBV), flock house virus (FHV), boolarra virus (BoV), Manawatu virus (MwV), *Lymantria ninayi* virus (Greenwood) (LNV_G), and New Zealand virus (NZV) which is also known as *Drosophila* line 1 virus (DLV). These viruses were isolated from mosquitoes (NOV), New Zealand black beetles (BBV), two species of grass grubs (FHV, BoV and MwV) and gypsy moths (LNV_G).

The beta nodaviruses were discovered only within the last few years, but distinct viral species have now been isolated from several marine fish: striped jack nervous necrosis virus (SJNNV, the type species of the genus), barfin flounder nervous necrosis virus (BFNNV), *Dicentrarchus labrax* (sea bass) encephalitis virus (DlEV), Japanese flounder nervous necrosis virus (JFNNV), Lates calcarifer (barramundi) encephalitis virus (LcEV), redspotted grouper nervous necrosis virus (RGNNV), and tiger puffer nervous necrosis virus (TPNNV). Typically, these viruses were isolated from immature fish larvae and hatchlings in commercial fish hatcheries where they can cause significant economic losses. Capsid protein sequences show that the two genera are distantly related, but the viruses within each genus share antigenic determinants although they all show distinct reactivities. NOV is more distantly related to the other alpha nodaviruses than are the other six. No serological relationship has been found to any virus outside the *Nodaviridae*.

Host Range

All the alpha nodaviruses were originally isolated from insects, and some of them can be transmitted experimentally to several different insect species, including mosquitoes, bees, ticks, beetles and moths. In the laboratory, larvae of the wax moth (*Galleria mellonella*) provide convenient hosts. SJNNV and the other beta nodaviruses were isolated from fish larvae,

but the breadth of their natural or experimental host ranges have not been determined. Although some fish cell lines that support virus replication have been identified, replication of the beta nodaviruses has yet to be carefully studied in cell culture.

In the region of Japan where NOV was isolated from a mosquito, high levels of neutralizing antibodies were found in pigs, suggesting that the natural host range of the virus may include these animals. In the laboratory, NOV replicates abundantly in suckling mice and hamsters, causing hind limb paralysis and 100% mortality five to seven days after inoculation. Adult animals are unaffected. NOV also kills adult bees, as well as some species of mosquitoes, giving it the very unusual property of causing lethal infections in both insects and mammals. On the other hand, FHV has no effect on suckling mice, but can productively infect plant protoplasts and even replicate in plant cells if they are inoculated with viral RNA. Indeed, FHV RNA can replicate well not only in insect, plant, and vertebrate cells, but also in the yeast *Saccharomyces cerevisiae*. *Drosophila* cells in culture support abundant replication of BBV, FHV or BoV, and FHV readily forms plaques on monolayers of these cells. Transfection of NOV RNA into baby hamster kidney cells in culture results in vigorous replication and the production of large amounts of infectious virus. No cellular receptors for nodaviruses have been identified.

Virion Composition

Nodavirus particles are composed of a protein shell surrounding one molecule of each of the two genomic RNAs. Evidence for copackaging of the RNAs comes from the uniform sedimentation coefficient (135–140S) and buoyant density (1.33–1.35 g ml^{-1}) of the particles, measurements of their particle weight (about 9×10^3 kDa) and total RNA content (about 16%), and the demonstration that the two viral RNAs can be crosslinked by UV irradiation of virus particles. Most compelling, however, is the linear relationship that exists between virus particle number and infectivity as determined by plaque assay of FHV. Since both viral RNAs are required for a productive infection, this result establishes that they are copackaged.

The protein shell is composed of 180 protomers arranged with icosahedral symmetry on a $T = 3$ surface lattice (i.e. the asymmetric structural unit contains three chemically identical protomers). In the alpha nodaviruses, each protomer is composed of one copy of the capsid proteins β (about 40 kDa) and γ (about 5 kDa), which are produced by a single proteolytic cleavage of the coat protein precursor, protein α (about 45 kDa). Cleavage occurs relatively slowly after virus assembly, so fresh virus preparations usually contain a small amount of uncleaved protein α. Since protein γ is small and stains poorly, it has been overlooked in the characterization of some of the nodaviruses, but in BBV, FHV and NOV it is present in virions in equimolar amounts with protein β.

The amino acid sequences of the coat protein precursors (α) of several of the alpha and beta nodaviruses have been inferred from the nucleotide sequences of the corresponding RNA 2 segments. They contain about 400 amino acid residues and clearly represent members of the same evolutionary lineage. The protein α sequences of BBV and FHV are 87% identical to one another, and about 50% identical to those of NOV and BoV. In contrast, they share only about 10% sequence identity with the capsid proteins of the beta nodaviruses. All α protein sequences have very basic regions near their N-termini, where about one-third of the first 50 amino acid residues are lysines or arginines. For the alpha nodaviruses, cleavage of protein α to form proteins β and γ occurs near the carboxy terminus, between asparagine 363 and alanine 364 in the BBV and FHV proteins, and the same two residues are present at the homologous positions in the proteins of NOV and BoV, suggesting evolutionary conservation of the cleavage site. Beta nodavirus particles contain roughly equal amounts of two related capsid proteins of 40 and 42 kDa. It has not yet been determined whether these correspond to the precursor protein α and its larger cleavage product β.

Three-Dimensional Structure of Virions

The three-dimensional structures of some of the alpha nodaviruses have been determined at atomic resolution by X-ray diffraction and also examined by cryoelectron microscopy and image reconstruction. The protomers of BBV consist of five distinct regions:

- a crystallographically disordered (and therefore mostly invisible) amino terminal domain of 60 amino acid residues which lies inside the virion;
- an eight-stranded anti-parallel β-barrel (as seen in the structures of many other animal and plant virus protomers);
- a surface protrusion composed mostly of β sheet and formed by three large insertions between strands of the β barrel;
- an internal domain that includes the carboxy terminus of protein β and the amino terminus of protein γ, and consists of two α helices that extend towards the interior of the virion;

- an invisible disordered region inside the virion that is composed of the carboxy terminal 28 amino acid residues of protein γ.

It seems probable that the highly basic amino termini of the nodaviral coat proteins interact with some of the phosphate residues of the viral RNAs. In the BBV virion, 180 protomers are arranged with icosahedral symmetry to form a shell that extends from an internal radius of 95 Å to an external radius of 170 Å . However, most of the β-barrel structure and the amino acid residues that are responsible for the interprotomer contacts lie between 120 Å and 145 Å from the center of the particle. The site of cleavage of protein α to form proteins β and γ lies within the central cavity of the virion.

Despite having identical amino acid sequences, the protomers adopt slightly different conformations depending on which of three quasi-equivalent positions they occupy in the icosahedral lattice. The most crucial difference is that in one-third of the protomers, the peptide region composed of residues 20–31 is icosahedrally ordered and can therefore be seen in the crystal structure, where it occupies an intersubunit groove on the internal face of the protein shell. Underlying this peptide, about 20 nucleotides of the viral RNA genome can be seen, in the form of helical duplexes that further fill one-third of the intersubunit grooves, 30 in each virion. Protomers that interact across such filled grooves lie in the same plane forming a dihedral angle of 180°, whereas those that interact across unfilled grooves form a dihedral angle of 136°. This topological difference in a subset of the interprotomer interactions is largely responsible for the overall size and shape of the protein shell.

Genome Structure and Coding Potential

The molecular genetics of the alpha nodaviruses are summarized in **Fig. 1.** Nodaviruses contain two single-stranded RNA molecules, both of which are necessary for productive infection. The larger RNA encodes RNA replicase functions and replicates autonomously when introduced into appropriate cells in the absence of the smaller genomic RNA. From this it has been inferred that RNA1 encodes the entire viral contribution to the RNA-dependent RNA polymerase. The smaller RNA encodes the coat protein precursor α. The RNA 5′ ends are capped but the structure of their 3′ ends, which are not polyadenylated, remains an enigma. Alpha nodavirus RNAs are unreactive with RNA ligase or poly(A) polymerase, enzymes that react with 3′ —OH groups on RNA molecules. The basis for this lack of reactivity is unclear, but covalent modification of

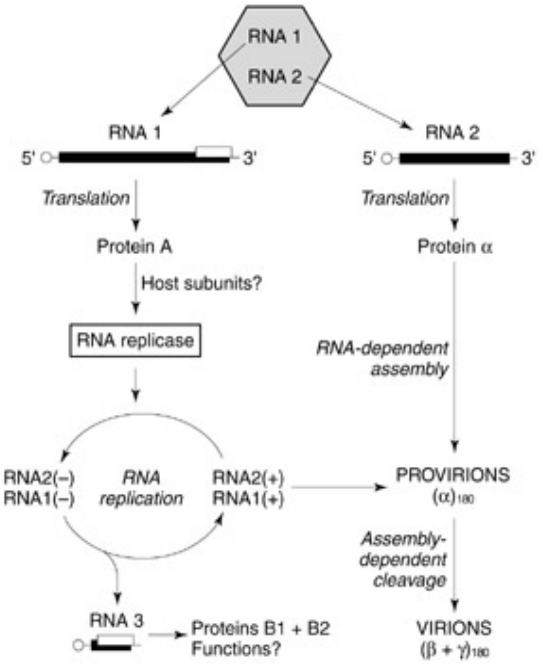


Figure 1 Genome strategy and replication cycle of the alpha nodaviruses. (Reproduced with permission from Ball and Johnson 1998.)

the 3′ end by a 'blocking group', perhaps a protein, has been proposed. However, RNA extracted from purified FHV does not incorporate a significant amount of radioactivity from labeled amino acids, nor does it yield an N-terminal sequence when subjected to Edman degradation. These results make it unlikely that the viral RNAs have a protein attached to their 3′ ends. Alternative possibilities are an unusually stable RNA secondary structure that masks the 3′ end, or perhaps covalent attachment to a small non-protein entity. The reactivity of the 3′ ends of beta nodaviral RNAs has not been reported.

A single long open reading frame starts at nucleotide 40 in FHV RNA 1 and ends at nucleotide 3033, encoding protein A (998 amino acid residues; 112 290 Da). The amino acid sequence of protein A contains a clear polymerase motif including glycine-aspartate-aspartate at positions 691–693, showing that it is the catalytic subunit of the viral RNA-dependent RNA polymerase. The carboxy terminus of protein A is overlapped by a short open reading frame that starts at nucleotide 2738 in FHV RNA 1, ends at nucleotide 3055, and encodes protein B2 of 106 amino acid residues (11 633 Da). The function of protein B2 is unknown, but its amino acid sequence predicts a high content of α-helix. It appears to be essential for the production of infectious virus, but not for RNA replication *per se*. Proteins A and B2 are

made in infected cells, but neither is found in virus particles.

The messenger RNA for protein B2 is a small RNA (RNA 3) that represents the 3′ terminal 387 nucleotides of RNA 1. It is produced during the replication of RNA 1, but is not packaged in virions. Like RNAs 1 and 2, its 5′ end is capped and its 3′ end is blocked. Ten nucleotides upstream of the AUG codon that initiates the protein B2 open reading frame, RNA 3 contains another AUG codon. This AUG lies in the same reading frame as protein A and initiates the synthesis of low levels of protein B1, which represents a carboxy terminal fragment of protein A. However, protein B1 is not essential for the replication of FHV, and BoV RNA 3 does not encode this protein.

Regulation of Viral Gene Expression

Despite their genetic simplicity, nodaviruses show temporal control of the synthesis of both their RNAs and proteins. As with other positive-stranded RNA viruses, the initial event after genome uncoating is the translation of the viral RNA, and the proteins produced from the input RNA can most easily be examined in cell-free translation systems. There, RNA 1 directs the synthesis of protein A and RNA 2 directs the synthesis of protein α. Neither B protein is synthesized by RNA extracted from purified viruses. In infected cells, the first synthetic events that can be readily detected are the replication of RNA 1 and the synthesis of RNA 3, shortly followed by the replication of RNA 2. These RNA syntheses occur in the cytoplasm and are catalyzed by the RNA-dependent RNA polymerase encoded in RNA 1. Synthesis of RNAs 1 and 2 in approximately equimolar amounts continues throughout the 24–36 h infectious cycle, whereas RNA 3 synthesis is transient and diminishes about 10 h after infection.

After amplification of the input RNA by replication, translation of protein A from RNA 1, and protein B2 from RNA 3 can be readily detected against a gradually diminishing background of host cell protein synthesis, which is progressively inhibited. Synthesis of protein α from RNA 2 starts 2–3 h later than that of proteins A and B2, but then increases rapidly and dominates the protein synthetic capacity of the infected cell for the remainder of the infectious cycle. By contrast, the synthesis of proteins A and B2 is transient and both are strongly repressed later in infection, despite the continued presence of their messenger RNAs. However, when RNA 1 replicates in the absence of RNA 2, proteins A and B2 and RNA 3 are synthesized continuously, and accumulate to unusually high levels. This result indicates that RNA 2 or its translation product is responsible for both the transcriptional and translational repression of proteins A and B2. In cell-free systems, RNA 2 is a much stronger message than RNA 1 and synthesis of protein α outcompetes the synthesis of protein A. A similar effect probably operates in infected cells, although it has been suggested that RNA 3 or protein B2 also contributes to the repression of protein A synthesis. Transcriptional repression of RNA 3 synthesis is mediated by RNA 2 itself, perhaps via a direct base-pairing interaction between RNA 2 positive strand and the promoter for RNA 3 synthesis on the negative strand of RNA 1. The net effect of these control mechanisms is that early in infection the major products are RNAs 1 and 3 and their encoded proteins A (RNA replicase) and B2 (unknown function), whereas later synthesis is dominated by the genomic RNAs 1 and 2 and the coat protein precursor α.

RNA Replication

Deproteinized RNA extracted from cells late in infection contains single-stranded, positive-sense RNAs 1, 2 and 3 in an abundance that approximates that of the ribosomal RNAs. Also detected are small amounts of double-stranded versions of these three RNA species, which amount to less than 5% of the total viral RNA. This demonstrates the presence of full-length negative strands that correspond to each of the three viral RNAs, but the intracellular state of these negative-strand RNAs before deproteinization is unknown.

Infected cells also contain a membrane-bound RNA-dependent RNA polymerase activity that can be partially purified and rendered template-dependent by treatment with micrococcal nuclease. This enzyme preparation can use positive-strand RNA 1, 2 or 3 as template and synthesize the corresponding negative-strand to produce double-stranded RNAs. However, complete RNA replication *in vitro*, with the net synthesis of RNAs 1 and 2 of the same sense as the input templates, requires the presence of intact membranes or a synthetic cationic lipid. Protein A copurifies with the RNA replicase activity, whereas most of protein B2 does not. However, the enzyme has not been purified sufficiently to enable its polypeptide composition to be determined, so the possible involvement of host cell subunits is an unresolved question. But since FHV replicase can function in cells of yeast, insect, plant or vertebrate origin, any required host cell components must be highly conserved.

Full-length cDNA clones of FHV RNAs 1 and 2 can be transcribed *in vitro* to produce infectious RNAs. Transcribed *in vivo* from an expression vector, these

cDNAs direct the synthesis of a self-replicating RNA 1 and a replication-competent RNA 2, respectively. Site-directed mutations that eliminate the protein B2 open reading frame or the initiation site for RNA 3 synthesis (but leave the protein A open reading frame intact), do not interfere with the autonomous replication of RNA 1, suggesting that protein B2 is not an essential component of the RNA replicase. Indeed, it is hard to see how protein B2 could be necessary for RNA replication since the production of its messenger RNA depends on replicase activity. However, protein B2 appears to fulfill an important function of some kind since mutations that eliminate its expression have invariably been found reverted in infectious viruses recovered from mutant cDNA clones.

The nature of the template for synthesis of positive-strand RNA 3 is unclear. During the initial stages of RNA replication, it is thought to be a partial or complete negative strand of RNA 1, which directs RNA 3 positive strand synthesis by internal initiation. This mechanism of subgenomic RNA synthesis resembles that seen in other RNA virus families, such as the togaviruses and bromoviruses. The presence in infected cells of double-stranded RNA 3 implies that the positive strand is a competent template for negative strand synthesis and this has been confirmed using partially purified RNA polymerase. However, it is unclear whether the RNA 3 negative strand can direct RNA 3 positive strand synthesis, i.e. whether RNA 3 truly replicates. Deletion analysis of the RNA 2 cDNA shows three regions of the template that are necessary for efficient replication: the 5′ and 3′ termini, and an internal region between nucleotides 520 and 720. The role of this internal region is unknown.

The unequal abundance of the positive and negative strands implies a difference in their efficiency of synthesis, presumably at initiation at the 3′ ends of the negative and positive strand templates, respectively. The 3′ terminal 65 nucleotides of BBV, FHV and BoV RNA 2 can be folded into similar secondary structures consisting of two adjacent hairpin loops that are preceded and connected by partially conserved sequences, and it is likely that these sequences and structures are involved in template recognition and the initation of negative strand synthesis. However, no corresponding structures exist at the 3′ terminus of the negative strand, so the elements involved in the more efficient recognition of this template are unclear. The only common feature is the resemblance between the 5′ sequences of RNAs 1, 2 and 3, which for FHV are 5′ GUUU..., 5′ GUAA..., and 5′ GUUA..., respectively. The RNA replicases from BBV, FHV and BoV can replicate each others' RNAs and genetic reassortants between these three viruses are viable. NOV RNA and replicase share a different specificity.

Virion Assembly

Assembly of FHV occurs in the cytoplasm in close association with intracytoplasmic membranes, and involves the coat protein precursor α and RNAs 1 and 2. Intermediates in the assembly pathway have not been identified, except for a 140S particle, the provirion, which differs from the mature virion in that it contains uncleaved protein α rather than proteins β and γ. Newly synthesized protein α is incorporated into provirions within a few minutes, whereas the incorporation of RNAs 1 and 2 occurs slowly, with a half-time of about 10 h. Following provirion formation, protein α slowly cleaves to form β and γ with a half-time of about 4 h at 22°C. This cleavage increases the stability of the virus particle substantially and renders it infectious. Since the site of cleavage lies within the interior of the virion, proteolysis is thought to be mediated by the coat protein itself, acting autocatalytically.

Nucleotides 186–217 of FHV RNA 2 are necessary for packaging the RNA into virus particles. This region forms a bulged stem–loop structure which may be the element recognized for nucleation of assembly on RNA 2. The mechanism responsible for copackaging one molecule of each of the two viral RNAs is not clear. A region of potential base-pairing between BBV RNAs 1 and 2 was proposed as a possible site of RNA–RNA interaction, but it is not conserved in the FHV sequences. Nevertheless, RNAs 1 and 2 are released as a 27S complex following gentle disruption of NOV, and purification of RNA 1 completely free of RNA 2 requires repeated separations, suggesting the existence of direct RNA 1–RNA 2 interactions that may be involved in copackaging.

Virus–Host Cell Interactions

In general, nodavirus infections of permissive cells in culture induce only mild cytopathic effects, which may in part account for the very high yields of virus that can be obtained from infected cultures (in excess of 10^6 virus particles per cell). Three days after infection of *Drosophila* line 1 cells with BBV, the progeny virus constitutes about 20% of the total cellular protein, and several of the *Nodaviridae* form paracrystalline arrays of virus particles in the cytoplasm of infected cells. Despite the high virus yields, most infected cells remain intact and metabolically active even late in infection, and little virus is released. However, a progressive inhibition of host cell protein

synthesis occurs during infection, due in part to the accumulation of large amounts of RNA 2 which is a very active messenger.

A small fraction of cultured *Drosophila* line 1 cells routinely survives infection with FHV and on examination the cells are found to be persistently infected. Viruses isolated from persistently infected cultures replicate poorly even in fresh cells, because their coat protein genes have accumulated multiple mutations. However, the establishment of persistence has been attributed to cellular changes rather than the mutations in the viral capsid gene which arise later. Defective viral RNAs that have suffered substantial deletions and rearrangements can be isolated from viruses harvested from persistently infected cultures, and the structures of these RNAs may provide insight into the *cis*-acting sequences necessary for RNA replication and encapsidation.

Epidemiology and Pathogenesis

The readiness with which alpha nodaviruses establish persistent infections in cell culture probably reflects their propensity to establish inapparent and persistent infections in their insect hosts. These infections can reduce egg viability and slow the development of the host without causing significant mortality. Under other conditions, such as overcrowding of the host, however, nodaviruses cause epizootic infections with high mortality. Such infections can be economically damaging in the case of SJNNV disease among hatchery-reared fish larvae. Large numbers of SJNNV particles are found in the brain and other cells of the central nervous system, and the infected larvae show abnormal swimming behavior and nervous necrosis.

Intraperitoneal inoculation of NOV into mice less than about 14 days old causes severe hind limb paralysis due to replication of the virus in the hind limb musculature and degeneration of spinal cord neurons. Mice inoculated when they are older than about 21 days show no signs of disease. It is not clear whether this change is due solely to the development of a responsive immune system, but 7-day-old mice born to or suckled by immunized mothers are completely resistant to intraperitoneal challenge with NOV. In wax moth larvae, NOV replicates predominantly in muscle cells, but cells of many other tissues are also susceptible to infection which kills the larvae.

See also: **Fish viruses; Persistent viral infection; Tetraviruses (*Tetraviridae*); Virus structure: Atomic structure, Principles of virus structure.**

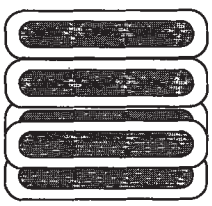
Further Reading

Ball LA and Johnson KL (1998) Nodaviruses of insects. In: Miller LK and Ball LA (eds) *The Insect Viruses*, New York: Plenum Press (in press).

Garzon S and Charpentier G (1992) Nodaviridae. In: Adams JR and Bonami JR (eds) *Atlas of Invertebrate Viruses*, p. 351. Boca Raton, FL: CRC Press.

Schneemann A, Reddy V and Johnson JE (1998) The structure and function of nodavirus particles: a paradigm for understanding chemical biology. *Adv. Virus Res.* 50: 381.

NONOCCLUDED BACULOVIRUSES



John P Burand, University of Massachusetts – Amherst, Amherst, Massachusetts, USA

History

The nonoccluded baculoviruses (NOB) are a loosely associated group of enveloped, rod-shaped viruses which, in many respects resemble the occluded baculoviruses except that they are not found associated with a protein crystal occlusion body. Only three NOBs have been studied to any significant extent. The first NOB to be identified was the *Oryctes* virus (Or-1V). The isolation of this virus resulted from a field study conducted throughout Southeast Asia in 1963, to find an agent that could be used to control the rhinoceros beetle, *Oryctes rhinoceros*, a pest on tropical palm plantations in the South Pacific. The virus was not naturally occurring in areas of the South Pacific where *O. rhinoceros* had been accidentally introduced and had become a pest. Since its initial discovery the virus has been found in insect populations in the Philippines, Sumatra, West Kalimantan, Thailand and India. Today, the virus is used to control beetle populations and serves as the best model for the use of a virus for insect pest control.

NORWALK AND RELATED VIRUSES (*CALICIVIRIDAE*)

Mary K. Estes and **Isabelle Leparc-Goffart**, Division of Molecular Virology, Baylor College of Medicine, Texas Medical Center, Houston, Texas, USA

History

Diarrheal illnesses of humans have been documented since the dawn of history. In spite of the discovery during the past century of numerous enteric bacteria and parasites, the etiology of a major portion of diarrheal illnesses remained unknown until the 1970s. It was assumed by exclusion that viruses were responsible for a significant portion of these illnesses. Thus, volunteer studies were initiated in the 1940s and 1950s because most viruses could not be propagated *in vitro* or in an animal model. These studies attempted to determine if illness could be induced with a presumptive nonbacterial viral agent by feeding bacteria-free fecal filtrates, derived from adults with acute nonbacterial gastroenteritis, to adult volunteers. Illness was induced demonstrating that the nonbacterial suspensions were indeed etiologic agents of acute gastroenteritis. The filtrates were named after the source of the original specimen, e.g. Marcy (Marcy State Hospital, Utica, New York), Niigata (Niigata Prefecture, Japan) and FS (Family Study, Cleveland, Ohio). These agents were passaged serially to other volunteers and also characterized antigenically in crosschallenge studies. However, because none of the infectious agents could be identified further *in vitro* or in an animal model, the etiology of acute nonbacterial gastroenteritis remained elusive. In the 1950s and 1960s, the introduction of tissue culture technology led to the discovery of over 100 new viruses, many of which were isolated in feces, but none of these new viruses could be implicated as an important etiologic agent of acute gastroenteritis. In the late 1960s, human embryonic organ cultures of the respiratory tract were used to successfully isolate several fastidious respiratory coronaviruses. Prompted by this success, renewed efforts were made to isolate viruses from fecal specimens of patients with viral gastroenteritis by utilizing human embryonic intestinal organ culture; these studies failed to identify an etiologic agent. It was considered that perhaps the lack of success was due to the absence of an infectious agent in the fecal specimen used as inoculum. Fecal suspensions prepared in the 1940s and 1950s, as described above, that were known to be infectious in challenge studies were either exhausted or not available at that time for evaluation with these newer techniques. Thus, in the early 1970s, a 'second generation' of volunteer studies was initiated. In one of these, a filtrate made from a rectal swab specimen from a secondary case of gastroenteritis from an outbreak of 'winter vomiting disease' that affected about 50% of the students and teachers in an elementary school in Norwalk, Ohio, was found to induce gastroenteritis in adult volunteers. This illness-inducing filtrate was serially passaged to other volunteers and was designated as the Norwalk agent. Attempts to cultivate or identify an infectious agent *in vitro* with the newer cell or intestinal organ culture techniques or in animals were unsuccessful.

In 1972, application of immune electron microscopy (IEM) led to the discovery of 27 nm virus-like particles in an infectious stool derived from the Norwalk outbreak and to the etiologic association of these virus-like particles with the Norwalk, Ohio outbreak. The 27 nm Norwalk particles thus became the first virus to be implicated as the cause of epidemic nonbacterial gastroenteritis; it was discovered without the use of any *in vitro* or animal model system. IEM also led to the discovery of other gastroenteritis agents such as the Montgomery County, Hawaii and Snow Mountain agents. Approximately 1 year after the discovery of the Norwalk virus, rotaviruses, the major etiologic agents of acute gastroenteritis of infants and young children, were discovered by electron microscopic techniques, again without the benefit of any *in vitro* system or animal model.

Taxonomy and Classification

The classification of viruses responsible for acute gastroenteritis was first based on morphology. For example, Norwalk virus was the prototype of a group of agents initially called small round-structured viruses (SRSVs). Recently, rapid advances in molecular biology have allowed these viruses to be classified based on their genome characteristics and most of the previously named SRSVs belong to the *Caliciviridae*.

The family *Caliciviridae* contains four genera: *Lagovirus*, 'Norwalk-like viruses', 'Sapporo-like viruses' and *Vesivirus*. The human caliciviruses responsible for epidemic gastroenteritis belong to

Table 1 Human calicivirus classification and antigenic relationships among the caliciviruses

		Antigenic relationships	
Genus	*Virus strain*[a]	*Determined by serologic IEM and/or crosschallenge studies in volunteers as described in the text*	*Determined by ELISA with hyperimmune antisera raised against recombinant Grimsby, Norwalk and Mexico virus-like particles*
'Norwalk-like viruses'	Norwalk	Distinct serotype	Distinct type
	Hawaii	Distinct serotype	Unknown
	Snow Mountain	Distinct serotype	Unknown
	Lorsdale	Unknown	Unknown (probably same as Grimsby)
	Southampton	Unknown	Unknown
	Mexico	Unknown	Distinct type
	Grimsby	Unknown	Distinct type
'Sapporo-like viruses'	Sapporo	Distinct serotype	Unknown
	Manchester	Unknown	Unknown

[a] Only a representative subset of virus strains in each genus ('Norwalk-like viruses' and 'Sapporo-like viruses'). See Green *et al.* (1999) for complete listing of strains.

the genus 'Norwalk-like viruses' (type species: Norwalk virus) and 'Sapporo-like viruses' (type species: Sapporo virus) (**Table 1**).

Properties of the Virion

These nonenveloped viruses contain a genome of single-stranded RNA of positive polarity. They have a diameter of 27–40 nm by negative stain electron microscopy and a buoyant density of 1.33–1.41 g ml^{-1}. These viruses, which are shed in the feces of individuals with gastroenteritis, cannot be propagated in cell cultures.

The virions are composed of a single capsid protein. Structural analysis of virus particles from stool is limited by the low number of particles present in these samples. However, by negative stain electron microscopy, the Norwalk virus has an indistinct 'feathery' outer edge and an indistinct surface substructure, although there is a suggestion of indentations on its surface (**Fig. 1A**). Expression of the capsid protein using the baculovirus system results in the self-assembly of the Norwalk virus protein into virus-like particles (VLPs) (**Fig. 1B**). By negative stain electron microscopy, these VLPs have a similar morphology to the native virus. The structure of these VLPs has been resolved by electron cryomicroscopy and computer image processing. The capsid exhibits a $T = 3$ icosahedral symmetry. The major structural protein folds into 90 dimers that form a shell domain from which arch-like capsomers protrude. A key characteristic of this architecture is 32 cup-shaped depressions at each of the icosahedral fivefold and threefold axes.

Genome Organization

In 1990, the genome of Norwalk virus was cloned and characterized. More recent studies have characterized and sequenced completely other viruses belonging to

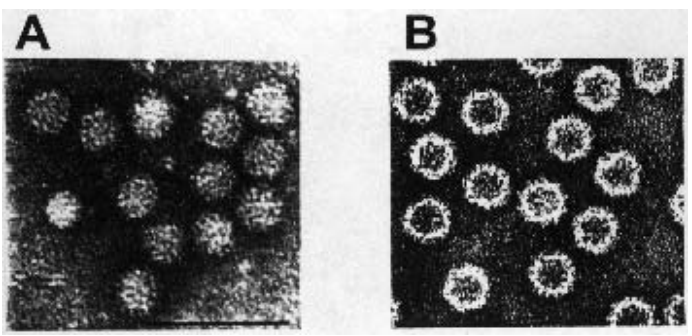


Figure 1 Negative stain electron micrographs of (**A**) Norwalk virus purified from stool of a volunteer (#547) given Hu/NVL/NV/1968/US, and (**B**) recombinant Norwalk virus-like particles produced and purified from insect cells infected with a recombinant baculovirus expressing ORF 2 and ORF 3 of Norwalk. Bar = 100 nm.

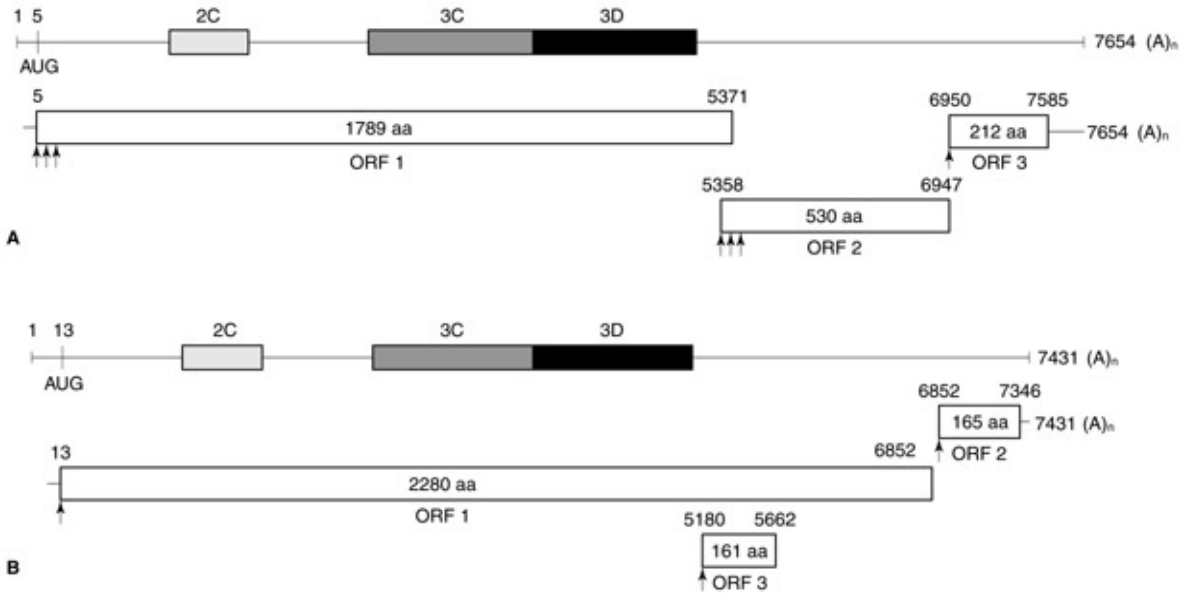


Figure 2 Genome organization of human caliciviruses. (**A**) Genome organization of Norwalk virus. The first ORF encodes the nonstructural proteins; ORF 2 encodes the capsid protein; and ORF 3 encodes a small basic protein. (**B**) Genome organization of Manchester virus (Liu *et al*, 1997). ORF 1 encodes the nonstructural proteins and the capsid protein, followed by ORF 2 (the homologue of the Norwalk virus ORF 3) encoding a small basic protein. ORF 3 is encoded by an out-of-frame sequence within ORF 1.

one of the two human calicivirus genera, either in the 'Norwalk-like viruses' (Norwalk virus, Lordsdale virus and Southampton virus) or in the 'Sapporo-like viruses' (Manchester virus). These viruses contain a positive-sense polyadenylated single-stranded RNA of approximately 7.6 kb (**Fig. 2**).

The genome of 'Norwalk-like viruses' is organized in three major open reading frames (ORFs). For Norwalk virus, the first ORF at the 5′ end encodes a large polyprotein of 1738 amino acids (aa) with a predicted molecular weight of 193.5 (193.5 K). This polyprotein contains short motifs of similarity with the 2C (helicase), 3C (cysteine protease), and 3D (RNA-dependent RNA polymerase) proteins of picornaviruses (**Fig. 3**). Thus, the 5′ end of the genome of the 'Norwalk-like viruses' codes for a precursor of

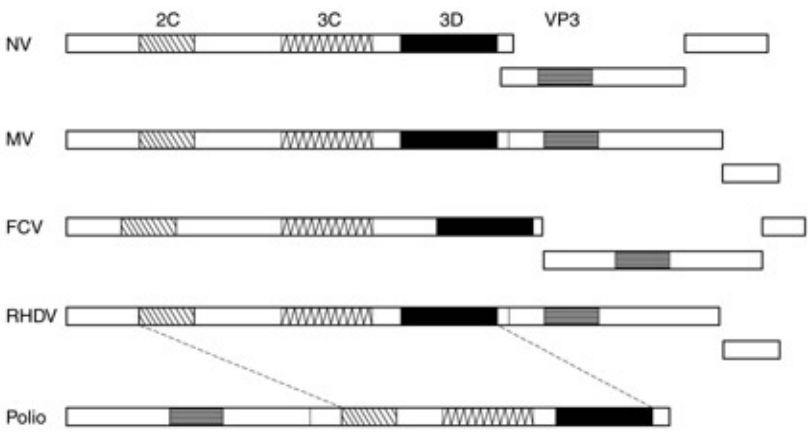


Figure 3 Comparison of the genomic organization between caliciviruses and picornaviruses. The genomic organizations of Norwalk virus (NV), Manchester virus (MV), feline calicivirus (FCV), rabbit hemorrhagic disease virus (RHDV), and poliovirus (Polio) are compared by showing the locations of the motifs within the genomes that are similar to the 2C helicase (▨), 3C cysteine protease (▩), 3D RNA-dependent RNA polymerase (■) and VP3 (▤) capsid protein of poliovirus.

the nonstructural proteins. ORF 2 encodes a 530aa (56.6 K) protein, the capsid protein. The ORF 2 protein expressed in insect cells self-assembles into virus-like particles as explained above (**Fig. 1B**). ORF 3 at the 3′ end of the genome is predicted to code for a small protein of 212aa (22.5 K) with a very basic charge (isoelectric point of 10.99). The ORF 3 protein does not have sequence similarity with any other proteins in the GenBank and its function remains unknown, although recently the ORF 3 protein has been found in VLPs expressed from cDNA constructs that contain both ORF 2 and ORF 3.

The genome of 'Sapporo-like viruses' is organized slightly differently. For Manchester virus, the first ORF codes for the nonstructural proteins as well as the capsid protein, which is found in-frame at the end of the nonstructural proteins. This genome organization is similar to that found in the animal calicivirus rabbit hemorraghic disease virus (RHDV) belonging to the genus *Lagovirus* (**Fig. 3**). ORF 2 encodes a predicted small highly basic protein of unknown function, similar to ORF 3 for Norwalk virus. Manchester virus contains a third ORF within the capsid protein which could encode another small basic protein. The significance of this ORF is unclear as it is not seen in any of the other calicivirus genomes sequenced thus far, and the small protein it potentially encodes shows no sequence homology to other viral proteins in the database.

Serologic and Phylogenetic Relationships

Four distinct serotypes of the 'Norwalk-like viruses' (Norwalk, Hawaii, Snow Mountain and Taunton) and one serotype of the 'Sapporo-like viruses' (Sapporo) were originally described by serologic IEM studies employing virus particles shed in stools as antigen and paired sera of infected individuals as the source of antibody. These serotype designations assumed that antibody reactivity by IEM reflects the reactivity of neutralizing antibodies. This may not be the case. Therefore, serotypes remain to be more clearly defined, a goal that is unable to be achieved due to the lack of a cultivation system. Early volunteer studies also examined the ability of different viruses to induce crossprotection. Based on these studies in addition to IEM, Norwalk, Hawaii and Snow Mountain viruses were defined as separate serotypes (**Table 1**). More recently, the antigenic relationships between these viruses have been examined by ELISA using hyperimmune antisera raised against virus-like particles. By this method, Norwalk, Mexico and Grimsby viruses are antigenically distinct.

The genetic relationships between the viruses within the calicivirus and the picornavirus families are being unraveled by sequence analysis of nucleic acids amplified directly from stools using reverse transcription–polymerase chain reaction (RT-PCR). Sequence comparisons of the full-length capsid protein of a number of viruses belonging to the four genera of the calicivirus family and of picornaviruses can be used to generate phylogenetic relationships but detailed understanding of the biological significance of these relationships awaits successful cultivation of these fastidious viruses.

Host Range and Virus Propagation

Attempts to cultivate the Norwalk and related viruses in tissue or organ culture have been unsuccessful. Efforts to find an animal model that develops illness after virus administration also have failed. However, chimpanzees become infected subclinically following administration of the Norwalk virus by the alimentary route; they shed soluble Norwalk antigen in the feces and develop serologic evidence of infection by IEM and radioimmunoassay (RIA).

Epidemiology

Human caliciviruses are the major known etiologic agents of acute nonbacterial gastroenteritis that cause clearly defined outbreaks that affect adults, school-aged children and family contacts; infrequently, these outbreaks involve infants and young children. Outbreaks occur in all seasons of the year in various communal settings, including schools, recreational and swimming facilities, cruise ships, restaurants, families and nursing homes. In an analysis of 74 gastroenteritis outbreaks from 1976 to 1980 (most of which were selected because they were nonbacterial), 42% were associated with Norwalk virus. These were minimal estimates because assays to detect evidence of infection were not available for most members of this group of agents until recently. Recent epidemiologic data using new assays to detect human caliciviruses indicate almost all (>90%) of reported gastroenteritis outbreaks in the United States and the Netherlands are caused by these viruses.

The prevalence of serum antibody to Norwalk virus in individuals in developed and developing countries is markedly different: in developing countries, antibody is acquired early in life and ≥90% of individuals have antibody by 2 years of age. In the United States, such antibody is acquired slowly during childhood and more rapidly in adulthood so that, by age 50, at least 80% of individuals have antibody. Although Norwalk virus may be associated with mild

Table 2 Response of 50 adult volunteers (19–39 years old) given Norwalk virus

Response	*% of infected volunteers[a] (n = 41)*	*% of uninfected volunteers (n = 9)*
Seroconversion	98	0
Antigen excretion	88	0
Infection	100	0
Asymptomatic	32	0
Symptomatic	68	0
Symptoms with clinical illness ($n = 28$)		
Diarrhea	86	0
Vomiting	57	0
Nausea	96	10
Abdominal cramps	96	0
Headache/bodyache	96	40
Chills	36	0
Fever (>37.8°C)	32	0

[a] Infection determined by antigen shedding and/or antibody response.
Data from Graham *et al* (1994).

gastroenteritis in infants and young children in developing countries, there is no evidence that it causes severe, life-threatening diarrhea in this age group.

Transmission

Human caliciviruses are highly contagious. Transmission of infection occurs by the fecal–oral route. The virus is found in the feces and has been detected in vomitus. A common source of infection, such as contaminated water or food, is frequently described. Person-to-person spread has also been demonstrated. Airborne transmission has been proposed because of the rapid spread of illness when a common source is not identified. Recent studies have shown that the virus may also be spread by individuals with subclinical infections, and virus can be shed for a longer time than previously recognized ($\geq$ 14 days postinfection).

Pathogenicity

Volunteers inoculated with Norwalk or Hawaii viruses develop characteristic transient histopathological mucosal lesions of the upper small intestine that includes broadening and blunting of the villi along with microvillus shortening on an intact mucosa. Mononuclear cell infiltration of the lamina propria and cytoplasmic vacuolization are also observed. Brush-border enzyme levels (alkaline phosphatase, sucrase and trehalase) are decreased during acute illness. Adenylate cyclase levels in the jejunal mucosa are not increased. The gastric and rectal mucosa are not affected histologically. Nausea and vomiting, common characteristics of this infection, may be caused by a delay in gastric emptying.

Clinical Features of Infection

Norwalk virus illness may begin abruptly with vomiting or diarrhea, or both. The spectrum of illness may vary widely in individual patients. For example, in experimentally infected adults, one volunteer vomited 20 times and required parenteral fluid therapy, whereas a second volunteer had no vomiting but eight diarrheal stools. The relative frequency of these and other symptoms was recently described for 50 volunteers challenged with Norwalk virus and is similar to those seen in natural outbreaks and in infection with other human caliciviruses. Of 50 volunteers orally administered Norwalk virus, 41 (82%) became infected; of these infections, 68% were symptomatic and 32% asymptomatic (**Table 2**). The most common symptoms with clinical illness are nausea, malaise and abdominal cramps. Diarrhea is usually watery without mucus, blood or leukocytes. Norwalk illness is usually mild, lasting 12–48 hours. It should be noted, however, that infrequently severe Norwalk virus gastroenteritis has been observed in middle-aged patients; in addition, Norwalk virus gastroenteritis has also been a contributing factor to the death of elderly, debilitated individuals.

Immune Response

The mechanism of immunity to human calicivirus remains an enigma as it deviates from the charac-

teristic pattern observed with most acute infectious illnesses in several ways: (1) adults are highly susceptible to both naturally occurring and experimentally induced Norwalk virus illness, as approximately 50% of unselected volunteers become ill after challenge; (2) the presence of preexisting serum antibody (measured by ELISA) does not appear to correlate with protection; and (3) short-term immunity has been demonstrated but long-term immunity may be absent. For example, 12 volunteers who were challenged with Norwalk virus on two separate occasions showed markedly different clinical responses: six became ill after initial and subsequent rechallenge 27–42 months later, whereas the others failed to develop illness following either challenge. Serum antibody did not correlate with protection. Paradoxically, individuals who became ill possessed higher levels of serum antibody to Norwalk virus than those who were resistant to challenge. The lack of correlation of serum antibody with protection may reflect the fact that the serum antibody detected was ELISA binding antibody and not neutralizing antibody. Correlation between antibody and protection may be seen when local intestinal neutralizing titers can be measured.

Diagnosis

A specific diagnosis of Norwalk virus gastroenteritis in an individual patient cannot be made on the basis of clinical signs and symptoms. Until recently, diagnosis remained essentially a research problem because reagents were not readily available. Diagnosis is made by detection of Norwalk virus in a stool specimen or the demonstration of a fourfold or more antibody increase in a patient's paired sera. Detection of a serologic response is more efficient than virus detection for demonstrating evidence of Norwalk virus infection. Early assays for these viruses included IEM (for the entire group), and RIA and/or ELISA for the Norwalk, Hawaii and Snow Mountain viruses, as well as immune adherence hemagglutination assay (IAHA) for the Norwalk virus. The cloning of Norwalk virus provided a major impetus to the study of the natural history of Norwalk virus infection because of the potentially unlimited source of antigen in the form of recombinant baculovirus-expressed virus-like particles (**Fig. 1b**). This capsid antigen shares antigenic specificity with native Norwalk virus by IEM and ELISA. The availability of animal antisera to recombinant Norwalk VLPs has also enabled the development of an ELISA for virus antigen detection. The antigen ELISA is highly sensitive but also highly specific. Similar antigen ELISAs using VLPs from Grimsby, Hawaii, Snow Mountain, Mexico and Sapporo viruses are being developed, as well as other assays that can detect more broadly cross-reactive antigenic epitopes. Until these assays become widely available, detection of the viral genome by RT-PCR is a favored method for diagnosis. Because diagnostic reagents previously were not available, it was suggested from analysis of numerous Norwalk virus outbreaks that a provisional diagnosis of Norwalk virus infection can be made if the following criteria are met: (1) bacterial or parasitic agents are not detected; (2) the incubation period is 24–48 hours; (3) vomiting occurs in at least 50% of ill individuals; and (4) the mean or median duration of illness is 12–60 hours.

Treatment

There is no specific antiviral therapy for Norwalk virus gastroenteritis: treatment therefore consists of fluid and electrolyte replacement therapy. Oral administration of isotonic fluids is usually sufficient for replacement of fluid and electrolyte loss. However, parenteral administration of fluids may be required if the vomiting or diarrhea is too severe to be managed by oral fluid replacement. Oral administration of bismuth subsalicylate can significantly reduce the severity and duration of abdominal cramps in volunteers challenged with Norwalk virus; the median duration of gastrointestinal symptoms was reduced from 20 hours to 14 hours, but this treatment did not affect significantly the number, weight and water content of stools and the level of virus excretion.

Prevention and Control

Because of the highly infectious nature of Norwalk virus infection, careful handwashing and effective disposal of contaminated material should help to reduce transmission. Careful attention to hygienic precautions in the preparation of food and monitoring the purity of drinking water or swimming facilities should also be practiced. Although the mechanisms of immunity to Norwalk virus remain unclear, the increasing recognition of the importance of these infections suggests that a vaccine might be useful if it is shown to be effective. Recombinant Norwalk virus-like particles represent one vaccine candidate. These VLPs are highly immunogenic when administered orally or intranasally to mice without adjuvant and they have also been shown to be safe and immunogenic when administered orally to volunteers. It is likely that evaluation of the immune response to these particles will help us understand immunity to these enteric infections.

Future Perspectives

Following the discovery of Norwalk virus and other human caliciviruses, a major impediment to their characterization was the inability to propagate them in tissue culture. Thus, all studies relied on the availability of particle-positive stools as the source of antigen. In spite of this limitation, important advances were made in the elucidation of the epidemiology, natural history, immunology and characterization of this group, with special emphasis on the prototype Norwalk strain. The cloning, characterization and expression of human calicivirus genomes represent another major breakthrough in the study of these viruses. Recent epidemiologic studies (allowed by new diagnostic assays) indicate that the epidemiology of infections with these viruses is not well understood, and infections are more common than realized previously. We predict that as human caliciviruses are studied, they will be found to play a significant role(s) in both acute and chronic human illness whose etiologies are currently unknown.

See also: **Diagnostic techniques: Detection of viral antigens, nucleic acids and specific antibodies, Isolation and identification by culture and microscopy; Enteric viruses.**

Further Reading

Ball JM, Hardy ME, Atmar RL *et al* (1998) Oral immunization with recombinant Norwalk virus-like particles induces a systemic and mucosal immune response in mice. *J. Virol.* 72: 1345.

Fankhauser RL, Noel JS, Monroe SS *et al.* (1998) Molecular epidemiology of 'Norwalk-like viruses' in outbreaks of gastroenteritis in the United States. *J. Infect. Dis.* 178: 1571.

Graham DY, Jiang X, Tanaka T *et al* (1994) Norwalk virus infection of volunteers: new insights based on improved assays. *J. Infect. Dis.* 170: 34.

Green KY, Lew JF, Jiang X, *et al.* (1993) Comparison of the reactivities of baculovirus-expressed recombinant Norwalk virus capsid antigen with those of the native Norwalk virus antigen in serologic assays and some epidemiologic observations. *J. Clin. Microbiol.* 31: 2185–2191.

Green KY, Ando T, Balayan MS, *et al.* (1999) Virus taxonomy: 7th report of the international committee on taxonomy of viruses. In van Regenmortel M, Fauquet C, Bishop D, Carsten D, Estes MK, Lemon S, Maniloff J, Mayo M, McGeoch D, Pringle CR, Wickener R (ed.). Academic Press, Orlando, Fla. (In Press).

Jiang X, Wang M, Wang K *et al* (1993) Sequence and genomic organization of Norwalk virus. *Virology* 195: 51.

Kapikian AZ, Estes MK and Chanock RM (1996) Norwalk group of viruses in virology. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology,* 3rd edn, p. 783. Philadelphia: Lippincott-Raven.

Liu B, Clarke IN, Caul EO and Lambden PR (1997) The genomic 5′ terminus of Manchester calicivirus. *Virus Genes* 15: 28.

Vinjé J, Altena SA, Koopmans MPG (1997) The incidence and genetic variability of small round structured viruses in outbreaks of gastroenteritis in The Netherlands. *J. Infect. Dis.* 176: 1374–1378.

Nucleopolyhedrosis Viruses

see **Baculoviruses**

O

Omsk Hemorrhagic Fever Virus *see* **Encephalitis Viruses**

O'nyong-nyong Virus *see* **Chikungunga, O'nyong-nyong and Mayaro Viruses**

ORBIVIRUSES AND COLTIVIRUSES (*REOVIRIDAE*)

Contents

General Features

PPC Mertens, Pirbright Laboratory, AFRC Institute for Animal Health, Pirbright, Woking, Surrey, UK

History

Orbiviruses are responsible for a number of economically important diseases of domesticated and wild animals, including African horse sickness virus (AHSV), epizootic hemorrhagic disease virus (EHDV) and bluetongue virus (BTV). Some orbivirus species (**Table 1**) (including Great Island virus, Changuinola virus and under special circumstances AHSV) include virus strains that can infect humans. However, the incidence of such infections is unusual or rare and their study is limited. Colorado tick fever virus (CTFV), which causes a rarely fatal disease of humans, was originally classified as an orbivirus (see Classification) and will also be considered here for historical reasons.

African horse sickness (AHS) was recognized as early as 1780, during the early days of European colonization and the importation of horses into southern Africa, which resulted in epizootics and high levels of mortality in the infected animals. The causative agent was shown to be filterable in the early twentieth century and has been studied in South Africa since 1887, with antigenic variation between strains recognized as early as 1908. The virus is considered enzootic only in sub-Saharan Africa but has also caused major epizootics in the Middle East, the Indian subcontinent, North Africa and the Iberian Peninsula.

Epizootic hemorrhagic disease (EHD) has occurred as periodic outbreaks in deer in the southeastern USA since 1890. The New Jersey and South Dakota strains were isolated in 1955 and 1956, respectively, and a second serotype was isolated in the Canadian province of Alberta in 1962. Types 3 and 4 were isolated in Nigeria in 1974. The virus has also been isolated from a range of other animals including cattle, which may represent a 'reservoir' host. Ibaraki virus causes an acute febrile disease of cattle, first recorded in Japan in 1959 and, although it is also classified within serotype 2, it is regarded as an atypical EHDV (see under Genetics). There are also at least six serotypes of EHDV in Australia (1, 2, 5, 6, 7 and 18), which are known to infect cattle, buffalo and deer without causing clinical disease.

Bluetongue is believed to have been originally described as a disease of sheep and cattle in South Africa in the late eighteenth century. However, it was not until the first decade of the twentieth century that the disease was reported in the scientific literature as 'malarial catarrhal fever'. The name 'bluetongue' was suggested in 1905 by Spreull, who showed that the agent was filterable and could be transmitted to goats and cattle, causing an unapparent infection.

Until 1943 bluetongue was regarded as a disease of ruminants that was exclusive to Africa. However, at this time an outbreak occurred in Cyprus, killing approximately 2500 sheep and causing 70% mortality in infected animals. It was subsequently suggested that less virulent strains had also caused earlier but unrecognized outbreaks in the island and subsequent outbreaks occurred in 1946, 1951, 1965 and 1977. Bluetongue was recorded in the 1940s in Palestine and Turkey and by 1950 it was present in Israel. In 1948 an apparently new disease known as 'sore muzzle' was recognized in Texas and in 1952 BTV serotype 10 was isolated from sheep with this disease in California. BTV serotypes 11, 17, 13 and 2 were subsequently isolated in New Mexico in 1955, Wyoming in 1962, Idaho and Florida in 1967 and Florida in 1983, respectively. Bluetongue serotype 10, which caused a single epizootic in Spain and Portugal in 1956, was reported in West Pakistan in 1958 and 1960 and in India in 1961. The disease has continued to occur at irregular intervals in the Indian subcontinent and therefore can be regarded as enzootic in this area. Before 1977 Australia was considered to be free of bluetongue virus. However, in 1978 a virus, which had been collected 2 years earlier in the Northern Territory, was reported to be indistinguishable from BTV. Eight virus serotypes have now been isolated in Australia.

Orbiviruses have the potential to cause very high levels of mortality on introduction into areas that are usually free of the disease and which therefore contain immunologically naive populations of susceptible host species. In endemic areas, they also cause a significant reduction in the productivity of domesticated animals. Infectious BTV has been detected in bull semen. Some early experiments (although apparently not reproducible and therefore of doubtful significance) indicated the possibility of long-term persistence of virus, coupled with immunotolerance, in cattle naturally infected *in utero*. There are therefore restrictions on import of both animals and germline materials (semen and ova) from infected areas, although the exact regulations, involving quarantine periods or even a complete import ban, differ from country to country.

The dangers posed by AHSV include high mortality in horses as well as economic losses associated with uncontested equestrian events and unfilled sporting contracts. Until it was controlled, the outbreak in Spain and Portugal (1987–1991) represented a very real threat to the staging of the equestrian events at the 1992 Barcelona Olympics.

Other orbivirus serogroups include viruses which affect a range of different animal species (**Table 1**).

Colorado tick fever (CTF) was first recognized in humans as a separate clinical entity in 1930, although cases of disease with a similar description had been recorded as early as 1850. The virus was isolated in 1948 from Rocky Mountain wood ticks (*Dermacentor andersoni*), confirming its status as a vector and indicating both long-term persistent infection of ticks and trans-stadial transmission. The infection of a number of rodent species was confirmed in 1960, and detection of prolonged viremia in hibernating *Citellus lateralis* (golden-mantled ground squirrel) suggested an alternative overwintering mechanism for the virus. Humans are considered as a 'dead-end' host, while rodents and other wild species, as the 'natural hosts', represent a reservoir for the virus in the field. Although CTF was originally considered as a relatively benign infection in humans, this view was modified in 1955 when a small number of more severe cases were recorded in children, which included central nervous system involvement and at least one death. An effective vaccine against CTF was prepared in the early 1960s but because of the usually benign nature of the infection it was not used.

Eyach virus (EYAV) was isolated in Europe in 1976 and, although serologically related, is distinct from the original American isolates of CTFV. Other less closely related coltiviruses have been isolated from humans and mosquitoes in China and Indonesia (including several isolates of Banna virus in 1987) (see Classification).

Classification

Orbivirus and *Coltivirus* are two of the nine genera (*Aquareovirus, Coltivirus, Cypovirus, Fijivirus, Orbivirus, Orthoreovirus, Oryzavirus, Phytoreovirus* and *Rotavirus*) within the family *Reoviridae* (**Tables 1** and **2**). The physical, serological and chemical properties of the virus particle, as well as its host range and genome characteristics, are all important factors in virus recognition and classification within these different genera. Mature virions of BTV (the type species of the genus *Orbivirus*) do not contain a lipid envelope. However, they can leave the infected host cell by budding through the cell membrane, transiently acquiring an unstable membrane envelope in the process. The intact virus particle has a double

Table 1 Viruses of the genus *Orbivirus* (total 158) by serogroup (species), serotypes, host species and principal vector

Virus serogroup (species)	*Serotypes*	*Host species*	*Principal vector*
African horse sickness virus species (AHSV)	AHSV 1–9	Equids, dogs, elephants, camels, cattle, sheep, goats, predatory carnivores and, in special circumstances, humans	*Culicoides*
Bluetongue virus species (BTV)	BTV 1–24	Cattle, sheep, goats, camels. elephants, (domestic and wild ruminants), predatory carnivores	*Culicoides*
Changuinola virus species (CGLV)	12 named serotypes	Humans, rodents, sloths	Phlebotomine flies, mosquitoes
Chenuda virus species (CNUV)	7 named serotypes	Seabirds	Ticks: *Ornithodoros*; *Argas*
Chobar Gorge virus species[a] (CGV)	2 named serotypes	Bats	Ticks: *Ornithodoros*
Corriparta virus species (CORV)	3 named serotypes	Humans, rodents	Culicine mosquitoes
Epizootic hemorrhagic disease[f] virus species (EHDV)	EHDV 1–8	Cattle, sheep, deer, camels, llamas, wild ruminants, marsupials	*Culicoides*
Equine encephalosis virus species (EEV)	EEV 1–7	Equids	*Culicoides*
Eubenangee virus species (EUBV)	4 named serotypes	Unknown[b] (isolated from insect vectors)	*Culicoides* and anopheline, culicine mosquitoes
Great Island virus species[c,d] (GIV)	36 named serotypes	Seabirds, rodents, humans	Ticks: *Argas*; *Ornithodoros*; *Ixodes*
Ieri virus species[e] (IERIV)	3 named serotypes	Birds	*Culex* mosquitoes
Lebombo virus species (LEBV)	Single serotype (LEBV-1)	Humans, rodents	Culicine mosquitoes
Orungo virus species (ORUV)	ORUV 1–4	Humans, camels, cattle, goats, sheep, monkeys	Culicine mosquitoes
Palyam virus species (PALV)	11 named serotypes	Cattle, sheep	*Culicoides* and culicine mosquitoes
Umatilla virus species (UMAV)	4 named serotypes	Birds	Culicine mosquitoes
Wad Medani virus species (WMV)	2 named serotypes	Domestic animals	Ticks: *Boophilus*; *Rhipicephalus*; *Hyalomma*; *Argas*
Wallal virus species (WALV)	2 named serotypes	Marsupials	*Culicoides*
Warrego virus species (WARV)	2 named serotypes	Marsupials	*Culicoides* and anopheline, culicine mosquitoes
Wongorr virus species[c] (WGRV)	8 named or numbered serotypes	Cattle, macropods	*Culicoides* and mosquitoes
Tentative species and unassigned viruses within the genus:			
Andasibe virus (ANDV)			Mosquitoes
Ife virus (IFEV)		Rodents, birds, ruminants	Mosquitoes
Itupiranga virus (ITUV)			Mosquitoes
Japanaut virus (JAPV)			Mosquitoes
Kammavanpettai virus (KMPV)		Birds	Unknown
Lake Clarendon virus (LCV)		Birds	Ticks
Matucare virus (MATV)			Ticks
Ndelle virus (NDEV)		Rodents	Unknown
Tembe virus (TMEV)			Mosquitoes

[a] Chobar Gorge and Fomede are related but distinct as analyzed by complement fixation tests. These viruses form the Chobar Gorge virus species (serogroup).
[b] There is serological evidence that members of the Eubenangee virus species may infect marsupials.
[c] The 36 virus isolates, currently grouped within the Great Island virus species, as well as the eight virus isolates in the Wogorr virus species have not been extensively compared in serum neutralization assays. However, preliminary serological studies of the Great Island Virus species indicate that they may represent different serotypes.
[d] The newly recognized Great Island virus species contains virus isolates previously classified in the Kemerovo virus and Great Island virus complexes of the Kemerovo serogroup.
[e] Ieri, Arkonam and Gomoka are closely related viruses and form the Ieri virus species (serogroup).
[f] Isolate '318' may represent another, ninth, serotype of EHDV.

Table 2 Viruses of the genus *Coltivirus*, by serogroup (species), serotypes host species and principal vector

Virus serogroup (species)	*Serotypes or nucleotypes*[a]	*Host species*	*Principal vector*
Coltivirus group A			
Colorado tick fever virus species (CTFV)	2 named serotypes (CTFV and S6-14-03)	Rodents, humans	Ixodidae ticks
Eyach virus species (EYAV)	3 named nucleotypes (Eyach virus, AR 577, AR 578)	Possibly humans	Ixodidae ticks
Coltivirus group B			
JKT-7075 virus species (JKT-7075)	1 named nucleotype (JKT-7075)		(Culex mosquitoes)
JKT-6423 virus species (JKT-6423)	4 named nucleotypes (JKT-6423, JKT-6969, JKT-7043, Banna virus)	Humans, other vertebrates	Culex and anopheles mosquitoes
Tentative species and unassigned viruses within the genus			
Chinese isolates that belong most probably to JKT-6423 species based on serological and electrophoretic profiles analyses: HN59, HN131, HN191, HN295			

[a] Nucleotypes distinguishable by RNA sequence analysis and differences in genome segment migration patterns (electropherotype).

protein capsid, with a M_r of approximately 10.8×10^7 and a diameter of 80 nm. The virion is spherical in appearance but has icosahedral symmetry, with an indistinct surface structure as viewed by negative staining and electron microscopy (**Fig. 1**). Removal of the outermost capsid layer releases 'core particles' which have an M_r of approximately 6.7×10^7 and icosahedral symmetry but also appear spherical. The core itself is composed of an outer core surface layer with a maximum diameter of 73 nm and an internal 'subcore' protein layer which has a maximum diameter of 59 nm (sizes determined for BTV cores by x-ray crystallography). The core surface is composed entirely of the VP7 protein, arranged as a lattice with $T = 13\ l$ symmetry, producing characteristic ring-shaped capsomeres that gave rise to the name for the genus, *Orbivirus* (Latin *orbis* = ring or circle). These rings, which can be seen by electron microscopy, are composed of either five or six separate wedge-shaped units, which represent trimers of VP7(T13) (illustrated for AHSV in **Fig. 1**).

The orbivirus genome is composed of ten linear segments of double-stranded RNA (dsRNA) that are identified as segment 1 to segment 10, in order of increasing electrophoretic mobility. A single copy of each segment is packaged per virus particle (ranging in size (BTV) from 3954 to 822 bp: total size of 19.2 kbp, total M_r of 13.1×10^6). The migration pattern of the genome segments during agarose gel electrophoresis (and therefore their relative sizes) appears to be characteristic of the virus serogroup (species). Replication of orbiviruses is associated with the formation of viral 'tubules' (composed of non-structural protein NS1) and viral inclusion bodies (VIBs), which are the site of virus morphogenesis, in the cytoplasm of infected cells.

Within each genus of the family *Reoviridae* the prime determinant for inclusion of virus isolates within a single virus species is their ability to exchange (reassort) genome segments during co-infection of individual cells, thereby exchanging genetic information and generating viable progeny virus strains. However, data providing direct evidence of segment reassortment between isolates is limited and other parameters can be used to examine the level of similarity that exists between isolates. These data can then be used to predict the compatibility of strains for reassortment and to identify the serogroups (orbiviruses) or nucleotypes (coltiviruses) that are recognized as distinct virus species. The different species parameters used to give this polythetic definition of virus species within the family *Reoviridae* include: serological comparisons (identification of serogroups); comparisons of RNA/protein sequences; crosshybridization analyses; analysis of conserved terminal regions on the RNA; identification as a recognized virus serotype (neutralization types) that is already classified within a known virus species; host range; and analysis of genome segment electrophoretic migration patterns (electropherotype) by agarose gel electrophoresis.

The orbiviruses were initially divided into serogroups (species) on the basis of crossreaction of isolates in complement fixation (CF), agar gel immunodiffusion (AGID) and fluorescent antibody tests. More recently enzyme linked immunosorbent assay (ELISA) and competition ELISA, using either serogroup specific monoclonal antibodies (to protein VP7

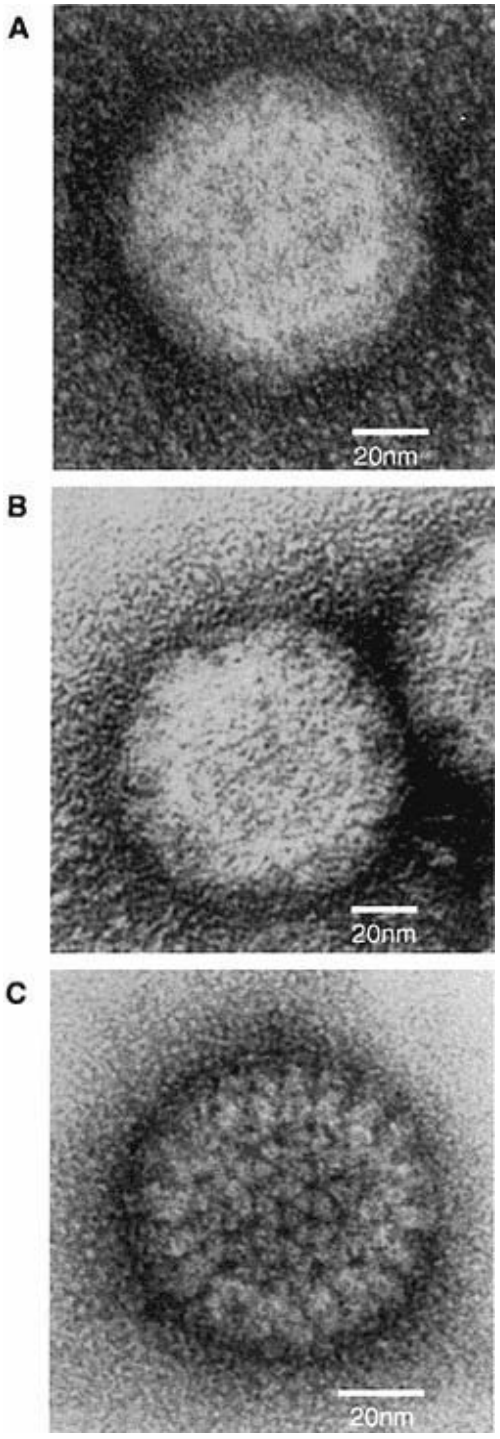


Figure 1 AHSV virus and core particles. Electron micrographs of AHSV serotype 9 particles, purified using CsCl gradient centrifugation and stained with 2% aqueous uranyl acetate. (**A**) Virus particles, showing the relatively featureless surface structure. (**B**) Infectious subviral particles (ISVP), containing cleaved outer capsid protein VP2 and showing some discontinuities in the outer capsid layer. (**C**) Core particles, from which the entire outer capsid has been removed, to reveal the structure of the VP7(T13) core surface layer and showing the ring-shaped capsomeres made up of VP7(T13) trimers, which give rise to the name 'orbivirus'.

(T13)) or polyclonal antisera, have been used to detect BTV, AHSV or EHDV specific antigens, or virus specific antibodies in sera from infected animals. Since these tests are accepted internationally for diagnostic purposes, they are therefore also being used for the classification of unknown viruses within specific orbivirus serogroups (species).

Low level but significant crossreactions can exist between some members of different serogroups, suggesting relatively closer relationships and a possible requirement for supergroups (for example a group to include AHSV, bluetongue virus, Eubenangee virus, equine encephalosis virus (EEV) and EHDV). The viruses previously classified within the Kemerovo serogroup of orbiviruses were originally separated into four antigenic complexes, which showed some serological crossreactions with each other and with the newly recognized Ieri virus species (**Table 1**). However, the viruses within these complexes have recently been reclassified, on the basis of compatibility for reassortment, as three distinct species. These are Great Island virus (containing the Great Island and Kemerovo virus complexes), Chenuda virus and Wad Medani virus (**Table 1**). There is also some evidence that larger differences can exist between serotypes within a single serogroup or species (for example EHDV), suggesting that more than one test system may be required to conclusively classify individual viruses within specific serogroups.

Colorado tick fever virus (CTFV) is the prototype species of the genus *Coltivirus* (sigla from Colorado tick fever virus). CTFV particles also have a double-layered capsid, with an estimated maximum diameter of 60–80 nm and a central core that is about 50 nm in diameter. Electron microscopic studies using negative staining have shown that particles have icosahedral symmetry and a relatively smooth surface structure. The majority of the viral particles are nonenveloped, but a few acquire an envelope during passage through the endoplasmic reticulum. Coltiviruses are found associated with intracytoplasmic granular matrices (which may be similar to VIBs of the orbiviruses), arrays of intracytoplasmic filaments or tubules and fine kinky threads. The coltivirus genome consists of 12 dsRNA segments that are named segment 1 to segment 12 in order of electrophoretic migration. The genome comprises approximately 28 kbp, with segment lengths ranging between 4.14 kbp and 675 bp (total M_r approximately 18×10^6). The difference in genome segment number was the primary reason for the reclassification of the coltiviruses as a separate genus within the family *Reoviridae*. Isolate S6-14-03, from a hare collected in California in 1976, is classified as a second serotype of CTFV (**Table 2**). Eyach virus (EYAV), isolated from *Ixodes* ticks in

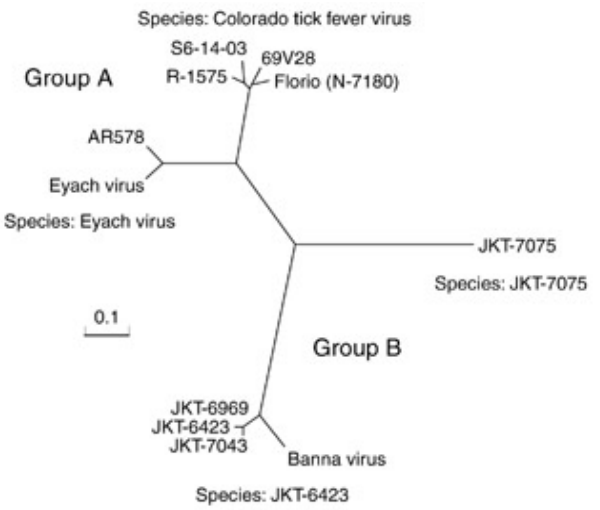


Figure 2 Unrooted neighbor-joining tree created using the program 'Clustal X', depicting groupings for the deduced amino acid sequence of translation products from genome segment 12 of different coltiviruses. The two groups (A and B) that are currently recognized are labelled. Bar marker represents the number of substitutions per site. The relationships illustrated were not corrected for multiple amino acid substitutions. (The RNA sequence data was kindly provided by Houssam Attoui.)

West Germany and France, represents a second distinct species within the group A coltiviruses, distinguished on the basis of serology and RNA sequence analysis (only 50–60% RNA sequence homology was detected in genome segment 12, between these European and American isolates; **Fig. 2**). However, EYAV does show some serological crossreaction with S6-14-03 in crossneutralization assays.

The Indonesian (JKT6423, JKT6969, JKT7041, JKT7075) and Chinese isolates of coltivirus (HN59, HN131, HN191, HN295, Banna virus) show no significant RNA sequence homology in the smaller genome segments with group A viruses but do show some significant amino acid sequence similarity and have been classified as group B coltiviruses (**Fig. 2**). Group B also contains two species (nucleotypes: JKT-7075 and JKT-6423; **Table 2**), which can be separated on the basis of RNA genome segment migration patterns (electropherotype) and RNA sequence analyses (sharing 40–60% RNA sequence homology in the smaller genome segments) (**Fig. 2**).

Geographic and Seasonal Distribution

Bluetongue virus is transmitted by *Culicoides* species (biting midges) and is only enzootic in areas where adults of the vector species are present and active for the majority or all of the year, thus maintaining a continuous series of virus infection cycles in vector and vertebrate host species. The species of *Culicoides* known to act as vectors for BTV are most active in the temperature range 18–29° and are almost inactive below 10° or above 30°. Relatively small rises in temperature within this active range can also increase their efficiency as vectors. Temperatures below 0°, which are maintained for approximately 2 hours or more, will kill adult *Culicoides*; therefore, in areas at relatively greater latitudes the vectors may not be present or active in significant numbers throughout the whole year, and the virus may be absent or only maintained at low levels for part of the year (usually in winter). Under these circumstances the incidence of infection and disease may be dependent on reintroduction in each year that it occurs, or can show major seasonal fluctuations in line with that of vector populations (see Epidemiology). Such a situation is illustrated by the outbreak of AHS in Spain, Portugal and Morocco (1987–1991), where cases were detected only in late summer and autumn. The effects of high temperatures together with relatively low humidity may be particularly significant in Africa, where it is evident that the Sahara represents an effective barrier to the spread of bluetongue and other orbiviruses, from sub-Saharan Africa to the Mediterranean region.

The distribution of bluetongue is effectively restricted to a band around the world between 50° North and 30° South in America and between 40° North and 35° South in the rest of the world (**Fig. 3**). Although the distribution of BTV and some other orbiviruses is relatively widespread, not all of the serotypes of each species are present at each location. In consequence the introduction of a new serotype into areas already enzootic for BTV can result in disease, even in host animals with neutralizing antibodies against those types already present.

Those orbiviruses and coltiviruses that are transmitted by tick vectors show geographic distribution which is also critically dependent on that of vector species. In the case of CTFV this is primarily the tick *Dermacentor andersoni*. Both vector and disease are principally limited to areas of the states and provinces in the western half of America and Canada (**Fig. 3**). The occasional isolation of virus outside this area may reflect a wider distribution or the significance of other vector species. The incidence of CTF shows seasonal variations in response to the peak activity of rodents and the adult tick population in spring and early summer. Overall, the geographic distribution of the coltiviruses is less well studied than that of the orbiviruses. However, the isolation of viruses of group A from America and Europe, and group B

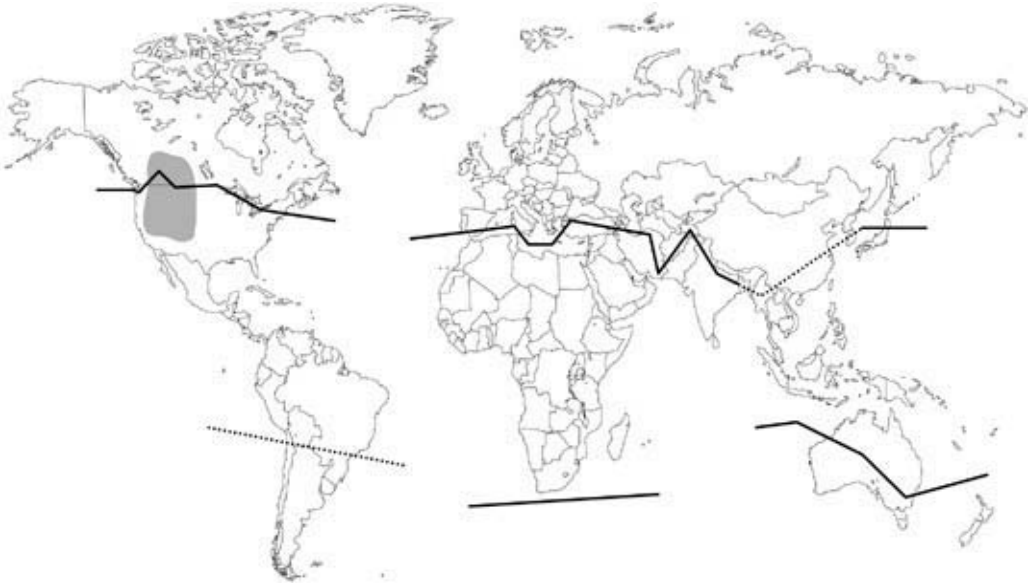


Figure 3 World distribution of the members of the bluetongue virus group and of Colorado tick fever virus (shaded area). The northern and southern limits of BTV distribution are indicated by a line. The dotted line indicates where the limit is uncertain. (From data supplied by R. F. Sellers (personal communication) and from Knudson DL and Monath TP).

viruses from Indonesia and China, suggests that they may be widely distributed.

Host Range and Virus Propagation

The importance of the diseases caused by some orbiviruses and coltiviruses has led to a tendency to consider them primarily in the context of their vertebrate hosts or in mammalian systems. However, the essential role of the vectors in transmission demonstrates the importance of virus infection and growth in insect or arthropod systems. This also suggests that some aspects of virus replication or its properties may be the result of adaptation to the vector rather than the vertebrate host species (see Transmission and Tissue Tropism). The host and vector range of the members of the different *Orbivirus* and *Coltivirus* species is outlined in **Tables 1** and **2**. In some cases the virus has been isolated from an invertebrate species only and no vertebrate host is known.

Information concerning the infection and replication of these viruses in the arthropod vectors is limited. The best studied of the orbiviruses in this respect is BTV and, although there are in excess of 1000 species of *Culicoides* worldwide, only 17 have been connected with BTV and six are known to be capable of transmitting the virus. These include *C. variipennis, C. imicola, C. fulvus, C. actoni, C. wadai* and *C. nubeculosus,* with possible involvement by *C. insignis* and *C. brevitarsis*. On purely epidemiological grounds it seems unlikely that many of the remaining *Culicoides* species will prove to be competent vectors and a large proportion of them may be refractory to infection. Confirmation of this and an understanding of the molecular basis for vector susceptibility and competence will depend on further study.

Members of the BTV and EHDV serogroups are normally restricted to domesticated and wild ruminants, including sheep, goats, cattle, camels, llamas, deer and antelope. African horse sickness is considered to be a disease primarily of horses and other equids. However, eating infected meat has been reported to cause a fatal viral pneumonia in dogs. BTV and AHSV have also been reported to infect large carnivores in Africa. There is serological evidence that AHSV can infect elephants and AHSV isolates have been made from camels, cattle, sheep and goats. Under unusual circumstances, involving inhalation of dust from freeze dried preparations of neurotropic vaccine strains, there is evidence to indicate that AHSV can infect humans, causing encephalitis and retinitis. Any possible risk to human health posed by consumption of AHSV-infected meat has not yet been fully evaluated.

Some orbiviruses can also infect marsupials, humans, rodents, bats, monkeys, sloths and (in the case of the Great Island, Ieri and Umatilla virus species) particularly birds. The coltiviruses include virus species which have been detected in ticks, rodents, humans and other vertebrates. Under experimental conditions many orbiviruses can infect mice or embryonated chicken eggs and these animal hosts

are routinely used for virus isolation from infected blood samples, or from insect vectors. The high levels of mortality in adult mice, if infected via a nasal route with some strains of AHSV, may have relevance to the reported cases of AHSV in humans believed to have become infected with neurotropic vaccine strains by the same route. Such experimental animal systems can provide useful models for studies of virulence factors in these viruses, as has been shown with members of the Great Island and AHSV serogroups (see Pathogenicity).

For most experimental purposes, including analyses of virus serotype in serum neutralization tests, the viruses are grown in tissue culture systems. Details of purification methods have been published for whole orbivirus particles, infectious subviral particles (ISVPs), core particles, attenuated vaccines, and antigens for diagnostic purposes, using BHK21, Vero, mouse myeloma (NSI) or other cell lines. Bluetongue virus has also been shown to grow in an *Aedes albopictus* and a *Culicoides* cell line (C6/36 cells and KC cells respectively).

Genetics

When two compatible orbiviruses or coltiviruses co-infect the same cell, their genome segments can reassort, so that the resultant progeny virus particles contain a mixture of segments derived from the two parental viruses (see Classification). Analysis by high percentage (e.g. 10%) polyacrylamide gel electrophoresis (PAGE) provides a simple method for separation of different dsRNA genome segments and can often be used to distinguish segments of identical size from even closely related viruses. This separation appears to reflect variation in the primary sequence of the RNA. It is therefore ideally suited for analysis of the parental origins of the genome segments present in reassortant progeny viruses. Using this technique the process of reassortment has been shown to occur in tissue culture systems, in some cases with sufficiently high frequency for the parental virus genotypes to be present as a minority in the progeny virus population. The process of reassortment has also been shown to occur in both vertebrate and invertebrate hosts and is therefore considered to be a potentially important factor in the generation of genetic diversity in virus populations in the field.

The coding relationships between the genome segments and viral proteins have been determined by direct *in vitro* translation of individually purified and denatured genome segments of BTV, EHDV and AHSV. Variation in dsRNA migration rates during PAGE, leading to changes in migration order of genome segments from different viruses, has led to some confusion over the coding assignments for BTV. However, the migration patterns of bluetongue virus genome segments, when analysed by agarose gel electrophoresis (AGE), appear to be solely dependent on molecular weight and are remarkably consistent, even for isolates of different virus serotypes or from different geographical origins. Using migration order during AGE to identify different BTV genome segments, it is therefore possible to produce a consistent coding assignment that is applicable to the BTV serogroup as a whole.

Other orbivirus species also produce variable genome profiles when analyzed by PAGE and more consistent profiles when compared by AGE. These include AHSV, Palyam, Corripata and EHDV serogroup members. The consistent nature of the AGE electropherotype has proved to be of real value in determinating the species of unknown or incorrectly classified virus isolates (results which have been confirmed by subsequent serological analyses). Virus isolates from the EHDV serogroup that have been compared by AGE analysis of their genome profile can be divided into two groups. The Ibaraki isolate (Japan) and isolates from Australia are distinct from the American, African and Indian isolates, giving an RNA migration pattern that shows greater similarity to that of the BTV serogroup members (particularly in the faster migration of genome segment 9). These data suggest that in relative terms the EHDV isolates from Australia and the Far East have a deletion in this genome segment.

By making reassortant viruses from two parental strains with distinct characteristics it is possible to relate differences in the properties of the individual progeny virus strains with specific genome segments and, by using genome segment to protein coding assignments, with specific viral proteins. This 'genetic' approach has been applied to studies of virulence factors in Great Island virus (GIV) and AHSV species (see Pathogenicity) and to analyses of outer capsid protein involvement in determination of virus serotype in both BTV and GIV species (see Evolution and Immune Response).

Although reassortment can take place with high frequency, the genome composition of orbivirus strains can apparently remain relatively constant over long periods in the field, even though cocirculating with similar viruses. This stability may result from incompatibility, in terms of their potential for reassortment, between certain viruses. There may also be functional constraints which affect the relative viability of reassortant progeny virus in the field. In this context it has been found that at least some reassortant viruses have increased replication times, or form smaller plaques in tissue culture systems.

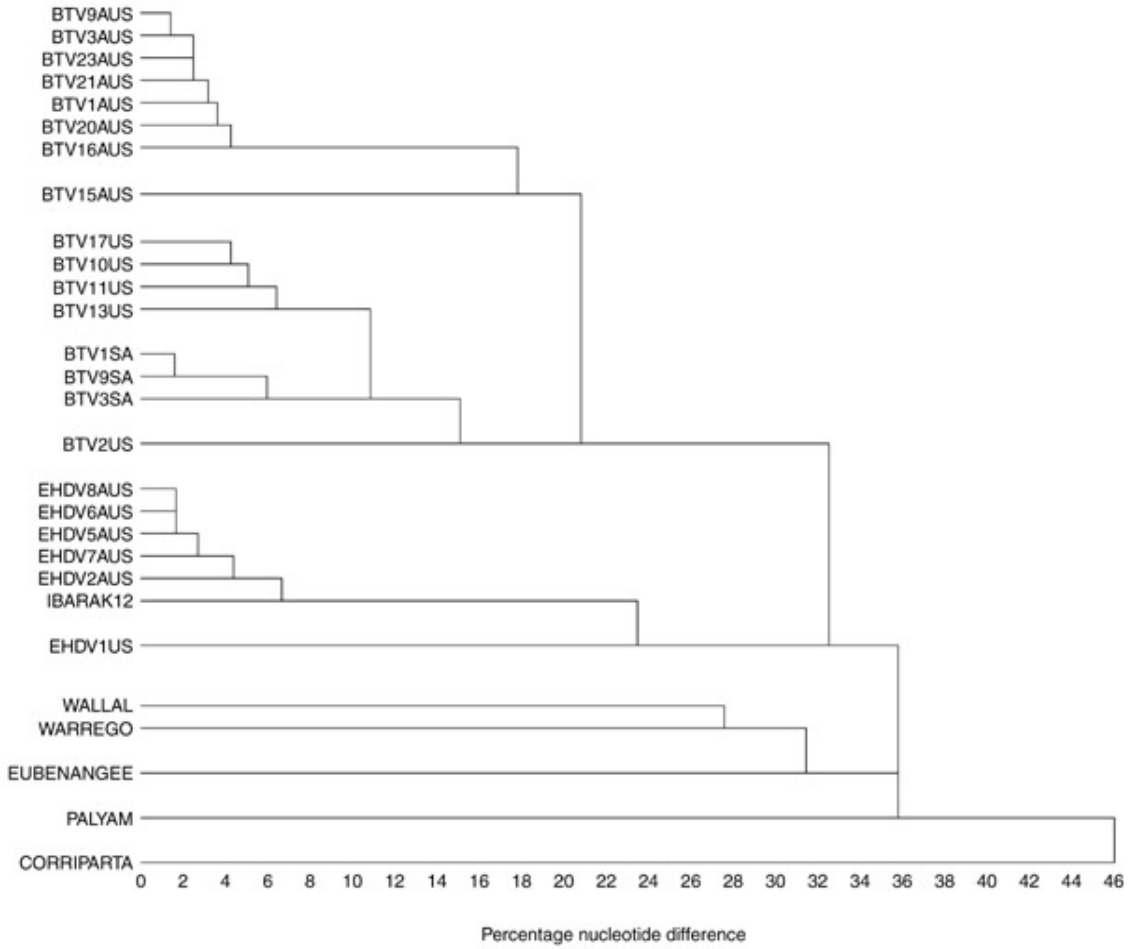


Figure 4 Phylogenetic associations of orbivirus isolates from Australia (AUS), South Africa (SA) and North America (US). Equivalent regions of the VP3 gene for the orbivirus isolates were compared using the DNADIST and KITSCH programmes to generate a single most parsimonious tree. (From RNA sequence data kindly provided by Allan Gould, from Gould A and Pritchard L I (1990).

Evolution

It has been suggested by several authors that the concept of the 'species' as 'the largest aggregate of individual organisms that evolves as a unit having a common gene pool' has direct relevance to these segmented genome viruses. The exchange of genetic information by reassortment of genome segments is in some ways directly analogous to sexual reproduction. Available information indicates that, although reassortment can occur within orbivirus serogroups, for example between members within the Great Island virus, Wallal virus, AHSV or BTV serogroups, there is no evidence for reassortment between members of different serogroups. These data provide the basis for the current classification of orbiviruses into species, primarily on their compatibility for reassortment.

Within the different orbivirus species (for example BTV) there is evidence of RNA and protein sequence variation depending on the geographical origin of the virus isolate, irrespective of virus serotype. This may reflect the accumulation of mutations, coupled with reassortment, resulting in coevolution of the common gene pool within separated locations (for example, Australia, Africa and North America). Gould and Pritchard have suggested that the term 'topotype' should be applied to each distinct group. The initial crosscomparisons, using representative RNA sequence data from genome segment 3 of BTV (**Fig. 4**), indicate that viruses can be grouped within a single 'topotype' if they show <8% sequence difference in this genome segment, while BTV or EHDV strains which contain >11% but <23% variation can be grouped within different topotypes. These data also demonstrate higher levels of sequence variation in genome segment 3 between members of different serogroups, in most cases >30%, although Wallal and Warrego virus species appear to have a relatively

closer relationship, with only approximately 26% variation. Further study is still required to determine both the number of different topotype groups (seven have been recognized) and to confirm that such sequence variation remains consistent across the majority of genome segments within a single virus isolate, despite the possibility of genome segment reassortment with other virus strains occurring in the field. These observations clearly demonstrate that not all isolates of a single *Orbivirus* serotype can be regarded as identical, or even necessarily very similar, and care must be taken to identify precisely each virus strain isolated or used for any research work.

The virus serotype within each *Orbivirus* serogroup is controlled by the specificity of interactions between neutralizing antibodies with the proteins that compose the outer capsid shell and therefore by the genome segments from which these proteins are translated. The two major outer capsid proteins of BTV (proteins VP2 and VP5) are encoded by genome segments 2 and 6, respectively (as separated by AGE). Segment 2 and to a lesser extent segment 6 exhibit a higher degree of RNA sequence variation than the other genome segments, which correlates with virus serotype. This reflects the interaction of VP2 with neutralizing antibodies and its involvement in determination of virus serotype. Reassortment studies have indicated that if the protein VP5 sequence is significantly different between parental strains, it may also have a role in controlling the progeny virus serotype. Although VP5 does not appear to contain neutralizing epitopes, it has been suggested that it may influence virus serotype indirectly by affecting the conformation of protein VP2. In cases where segment 2 and 6 reassortment has been shown to have such an effect, there is at least some evidence of one-way serological crossreactions with the parental virus types, indicating the exposure of 'new' neutralizing epitopes in the progeny particles. Considerable difficulties have been encountered in producing and isolating such VP2/VP5 reassortants, and a longer replication time has been observed in at least one such case, suggesting that a functional linkage may exist between the outer capsid proteins. Production and survival of this type of reassortant between parental viruses containing significantly different forms of genome segments 2 and 6 may therefore be particularly rare in the field. However, it is conceivable that any consequent exposure of new neutralizing epitopes could play a very significant role in the generation and evolution of new virus serotypes.

Serologic Relationships and Variability

Orbivirus genome segments coding for the non-structural proteins (segments 5, 8 and 10 of BTV, encoding proteins NS1(TuP), NS2(ViP), and NS3/NS3a respectively) and the core structural proteins (segments 1, 3, 4, 9 and 7 of BTV, encoding proteins VP1(Pol), VP3(T2), VP4(Cap), VP6/VP6a(Hel) and VP7(T13), respectively), are usually more conserved within serogroups than those coding for the outer capsid proteins (segments 2 and 6 of BTV, encoding proteins VP2 and VP5, respectively); however, in the AHSV species, genome segment 10 (coding for protein NS3) shows a relatively high level of sequence variation (up to 35%) that does not correlate with virus serotype. (Note: TuP, tubule protein; ViP, viral inclusion body matrix protein; Pol, polymerase; T2, major virion structural protein with $T = 2$ icosahedral symmetry; Cap, capping enzyme (guanylyltransferase and transmethylase); hel, Helicase; T13, major virion structural protein with $T = 13$ icosahedral symmetry.) On the basis of RNA sequence analyses and serology, genome segment 10 and protein NS3 of AHSV can be divided into three groups (α, β and γ) (**Fig. 5**). Reassortment studies using a mouse model system have demonstrated an effect of NS3, from different groups, in the control of virulence. However, these studies have also indicated that variations in NS3 can influence the timing of progeny virus particle release from infected insect cells. The vector status of individual insects is at least partially determined by 'barriers' that influence the release and dissemination of virus from the initial site of infection in the gut (see Transmission and Tissue Tropism). The nature of genome segment 10 could therefore also have some effect on the ability of *Culicoides* to transmit AHSV.

The different *Orbivirus* species (serogroups) can

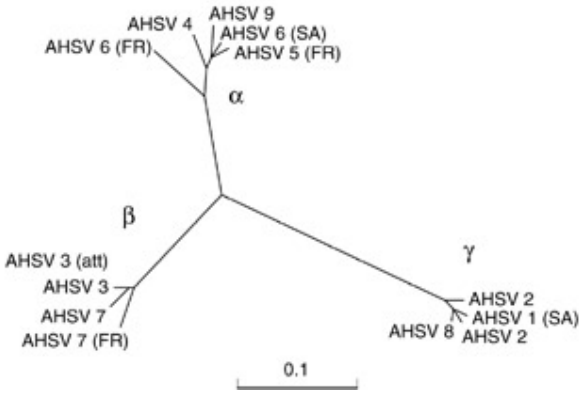


Figure 5 Unrooted neighbor-joining trees depicting groupings for genome segment 10 from isolates of various African horse sickness virus serotypes based on nucleotide differences. Three groups are evident, labeled as α, β and γ. Bar represents 1% nucleotide variation. (The RNA sequence data was kindly provided by Lesley-Ann Martin. Nick Knowles constructed the phylogenetic trees.)

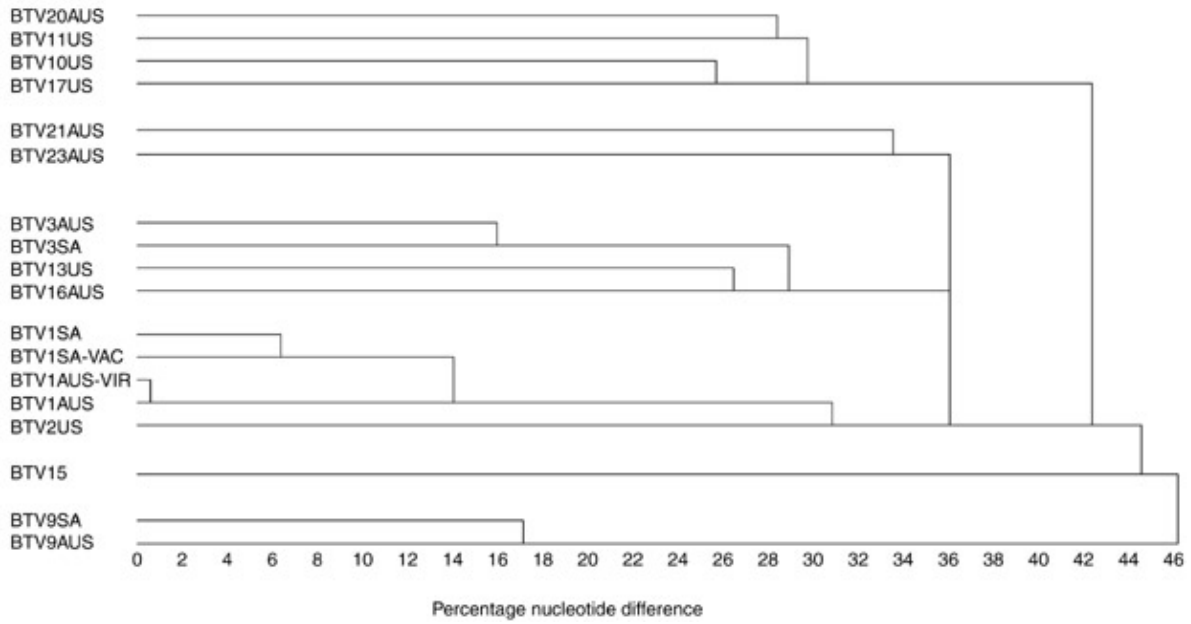


Figure 6 Phylogenetic associations of BTV serotypes from different geographic locations of the world. Equivalent regions of each VP2 gene (from nucleotide 975 to 1190; relative to BTV1AUS VP2) was used to calculate the interrelationships between each BTV serotype. (Data kindly provided by Allan Gould.)

be distinguished by complement fixation, agar gel immunodiffusion, fluorescent antibody staining, ELISA and competition ELISA, using reactions with 'reference' preparations of group specific antibodies. In several of these test systems, VP7(T13), which is the protein on the surface of the virus core of BTV, has been shown to be immunodominant and of major significance. However, RNA sequence data indicate that the gene coding for VP7(T13) is more variable within a serogroup (species) than those encoding some of the other core and nonstructural proteins. This may indicate some partial exposure of VP7(T13) to antibody selective pressure. It has been reported that some VP7 specific monoclonal antibodies can react with whole virus particles, leading to suggestions that this core surface protein is partially exposed in the intact virion. However, more recent studies indicate that this reaction may result from damage to the outer capsid during sample preparation and that the VP7(T13) core surface layer is not normally exposed in the intact virion.

From the practical point of view, sequences from genome segment 5 (coding for nonstructural protein NS1(TuP)) may be most suitable for use as serogroup specific nucleic acid probes or in group specific PCR-based test systems. This is partly because this genome segment is transcribed into the most abundant viral mRNA in infected cells but also because it is reported to be highly conserved within an orbivirus species (serogroup), while displaying >40% sequence variation between species.

The most variable but serotype-specific of the BTV genome segments and proteins are segment 2 and VP2 (the major neutralization antigen), respectively. From the limited data that are currently available for BTV (for example, between bases 975 and 1190 of segment 2; **Fig. 6**), viruses within the same serotype, regardless of geographic origin or 'topotype', appear to contain <19% RNA sequence variation in genome segment 2. In contrast, viruses that are classified as different serotypes appear to contain >26% RNA sequence variation in the same region. However, additional studies using crosshybridization of nucleic acid probes generated from genome segment 2 have demonstrated that at high washing stringencies, although such probes can be used to detect some isolates of the same serotype, they do not detect them all, while at lower stringencies they will also bind to RNA from other distinct but closely related serotypes. It may not therefore be possible to produce 'serotype specific' nucleic acid probes from segment 2 that are capable of simultaneously detecting all and only members of each individual serotype.

The variation in segment 2 that is observed within a serotype may reflect mutations, like those between 'topotypes', that have accumulated in geographically separated viruses. There is also a wide range of variation in the percentage difference in segment 2

nucleotide sequence that is observed between viruses from different serotypes. This provides evidence for relatively closer grouping of some serotypes (for example BTV 3, 13 and 16, with <32% RNA sequence difference; **Fig. 6**), as well as and relatively more distant relationships between 'nucleotype' groups, with higher levels of variation (>34%, <46%). The limited data presented in **Fig. 6** suggest that each genome segment 2 from viruses within a single serotype may have a common ancestry. These data also suggest relatively closer ancestral relationships for genome segment 2 from virus serotypes within a single 'nucleotype' and may have important implications for the origins and spread of different virus serotypes found in different parts of the world.

Data from the analysis of reassortant viruses demonstrate that variation in genome segment 6 (coding for VP5, the other major outer coat protein of BTV) can also have a significant influence on virus serotype. In viruses containing relatively similar VP2 proteins, the influence exerted by VP5 on serotype may further complicate attempts to develop serotype specific nucleic acid probes based on the RNA sequence of genome segment 2 alone.

Epidemiology

Competent individuals of vector species are thought to become infected with orbiviruses for life. However, adults of many vectors are relatively short lived and there is no evidence for transovarial transmission in the vector, or for high levels of persistent viremia in the vertebrate host. BTV and the other orbiviruses, which are transmitted by short-lived *Culicoides* species, are therefore thought to be maintained in the field primarily by continuous cycles of infection through the host and vector. In consequence, their distribution is dependent on the abundance of both susceptible hosts and the competent adult vectors. The vertebrate hosts have a wide distribution and relatively stable population numbers throughout the year. The distribution of the vectors as well as their level of activity and longevity is therefore a critical factor for both the spread and maintenance of these viruses in the field (see Seasonal and Geographic Distribution).

The most significant factors affecting vector activity and distribution are climate and the availability of suitable breeding sites. In areas where cold weather conditions (particularly frosts) occur during part of the year, adult individuals of many vector species may be absent or inactive, in terms of virus transmission (see Seasonal and Geographic Distribution), for periods longer than the 'normal' viremia in the infected vertebrate host (up to 100 days in cattle and 30 days in sheep; see Transmission and Tissue Tropism). Under these conditions the virus cannot reinfect emerging flies and is unable to persist in the field; outbreaks of disease will therefore be dependent on reintroduction during each year that it occurs. In consequence there are geographically isolated areas of the world, for example the UK, where susceptible and serologically naive host populations coexist with seasonally variable but competent populations of vector species. Introduction of the virus into such an area could result in a devastating, even if short-lived, epizootic.

Viruses, including orbiviruses, can be introduced into new areas by importation of infected animals (for example import of an AHSV infected zebra into Spain in 1987). However, there is evidence to indicate that introduction of infected individuals of vector species can also result in outbreaks of disease. *Culicoides* species and other flying insects can be moved large distances, even across oceans, by wind currents and, if infected, can result in wind-borne spread of these viruses. Transmission by this route is believed to have occurred during movement of BTV serotype 4 from Turkey/Eastern Mediterranean region/Syria to Cyprus in August 1977, from Cyprus to Turkey in October 1977 and movement of AHSV from Turkey to Cyprus in 1960.

Ticks have a longer lifespan (>1 year) than mosquitoes or *Culicoides* species (<2 months); therefore, although individuals of all vector species are believed to become infected for life, only the tick vectors can provide a reliable overwintering mechanism for virus. Seasonal distribution of CTFV and the tick-borne orbiviruses is therefore less dependent on continuous reinfection of the emerging vector population, but is dependent on seasonal activity of individuals of the vector species.

The importance of bluetongue as a disease is most clearly illustrated on those occasions when the virus has been introduced into areas from which it is normally absent, containing both competent vector species and serologically naive but susceptible populations of mammalian hosts. Arguably the most extreme example of this is the major epizootic of bluetongue caused by a serotype 10 virus in the Iberian peninsula in 1956. Within the first 4 months the disease had killed approximately 180 000 sheep, with a mortality of close to 75%. Policies of quarantine, slaughter and compulsory annual vaccination (subsequently discontinued) led to the eradication of the disease from this area within 4 years and no subsequent cases have been reported. There is evidence that bluetongue virus may also cause significant economic losses in enzootic situations around the world, although these are usually less dramatic,

resulting in part from decreased productivity and reduced milk yield rather than extensive fatalities. AHSV is believed to persist in Africa as the result of a normal infectious cycle through an unknown mammalian host species (probably zebra) and *Culicoides*. In this context the horse can be regarded as simply an indicator host for the presence of the virus. The dangers posed by AHSV are also illustrated by the results of its recent introduction into Spain (1987), causing an outbreak of AHS that continued for 4 years and required extensive human intervention to prevent its long-term persistence in the southern part of the Iberian Peninsula. The virus spread to Portugal and Morocco, persisting in Mediterranean Africa up to 1991, causing the loss of more than 3000 horses with a mortality of greater than 95% in infected animals. Earlier epidemics in 1959–1961, in the Middle East and Indian subcontinent, were reported to have killed as many as 300 000 horses, donkeys and mules. The epizootic of bluetongue in Spain in 1956–1960 and the persistence of AHS in that area both illustrate the importance of import/export regulations that are designed to prevent the spread of new viruses (e.g. AHSV) to countries currently free of the disease, for example Australia and the Americas.

EHDV causes disease and significant levels of mortality in wild white-tailed deer populations in North America but does not appear to severely affect sheep or cattle. Ibaraki virus, which is classified as an EHDV, albeit atypical, is serologically similar to EHDV serotype 2 (Alberta strain) but does not cause disease in sheep. However, Ibaraki virus has produced symptoms similar to clinical bluetongue in Japanese cattle. There are also at least six serotypes of EHDV in Australia known to infect cattle, buffalo and deer without causing clinical disease. Isolations of EHDV have been made in Africa and India and serological surveys have indicated that it is present in the Far East (Japan and Korea) in the eastern Mediterranean region, South America and Indonesia.

Transmission and Tissue Tropism

Multiplication of bluetongue virus takes place in spleen, bone marrow, macrophages and vascular endothelial cells. BTV has been isolated from blood for up to 30 days postinfection in sheep and up to 100 days in cattle. A short-lived viremia has also been observed in newborn lambs naturally infected *in utero*, which may effectively extend the normal period of viremia in sheep. This may also have significance in overwintering of the virus (see Epidemiology).

Individuals of vector species become infected by ingestion of viremic blood from infected vertebrate hosts. *Culicoides* can ingest approximately 0.1 μl of blood, which may contain approximately 100 $TCID_{50}$ (50% tissue culture infectious dose) of virus. BTV is believed to initiate infection via the cells of the midgut wall. Once infection of these cells is established, progeny virus particles are released into the insect hemocele. Secondary targets may then become infected, including the fat body and, most significantly from a transmission point of view, the salivary gland. The release of virus into saliva is thought to be essential for reinfection of the vertebrate host during subsequent blood meals. At ambient temperatures of 25°, productive infection of the salivary glands and the ability to transmit the virus takes approximately 7 days after ingestion of the original viremic blood meal. Females of the *Culicoides* vectors take blood meals from vertebrate hosts prior to egg laying. They can be expected to take a blood meal approximately 3 days after they emerge, laying eggs for the first time 2 days later. Although they can feed again 1 day after egg laying, the earliest they will be able to transmit the virus is approximately 7 days after infection (10 days after emergence), assuming that conditions remain favourable for relatively active behaviour (see Geographic and Seasonal Distribution).

There is considerable variation between different species of *Culicoides*, and even between populations or individuals within the same species, in terms of their susceptibility to infection and ability to transmit BTV or presumably other orbiviruses. Much of this difference appears to reside at the initial stages of infection of the midgut epithelium (the gut barrier), as even some normally 'nonvector' species can support replication and transmit the virus if infected by direct injection into the hemocele. The barriers to transmission can be divided into a mesenteron infection barrier that can prevent even initial infection of the gut cells, a mesenteron escape barrier that prevents release of progeny virus particles into the hemocele and a dissemination barrier that prevents released virus from infecting secondary target tissues such as the salivary glands. There is no evidence for either a salivary gland infection or escape barrier.

The BTV particle can be modified by treatment with proteolytic enzymes (such as trypsin, chymotrypsin or plasmin) resulting in cleavage of one of the proteins (VP2) in the outer capsid and production of infectious subviral particles (ISVPs) (**Fig. 1**). This modification increases particle infectivity by approximately 100 times for *C. variipennis* or *Aedes albopictus* cell lines (KC cells and C6/36 cells) and results in a similar increase in oral infectivity for adults of *C. nubeculosus* and *C. variipennis* (compared to either intact virus particles or cores). It is considered likely that serum proteases from the mammalian host, or insect gut proteases, can modify the virus particles,

leading to infection of the insect vector primarily by ISVPs. Core particles of BTV have a much lower specific infectivity for mammalian cell lines, approximately 10^5 times lower as compared with either intact virus particles or ISVPs. However, core particle infectivity is also much higher (increased by approximately 10^4) for a *C. variipennis* cell line and is comparable to that of intact virus particles. These observations suggest that host cell binding and penetration functions of the virus are not entirely limited to the BTV outer capsid proteins but are also properties of the outer core protein layer (VP7(T13)). This conclusion is supported by core particle/cell binding studies with both insect and mammalian cell lines, and the neutralization of core particle infectivity by antibodies to VP7(T13).

Although detailed information concerning the replication of orbiviruses and coltiviruses in their arthropod vectors is limited, there is little evidence for significant changes in fecundity, lifespan or behavior as the result of orbivirus infection in any of the known vector species. In mammalian cells (BHK21) 100% cytopathic effect (CPE) and cell death have been observed within 24 h of BTV infection. In contrast, although BTV replicates in both *Aedes albopictus* and *Culicoides* cell lines, with continuous release of virus into the media, little or no CPE or cell lysis is observed. It appears likely that orbiviruses infect susceptible cells within the insect vector throughout their normal lifespan, resulting in persistence of virus replication. In the absence of cell lysis as a result of infection, an alternative mechanism of virus release from *Culicoides* cells appears to be essential for dissemination of virus from the gut to the salivary glands, and therefore for transmission between individual mammalian hosts. The orbiviruses express a small nonstructural protein (NS3), encoded by genome segment 10, that can mediate the release of virus particles from the infected cell. Although this property of NS3/NS3a may have relevance to infection in mammalian systems (see Pathogenicity), resulting in rapid spread and superinfection of cells, it may have originally evolved as an essential component of the infection strategy of the orbiviruses for the insect vector species (see Serologic Relations and Variability).

Pathogenicity

Members of the BTV and EHDV serogroups are normally considered to infect domesticated and wild ruminants, while AHSV primarily infects horses and other equids. The severity of the disease varies from fatal to unapparent in both domesticated and wild animals, depending not only on the virus strain but also on the breed or species of host animal. Most cases of BTV infection in sheep are subclinical or relatively mild and complete recovery occurs within a few weeks. Mortality rates of 2–30% have been reported in South Africa, while estimated mortality rates of 5%, with morbidity rates averaging 10%, have been reported for outbreaks in North America. However, much higher mortality rates (as high as 75%) have been reported on introduction of the virus into serologically naive populations of sheep in nonendemic areas (for example, in Portugal and Spain in 1956). Although bluetongue virus is believed to infect many, if not all, African antelope species, these infections tend to be mild or remain inapparent. In contrast, epizootics were described in 1976 and 1984 in American pronghorn antelope in Wyoming, which killed approximately 3200 and 300 animals, respectively. Although African breeds of sheep show greater resistance to BTV, breeds from areas normally free of the disease can be highly susceptible and suffer a high rate of mortality on infection with virulent strains. The breeds of sheep that are most susceptible to BTV are believed to be Dorset horn and Soay, both of which are of British origin. In contrast, cattle and goats infected with BTV will normally show a relatively mild or inapparent infection.

The variation of pathogenicity for different species is particularly evident with AHSV. Although zebra have been suggested as an important host involved in virus maintenance in the field and in importation of the disease to new areas (Spain, 1987), the virus usually only causes a mild or unapparent infection. However, in the indicator host (the horse) the virus can cause mortality of over 95%. African breeds of horses have lower susceptibility to the disease than those from Europe. This may be partly due to protection by maternal antibodies for sufficient periods for younger animals to be challenged naturally and acquire their own immunity. It may also result from selection of more resistant individuals. Mules and donkeys can also be infected by AHSV and there is evidence that the African breeds are relatively resistant, while those in Europe show mortality thought to be in excess of 75%. The presence of maternal antibodies and selection of resistant individuals may also explain the relatively low incidence of clinical BTV in sheep in some enzootic areas. Overt disease is more frequently the result of introduction of a new strain, particularly of a different serotype, from another area.

External factors and stress not normally encountered under laboratory conditions can also significantly affect the final outcome of infection. In arid areas any reduction of an animal's ability to reach water and food supplies, caused for example by BTV

infection in sheep, may result in a significant rise in mortality.

Under experimental conditions a virulent strain of BTV 3 was shown to become nonlethal after a single passage in tissue culture cells. This may result from specific selection and replication of nonlethal components of the virus population in this system. Unfortunately these observations have limited studies of virulence factors associated with specific genome segments by reassortment, as such studies require the use of tissue culture systems. A similar loss of pathogenicity was not observed with AHSV isolates, and studies of virulence using genome segment reassortment and a mouse model system have now been completed. Genome segment 2, encoding the larger outer coat protein VP2, appeared to have a primary role in the control of virulence. However genome segment 5, encoding the smaller outer capsid protein VP5, also appeared to play a role, and segment 10, encoding the other variable protein of AHSV (NS3), was involved in generation of an intermediate, partially virulent phenotype. Other reassortment studies using horses have confirmed the involvement of NS3 in control of virulence. It appears likely that cell infection and tropism (controlled by outer capsid proteins), as well as the rate and timing of cell release (controlled by NS3), may be important factors in the spread of infection within the host.

Neurovirulence of the Great Island virus species has also been analyzed in mice, using reassortant viruses. These studies showed involvement of genome segments 4 and 5, which encode the two major outer capsid proteins. It was also demonstrated that reassortant viruses could have modified or even increased virulence characteristics.

Alteration of virus strains by many repeated passages in mouse brain and/or tissue culture systems are the most important methods for production of the live but attenuated *Orbivirus* vaccines. Some of these vaccine strains have significantly increased infectivity for mice via an oral route and are regarded as neurotropic (see Host Range and Virus Propagation). Live vaccines also have the potential to exchange genome segments with other vaccine or wild-type viruses and thereby generate progeny virus strains which may have novel and even more dangerous characteristics. Killed vaccines have also been developed for CTFV and AHSV.

Clinical Features of Infection

There is great variation in the response of the vertebrate host to orbivirus infection, ranging from a subclinical or mild febrile response to more severe and even fatal infection.

Bluetongue in sheep is characterized by a rise in body temperature, which may last for up to 14 days, accompanied by an increase in respiration rate. Edema and inflammation around the face, mouth and nose are often observed, accompanied by hemorrhages in the mucous membranes of the mouth, which range from petecheal to ecchymotic. In a few cases the tongue becomes swollen, markedly congested and may protrude from the mouth. Although this feature of infection is transitory, the cyanotic appearance of the tongue in such cases gave rise to the name for the disease. The epithelial lesions of the mouth may become chronic and ulcerated, resulting in bacterial infection and necrosis. Coronitis or inflammation and degeneration of skeletal muscles can result in lameness and an unwillingness or inability to stand. Vomiting may result from smooth muscle lesions of the esophagus and pharynx, leading to pneumonia, which is frequently fatal. Other features include hemorrhages at the skin–horn junction (periople) and in some cases torticollis, which is usually terminal. Transmission of BTV from infected ewes to the ovine fetus, resulting in infection, may cause abortion and malformation, particularly with attenuated vaccine strains (see Pathology and Histopathology). Although direct (experimental) infection of the fetus *in utero* in cattle will have similar results, the virus is not thought to cross the bovine placenta under normal circumstances. EHDV and BTV are reported to produce essentially similar symptoms in deer, and Ibaraki virus is reported to produce symptoms, similar to clinical bluetongue, in cattle.

Four forms of AHS are recognized. These include the febrile type, which is relatively mild, resulting in a complete recovery within a few days. The pulmonary type is more severe and is nearly always fatal. It is characterized by rapid temperature rise, rapid respiration rate, breathing difficulties, a dry painful cough and nasal discharge of serum that has leaked from damaged pulmonary blood vessels. This also results in frothing in the lungs, causing suffocation from blockage of the pulmonary alveoli and subsequent death. The third form is the cardiac or edematous type and is characterized by temperature rise and very marked edema of the head and neck, including notably the supraorbital fossae, which may also extend throughout the whole body but not the limbs. The heart is affected, causing an increase in pulse rate. The fourth, mixed type of disease is rarely seen in the living animal because of superimposition of the symptoms of the other types and is usually recognized by postmortem examination.

Colorado tick fever is characterized in humans by

abrupt onset of fever, chills, headache, retro-orbital pain, photophobia, myalgia and generalized malaise. Abdominal pain occurs in about 20% of patients and a rash is relatively uncommon (<10%). Other symptoms include diarrhea or constipation and loss of appetite for tobacco. A diphasic or even triphasic febrile pattern has been observed, usually lasting 5–10 days. Severe forms of the disease involving infection of the central nervous system or hemorrhagic fever, or even both, have been infrequently observed, nearly always in children under 12. At least three such cases have proved to be fatal. Although congenital infection with CTFV does appear to occur, the risks of abortion and congenital defects remain uncertain.

Pathology and Histopathology

In sheep the replication of BTV takes place in hemopoietic and lymphoid tissues, including the spleen, bone marrow, monocytes, macrophages, neutrophils, vascular endothelial cells and draining lymphoid tissues. Lesions in acute infections are caused primarily by vascular thrombosis occurring in the capillaries and small blood vessels, resulting in hemorrhage and edema within the surrounding tissues. In the squamous layers of the skin and mucosa, vacuolation and necrosis of the epithelial cells can result, developing into erosions and ulcerated areas in more chronic cases. Skeletal muscle degeneration is frequently seen as focal areas within the muscle mass, associated with hemorrhages and edema. Lesions have been described in many other organs and appear to relate primarily to breakdown in the microvasculature, resulting in thrombosis, hemorrhage, edema, ischemia and necrosis of surrounding tissues. Affected organs include the heart, lungs, spleen, thymus, kidneys, skeletal muscles and a range of other tissues. In fetuses infected at the 5 and 6 week stage, encephalopathies and malformation occur, particularly with an egg adapted vaccine strain of serotype 10. The primary importance of virus replication in the vascular endothelium and the resultant damage to the microvascular system is also very clear in both EHDV and AHSV infections.

At the intracellular level, BTV replication is characterized by the production of viral inclusion bodies (VIBs) that are the site of core particle replication and assembly. There is evidence from electron microscopy studies of both mammalian and insect cells that the outer capsid proteins are added only at the periphery of the VIBs. In persistently infected insect cells in cell culture, VIBs continue to grow and by 21 days may occupy up to 25% of the cell volume. Although release of virus particles and reinfection of cells appears to be a feature of orbivirus replication, the presence of only a single VIB in these persistently infected cells suggests that VIBs may fuse, or may absorb the 'superinfecting' particles. Reassortment of genome segments between virus strains may therefore occur within such fused VIBs. Progeny virus particles attach to filaments of the cytoskeleton, producing an effect like strings of beads when viewed by electron microscopy. During replication of BTV and other orbiviruses, long tubular arrays of viral protein NS1(TuP) are formed. These 'tubules' have a helical structure and vary in diameter between different *Orbivirus* species but have an (as yet) unknown role in virus replication. However, virus particles are sometimes seen to be attached at regular distances along the length of the tubules within the cytoplasm of the infected cell, suggesting a specific and functional association.

Within the cytoplasm and sometimes also in the nucleus of CTFV infected cells, bundles of filaments characterized by cross-striations are observed. These structures may be analogous orbivirus 'tubules'. CTFV infected cells also contain intracytoplasmic granular matrices that may be analogous to orbivirus VIBs.

Immune Response

The humoral response to orbiviruses includes the development of relatively long lived serotype specific neutralizing antibodies, usually within 2 weeks of infection and always detectable in animals that develop viremia. There is evidence to indicate that these antibodies, which are directed against epitopes on one of the outer capsid proteins, are involved in protection against challenge with the same virus serotype. In the case of BTV the neutralization epitopes are located on the larger outer protein (VP2), although reassortment studies indicate that the smaller outer capsid protein (VP5) also exerts some influence on virus serotype (see Serologic Relationships and Variability). In members of the Great Island virus species there is evidence (also from analyses of reassortant viruses) that genome segment 5 or 6, which encodes the smaller outer coat protein, has a primary role in neutralization and determination of serotype. From monoclonal antibody studies with BTV, there is evidence of neutralizing epitopes that partially crossreact between at least some virus serotypes. It has been observed that sequential infection of sheep with different BTV serotypes results in production of a progressively broad spectrum of neutralizing antibodies against a number of additional BTV serotypes. This production of neutralizing antibodies to virus serotypes not previously encoun-

tered may also reflect the involvement of serotype crossreactive sites in BTV neutralization.

Antibodies against outer capsid components form the basis of neutralization assays to determine virus serotype. However, antibodies are also produced against the other core structural proteins and nonstructural proteins, which are more conserved between serotypes. These antibodies, particularly those against the protein surface of the core VP7(T13), can be used to determine the virus serogroup, which correlates with virus species. The BTV core particle is itself infectious, particularly in vector insect cell systems, and can be neutralized by polyclonal or monoclonal antibodies to VP7(T13) that crossreact between serotypes.

Killed virus vaccines have been developed for AHSV, some other orbiviruses and CTFV. Significant levels of antibodies to nonstructural virus proteins are not produced in response to these vaccines, as their presence is dependent on *de novo* protein synthesis and virus replication. In these circumstances detection of antibodies to NS1(TuP) of BTV has been used to distinguish vaccinated (killed virus) from infected animals. However, in view of the antigenic variability of NS3 of AHSV (see Serologic Relationships and Variability), care must be taken in the development and validation of any assay system designed to detect virus replication.

Protection against challenge with BTV and AHSV can occur in the absence of neutralizing antibodies. BTV induces virus specific cytotoxic T cells in both sheep and mice, which can partially protect animals from BTV challenge (using adoptive transfer techniques in monozygotic sheep). Most BTV specific T cells appear to be serotype crossreactive as assessed by both antigen specific proliferation assay and cytotoxicity assays. However, T cell lines and clones have been isolated that are BTV serotype specific. One such clone with a helper T cell phenotype (CD5+, CD4+, CD8−, T-19−) was shown to react with a site on outer capsid protein VP2 and produced large amounts of interleukin 2 and interferon γ when stimulated with either intact virus particles or with purified VP2 alone. BTV serotype specific antigenic sites for B cells and at least one site for ovine helper T cells are therefore both located within VP2 (the major neutralization antigen of BTV). Ovine cytotoxic T cell lines (CD8+) which are BTV serotype crossreactive have been isolated, although the protein specificity involved has not been determined. Separately, an ovine CD8+ T cell line has been isolated in which the majority of cells appear to be specific for BTV core proteins, but the function of the cells has yet to be determined.

The outer core protein VP7(T13) of AHSV forms crystalline arrays within the cytoplasm of infected cells. These crystals, which are relatively simple to purify, can be used to raise a serotype crossreactive and protective immune response that is not dependent on antibodies to outer capsid proteins or antibodies that can neutralize intact virus particles.

B cells require antigen specific helper T cells in order to produce virus specific antibodies. The isotype of antibodies is influenced by lymphokines produced by these cells. Production of cytotoxic T cells also appears to form a significant part of the host response to orbivirus infection. It is therefore clear from the available data that the T cell response as a whole also plays an important role in protection against BTV and other orbiviruses.

Prevention and Control

Prevention and control measures are applied to those orbiviruses that cause economically important disease in domesticated animals. The most significant of these measures are intended to prevent introduction of viruses not already present into areas that may contain both susceptible hosts and competent vector populations. The results of such introductions were illustrated in the Iberian peninsula in 1956–1960 with BTV and 1987–1991 with AHSV. The measures used to control the import of virus vary from country to country, involving restrictions on movement of animals and germline material (semen and ova) from infected areas. These restrictions, which may include quarantine periods, or even a complete ban on importation, are inevitably dependent on sensitive tests for both virus and antibodies. With the inability to confirm a carrier state for BTV in cattle, there is pressure for some relaxation of the current import/export restrictions, particularly if coupled with adequate testing of individual animals. Within endemic and epizootic areas other control measures are used; these may include restriction of animal movement, vaccination, control of insect vector populations and slaughter. Rapid and sensitive species (serogroup) specific diagnostic tests (ELISA) for both virus antigens and antibodies form an essential part of control and eradication programmes. However, serum neutralization assays are still used identify virus serotype. More rapid serotyping tests are not yet reliable or generally available but would also be a valuable tool in the overall control of these viruses.

Live attenuated vaccines have been developed for many BTV serotypes. A BTV serotype 4 strain was used as a monovalent vaccine in South Africa, as early as 1907, for more than 40 years. More recently, polyvalent attenuated vaccines containing a total of 15 serotypes have been administered in South Africa as three, weekly pentavalent vaccinations. The seg-

mented nature of the BTV genome and the ability of the genome segments to reassort suggest that the use of such polyvalent vaccines could potentially give rise to problems of increased antigenic diversity. Reassortment studies also indicate that reassortant virus strains may have different, or even enhanced, virulence characteristics in comparison to either parental strain. The original use of a serotype 4 BTV strain for vaccination could have some connection with the central antigenic relationship believed to exist between BTV 4 and other serotypes isolated in Africa.

Polyvalent live AHSV vaccines are used in South Africa as two separate preparations containing serotypes 1 to 6, or 7 and 8. Serotype 9 is not included because protection for this type is believed to be conferred by type 6. All of the viruses used were attenuated by serial passage in BHK-21 cells, apart from serotype 3 which was attenuated by passage in mouse brains. A polyvalent AHSV vaccine containing all nine serotypes is also being used in Egypt. A monovalent AHSV serotype 4 vaccine was used in Portugal, Morocco and Spain to help control the recent outbreak of disease caused by this serotype. The vaccine has been used in horses, both in the affected area and in a surrounding 'buffer zone', together with restrictions on animal movement and controls of insect vector populations in the immediate vicinity of animal accommodation.

A vaccine strain of BTV serotype 10 has been licensed in America for use in sheep only. Although vaccination gives protection of the animal, its use has been shown to be linked to cases of abortion and malformation of the ovine fetus. No vaccines are generally available for EHDV, although a vaccine was developed and used for the control of Ibaraki virus in Japan and experimental vaccines have been developed for some EHDV strains (for example strain 318).

In the case of CTF, personal protection against tick bite is the most effective control measure against virus infection and disease. In recreational areas such as camp grounds and parks, frequent inspection for ticks and their removal before attachment, together with the use of repellents, such as permethrin, and protective clothing are all recommended. Control measures against the tick vector have been considered and an inactivated vaccine was tested in volunteers in the early 1960s. However, because of the usually benign nature of CTFV infection and the limited numbers of cases reported each year (less than 200 cases per year in North America between 1976 and 1990), the use of the vaccine as a public health measure was not considered appropriate. Since CTFV can be transmitted by infected blood transfusion, it is recommended that CTF patients do not give blood for at least 6 months.

Future Perspectives

Potentially one of the most important, although uncertain, future developments associated with the orbiviruses, coltiviruses and other viruses with arthropod vectors is the effect that global warming may have on the distribution and overwintering of the vector populations. It is conceivable that the predicted changes in world climate will increase the spread of competent vectors and reduce periods of the year during which the adults are absent or inactive. This may increase the size of the enzootic areas to include areas or even countries that are now normally free from disease but which contain large populations of highly susceptible and serologically naive host animals.

New virus species (serogroups) and serotypes within existing serogroups continue to be isolated and recognized. It seems likely that new host and vector species may be demonstrated for both existing and newly isolated virus strains. Studies are currently underway to analyze the molecular basis of both insect and viral factors that may be involved in vector status of species, populations and individuals of *Culicoides*. New diagnostic tests, for example competition ELISA using polyclonal or monoclonal antibodies, are being developed for additional orbivirus serogroups (other than those already available for BTV, AHSV and EHDV). The sensitivity of polymerase chain reaction-based tests may also have important practical applications in both sensitive detection systems and characterization tests for these viruses, particularly if coupled with a crosshybridization assay, such as dot or northern blots.

The development of tests that can distinguish between vaccinated and infected animals may prove to be significant. These tests, which detect antibodies to serotype crossreactive nonstructural proteins in animals that have been infected but not in those that have received an inactivated or recombinant vaccine, may remove the understandable resistance to some vaccination programs. The currently used attenuated vaccines cause seroconversion that is indistinguishable from that in naturally infected animals. As a result of import/export restrictions, the use of such vaccines can lead to a drop in the animal's value, either for export or as a source for germline material. The use of killed vaccines and suitable tests could avoid these problems.

Molecular biology techniques have been used to express individual orbivirus proteins, both singly and in various combinations. These proteins represent a valuable resource for research, and together with the particles into which they self-assemble, may also form the basis of both safe and effective recombinant

vaccines, or noninfectious diagnostic antigens. However, the cost of the reagent or vaccine, if high relative to the value of the animal, may remain a significant consideration limiting their use.

The analysis of the entire genome RNA sequence for representative strains of BTV and subsequently of AHSV, together with the deduced amino acid sequences, have been major steps forward in orbivirus research (see Molecular Biology, below).

Recently, baculovirus-expressed VP7 proteins of BTV and AHSV have been crystallized for use in x-ray diffraction studies, resulting in the resolution of their organization and atomic structure. The resulting model for VP7, inserted into a structural model derived by cryoelectron microscopy, was used to produce a structural model for the entire VP7(T13) surface layer of the BTV core particle (see Molecular Biology). Recently BTV cores have also been crystallized and used for x-ray diffraction studies, allowing resolution of the atomic structure of both the entire 780 copies of VP7(T13) in the outer layer of the native particle, as well as the 120 copies of VP3(T2) in the underlying subcore layer. Collectively these structural studies have provided information on the organization of the dsRNA genome and the location of the core associated enzymes within the central space of the subcore, at the vertices of the icosahedron. Further crystallographic and electron cryomicroscopy studies have indicated that the mRNA synthesized by the core is extruded via a pore in the subcore shell (VP2(T2)) and released from the particle.

Structural data, together with results from ongoing studies – to identify and characterize enzymatic functions of the minor viral proteins, as well as their interactions, properties and active sites; to analyse protein–protein and protein–RNA recognition sites involved in replication and assembly; to characterize individual T cell and B cell epitopes; to identify virus–cell binding sites in both vertebrate and invertebrate systems; and to identify alternative overwintering mechanisms for the virus – will inevitably lead to a better understanding of virus replication, transmission and interaction with the vector and with the mammalian host's immune system. These data will also help in the design of methods and reagents for virus control and diagnosis.

Further studies of RNA and protein sequence variation between virus isolates will improve our understanding of the evolutionary relationships within the different virus groups and their movement on a global basis, and will help to confirm the status of 'topotypes' or 'nucleotypes' as potential components of a more comprehensive and accurate orbivirus nomenclature system.

The most important future research development affecting viruses from each of the different genera and species within the family *Reoviridae* as a whole will be the development of effective 'reverse genetics' or 'rescue' systems to facilitate the direct manipulation of virus gene sequences. This will allow the roles of different RNAs, proteins and even individual motifs to be analyzed with unprecedented precision and would lead to our better understanding of these complex and economically important viruses.

See also: **Orbiviruses and coltiviruses (*Reoviridae*): Molecular biology; Genetics of animal viruses; Immune response: Cell mediated immune response, General features; Pathogenesis: Animal viruses; Reoviruses (*Reoviridae*): General features, Molecular biology, Plant reoviruses; Replication of viruses; Vaccines and immune response; Vectors: Animal viruses.**

Further Reading

Brown SE and Knudson DL (1995) Coltivirus infections. In: Porterfield JS (ed.) *Exotic Viral Infections,* p. 229. London: Chapman and Hall.

Joklik WK (ed.) (1983) *The Reoviridae,* p. 287. New York: Plenum Press.

Mellor PS, Baylis M, Calisher C, Hamblin C and Mertens PPC (eds) (1998) African horse sickness. *Arch. Virol. Suppl.* 14.

Mertens PPC *et al.* (1999) Family *Reoviridae.* In: Murphy FA, Fauquet CM, Bishop DHL *et al* (eds) *Virus Taxonomy: Classification and Nomenclature of Viruses.* Seventh Report of the International Committee on Taxonomy of Viruses. (in press).

Sellers RF (1990) Orbiviruses. In: Collier LH and Timbury MC (eds) *Topley and Wilson's Principles of Bacteriology, Virology and Immunity.* Vol. 4: *Virology* 8th edn. London: Edward Arnold/Hodder and Stoughton.

Walton TE and Osburn BI (eds) (1992) *Bluetongue, African Horse Sickness and Related Orbiviruses.* Boca Raton: CRC Press.

Zeller HG, Karabatsos N, Calisher CH *et al* (1989) Electron microscopic and antigenic studies of uncharacterized viruses III. Evidence suggesting the placement of viruses in the family *Reoviridae. Arch. Virol.* 109: 253.

Molecular Biology

Polly Roy, Department of Biochemistry, University of Oxford, NERC Institute of Virology and Environmental Microbiology, Oxford, UK, Department of International Health, University of Alabama at Birmingham, Birmingham, Alabama, USA

Peter P. C. Mertens, Institute of Animal Health, Pirbright Laboratory, Woking, Surrey, UK

Properties of the Virion

Members of the genus *Orbivirus* within the family *Reoviridae* are currently classified within 19 distinct serogroups (species). Details of the structure, genetics and molecular properties of orbiviruses have largely been gleaned from studies of one serogroup, the bluetongue virus (BTV), the type species of the genus.

Orbiviruses are nonenveloped with two protein shells and a genome consisting of ten double-stranded RNA (dsRNA) segments. In the electron microscope when viewed by negative staining, orbivirus particles (70–85 nm diameter; sedimentation coefficient 570S) have a poorly defined surface structure exhibiting a 'fuzzy' appearance. Beneath this ill-defined outer virion structure, is a 70 nm diameter icosahedral inner shell, termed the 'core' (470S) which has a well-defined capsomeric structure. In contrast to conventional microscopy, cryo-electron microscopy of BTV particles reveals that the outer capsid also has an icosahedral morphology with a diameter of 86 nm diameter. The outer capsid consists of two proteins of distinctive shape, one globular and almost spherical, the other sail-shaped. The sail-shaped spikes project 4 nm beyond the globular protein. The capsomeric surface of the core, underneath the outershell, exhibits icosahedral symmetry with a triangulation number (T) of 13 in a left-hand configuration. The distinctive feature of the surface structure of the core is the presence of 132 aqueous channels at all 5- and 6-coordinated positions and 260 prominent knob-like structures at all the local and strict threefold axes (**Fig. 1**). These knobs which are about 40 Å (4 nm) in length and 40 Å (4 nm) in diameter at the surface of the core, are arranged as six-membered rings, with five-membered rings at the vertices or the icosahedron. These rings give rise to the genus name (*orbis*: Latin: ring or circle). The aqueous channels are about 80 Å (8 nm) deep and 80 Å (8 nm) wide at the core surface. The knobbly morphological units are connected at a lower radius at all the local and strict twofold axes forming saddles between the knobs. The protruding knobs give a bristly appearance to cores by cryomicroscopy. The knobbly capsomers are assembled on an inner layer (210–290 Å, 21–29 nm). The exterior surface of the innermost layer is almost spherical but it has an angular appearance due to the variations in thickness of the protein shell. The viral nucleocapsid and the segmented dsRNA genome are located at a radius of <210 Å (21 nm). More defined description has been discussed under the section of x-ray crystallography (see later).

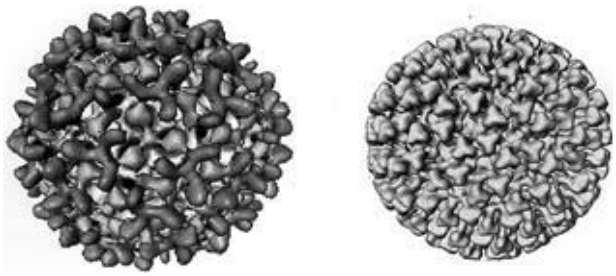


Figure 1 Surface representation of the three-dimensional structure of the BTV virion (left) and cores (right) as deduced by cryoelectron microscopy. (Reproduced from Hewat *et al* (1992) *J. Struct. Biol.* 109: 61; Grimes *et al* (1998) *Structure* 5: 885.)

The Virus Genome

The BTV virus genome is composed of 10 dsRNA segments in equimolar amounts, representing a single copy of each segment per particle. The RNA, when extracted under acidic conditions (pH 4.0) with sodium dodecyl sulfate (SDS) and phenol, occasionally appears as an unfragmented continuous structure. Under the electron microscope this structure has a rosette configuration with 10 varying length loops that emanate from a central area resembling a doughnut. The relative size of each of the 10 loops is approximately equivalent to the sizes of the individual gene segments. The RNA segments are numbered 1 to 10 in order of migration in SDS-polyacrylamide gel electrophoresis (PAGE) and are also referred to as large, medium and small segments (i.e. L1–L3, M4–M6 and S7–S10). Their relative order of mobility may vary according to the electrophoretic conditions used (agarose or acrylamide gel electrophoresis, etc). The dsRNA profiles of members representing each orbivirus serogroup are generally distinctive by PAGE and also differ from those of other members of the *Reoviridae* family.

The complete sequences of all 10 dsRNA segments of BTV serotype 10 (BTV-10) have been determined. In addition, a number of RNA segments of other BTV serotypes and those of other BTV serotypes and those of other orbiviruses (e.g. epizootic hemorrhagic disease virus (EHDV) and African horse sickness virus (AHSV)) have been sequenced.

Table 1 Coding assignments (proteins) of the BTV-10 viral RNA segments

Genome segment[a]	*No. of bp*	*Protein*	*No. of aa*	*Protein size*	*Location*
L1	3954	VP1	1302	149588	Core
L2	2926	VP2	956	111112	Outer shell
L3	2772	VP3	901	103344	Core
M4	2011	VP4	654	76433	Core
M5	1639	VP5	526	59163	Outer shell
M6	1770	NS1	552	64445	Tubules
S7	1156	VP7	349	38548	Core
S8	1123	NS2	357	40999	Nonstructural
S9	1046	VP6	328	35750	Core
S10	822	NS3	229	25572	Nonstructural

[a] Genome segment nomenclature based on order of migration during age and on descending order of size. During PAGE the migration of segments 5 and 6 is frequently but not always reversed.

The 10 dsRNA segments of BTV have conserved end sequences. For the mRNA sense strand of each duplex, these involve six nucleotides at the 3′ ends and eight nucleotides at the 5′ ends of BTV-10. For BTV-10, the genome is 19 218 bp in length with molecular weight 13×10^6. The sizes of the BTV-10 RNAs range from 3954 bp (L1, mol. wt. 2.7×10^6) and 822 bp (S10, mol. wt. 5×10^5). The overall base ratio of the genome is 28.1% A, 28.1% U and 21.9% C, 21.9% G. The base ratios of the individual segments are quite similar (i.e. A and U range from 29.3 to 25.4%; G and C from 24.6 to 20.7%). The 5′ noncoding regions range from 8 nucleotides (nt) (M4) to 34 nt in length (M6). The 3′ noncoding sequences are generally longer, i.e. from 31 nt in length (M5) to 116 nt (S10).

Other conserved features can be recognized in most of the segments. For example, nine of the ten segments of BTV-10 have another A following the 5′ conserved sequence (i.e. at position 7), six of the segments have two A residues at positions 7 and 8. At the 3′ ends of the positive-sense strands the conserved sequence is preceded by AC in 7 of the 10 segments, and in the remaining three segments (M4, M5 and M6) it is preceded by CA or CC. Also UU dimers are found proximal to the 3′ ends in seven of the segments (L1, L2, M5, M6, S7, S8 and S9). The 3′ noncoding regions of seven of the segments (L3, M4, M5, S7, S8, S9 and S10) are purine-rich. The 5′ and 3′ terminal sequences of the 10 mRNA strands of BTV and other orbiviruses (EHDV, AHSV, etc.) have inverted complementary sequences capable of forming intramolecular hydrogen bonds (i.e. end-to-end hydrogen bonding) and share some common features (e.g. a looped out sequence proximal to the 3′ termini). A high percentage of the nucleotides (e.g. 379 out of a possible 578 bp of S7 of BTV-10) of each RNA strand can form intramolecular duplex structures (stem–loop configurations) that are not only similar between the corresponding segments of various BTV serotypes but also similar to those of EHDV and AHSV.

Apart from the BTV-10 L1 segment the first AUG codon on the positive-sense mRNA strand of each of the segments initiates a long open reading frame. The L1 segment has an additional AUG codon upstream of the codon which initiates the open reading frame (residues 7–9). There are two methionine codons in the same reading frame in the S10 RNA sequences, one at position 20–22, the other at nucleotides 59–61. Four of the BTV-10 gene products end at the termination codon UGA (L1, M5, S8 and S10), four at UAG (L2, L3, M6 and S7) and the remaining two at UAA codons (M4 and S9). **Table 1** summarizes the coding assignments of the 10 genome segments of BTV-10.

The evolutionary relationships of the 24 BTV serotypes and related orbiviruses have been studied extensively at the genetic level, using a number of experimental approaches, including sequence comparisons. Data demonstrate that apart from RNA segments L2 and M5, all other eight RNA segments are highly conserved. Despite this sequence conservation, variations do exist for each RNA segment representing the various BTV serotypes as well as for various isolates within a serotype. In addition, high frequency of segment reassortment can occur between different BTV serotypes (in cell cultures, vertebrate hosts, or *Culicoides* vectors), generating new genotype combinations.

The Virus Proteins

Each dsRNA genome segment of BTV codes for the synthesis of one major protein. The outer capsid of

the virion contains two major proteins, VP2 and VP5 protein, which constitute approximately 40% of the total protein content of the virus. The core particle contains two major (VP3, VP7) and three minor proteins (VP1, VP4, VP6). The structural proteins are numbered VP1 to VP7 in order of their decreasing sizes on PAGE. Three nonstructural proteins (NS1, NS2, NS3) are also synthesized in infected cells, in addition to the seven structural proteins. The amino acid sequences of each of the 10 proteins of BTV-10 and various proteins of other serotypes have been deduced from cDNA analyses of the viral RNAs. The genes have been expressed in insect cells by recombinant baculoviruses and the proteins analyzed in terms of structure and function. The characteristics of proteins are summarized below.

The two outer capsid proteins

A total of 180 molecules of VP2 are present per BTV virion and form spike-like structures on the outer shell. The VP2 protein is the product of the L2 segment and has a molecular size of 111 kDa. The VP2 protein appears to be hydrophilic and contains many charged residues. It is rich in aromatic residues and conserved cysteines which may indicate a highly ordered, disulfide-bonded structure. The VP2 proteins of at least six different serotypes (BTV-1, -2, -10, -11, -13, -71) have been sequenced. It exhibits the greatest sequence variation of all the structural proteins. The VP2 protein of these six BTV viruses share 32–72% identical amino acids. Sequence alignments reveal distinct variable and conserved regions. For example, the C-terminus and a middle region of the molecule are well conserved with the absolute conservation of an octapeptide (NPYPCLRG) at position 360–367. The overall predicted secondary structures and functional plots of the VP2 proteins exhibit remarkable similarities among the serotypes reflecting limited evolution. Low levels of VP2 sequence homology can also be identified among BTV, EHDV and AHSV, particularly at the N- (50%) and C- (60%) terminal regions (20 amino acids at each end).

The VP2 protein is the principal antigen that determines serotypes. It is also the hemagglutinin protein. It elicits serotype-specific neutralizing antibodies which confer protection against homologous virus infection in vertebrate hosts. However, the neutralizing epitopes of VP2 are conformation dependent. VP2 purified from SDS-PAGE failed to induce neutralizing antibodies. Recombinant baculoviruses that express VP2 proteins induce antibodies that neutralize the infectivity not only of the homologous virus, but also to a lesser extent of some other heterologous BTVs, reflecting their closer relationship (e.g. BTV-4, -10, -11, -17). The immunity elicited in sheep provides protection against virulent virus challenge. VP2-specific monoclonal antibodies neutralize virus infectivity and provide passive protection against homologous virus challenge. The regions of VP2 that are involved in determining serotype specificity, neutralization and protective activities, have not yet been identified.

The VP2 protein is involved in the attachment of virus to cells. When VP2 is removed, the infectivity and the binding of the derived particles are significantly less than for complete virion particles. Intermediate subviral particles (ISVP) of BTV that still contain the chymotrypsin, tryspin or plasmid cleavage products of VP2 are, however, fully infectious. ISVP also have increased infectivity for insect vectors or insect cells. Although cleavage of VP2 does not affect cell attachment, ISVPs lack hemagglutination activity.

The second outer capsid protein, VP5 which appears to have a globular structure, is composed of 526 amino acids with molecular weight 59 kDa. In contrast to VP2, the primary sequences of the VP5 protein are much more conserved (in some cases up to 94%) among the various BTV serotypes and distinct variable regions are not easily discernible. The protein also shares relatively strong sequence homology (59–62%) with the VP5 protein of other orbiviruses, particularly EHDV. The BTV VP5 protein is rich in certain nonpolar amino acids such as alanine and isoleucine and has low content of tryptophan. It is noteworthy that following the methionine at the N-terminus, a single glycine residue is shared by the VP5 proteins of all BTV and EHDV analyzed so far. However, the protein is not myristoylated. .

The BTV VP5 may also contribute in the induction of neutralization-specific immune responses. A mixture of lysates of insect cells infected with two recombinant baculoviruses, one expressing VP2 and the other VP5 induces higher neutralizing antibodies in sheep than the single recombinant virus expressing VP2.

The two major core proteins VP3 and VP7

The BTV VP3 protein (103 kDa) is coded by the L3 segment and is a major structural protein of the inner capsid. It contains group-specific antigenic determinants. The L3 RNA (and VP3 protein) sequences are highly conserved among the various BTV serotypes analyzed so far. For example, there are 126 differences between the nucleotide sequences of BTV-10 and BTV-17 (out of a total of 1772 nucleotides), and only nine amino acid changes (out of 901), i.e. 0.15% of the possible sites where a single nucleotide change would

cause an altered amino acid. High levels of amino acid sequence homology have been demonstrated among the VP3 proteins of BTV, EHDV and AHSV reflecting the structural constraints in VP3. The baculovirus-expressed VP3 recognizes not only antibodies of all BTV serotypes, but also those of EHDV and AHSV. The VP3 protein is generally hydrophobic in character with relatively few hydrophilic regions. It contains few cysteine residues, but is abundant in nonpolar amino acids.

The protein VP7 is encoded by the BTV M7 RNA segment and is the major structural component of BTV cores comprising at least 36% of the total core protein. The sequence data obtained for the VP7 proteins of BTV, AHSV and EHDV indicate that the three viruses are closely related at the genetic level. Some 64% of the BTV-10 and EHDV-1 VP7 amino acids are identical in type and position, 44% of the EHDV and AHSV VP7 sequences and 44% of the BTV-10 and AHSV-4 sequences. The amino acid composition of the BTV VP7 protein differs considerably from that of the other BTV gene products. It is deficient in charged amino acids (19–20%) and rich in hydrophobic amino acids. VP7 has only one lysine which is remarkable since the other gene products are lysine-rich.

The VP7 protein has been synthesized by recombinant baculoviruses. The protein is highly immunogenic and reacts with antibodies raised to BTV, AHSV and EHDV. x-ray diffraction studies of crystallized VP7 obtained from recombinant baculoviruses exhibit two different symmetries, hexagonal and monoclinic. These crystals can diffract up to 2 Å (0.2 nm) resolution. The expressed proteins in crystals are clustered in trimers. The atomic structure of VP7 trimer is discussed later.

When VP3 and VP7 are synthesized simultaneously by a recombinant virus or *in vitro* (by transcription–translation of mRNA), they form icosahedral core-like particles (CLPs) as shown in **Fig. 2**. These resemble authentic BTV cores in size, shape and in the VP7:VP3 stoichiometry. It appears that 780 copies of VP7 forms 260 trimers or knobby capsomers of the core surface and for 260 trimers there are 120 copies of VP3 in the core. However, unlike the rotavirus VP6 (which is equivalent to the BTV VP7), the BTV VP7 does not self-assemble to form a morphological (core-like) structure. VP3 serves as a scaffold for the assembly of the VP7 trimers. VP7 of certain orbiviruses (e.g. AHSV) can self-assemble to form crystals composed of flat sheets of hexagonal rings. These rings are similar to the six-membered rings of VP7 trimers seen in the core surface layer. VP3 on the other hand, can form icosahedral subcore-like structures alone, and can interact with each of the three minor proteins (VP1, VP4 and VP6) indicating that it serves a primary role in formation of the virus capsid. The VP7 layer not only gives increased rigidity and stability to the core particle, it can also play a role in cell entry and infection by the core, in particular, in insect vector/cells. Both outer capsid proteins, VP2 and VP5, interact with each other and as well as with VP7 to form the double-capsid structure. Without the core, the outer capsid cannot be formed. The topography of the four major proteins in the virion that have been obtained from recent data is shown in **Fig. 3**.

The three minor core proteins VP1, VP4 and VP6

The RNA segments L1, M4 and S9 encoding the three minor proteins, are highly conserved among BTV serotypes. The largest RNA segment (L1) codes for the minor core protein, VP1 (150 kDa). This basic protein has a positively charged C-terminus, and the highest predicted net positive charge (+27.5). It exhibits a number of hydrophobic domains, based on its predicted amino acid sequence, size, location and molar ratio in the core, VP1 protein is the virion-associated RNA-directed RNA polymerase. The protein shares some sequence homology with the DNA- and RNA-directed RNA polymerases of eucaryotic, prokaryotic polymerase and those of other viruses. It contains a GDD motif characteristic of RNA polymerases. In addition, cell lysates infected with a recombinant baculovirus expressing VP1 protein catalyze the synthesis of polynucleotide chains in an *in vitro* system. The protein is also capable of binding BTV mRNA.

The second minor (estimated 20–24 copies per virion) core protein VP4 (76 kDa), is rich in charged amino acids and hydrophilic. It has a particularly strong hydrophilic domain at the C-terminus. VP4 exists as a dimer in solution and in baculovirus core-like particles (also likely within the core). A leucine zipper in the center of the molecule is responsible for the dimerization of VP4. The VP4 protein possesses various enzymatic activities. It has a nucleoside triphosphatase activity, the significance of which is not clear. The VP4 protein has a guanylyl transferase activity (i.e. an RNA capping enzyme). It binds GTP and transfers the GMP moiety to *de novo* synthesized uncapped BTV ssRNA to produce capped products. The protein also has methyltransferase activities which catalyze the methylation of capped BTV RNA to form methylated cap1 structures (m^7GpppGpm..) at the 5′ termini. Both methyltransferase type 1 and type 2 activities are associated with VP4, indicating that it is the only protein required for the complete BTV capping reaction.

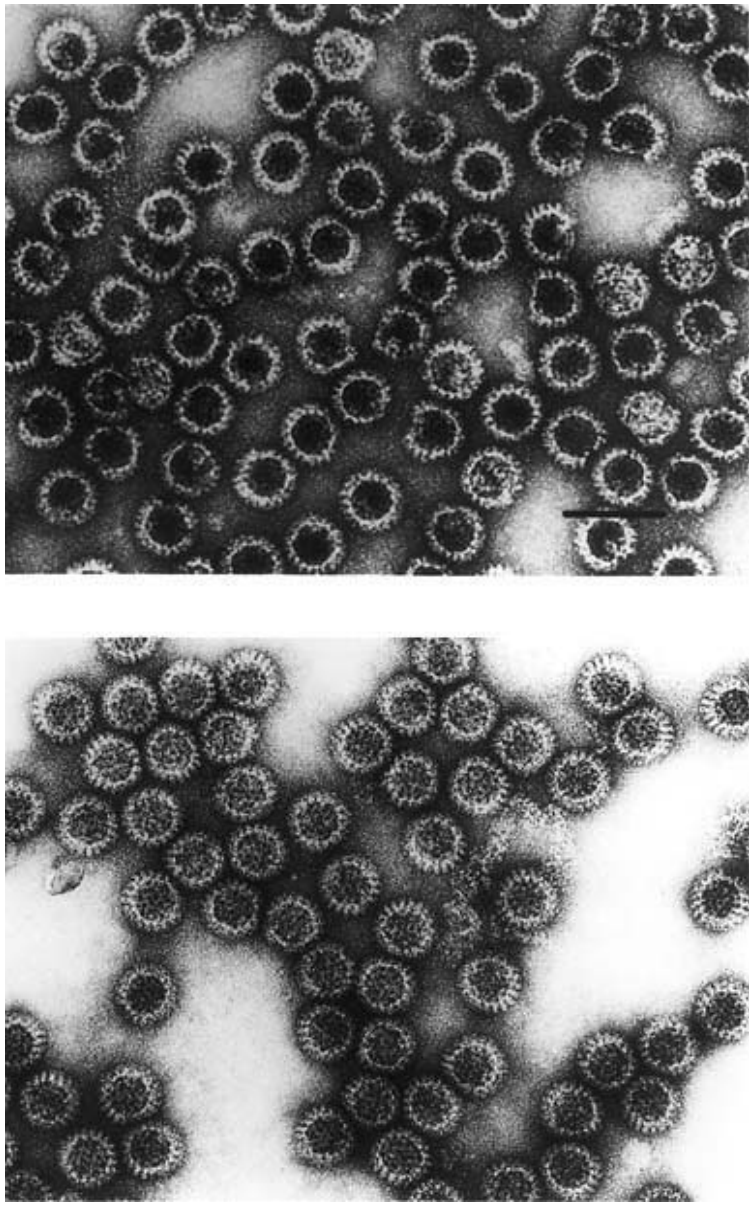


Figure 2 Lower micrograph consists of BTV cores derived from BTV virions. Top micrograph shows cores made by coexpression of VP3 and VP7.

The third minor core protein VP6 (35.8 kDa), is rich in basic amino acids (Arg, Lys and His). It has only one cysteine and a low proportion of aromatic amino acids, or asparagine or glutamine residues. VP6 is rich in glycine residues (40 out of 328 amino acids, 12%) with 12 glycines located between residues 88 and 116. The VP6 protein has been demonstrated to have high binding affinity for both ss- and dsRNA and DNA suggesting its close association with the viral genome in virions. In addition, RNA-dependent ATPase and helicase activities have been identified associated with the purified VP6 protein. The protein also has an ATP binding activity. RNA unwinding of duplexes which occurred with both 3′ and 5′ overhand templates, as well as with blunt-ended dsRNA is thought to be essential during the synthesis of RNA within viral particles, as part of the virus replication cycle.

The three nonstructural proteins, NS1, NS2 and NS3

The nonstructural protein NS1 forms tubules in the cytoplasm of cells infected with orbiviruses. It is

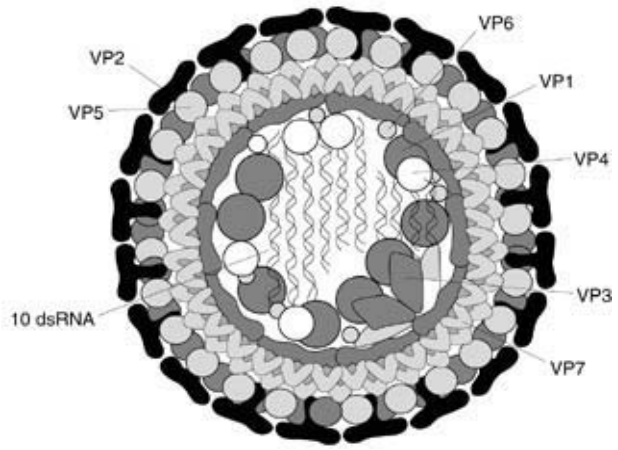


Figure 3 Schematic of BTV showing the topography of the seven structural proteins.

encoded by the M6 RNA segment. The protein is synthesized in large amounts, comprising some 25% of the virus-specified proteins in the infected cells. The protein converts rapidly into high-molecular-weight tubular structures with a sedimentation coefficient 300–500S. These tubules are predicted to be involved in virus assembly, although the exact nature of their role remains unknown. They are characteristic features of orbivirus-infected cells (see 'Replication'). The NS1 protein (64 kDa) is rich in cysteines (16 residues) which suggests a highly ordered, disulfide bond-linked structure. The protein is highly conserved (up to 96%) among the various stereotypes of BTV, but distinct from those of EHDV and AHSV. However, all 16 of the cysteine residues in the NS1 sequence are conserved between the three orbiviruses, suggesting that they are important for maintenance of structure. The NS1 protein has several regions of hydrophobic amino acids distributed throughout the molecule, particularly in the C-terminal half. Mutational studies demonstrated that both N- and C-termini and the cysteines at amino acids (aa) 337 and 340 are essential for tubule formation. Analysis of a panel of monoclonal antibodies has established that an NS1 antigenic site is located near to the carboxy terminus of the protein which is accessible to the surface of the tubules. Expression of the NS1 gene in insect cells by a recombinant baculovirus yields large amounts of NS1 tubules which are morphologically and immunologically similar to the authentic BTV-derived tubules. The average diameter of these tubules appears to be 52.3 nm. They can be up to 1000 nm long. The structure of the helical surface lattice of tubules has been determined using cryoelectron microscopy and computer-image processing to a resolution of 40 Å (4 nm). The NS1 protein molecule is about 5.5 nm in diameter and forms a dimer-like structure, so that the tubules are composed of helically coiled ribbons of NS1 dimers, with 21 or 22 dimers per turn. The surface lattice displays P2 symmetry and forms a one-start helix with a pitch of 9.1 nm. The tubules exist in two slightly different pH-dependent conformational states.

NS2, the second largest nonstructural protein (41 kDa), is encoded by RNA segment S8. It is the only viral-specific phosphoprotein. The protein has the lowest proportion of threonines of all the BTV gene products, but an average content of serine. Both mammalian and recombinant baculovirus-derived NS2 proteins are phosphorylated at serine residues only. NS2 has a high content of cysteines, mostly located near the C end, which suggests a highly ordered structure in that region. Overall, the protein is hydrophilic and rich in charged amino acids. The protein possesses ssRNA-binding ability, a property which is independent of its phosphorylation state. Deletion of up to 130 amino acids (out of 357) from the C-terminus yield a protein that can still bind RNA. It appears that the first 40 amino acids of the N-terminus of the molecule are essential for RNA binding. In particular, the arginines at amino acids 6 and 7 and a lysine at amino acid 4 are required for ssRNA binding. By immunoelectron microscopy, it has been demonstrated that NS2 protein is located within the BTV-induced viral inclusion bodies (VIB). However, it is not associated with virus particles in infected cells. Recombinant baculoviruses that express NS2 form morphological structures similar to VIBs found in BTV-infected cells.

The third nonstructural protein is NS3 (25.6 kDa). It is encoded by the smallest RNA segment, S10, and is highly conserved among BTV serotypes, although it is more variable among different AHSV serotypes. A second protein (NS3A) of 24 kDa is also detected in low abundance in infected cells. These two proteins are related to each other. NS3A is synthesized from the second in-frame translation initiation codon. Both proteins are integral membrane glycoproteins and are involved in virus egress from infected cells. The NS3 sequence contains two putative transmembrane domains at amino acids 118–141 and 162–182 and two potential asparagine-linked glycosylation (aa 63 and 150), one of which is located between the two hydrophobic domains. Both hydrophobic domains span the cell membrane, however, only the site at aa 150 is responsible for N-linked glycosylation of the NS3 protein. It is noteworthy that NS3 proteins of certain AHSV serotypes do not possess such potential glycosylation sites.

Three-dimensional x-ray Crystallographic Structure of Orbivirus Proteins and Particles

VP7

The BTV-10 VP7 crystal structure at 2.5 Å (0.25 nm) resolution revealed a unique molecular architecture of trimeric nature. Each monomer consists of two distinct domains, the 'upper' and 'lower' and are twisted such that the top domain of one monomer rests on the lower domain of a threefold related subunit in a clockwise direction (see **Fig. 4**). The interactions between subunits are extensive and there are contacts between the top domains related by threefold symmetry and also with the symmetry-related bottom domain. There are both hydrophobic interactions and specific hydrogen bond interactions which bind the two domains strongly. Despite the extensive interactions there are cavities at the center of the trimer (along the threefold axis) surrounded by predominantly uncharged residues.

The smaller upper domain of VP7 forms the 'head region' or the VP7 trimer which forms the knobby projections seen by cryoelectron microscopy on the outer surface of the core and contains the central one-third of the polypeptide chain of the molecule (aa residues 121–249). In the trimer, the three upper domain subunits, each of which is folded into an antiparallel β-sandwich, are intimately associated via two extended loops from each subunit, both with each other and with all possible threefold related subunits. The topologies of the two groups of β-sheets are A'BIDG and CHEF, corresponding to a jelly roll structure. One loop (the D–E loop), consisting of aa 198–208, interacts with both trimeric partners at the top of the molecule. Below this loop, aa 133–141 form another loop (the A–B loop), clipping onto the inner-face of the β-sandwich of an adjacent subunit. The x-ray structure of upper domain of AHSV-4 VP7 has also been solved and has shown similar structural arrangement. The characteristic feature of the upper domains of both molecules is the presence of an Arg-Gly-Asp (RGD) motif which may be responsible for binding of the orbivirus core to a cellular integrin receptor. The lower domain of the VP7 trimer is composed entirely of α helixes and long extended loops. This domain contains the N-terminus (1–120) and the C-terminus (250–349) of the molecule (**Fig. 4**). There are nine helices, five are in the N-terminal half and four in the C-terminal half. In the center of the domain is an antiparallel, four-helix bundle composed of amino acid residues 2–17 and 54–73 from the N-terminus and amino acids 276–288 and 304–322 from the C-terminus. The connection between the two domains is exposed, and is easily accessible to proteolysis. The single lysine residue of BTV-10 VP7 is situated in the junction of the two domains. The helices in the bottom domain pack together tightly.

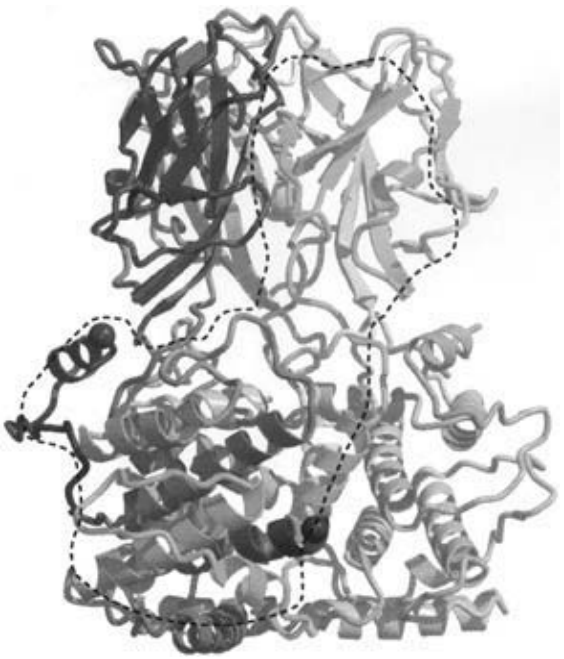


Figure 4 The structure of VP7 trimer rendered from x-ray crystallographic analysis. The threefold symmetry axis of the trimer is disposed vertically so that the flat base at the bottom of the image forms the inner surface of the outer VP7 layer of the core, which adheres to the VP3 subcore. Note the clear division into two domains, the upper domain contains mainly β-strands (shown as arrows), whereas the lower domain is constructed of a number of closely packed helices. (Reproduced from Grimes *et al* (1995) *Nature* 373: 167.) One subunit is outlined with a dashed line to clarify the twist of the subunit trimer with respect to the molecular 3-fold axis. **(For color references see Color Plate 13.)**

Core particle

High resolution (23 Å) cryoelectron microscopy and x-ray crystallographic structures at 3.5 Å resolution have been determined for two different BTV serotypes (BTV 1 SA and BTV-10). From these data an atomic model has been constructed for the structure and organization of the major protein components within the core particle.

The organization of 260 VP7 trimers follows precisely $T = 13$ icosahedral lattice, in accordance with the theory of quasi-equivalence. By fitting the x-ray structure of an individual VP7 trimer onto the cryoelectron microscopy BTV core structure it is clear that the threefold axis of the trimer (85 Å (8.5 nm) long) is perpendicular to the core surface and the broader base of the trimer (65 Å (6.5 nm)) contacts

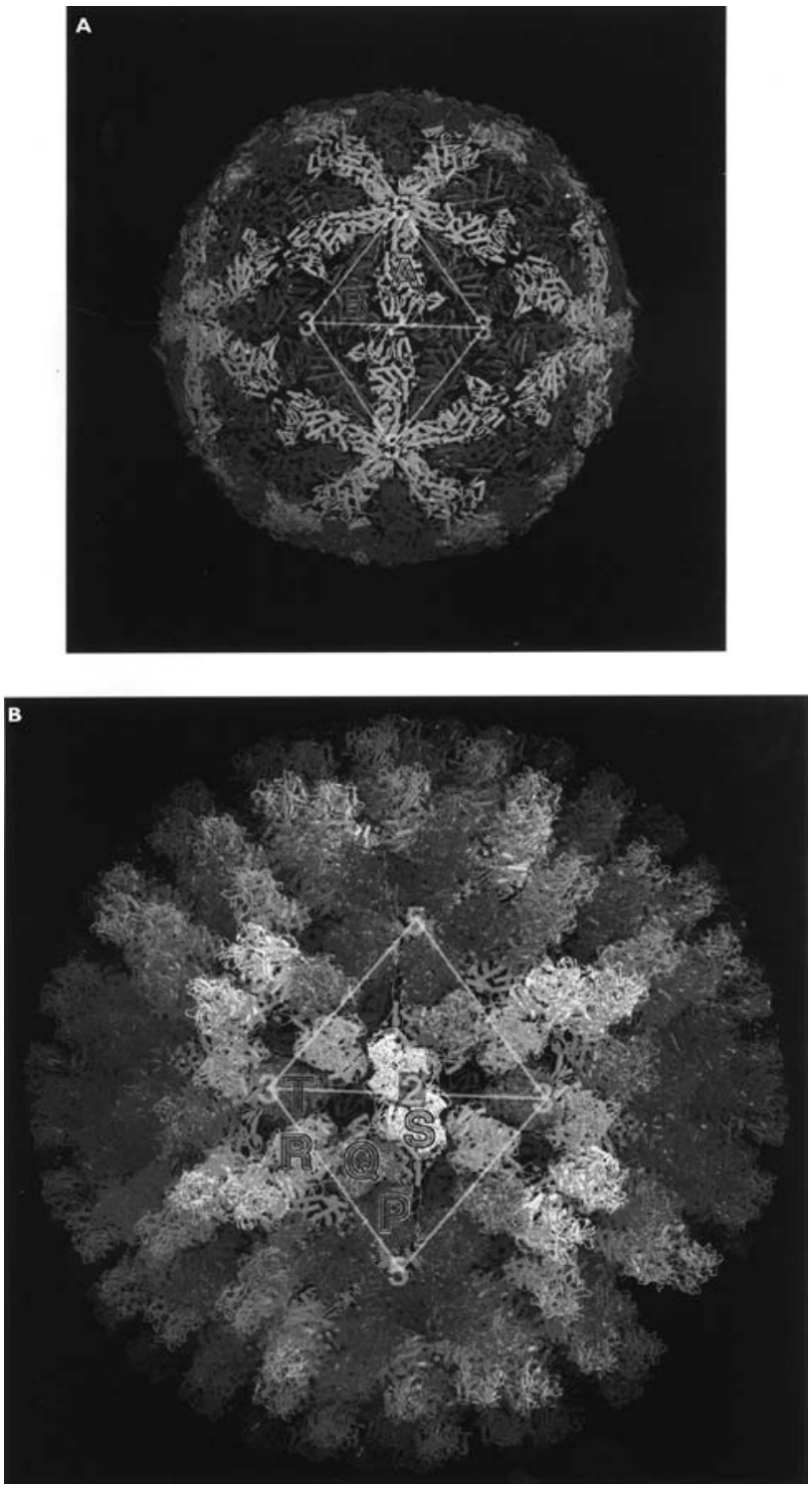


Figure 5 The essential features of the architecture of the native BTV core as determined by x-ray crystallography and cryoelectron microscopy. The icosahedral asymmetric unit is the triangular area defined (as marked) by the symmetric axes of the icosahedron. (**B**) The core surface layer composed of 780 copies of VP7, arranged as 260 trimers with $T = 13$ symmetry. The asymmetric unit contains 13 copies of VP7, arranged as five trimers P, Q, R, S and T: colored red, orange, green, yellow and blue, respectively. Trimer 'T' sits on the icosahedral threefold axis, and thus contributes only a monomer to the unique portion of the capsid. (**A**) The inner capsid layer of the BTV core (the subcore) composed of 120 molecules of VP3, arranged with $T = 2$ symmetry according to the principles of 'geometrical quasi-equivalence'. The icosahedrally unique molecules, of VP3 A and B, are colored in red and green, respectively. Note the completely different structural environment of the A and B molecules. (Reproduced from Grimes *et al* (1998) *Nature* 395: 470–478. Copyright 1998, Macmillan Magazines Ltd. **(For color references see Color Plate 17.)**

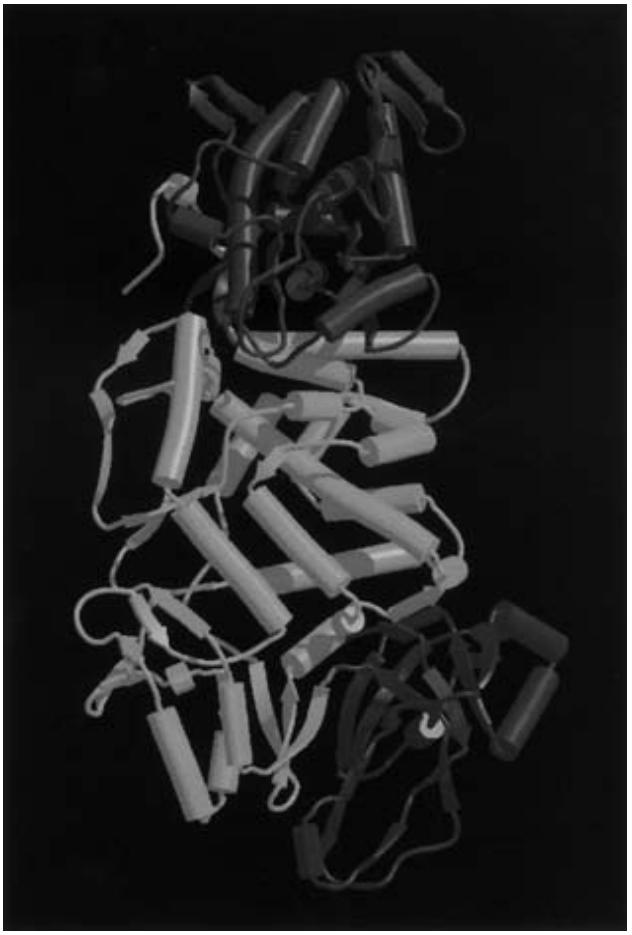


Figure 6 A representation of the VP3 B molecule, color-coded by domain. The 'apical' domain is shown in blue (residue 297–588), the 'carapace' domain in green (residues 7–297, 588–698 and 855–901) and the 'dimerization' domain in red (residue 698–855). (With acknowledgement to J. Grimes and D. Stuart for provision of the image.) **(For color references see Color Plate 18.)**

the internal network of the VP3 of the subcore. The VP7 layer occupies between 295 Å and 365 Å (29.5–36.5 nm) from the center of the core. As shown in **Fig. 5** (see also Plate 17 for color) five VP7 trimers at different distances from the fivefold axis form a protomeric unit (labeled as P to T: red, orange, green, yellow and blue). These trimers show relatively little variation in their structure throughout the core surface layer.

The 260 trimers of VP7 adhere to a scaffold that is made up of only 120 VP3 molecules indicating a symmetry mismatch in the interactions between the bases of the 260 trimers of VP7 and the 120 molecules of VP3. Indeed, the interactions between VP7 and VP3 occur in 13 different relative positions. The flat base of VP7 trimers, with a large number of hydrophobic residues, are mainly responsible for the interactions with VP3. The interacting base of the trimers is formed largely by the three copies of helix 2 and the segments of the chain (aa 22–27 and aa 44–52) leading into and out of this helix.

The helical lower domains are involved in extensive interactions with the lower domains of the other VP7 trimers. These interactions are mediated through helix–helix packing and helix–loop binding. The interaction between the three VP7 lower domains in the trimer are complex.

The inner protein shell known as the 'sub core', is composed entirely of 120 copies of the VP3 protein, that are organized as an icosahedral lattice with $T = 2$ symmetry. The VP3 subcore shell has a maximum diameter of 59 nm, forming a central space with an internal diameter of 38 nm, that contains both the genome and the minor viral proteins.

The individual VP3 molecule forms a flat, elongated wedge shape (13–35Å thick × 75 Å wide × 130 Å long (1.3–3.5 × 7.5 × 13 nm)), which contains many α helices as well as β strands and is made up of three distinct domains (**Fig. 6**) (see also Plate 18 for color). The apical domain (residues 298–587) is situated closest to the fivefold axes in the intact particle. The carapace domain (residues 7–297, 588–698 and 855–901) forms a rigid plate that represents the majority of the surface of the subcore shell. The dimerization domain (residues 699–854), which forms quasi-twofold interactions between individual A group molecules, is situated furthest away from the fivefold axes.

The 120 copies of VP3 are arranged as two groups (A and B) to form the subcore layer (**Fig. 7**) (see also Plate 20 for color). Five of the A group molecules (shown in green) are arranged as a five-pointed star around each fivefold axis. Five additional B group molecules (shown in red) are positioned, one between each of the points of the star, but at a greater distance from the fivefold axis. In this way the VP3 molecules form a convex dish-shaped decamer. Twelve decamers, positioned one at each of the fivefold axes are arranged edge to edge, so as to form the complete subcore VP3 shell. The A and B forms of VP3 interact between different decamers via the dimerization domain. These interactions and interactions between the three different 'B form' molecules of VP3 around the icosahedral threefold axis (again between decamers) appear likely to play a role in holding the decamers together to form the subcore shell. It is possible that the formation of dimers, trimers, pentamers or decamers of VP3 could represent assembly intermediates but their relative significance is as yet unknown.

The inner surface of the VP3 shell interacts directly with the minor protein components (VP1, VP4 and

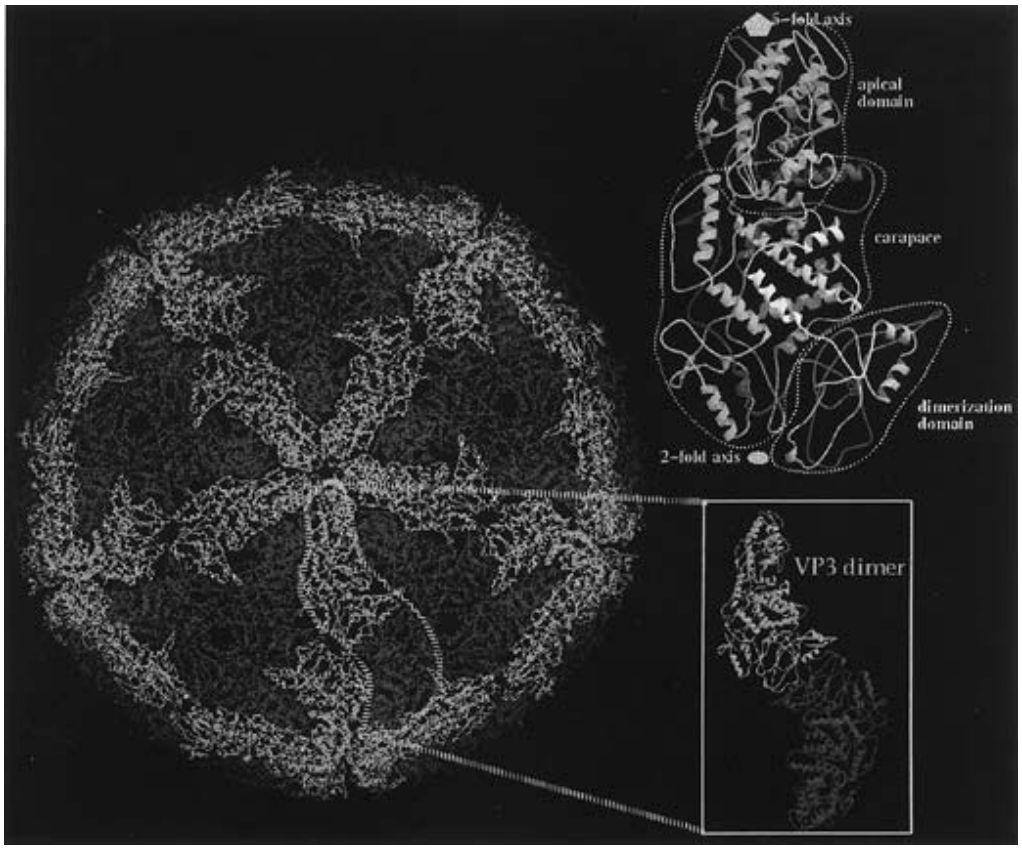


Figure 7 Overall structure and architecture of the VP3 layer of the BTV subcore. Each of 120 molecules has a structure similar to that illustrated on the right of the image. (Reproduced from Stuart *et al* (1998) African Horse Sickness Virus. *Arch. Virol. Suppl.* 14: 235–250.) **(For color references see Color Plate 20.)**

VP6) of the transcriptase complexes (TCs), which are situated at the fivefold axes, within the central space. The TCs (10–12 in total) appear to be present as a single complex at the vertices of the icosahedral particle and therefore do not have strict icosahedral symmetry. As a consequence, their density as viewed by crystallography is averaged around the fivefold axis and appears smeared. It has not yet been possible to resolve their structure, or their interactions with the dsRNA in atomic detail.

The inner surface of the VP3 subcore shell has relatively few charged residues and has a series of shallow grooves. These features may be essential to organize the dsRNA molecules of the genome and to allow them to be transported rapidly and simultaneously through the active sites of the TCs and then repackaged during each round of transcription. Much of the genomic RNA can also be detected by x-ray crystallography, as electron density within the central core space (**Fig. 8**) (see also Plate 19 for color). The dsRNA appears to be highly ordered and approximately 80% of the entire genome can be modeled as four distinct concentric layers that have center to center spacing between RNA strands of 26–30 Å (2.6–3.0 nm). This spacing is consistent with the formation of the RNA into a liquid crystal array, with interactions between the individual layers lubricated by counterions. This would retain the RNA molecules in a highly ordered and closely packed structure but would also allow them a high level of fluidity, that is necessary for their rapid transport and processing by the TCs. As a consequence of the spacing of the A and B forms of VP3, the grooves in the inner surface of the subcore shell form a spiral around the fivefold axis. The outer dsRNA layer appears to interact with these grooves and can also be modeled as a spiral extending outwards from the TC. It is uncertain how the RNA molecules pass inwards to the next and subsequent concentric shells but it seems likely that simple steric clashes with near neighbors and possibly interactions with a lysine residue (807) on a flexible loop of VP3 B, may be important factors in their organization. The density detected in the inner layers of RNA gets progressively weaker, suggesting that its organization

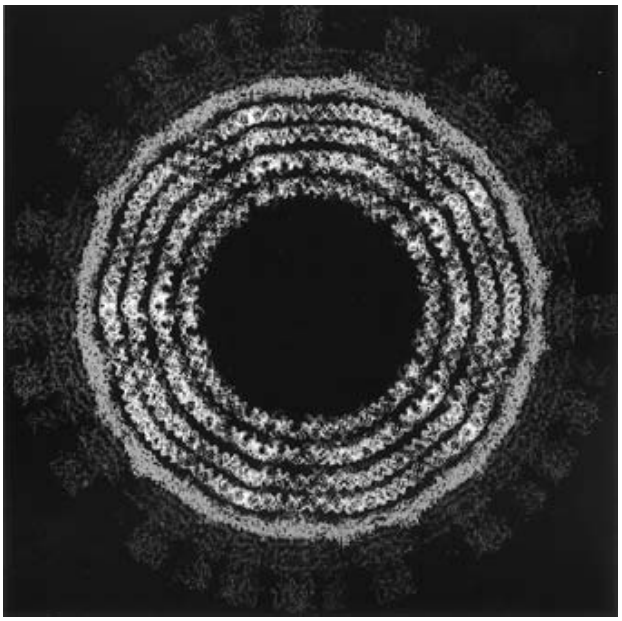


Figure 8 Thin section through the structure of the core of BTV (as determined by x-ray crystallography of native cores of BTV1 and BTV10). The VP7 outer layer of the core is shown in dark blue, the thin VP3 subcore layer in pale blue and the four layers of the genomic dsRNA are predominantly yellow. (Reproduced from Stuart *et al.* (1998) African Horse Sickness Virus. *Arch. Virol. Suppl.* 14: 235–250.) **(For color references see Color Plate 19.)**

and icosahedral symmetry is progressively less rigid. Nevertheless there does appear to be some elements of spiral organization to each of the RNA layers.

Activation of the core-associated enzymes in the presence of magnesium ions and NTP substrates causes conformational changes around the fivefold axis of the core, resulting in movement outwards of the major proteins (VP3 and VP7) and opening of a pore in the VP3 layer at the fivefold axis. The mRNAs synthesized by the core-associated transcriptase complexes are extruded through this pore, then pass out through the central space in the pentameric rings of VP7 trimers. There are also pores in the VP3 layer at the threefold axes but in the intact core particle these are completely blocked by the T trimer of VP7.

Replication

Although field isolates of BTV from infected ruminants or insects often replicate poorly in cultured cells, they can readily be adapted to growth *in vitro*. Such viruses replicate in the cytoplasm of a wide variety of vertebrate and invertebrate cells in culture. No direct proof is available on the nature of the receptor to which BTV binds although analysis of BTV–erythrocyte interaction has implicated specific sialic acid-containing oligosaccharides in the glycophorin of human and animal erythrocytes. Binding to both erythrocytes and vertebrate cells is mediated by the BTV outer coat protein VP2.

Following adsorption, BTV enters vertebrate cells through the normal cellular process of receptor-mediated endocytosis. Thus, shortly after infection the virus is found in vesicles that lead to degradation and removal of at least one, and perhaps both, of the outer coat proteins of the virus. The outer capsid layer can also be removed *in vitro* by treatment with metal ions (Mg^{2+}), or by reduced pH. An increase in endosomal (and lysosomal) pH by addition of lysomotrophic weak bases or acidic ionophores to cells before infection, results in the failure of endocytosed virus particles to initiate replication. Current data imply that core particles enter the cytoplasm by penetration of the endosomal membrane. In the cytoplasm, the partially or completely uncoated particles bind to the cell's intermediate filaments and activate the transcription of viral RNA. *In vitro*, mRNA synthesis can be activated by proteolytic digestion and removal of the outer capsid of the virus. This modification allows free access of the nucleoside triphosphates to the genome and the extrusion of the newly synthesized mRNA to the cytoplasm.

The BTV RNA polymerase itself has not been fully characterized. More than one protein may be involved in transcription. The structural integrity of the core particle may be essential for maintaining the full transcriptional activity of the virus.

The molar ratios in which the different mRNAs are transcribed remain the same throughout the infection cycle. The ratios of mRNAs synthesized *in vivo* and *in vitro* are also identical. Unlike reovirus, these molar ratios are not inversely proportional to their molecular weights. For example, segment M6 encoding the NSI protein is transcribed much more rapidly and efficiently than the segment S10 encoding the NS3 protein.

Replication of orbivirus dsRNA has not been extensively studied. It is presumed that the overall strategy of replication and transcription are similar to those of reoviruses and rotaviruses. The mRNA of BTV is capped and methylated during the transcription process. The minor protein VP4 is responsible for the modification of the mRNAs. During transcription the negative-sense strand of each duplex is the template for the synthesis of mRNA species. These are synthesized and extruded from cores and program the synthesis of virus proteins. After *de novo* synthesis, structural and nonstructural viral proteins (e.g. NS2) associate with the viral mRNA species which leads to formation of nascent progeny subcore,

core particles and concomitant synthesis of dsRNA. The two outer capsid proteins (VP2 and VP5) are subsequently added onto the nascent core particles prior to the release of the newly formed virions.

It has been shown using the appropriate recombinant baculovirus expression vectors that the BTV minor proteins (VP1, VP4 and VP6) are incorporated within synthetic BTV CLPs and virus-like particles (VLPs) in the absence of mRNAs (or genomic RNA) or nonstructural proteins. A specific mechanism must exist for the recognition and incorporation of mRNA species. Such a mechanism could involve not only the virus-induced nonstructural proteins, such as NS2, but also particular sequences and structures of the mRNA species themselves. The conservation of the terminal regions of these RNAs may be of relevance to the question of their recruitment to the sites of particle morphogenesis and encapsidation into cores and eventually virions. The secondary structures of the mRNAs, if formed within viral cores that are transcriptionally active within cells, may facilitate mRNA egress from particles and may also contribute to the overall stability of the viral mRNA species within the cytoplasm of infected cells and its interaction with ribosomal components for the purpose of translation.

Infection of cells with BTV leads to a rapid inhibition of cellular macromolecular synthesis. The first sign of virus replication is the accumulation of a fibrillar network around the infecting core particles in the cytoplasm resulting in formation of a viral inclusion body (VIB). With increasing time postinfection, the size and number of the VIBs increase. Immunogold electron microscopy has confirmed that VIB are rich in ssRNA and the phosphorylated NS2 protein. In addition, the VIB matrix contains the two major core proteins VP3 and VP7. Subcore and core particles are made within the VIB matrix and interact with outer capsid proteins only at the periphery of VIBs. The major outer coat protein VP2 is not a VIB constituent. Indeed, it is only found at the surface of the VIB structure, at the locations where core particles mature to form progeny virus particles.

NS1 protein is the predominant constituent of viral-specific tubules found throughout the cytoplasm. Although tubules are the hallmark of orbivirus-infected cells, their function in virus replication is unknown. NS1 is also found associated with core-like structures within VIBs, as well as intracellular and released virus particles. However, tubules can be removed from virus particles during purification. VIBs, tubules and intracellular virus particles bind to the vimentin-rich, intermediate filament networks of the cell's cytoskeleton.

Immunoelectron microscopy indicates that VP2 is added to developing virus particles either at the periphery of VIBs or following binding to the cytoskeleton. Analyses of intracellular virus particles have revealed significant differences in the organization and/or the amount of VP2 associated with cytoskeleton-associated or free virus particles in the cell cytoplasm. Although the majority of newly synthesized virus particles remain cell-associated, a proportion leave infected cells (depending on the cell type). Released viruses also differ from their intracellular counterparts with respect to the organization of the VP2 protein in the outer coat.

Large accumulations of virus particles are found on the surface of BTV-infected cells and retain an association with the underlying cortical layer of the cytoskeleton. Virus particles are observed to leave infected cells in either of two ways. First, and particularly early in infection, particles can be seen to bud through the cell membrane and acquire, at least temporarily, an envelope. However, no virus antigen can be detected at the surface of such enveloped particles. Second, egression of virus particles from infected cells is accomplished by a process of extrusion where individual and groups of virus particles move through the plasma membrane. This appears to happen without a significant effect on host cell viability. The minor nonstructural protein NS3 has been located in areas of the plasma membrane where virus particles are leaving, suggesting a role for this protein in this process.

Future Perspectives

Although significant progress has been made on elucidating structure–function of BTV genes and gene products, little is known about the intracellular assembly process and the stoichiometries of the viral components in the morphogenetic pathway of virion assembly. The baculovirus expression system has provided a method to understand how and which viral proteins interact and assemble to form virus particles. It can be anticipated that the availability of large quantities of viral components and the core- and virus-like structures will not only lead to further understanding of their three-dimensional structure at the atomic level, but also facilitate future investigations of the mechanisms of viral replication.

See also: **Orbiviruses and coltiviruses (*Reoviridae*): General features; Reoviruses (*Reoviridae*): General features; Molecular biology; Replication of viruses; Virus structure: Atomic structure; Principles of virus structure.**

Further Reading

Monath TP and Guirakhoo F (1999) Orbiviruses and Coltiviruses. In: Fields BN *et al* (eds) *Fields Virology*, 4th edn, pp. 1735–1760. New York: Raven Press.

Roy P (1996) Orbiviruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 1709. Philadelphia: Lippincott-Raven.

Roy P and Gorman BM (eds) (1990). Bluetongue viruses. *Current Topics in Microbiology and Immunology*. Berlin: Springer-Verlag.

ORGAN SYSTEM INFECTIONS

Jangu E Banatvala, Guy's, King's College and St Thomas' School of Medicine, St Thomas' Campus, London, UK

Felicity Nicholson, c/o Trailfinders Travel Clinic, Kensington, London, UK

Viruses Affecting the Heart

Introduction

Viruses may affect the heart in a number of different ways. Developmental defects may occur as a result of intrauterine infection by such viruses as rubella, and infection of the myocardium may be induced by a large number of viruses in different groups, although infection by enteroviruses probably represents the commonest cause of acute and chronic heart muscle disease. As yet, there is little evidence to support the role of viruses as a cause of endocardial disease.

Although the myocardium is exposed during the viremic phase of infection to a large number of viruses, only a minority give rise to clinically apparent cardiac disease. Nevertheless, asymptomatic myocarditis is probably relatively common since histological studies of myocardial tissue obtained from young adults killed accidentally reveal a focal myocarditis in 0.8–3% of tissues. Since a focal myocarditis may induce a fatal arrhythmia, it is perhaps not surprising that autopsies carried out on young persons who died unexpectedly have shown that about 15% have evidence of myocarditis. In a small proportion of those experiencing a subclinical myocarditis, it is possible that virus may not be cleared by immune mechanisms and persist to result in the development of chronic heart muscle disease over a period ranging from a few months to several years. Some studies suggest that approximately 25% of patients with acute myocarditis fail to recover fully and develop chronic cardiac disease.

Etiology

Table 1, although not comprehensive, lists viruses that may induce cardiac disease. Some are involved commonly [e.g. enteroviruses, human immunodeficiency virus (HIV) and, if acquired congenitally, rubella] whereas others are uncommon [e.g. varicella-zoster, cytomegalovirus (CMV) and hepatitis B]. Many of these viruses involve the heart as part of a clinically obvious generalized infection (e.g. varicella, mumps and rabies) whereas in others, cardiac disease is the primary presenting clinical feature. The more common causes of viral heart disease are discussed below, but some of those occurring less commonly are listed in **Table 1**.

Cases categorized as 'cardiovascular' in the WHO *Global Surveillance of Virus Diseases Programme* from 1978 to 1985 shows that enteroviruses, particularly coxsackie B viruses, followed by influenza B and A viruses are the commonest viruses associated with cardiac disease; in most cases the association is likely to relate to acute myocarditis or pericarditis.

Intrauterine and perinatal infections

Intrauterine or perinatal infections may induce developmental defects of the heart. Thus, if acquired during the first trimester, maternal rubella may result in a number of cardiac abnormalities, particularly a patent ductus, septal defects or pulmonary stenosis. A focal myocarditis may also be present. If acquired in pregnancy, human parvovirus infection (B14) infects the fetus and induces cardiac failure as a result of a severe anemia caused by viral destruction of fetal erythrocyte precursors. This may result in a hydrops fetalis and cause miscarriage or stillbirth.

Enteroviruses, particularly coxsackie B viruses, may cause severe neonatal disease with mortality rates approaching 40–50%. Infection may be acquired transplacentally, perinatally via contamination with maternal fecally excreted virus or via other infants in

Further Reading

Monath TP and Guirakhoo F (1999) Orbiviruses and Coltiviruses. In: Fields BN *et al* (eds) *Fields Virology*, 4th edn, pp. 1735–1760. New York: Raven Press.

Roy P (1996) Orbiviruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 1709. Philadelphia: Lippincott-Raven.

Roy P and Gorman BM (eds) (1990). Bluetongue viruses. *Current Topics in Microbiology and Immunology*. Berlin: Springer-Verlag.

ORGAN SYSTEM INFECTIONS

Jangu E Banatvala, Guy's, King's College and St Thomas' School of Medicine, St Thomas' Campus, London, UK

Felicity Nicholson, c/o Trailfinders Travel Clinic, Kensington, London, UK

Viruses Affecting the Heart

Introduction

Viruses may affect the heart in a number of different ways. Developmental defects may occur as a result of intrauterine infection by such viruses as rubella, and infection of the myocardium may be induced by a large number of viruses in different groups, although infection by enteroviruses probably represents the commonest cause of acute and chronic heart muscle disease. As yet, there is little evidence to support the role of viruses as a cause of endocardial disease.

Although the myocardium is exposed during the viremic phase of infection to a large number of viruses, only a minority give rise to clinically apparent cardiac disease. Nevertheless, asymptomatic myocarditis is probably relatively common since histological studies of myocardial tissue obtained from young adults killed accidentally reveal a focal myocarditis in 0.8–3% of tissues. Since a focal myocarditis may induce a fatal arrhythmia, it is perhaps not surprising that autopsies carried out on young persons who died unexpectedly have shown that about 15% have evidence of myocarditis. In a small proportion of those experiencing a subclinical myocarditis, it is possible that virus may not be cleared by immune mechanisms and persist to result in the development of chronic heart muscle disease over a period ranging from a few months to several years. Some studies suggest that approximately 25% of patients with acute myocarditis fail to recover fully and develop chronic cardiac disease.

Etiology

Table 1, although not comprehensive, lists viruses that may induce cardiac disease. Some are involved commonly [e.g. enteroviruses, human immunodeficiency virus (HIV) and, if acquired congenitally, rubella] whereas others are uncommon [e.g. varicella-zoster, cytomegalovirus (CMV) and hepatitis B]. Many of these viruses involve the heart as part of a clinically obvious generalized infection (e.g. varicella, mumps and rabies) whereas in others, cardiac disease is the primary presenting clinical feature. The more common causes of viral heart disease are discussed below, but some of those occurring less commonly are listed in **Table 1**.

Cases categorized as 'cardiovascular' in the WHO *Global Surveillance of Virus Diseases Programme* from 1978 to 1985 shows that enteroviruses, particularly coxsackie B viruses, followed by influenza B and A viruses are the commonest viruses associated with cardiac disease; in most cases the association is likely to relate to acute myocarditis or pericarditis.

Intrauterine and perinatal infections

Intrauterine or perinatal infections may induce developmental defects of the heart. Thus, if acquired during the first trimester, maternal rubella may result in a number of cardiac abnormalities, particularly a patent ductus, septal defects or pulmonary stenosis. A focal myocarditis may also be present. If acquired in pregnancy, human parvovirus infection (B14) infects the fetus and induces cardiac failure as a result of a severe anemia caused by viral destruction of fetal erythrocyte precursors. This may result in a hydrops fetalis and cause miscarriage or stillbirth.

Enteroviruses, particularly coxsackie B viruses, may cause severe neonatal disease with mortality rates approaching 40–50%. Infection may be acquired transplacentally, perinatally via contamination with maternal fecally excreted virus or via other infants in

Table 1 Viruses affecting the heart

Viruses	*Clinical features*
Enteroviruses	Acute myopericarditis (postnatally acquired) Acute myocarditis during generalized intrauterine or perinatal infection DCM
Influenza A and B	Myocarditis as one feature
HIV	Myocarditis, cardiomyopathy
Mumps	Myocarditis (seldom symptomatic but electrocardiograph changes may occur in about 5–15%)
Measles	Myocarditis (very rare)
Rubella	Developmental defects if congenitally acquired, sometimes with focal myocarditis
Arboviruses: Dengue Yellow fever Chikungunya	Myocarditis as one feature
Rabies	Myocarditis as one feature
Hepatitis B	Myocarditis as one feature (very rare)
Varicella-zoster	Myocarditis as one feature
CMV	Myocarditis may occur in transplanted allografts (rare)
Herpes simplex	Myocarditis during generalized neonatal infection
Epstein–Barr	Myopericarditis as one feature (rare)
Adenoviruses	Myocarditis (very rare)
Vaccinia	Myocarditis (rare)

the nursery who are excreting enteroviruses. A severe generalized infection with multisystem involvement may be evident at birth or a few days afterwards; death often results from heart failure due to severe myocarditis.

More recently, it has been recognized that, if acquired *in utero* or perinatally, HIV 1 may induce a cardiomyopathy in infancy which may result in arrhythmias and sudden death.

Acute myopericarditis

Acute myocarditis is defined as an 'inflammatory cell infiltration of the myocardium with necrosis and/or degeneration of adjacent myocytes, not typical of ischemic damage associated with coronary arterial disease'. There is invariably some pericardial involvement in patients with myocarditis and vice versa; because of this, they will be considered together, although the clinical features of one may predominate over the other.

Role of enteroviruses

Although many of the viruses listed in **Table 1** may cause acute myopericarditis, the accumulated data from a number of virological studies suggest that enteroviruses are the commonest cause of this disease. Serological studies employing techniques to detect virus-specific immunoglobulin M (IgM) indicate that about 33% to almost 50% of cases of acute myopericarditis are induced by enteroviruses, these findings being supported by the fact that enteroviral RNA has been detected in 20–40% of endomyocardial biopsies. As there are over 70 enterovirus serotypes, molecular biological studies generally employ sequences for hybridization or primers for polymerase chain reaction (PCR) which are highly conserved among this group of viruses. Because of this, and as virus-specific IgM responses may be broadly reactive against a number of enteroviruses, it is difficult to establish which particular enteroviruses are involved. However, the finding that many patients with myopericarditis often have high neutralizing antibodies to coxsackie B serotypes, and that this group of viruses is cardiotropic in certain strains of mice, suggests that coxsackie B viruses are more likely to be involved than other enteroviruses in the pathogenesis of acute myopericarditis.

HIVs may cause myocardial damage, although the prevalence of myocardial disease in patients with acquired immune deficiency syndrome (AIDS) has not been clearly established. However, one series suggested that it occurs in as many as 50% of cases. *In vitro* studies suggest that HIV is unable to gain entry into myocytes, and studies attempting to detect HIV in myocardial biopsies employing hybridization studies have produced conflicting results. Nevertheless, although myocarditis may be induced by HIV itself,

since the virus has been isolated from heart muscle, it is also possible that one or more opportunistic infections may also induce myocardial damage.

Chronic cardiac disease

Apart from enteroviruses and more recently HIV, there is as yet little information relating to the role of viruses in chronic cardiac disease. Two forms of chronic cardiac disease are associated with enterovirus infection, chronic relapsing myopericarditis and dilated cardiomyopathy (DCM).

Chronic relapsing myopericarditis About 20% of patients who develop acute myopericarditis in which the features of pericardial involvement predominate experience a relapsing course which may extend over a period of months or years. About 60% of such patients have a persistent enterovirus-specific IgM response during the relapsing course of the disease, which is indicative of a persistent antigenic stimulus. However, it is seldom possible to isolate virus from patients' stools or nasopharyngeal secretions during the acute phase and hardly ever during the relapsing course.

DCM DCM is defined as 'heart disease with intrinsic muscle dysfunction, excluding such causes as valvular heart disease and ischemic heart disease'. In developed countries, the disease has an estimated annual incidence of 100 000 and end-stage disease is, next to ischemic heart disease, the commonest cause of cardiac transplantation. The results of serological and nucleic acid hybridization studies suggest that enteroviruses may be involved in 20–40% of cases. *In situ* hybridization shows that replication of enteroviral RNA is focal and more restricted than in acute myocarditis and that the number of infected myocardial cells relates to the severity of disease.

Chronic heart muscle disease has been detected in children as well as adults with HIV 1 infection, including patients with different risk-behavior patterns. In general, cardiac disease is more likely to be present in patients with the later stages of HIV-associated disease. As specific antiviral chemotherapy and the prophylaxis and treatment of opportunistic infections improve survival time, it is possible that more cardiac disease will be seen to be the cause of death among HIV-infected patients.

Infection following cardiac transplantation

CMV infections occur commonly following cardiac transplantation. The severity of the infection is dependent on the severity of the immunosuppressive regime and the serological status of the donor and recipient. Thus CMV infections, often severe, occur in up to 60% of seronegative recipients transplanted with allografts from CMV-positive donors, infection usually being acquired via the allograft and occurring 6–8 weeks post-transplantation. If both donor and recipient are seropositive for CMV, infection is likely to be less severe and may result from infection via the allograft or re-activation of the patient's own CMV. Infection is associated with a significantly higher rate of graft rejection and graft-associated atherosclerosis. It is important to establish a diagnosis rapidly, since patients usually respond well to treatment with ganciclovir. Ganciclovir may also be used prophylactically in order to prevent seronegative recipients developing severe CMV following transplantation with an allograft from a seropositive donor.

Atherosclerosis

Atherosclerosis, particularly coronary heart disease, represents one of the leading causes of death in most industrialized countries. Furthermore there is now an increasing prevalence of disease in developing countries who are adopting a Westernized lifestyle. It has been recently postulated that viruses, particularly CMV, may play a role (see under Pathogenesis below).

Epidemiology

The epidemiology of influenza and other viruses listed in **Table 1** is described in entries on each of the specific viruses.

Since the accumulated data from a number of studies suggest that enteroviruses are the most frequent group of viruses involved in the pathogenesis of acute and chronic cardiac disease, it is appropriate to comment briefly about the epidemiology of this group of viruses. Enteroviruses are ubiquitous, and infection is spread by the fecal–oral route. In countries with poor standards of hygiene and sanitation, infection occurs in infancy and childhood, often subclinically. In industrialized countries, exposure to infection is often delayed and adults may experience some more severe manifestations of infection, including myopericarditis. In tropical climates where overcrowding and poor sanitation prevail, infection occurs throughout the year; up to 30% of infants under the age of 2 may be found to be excreting enteroviruses at any one time. In temperate climates enterovirus infections occur more commonly in summer and autumn, and epidemics with one or more serotypes tend to occur in 3–5 year cycles.

Pathogenesis

With the exception of enteroviral myocarditis, there

is comparatively little information relating to the pathogenesis of virally induced acute cardiac disease. However, since influenza A has been detected in cardiac tissues of a few patients with influenza who develop myocarditis, it is possible that damage may be induced directly by viral destruction of myocytes, although the possibility of immunopathological mechanisms cannot be excluded, particularly as membrane-bound viruses contain host antigens.

Whether or not patients experiencing enterovirus infection develop an acute myopericarditis depends on a complex interplay between viral and host factors. Experimentally induced acute enterovirus myocarditis in the mouse is histologically similar to human myocarditis in many respects, with extensive infiltration of lymphocytes, particularly T cells. However, murine myocarditis can only be induced if selected mouse strains and cardiotropic coxsackie B viruses, e.g. CVB-3m, are used. However, it is now known that enteroviruses have a particularly high mutation rate; some natural isolates of coxsackie B virus strains differ from the prototype by as many as 12 epitopes and the rate of mutation may be high as 10^{-4}. Minor but subtle changes in polypeptides expressed on the viral capsid may affect tissue tropism markedly. The accumulated data from a number of experimental studies in mice, together with the finding that virus is seldom isolated from patients' secretions or excretions recovered from cardiac tissue, suggest that enterovirus-induced myocardial damage is likely to represent a postinfectious autoimmune disease. Viral clearance probably results from synergistic responses between neutralizing antibodies, NK cells and macrophages, cardiac damage resulting from destruction of heart muscle fibers mediated by cytotoxic T lymphocytes recognizing virally induced neoantigens on monocyte membranes.

Two hypotheses have been suggested to explain the pathogenesis of enterovirus-induced dilated cardiomyopathy, the first being that disease results from persistent but defective viral replication, which may disrupt myocyte gene expression, impairing the function but not destroying the myocytes. The presence of relatively high amounts of viral RNA template strands in cardiac tissue of patients with dilated cardiomyopathy lends support to this hypothesis and may explain why infectious virus or viral antigens cannot be detected in the cardiac tissue of patients with dilated cardiomyopathy. The second hypothesis proposes that dilated cardiomyopathy has an autoimmune basis; virally induced alterations in MHC antigen expression resulting in exposure of neoantigens of the immune system represent a possible mechanism. Sharing of viral antigens with host cardiac tissue (molecular mimicry) may provide an alternative mechanism. Although a number of autoimmune phenomena have been detected in patients with dilated cardiomyopathy, it remains to be established whether they are involved in the pathogenesis of disease or merely represent epiphenomena.

The role of viruses in the pathogenesis of atherosclerosis among humans is speculative. However, Marek's disease virus (MDV), which is a herpesvirus affecting fowls, induces atherosclerosis in chickens; feeding on cholesterol-rich diets accelerates the disease progress. The administration of a live attenuated vaccine does not result in the development of atherosclerosis but prevents development of MDV-induced arterial lesions in chickens exposed to MDV. Among humans, evidence that CMV induces atherosclerotic change is much more circumstantial. CMV nucleic acid sequences and viral antigens have been detected in arterial smooth muscle including the coronary arteries of young adults experiencing traumatic deaths. Such lesions have been described in areas of the arterial wall showing early atherosclerotic changes. There is also some evidence that suggests that patients having transplants who become infected with CMV are particularly likely to develop accelerated atherosclerosis in the coronary arteries of the transplanted organ.

Clinical features

Acute myopericarditis Some patients present with a 3–4-day history of such nonspecific clinical features as malaise, myalgia, sore throat and fever, before developing the clinical features of the disease whereas others may present initially with cardiac manifestations including chest pain, tachycardia out of proportion to fever, gallop rhythm and breathlessness. A pericardial friction set may be available. Death may result from arrhythmias or from circulatory collapse. Raised cardiac enzymes and electrocardiograph (ECG) changes may resemble those seen in acute myocardial infarction if the myocarditis is restricted to a localized region of the myocardium. More commonly, the ECG is nonspecific but patients in whom the features of pericarditis predominate may have ST elevation. Some 80–90% of patients make a full recovery although a few die in the acute stage and others develop DCM, the features of which may appear rapidly or occur after many years following a prolonged latent phase.

Pericarditis in patients with AIDS Pericarditis is not uncommon in patients with terminal AIDS although this disease rarely contributes to the patient's death. However, pericarditis may occur

earlier and this may be neoplastic or infectious in etiology. A number of different infectious agents may be involved, including viruses, particularly those that cause opportunistic infections in patients with severe immunodeficiency disorders. It is important to distinguish between a neoplastic and an infectious cause since specific antimicrobial therapy may affect the patient's prognosis favorably.

DCM DCM often presents insidiously with increasing shortness of breath, lethargy and clinical features associated with cardiac failure since the heart has an impaired pump function. The absence of heart-valve, coronary-artery or lung disease is suggestive of DCM, which should be confirmed histologically by examination of an endomyocardial biopsy. In contrast with acute myopericarditis, the prognosis is poor, the 5-year survival rate being about 50% in the absence of cardiac transplantation.

Laboratory diagnosis

Specific virological investigations for many of the viruses listed in **Table 1** are described in the respective entries dealing with these infections.

Enteroviruses are the commonest cause of acute myopericarditis and, although attempts should be made to isolate virus from stools and nasopharyngeal washings, virus excretion is only rarely present when patients present with symptoms, as cardiac events occur relatively late in the course of infection, since they are probably induced by immunopathological mechanisms. By the time patients present with cardiac disease, neutralizing-antibody responses have usually reached maximum levels. Although high titers may provide circumstantial evidence of recent infection, such titers may persist for a varying but often prolonged time after infection, which may be subclinical and therefore unrelated to the patient's current illness. Detection of enterovirus-specific IgM may be useful, since its presence is indicative of a current or recent antigenic stimulus; such responses usually persist for 3–6 months but occasionally longer following an acute infection. Although up to 50% of patients with acute myopericarditis may have an enterovirus-specific IgM response, such responses may be detected in 5–10% of apparently healthy persons, presumably the result of a recent subclinical enterovirus infection. In patients from whom endomyocardial biopsies are obtained, attempts may be made to detect enterovirus RNA by hybridization studies employing cDNA-labeled probes or by PCR.

Enteroviruses may be readily isolated from excretions and secretions as well as organs obtained at autopsy from infants with severe generalized neonatal enterovirus infection.

Although evidence of possible enterovirus infection in patients with DCM may be obtained by detecting enterovirus-specific IgM responses, some workers have been unable to demonstrate consistent differences between patients and controls. Examination of endomyocardial biopsies or cardiac explants using molecular biological techniques to detect enteroviral RNA are, of course, more reliable but, because of sampling errors, it may be necessary to examine a large number of sections.

A diagnosis of infection by CMV in cardiac-transplant patients may be achieved either by isolating virus from the patient's urine or nasopharyngeal secretions or, more rapidly, by detecting the presence of CMV antigens by immunofluorescence in urine or nasopharyngeal secretions or in the buffy coat. In patients who develop pneumonitis, viral antigens may be detected in washings obtained by bronchoalveolar lavage.

Viruses Affecting the Pancreas

Introduction

During the viremic phase of many systemic infections, pancreatic infection is likely to be common. However, as most viral infections are not fatal and because pancreatic changes may be subtle and therefore not adequately investigated, less attention has been directed towards the viral effects of pancreatitis than is the case for many other organs.

Nevertheless, studies on children with disseminated fatal viral infections showed that pancreatic lesions, often with islet cell destruction, were present in patients with coxsackie B, CMV, varicella-zoster and congenitally acquired rubella. In addition, pancreatitis has been observed following mumps and, less commonly, following Epstein–Barr virus (EBV) infection and hepatitis B virus infections. The finding that hepatitis B surface and core antigens have been detected in the acinar cells of patients with hepatitis B antigenemia suggests that this virus may replicate in pancreatic tissue. Although such filoviruses as Marburg and Ebola induce generalized infections with severe hepatic involvement, some patients develop clinical features of acute pancreatitis, and post-mortem studies have demonstrated areas of focal pancreatic necrosis.

EBV infections are closely involved in the pathogenesis of Burkitt's lymphoma and this lymphoma may be found at autopsy to involve the pancreas relatively commonly. It may present clinically as a diffuse enlargement of the pancreas, this being due to

the tumor itself, or a pancreatitis resulting from tumor lysis induced by chemotherapy.

Mumps

The exact frequency of pancreatitis complicating mumps is not known although a figure of about 5% has been suggested. Experimental studies employing human cell cultures have shown that mumps virus may destroy islet cells.

Patients present with epigastric pain, often severe, and vomiting, during which time serum amylase levels are markedly raised. In general, patients make a good recovery although rarely a severe hemorrhagic pancreatitis may occur.

Enteroviruses

Some studies have suggested that enteroviruses, particularly coxsackie B viruses, may cause acute pancreatitis although the commonest etiological factors are biliary disease and alcohol abuse. In order to assess the incidence of enteroviruses as a cause of acute pancreatitis, large-scale multicenter clinical and virological studies are required, employing some newly developed virological techniques.

CMV

Postmortem studies have shown histological changes in the pancreas consistent with CMV infection in infants dying with severe congenitally acquired CMV. Immunocompromised patients, including those with AIDS, may develop severe disseminated opportunistic infections by herpes, particularly CMV infections in which pancreatic lesions may be detected. However, pancreatitis is not generally a prominent feature of post-transplant CMV infection but, when it occurs, is often associated with a fatal outcome.

Role of viruses in the pathogenesis of insulin-dependent (Type I) diabetes mellitus (IDDM)

In recent years, considerable attention has been focused on the role of viruses in the pathogenesis of IDDM. It occurs in about 1 in 1000 children in industrialized countries and there is some evidence that the incidence is increasing. This disease, which has a strong autoimmune component, represents a complex interplay between genetically determined and environmental factors, and viruses may play a role. That environmental factors are important is suggested by the finding that IDDM is discordant in approximately 50% of identical twins when disease is initially diagnosed among persons aged less than 40.

Experimental studies in mice, case reports and seroepidemiological studies have implicated such viruses as coxsackie B, mumps, rubella, CMV and reoviruses.

Experimental studies

Picornaviruses may induce diabetes in certain inbred strains of mice. It is probable that susceptibility is conferred by an inherited autosomal trait. The gene product controlling susceptibility may be a virus receptor on the plasma membrane of β cells complementary to epitopes on certain picornaviruses. Encephalomyocarditis virus (EMCV) and strains of coxsackie B viruses induce lytic infections in islet cells, and infected mice develop abnormal glucose tolerance and hyperglycemia. Two variants of EMCV, D and B, have been identified, the D variant inducing prolonged hyperglycemia as well as retinal and renal damage resembling diabetic complications in humans. The B variant is non-diabetogenic, but prior infection protects mice from infection by the D variant. The B variant is deficient in at least one oligonucleotide, 20–25 nucleotides in length. Coxsackie B viruses usually infect acinar cells but β-cell tropism can be induced by passage in pancreatic cultures rich in islet cells. Such virus strains induce a transient hyperglycemia in susceptible mice.

In vivo, lymphocytic choriomeningitis virus (LCMV) induces a persistent infection in the absence of cytocidal changes in newborn mouse β cells. Infected mice develop hyperglycemia and abnormal glucose tolerance, possibly as a result of viral gene products competing with insulin synthesis.

All three reovirus serotypes may induce pancreatic damage involving the acinar cells in newborn mice. However, reoviruses 1 and 3 can infect and damage islet cells. Mice infected with reovirus 1 are runted and develop polyendocrine disease with a transient diabetes, developing autoantibodies directed not only against islet cells but the other endocrine organs including anterior pituitary and thymus as well as the gastric mucosa of uninfected mice. Polyendocrine disease associated with autoantibodies may also be seen in patients with recently diagnosed IDDM.

Evidence linking virus infection with IDDM in humans

Congenitally acquired rubella Follow-up studies on children with congenitally acquired rubella have shown that 20% of infants whose mothers were infected during the 1941 Australian epidemic and 12.5% during the 1963–1964 USA epidemic developed IDDM, islet cell antibodies being present in 20%. This suggests that more patients will develop IDDM in due course. Clinical manifestations of disease are often delayed until adolescence or adult life.

The distribution of human leucocyte antigen (HLA) types is typical of those among patients with autoimmune disease, including patients with IDDM that is not associated with congenitally acquired rubella. Thus, there is a significant increase in HLA DR3, some increase in HLA DR4 and a virtual absence of HLA DR2. Islet cell surface antibodies, which precede the onset of IDDM by months or years, have a cytotoxic effect on islet cell cultures. Although autoimmune mechanisms undoubtedly play an important role in the pathogenesis of IDDM following congenitally acquired rubella, the method by which this virus triggers this has not been established.

Anecdotal reports of IDDM associated with acute viral infections A small number of cases of IDDM have occurred in which the onset of diabetes was associated with well-documented infection by mumps or coxsackie B viruses. Although this association may be merely temporary since such infections are common in childhood, detection of coxsackie B4 virus or its antigens in pancreatic tissue of a fatal case and the finding that when this virus as well as a coxsackie B5 virus isolated from a nonfatal case induced hyperglycemia in mice, suggests that these virus strains have diabetogenic potential. The virus from the fatal case induced infiltration of inflammatory cells and β-cell necrosis in the mouse pancreas, coxsackie B antigen being detected in the β cells. Five cases have so far been reported in which laboratory-confirmed coxsackie B virus infection occurred contemporaneously with the onset of the symptoms of IDDM.

Despite these observations, it must be appreciated that IDDM has many of the features of a classical autoimmune disease, the presence of islet cell antibodies and of abnormal glucose tolerance preceding the onset of IDDM by months or years. This suggests that perhaps only exceptionally can acute viral infections cause such extensive destruction of β cells to precipitate the rapid development of IDDM.

IDDM has also occasionally been reported among children who have recently developed mumps, particularly if there has been evidence of an acute pancreatitis. Studies carried out in Norway in the earlier part of this century drew attention to the finding that epidemics of mumps were followed by an increased number of deaths from juvenile diabetes within a 4-year period. More recently, islet cell antibodies have been detected in a high proportion (46%) of patients with mumps and infectious mononucleosis (over 50%), although not in patients infected with enteroviruses, measles and postnatally acquired rubella. However, if mumps virus is significantly related to the pathogenesis of IDDM, the extensive use of attenuated mumps vaccine in the USA, and now elsewhere, should result in a marked decline in the incidence of IDDM; this has not been observed in the USA.

Seroepidemiological studies

A number of studies have been conducted comparing serological responses to coxsackie B viruses among patients with IDDM and matched controls. Most of the earlier studies employed neutralization, but, more recently, tests to detect enterovirus-specific IgM have been used. Although there is a tendency for a greater proportion of patients with IDDM to have higher neutralizing-antibody responses, this pattern is inconsistent. Tests to detect enterovirus-specific IgM responses have been carried out in the UK and Scandinavia; in certain years, there appears to be a close correlation between virus-specific IgM responses and the onset of IDDM but not in others. The accumulated data from serological studies suggest that if enteroviruses are involved in the pathogenesis of IDDM, they may precipitate symptomatic disease in patients who already have compromised β-cell function rather than initiate the train of events that results in autoimmune mechanisms that progressively destroy β cells over a prolonged period. This hypothesis is supported by the finding that mice given a subdiabetogenic dose of streptozotocin, a β-cell poison, develop β-cell destruction, abnormal glucose tolerance and hyperglycemia when challenged with a nondiabetogenic strain of a coxsackie B virus.

Hypotheses to explain virally induced IDDM in humans

In addition to IDDM very occasionally resulting from an acute lytic viral infection or viral infection precipitating disease in patients with already compromised β-cell function, alternative and perhaps more plausible mechanisms are likely. Firstly, persistent viral infections may occur, resulting in the conservation of the vital but not specialized functions of β cells. The possibility that such phenomena may be induced by enteroviruses, for example coxsackie B viruses, should not be discounted since picornaviruses are now established as causes of persistent infections in animals (e.g. foot and mouth disease, Theiler's virus infections) and man (chronic cardiac disease, chronic fatigue syndrome). The finding that immunoreactive α-interferon has been found in pancreatic tissue removed at necropsy from patients with IDDM may support the presence of virally induced disease. Most of these specimens also showed hyper-expression of MHC Class I antigens, similar findings being present in cases of acute infantile pancreatitis, most of

which were caused by coxsackie B virus infections. Molecular biological techniques, particularly PCR, will be useful in determining whether viruses can be detected and shown to persist in residual β cells among patients with IDDM.

Secondly, viral infection may induce IDDM by triggering autoimmune responses; a persistent infection is also consistent with such a hypothesis. Autoimmune responses may result from epitope sharing between viral and cellular components (a molecular mimicry), the induction of anti-idiotype responses which may react not only with viral antibody but also viral receptors on the cell surface, or the aberrant expression of MHC Class II antigens on epithelial cell surfaces. Thus, viral infection, perhaps asymptomatic, may induce γ-interferon which in turn may result in the expression of aberrant HLA DR antigens on the surface of endocrine epithelial cells which will enable them to function as antigen-presenting cells, presenting cell antigens to autoreactive T cells. This hypothesis is consistent with the prolonged latent phase between infection with a virus and the clinical manifestations of IDDM.

Viruses Affecting the Kidney

Viruses may reach the kidney during the viremic phase of infection, and replicate within the organ. A number of outcomes are possible including asymptomatic replication, direct virally induced specific damage, the establishment of viral latency or indirect damage via such secondary mechanisms as hypovolemia and immune-complex deposition.

It is probable that few viruses exert a direct effect on the kidney, but this may merely reflect the lack of appropriate investigations. The extent of asymptomatic infection is also unknown as postmortem studies are only likely to be conducted if macroscopic changes are evident, or if antemortem biochemical or physiological indicators are suggestive of renal pathology. However, indirect evidence of asymptomatic renal involvement can be found by the detection of whole virus or viral antigen in the urine.

Following certain infections, most notably with CMV, certain adenoviruses and human polyomaviruses [JC virus (JCV) and BK virus (BKV)] latency is established and re-activation as evidenced by excretion in the urine may occur at intervals throughout the patient's life. In an immunocompetent person, such re-activations lead only rarely to symptomatic infection, but, in immunosuppressed patients, such latent viruses may play a significant part in their morbidity and even mortality.

Certain viruses may cause renal damage indirectly. Such secondary mechanisms include hypovolemia following hemorrhage, or alteration in endothelial permeability, leading to oliguria, anuria and increased blood urea nitrogen (BUN), or immune-complex deposition in the glomerular basement membrane with subsequent alteration of glomerular function.

Table 2 Categorization of viruses affecting the kidney

Viruses causing direct damage
Hantaviruses
Arenaviruses (Lassa, Junin, Machupo, LCMV)
Flaviviruses
CMV
HIV
Viruses causing asymptomatic replication
Mumps
CMV
Viruses causing latent infection
CMV
Adenoviruses (11, 14, 34, 35 Subgenus B2)
Human polyomaviruses (BK, JCV)
Viruses causing secondary damage
Hypovolemia
Hantaviruses
Yellow fever
Immune-complex deposition
Hepatitis B
CMV
HIV?
Arenaviruses

Table 2 summarizes the viruses that fall into the above categories. However, as a virus may fall into more than one category, such divisions, although helpful, should not be treated as discrete entities.

CMV

CMV belongs to the family *Herpesviridae* and has a morphology by electron microscopy indistinguishable from the other members of the family. A detailed account of the viral properties and epidemiology can be found elsewhere in this volume. Like other herpesviruses it has the capacity to incorporate its viral DNA into host cells and can therefore establish a latent infection in salivary gland epithelium, lymphocytes, kidneys and probably other organs. Occasionally the virus re-activates and infectious virions can be found in the urine and/or saliva. Such re-activations are usually asymptomatic, thus allowing horizontal spread within the community. Indeed, in the immunocompetent patient, even primary CMV infection causes only minimal morbidity thus maximizing contact of the infected host with other susceptible

individuals. In primary infections, viruria provides evidence of renal involvement, cytomegalic cells being most pronounced in the proximal tubules of the cortex; these cells may also be found in the loop of Henle and collecting tubules. Infiltrates containing early and late CMV antigens are commonly seen and in severe disease immune complexes may be deposited in the glomeruli. Although kidney infection may be prolonged, it rarely results in renal dysfunction. However, CMV plays an important role in the morbidity and mortality of transplant patients. As the virus remains latent in the distal tubular epithelium, it is possible to transmit infection following transplantation. In patients who are seronegative prior to the operation, there is an estimated 70–80% acquisition of primary CMV if the donor organ is CMV positive but only 5% if the donor organ is CMV negative. However, there is also a degree of morbidity amongst seropositive recipients of seropositive kidneys. Between 60 and 95% experience either reactivation or re-infection following transplantation; restriction enzyme analysis may help to distinguish between these. The extent of the disease (e.g. pneumonitis, gastroenteritis, leukopenia, enhanced graft rejection) is in part governed by the degree of immunosuppression, and patients with a poor transplant history who receive additional treatment are at greater risk from CMV. In patients experiencing graft rejection there is a distinct glomerulopathy in the presence of CMV viremia, characterized by enlargement of endothelial cells, mononuclear cell accumulation and fibrillar material in the glomerular capillaries. It is not certain whether CMV is directly responsible for this pathology. Other possible mechanisms include immune-complex disease and immunological cross-reactivity (molecular mimicry) between CMV and HLA DR β chain which share sequence homology. Prophylaxis with acyclovir and more recently gancyclovir, which is currently reserved in the UK for seronegative recipients of seropositive kidneys and seropositive recipients of seropositive kidneys who are receiving additional immunosuppresive therapy, has shown a decrease in morbidity and mortality from CMV in these patients.

Hepatitis B

Details of the properties and epidemiology of hepatitis B can be found elsewhere in this volume.

Extrahepatic manifestations of viral hepatitis are seen in approximately 10–20% of patients, the pathogenesis of which is thought to be immune-complex-mediated. Glomerulonephritis and nephrosis associated with hepatitis B has been described in both adults and children in numerous studies. Children, in particular, may not exhibit liver disease and in some adults it may also be minimal or absent. Complexes of hepatitis B antigen and immunoglobulin have been demonstrated in the serum and glomeruli of patients with membranous nephropathy, thus supporting the hypothesis that the disease is immune-complex-mediated. A membranous glomerulonephropathy is more likely to develop in adults who acquire the disease in childhood and who are hepatitis e antigen positive. The prognosis in children tends to be good but, approximately 10% of adults are given maintenance dialysis and about one-third show poor progress despite attempts to treat with interferon.

HIV

A detailed account of the viral properties and epidemiology is given elsewhere in the Encyclopedia.

Patients with HIV infection can manifest a spectrum of potentially reversible forms of acute renal failure and a unique form of glomerulonephropathy. The entity of HIV-associated nephropathy was first described in 1984 and is increasingly being recognized. It is characterized by distinct clinical and pathological features thought to be caused by direct viral damage. It causes irreversible renal failure and this provides the additional problem of managing HIV patients on dialysis units.

A variety of renal lesions, particularly tubulointerstitial, have been found at postmortem. These include, for example, nephrocalcinosis and interstitial nephritis which are associated with an antemortem history of electrolyte disorders. Evidence of glomerular involvement in the form of focal segmental glomerulosclerosis has also been found. This is associated with heavy proteinuria in excess of 3.5 g per day and rapidly progressive renal failure. It is more commonly found in young black men, about half of which are intravenous drug users, the remainder belonging to other well-recognized risk groups.

Another postmortem finding is that of diffuse mesangial hyperplasia associated with deposits of IgM and C3. An immune-complex mechanism has been proposed but the antigen involved remains elusive. Such lesions are rarely associated with overt disease.

Adenoviruses

The family *Adenoviridae* comprises over 47 human serotypes which have been divided into six subgenera based on the sequence homology of their DNA. A further division of the subgenus B (B1, B2), based on differences in DNA-restriction sites, has identified

Table 3 Hantaviruses that cause human disease

Genus	*Host*	*Human disease*	*Geographical distribution*
Hantaan	*Apodemus agrarius* (field mouse)	Hemorrhagic fever with renal syndrome	Korea, E. USSR, Manchuria, China
Seoul	*Rattus* species (wild/lab)	Hemorrhagic fever	East Asia, world-wide, seaports
Puumala	*Clethrionomys glareolus* (bank vole)	Nephropathia epidemica	Scandinavia, Europe, W. USSR
Prospect Hill	*Microtus pennsylvanicus* (meadow vole)	Infection only	USA

four serotypes (11, 14, 34, 35) known to establish low-grade persistent infections of the kidney. It is likely that viral replication proceeds at a reduced rate in this situation, since, during acute infection, the adenovirus switches off expression of host mRNA leading to an unregulated production and consequent accumulation of viral protein in the cell nucleus, with destruction of normal cell function. These latent viruses are most commonly re-activated in immunosuppressed patients, such as those with AIDS, and in bone- and renal-transplant recipients. There have also been occasional reports of adenovirus 11 being transmitted during kidney transplantation leading to hemorrhagic cystitis in the recipients.

Human polyomaviruses

BKV and JCV are the two polyoma viruses (family *Papovaviridae*) known to infect man. These non-enveloped icosahedral double-stranded DNA viruses are antigenically distinct and exhibit minimal cross-reactivity in serological tests. BKV is acquired in early childhood, about 90% by 5 years, most people having antibodies that persist through adult life. The acquisition of JCV is less clear cut, with rates varying greatly among different populations. In the UK, the virus is acquired more slowly with only 5% of 5 year olds and 50–60% of adults showing evidence of infection. In the USA, initial acquisition is higher and there is a further increase after 50 years with the level reaching about 75%. Infection is thought to be transmitted via the respiratory route, with virus reaching target organs via the blood.

In the immunocompetent host, following primary infection, virus remains latent in the kidney, nucleotide sequences having been found in a number of kidneys at postmortem. Immunosuppression, particularly if it leads to a deficiency of T cells, can result in virus re-activation with excretion of virus in the urine. In renal-transplant recipients and pregnant women, both viruses re-activate frequently, but in bone-marrow-transplant recipients, BKV re-activation greatly exceeds that of JCV.

Some 25–45% of renal-transplant patients excrete virus in the urine postoperatively which may last from weeks to months. As with CMV, infection may be primary, or result in re-activation or re-infection. BKV is more likely to produce a re-activation/re-infection and JCV, a primary infection. However, unlike CMV these viruses mainly cause asymptomatic infection. If infection leads to renal dysfunction, it may be misdiagnosed as graft rejection and consequently immuno-suppressive therapy increased. This may result in the development of ureteric stenosis in the transplant recipient.

About 40–50% of bone-marrow-transplant patients have been found to excrete polyomavirus, mainly BKV, in the urine about 2–8 weeks after transplant. Again most infections are not associated with illness. However, late-onset BK viruria has been associated with late-onset hemorrhagic cystitis, the frequency being greater in autologous transplant patients.

Hantaviruses

The genus *Hantavirus* (family *Bunyaviridae*) has four members known to cause human disease, namely Hantaan, Seoul, Puumala and Prospect Hill virus. These segmented single-stranded negative-sense RNA viruses are enzootic in wild rodents and cause persistent asymptomatic infection with excretion in body fluids despite high titers of neutralizing antibodies. There is no evidence of vertical transmission in animals. Transmission to humans is by inhalation of aerosolized excreta. Although this therefore requires close contact, there is no evidence of person to person spread.

Table 3 summarizes the enzootic host, nature of human disease and geographical distribution of each genus.

Korean hemorrhagic fever (KHF) or hemorrhagic

fever with renal syndrome (HFRS) was first described in 1951 and is by far the most severe of the diseases. About 33% of those infected experience hemorrhagic manifestations, the remainder having an influenza-like illness. Some 10–15% experience hypovolemic shock and 5–10% die. Clinical features of a typical severe case include a sudden onset of high fever, myalgia and malaise which lasts approximately 3–7 days, followed by a relatively short hypotensive phase characterized by petechial hemorrhages, proteinuria and thrombocytopenia. About one-third of deaths occur as a result of hypovolemic shock at this stage. During the oliguric phase, which can last up to 1 week, a further 50% of deaths occur. The final phase of diuresis may last for weeks, with full recovery taking about 6 months.

Undoubtedly some of the renal damage is the result of hypovolemia but evidence of direct viral damage has also been found at the microscopic level. Renal tubules show evidence of congestion, desquamation and necrosis.

The Seoul virus produces less severe disease with a lower fatality rate than Hantaan virus infection.

Although nephropathia epidemica, an acute fever with renal involvement, has been known for years in Scandinavia, it has only recently been attributed to the Puumala virus. Again the disease is relatively mild with a mortality rate of less than 1%.

Prospect Hill virus, which is enzootic in meadow voles in the USA, very rarely produces human disease.

Colonized experimental rats may act as a reservoir of Hantaan virus and have been responsible for a number of outbreaks of HFRS among laboratory personnel in Japan, Korea and Belgium. Worldwide surveys have shown evidence of urban and laboratory rats infected with hantaviruses in parts of Asia, Europe and the Americas where as yet no disease has been reported, suggesting that such agents exist all over the northern hemisphere where rodents can be found. It may, therefore, be worth considering the diagnosis of hantavirus infection in cases of idiopathic glomerulonephritis.

Arenaviruses

These are a group of enveloped single-stranded RNA viruses, three of which cause hemorrhagic fever in man: Lassa in West Africa, Junin in Argentina and the Machupo virus in Bolivia. These viruses, like the hantaviruses, are also enzootic in wild rodents causing asymptomatic infection with persistent virus excretion in the urine. Spread to humans is via contact with the infected excreta, which may occur both in the natural setting, when rodents are caught for food, and in the laboratory. Cases of laboratory acquired infection, although few in number have been associated with considerable mortality. Although disease induced by these viruses is probably due to their direct cytolytic action, LCMV, another arenavirus, is known to exert its effects by immunologically mediated mechanisms.

The hemorrhagic arenaviruses produce a similar disease pattern in man, with an insidious onset of chills, malaise, headache, retroorbital pain, myalgia and nausea. This is followed by fever, conjunctival injection rash and edema of the face, neck and lower thorax. Hypotension with oliguria and bleeding from the gums and nose, hematemesis, hematuria and malena then develop. The oliguria may lead to anuria and profound neurological problems may also develop.

Evidence for direct viral replication and subsequent renal involvement is the development of renal tubular acidosis, indicative of tubular lesions. Viral antigens have also been demonstrated in tubular epithelium. Postmortem examination of the kidneys in Argentinian hemorrhagic fever shows patchy tubular and papillar necrosis together with other less-specific changes of generalized vasocongestion and multiple hemorrhages.

Glomerular function appears to be directly related to cardiac output and is reduced in consequence of the hypovolemia associated with changes in capillary endothelial cells. Despite the fall in serum Na^+ and K^+ concentrations, there is an increased excretion of these electrolytes producing hyperosmotic urine. This is due to an increased production of antidiuretic hormone (ADH), either autonomously or as a result of the hypovolemia.

There is little evidence to support the development of immune complexes during infection with the hemorrhagic fever arenaviruses, there being no glomerular deposition of immunoglobulin, complement (C3) or viral antigens, this being in sharp contrast to infection with LCMV. It would seem, therefore, that renal damage is the result of a combination of direct viral damage to the renal tubules and hypovolemia with consequent reduction in glomerular function.

Diagnosis

A variety of methods can be employed such as direct visualization of virus by electron microscopy, viral antigen detection by immunofluorescence, virus isolation and serology. Close consultation with laboratory personnel ensures that the appropriate specimens are collected and the right tests are carried out.

CMV can be isolated from a variety of specimens including urine, nasopharyngeal secretions, periph-

eral blood lymphocytes and others such as bronchoalveolar lavage and lung biopsies. Demonstration of a cytopathic effect in a human fibroblast cell line may take up to 3 weeks, which is inappropriately long if prompt treatment is needed. In renal-transplant patients, it may be critical to distinguish rapidly between graft rejection and acute CMV where management of immunosuppressive drugs would be diametrically opposed. In this situation, the detection of early antigen fluorescent foci (DEAFF) test can be employed which gives a result in 24 h. In this technique, samples of urine, peripheral blood lymphocytes and nasopharyngeal or bronchial washings are absorbed on to a monolayer of human fibroblasts (e.g. HELS), incubated overnight and the presence of early antigen detected by a series of specific antibodies, the last of which is labelled with fluorescein. CMV-specific nuclear antigen may then be demonstrated with the aid of a fluorescence microscope. Results should be interpreted with caution before the recommendation of specific treatment, as a positive urine or throat swab alone may be due to asymptomatic re-activation and not represent active disease. The finding of a positive result in peripheral blood lymphocytes is, however, highly significant.

Serological tests may also aid diagnosis, for example, demonstrating a rising complement-fixating titer (CFT) of antibodies to adenoviruses. In primary CMV infection, seroconversion can be demonstrated using a latex test or CFT, but, for re-activation/re-infection, it is necessary to demonstrate rising titers using CFT. In an immunocompetent patient, with the use of a sensitive and specific solid-phase radioimmunoassay, CMV-specific IgM can be detected in about 90% of primary infections but not in re-activation infections. However, in the immunosuppressed patient, results are less clear cut, with some patients with a primary infection failing to produce CMV-specific IgM and approximately one-third producing IgM antibodies during re-activation or re-infection.

Viruses Affecting the Thyroid

The effect of viruses on the thyroid is an area that has been underresearched; there are few reports of specific viruses being associated with thyroid disease.

Both acute and subacute thyroiditis have long been thought to have a viral etiology, but no specific association has been found despite numerous studies measuring broad spectra of viral antibodies in paired serum samples. However, an association between antibodies reactive with microsomal and/or thyroglobulin fractions of thyroid tissue has been found in 20–40% of congenital rubella sera. Thyroid function was found to be abnormal (showing both hypo- and hyperthyroidism) in 5% of all congenitally infected children being restricted to those with thyroid autoantibodies. The finding of rubella virus antigen in a biopsy of thyroid tissue from a 5-year-old boy with congenital rubella and Hashimoto's thyroiditis suggests that virus may persist in the tissue even though no virus is isolated. It is interesting to note that there is a certain degree of sequence homology between the N terminus of the C protein of rubella virus and human thyroglobulin, and it is therefore possible that antibodies result from molecular mimicry.

Although a study of HIV-positive children showed them to be euthyroid, approximately one-third of the patients had elevated baseline and peak thyrotropin levels in response to thyroid-releasing hormone (TRH), suggesting a state of compensated hypothyroidism. It is possible that suitable alterations in thyroid regulation may contribute to failure to thrive in some HIV-positive children. A study comparing adults with AIDS, asymptomatic HIV-positive patients and healthy controls showed that there was a decreased tri-iodothyronine uptake and free thyroid hormone in AIDS and HIV-positive patients in comparison with controls, this being inversely related to an elevated serum thyroid-binding globulin (TBG) in the same patients. There was no correlation between thyroid function and the T helper/suppressor cell ratio. It would appear that any viral effects on the thyroid gland are minimal, and overt disease is as yet undescribed.

See also: **Adenoviruses (*Adenoviridae*): General features; Coxsackieviruses (*Picornaviridae*); Cytomegaloviruses (*Herpesviridae*): General features (human); Enteroviruses (*Picornaviridae*): Human enteroviruses (serotypes 68–71); Hantaviruses (*Bunyaviridae*); Human immunodeficiency viruses (*Retroviridae*): General features; Mumps virus (*Paramyxoviridae*); Polyomaviruses – murine (*Papovaviridae*): General features; Rubella virus (*Togaviridae*).**

Further Reading

Melnick JL (1987) Insulin dependent (juvenile onset, type 1) diabetes mellitus–coxsackie B viruses revisited. In: Banatvala JE (ed.) *Progress in Medical Virology*, vol. 34, p. 33. Basel: Karger.

Muir P (1992) The association of enteroviruses with chronic heart disease. *Rev. Med. Virol.* 2: 9.

Schmidt NJ and Emmons RW (eds) (1989) *Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections*, 6th edn. New York: APHA.

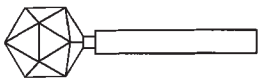
Schultheiss HP (ed.) (1988) *New Concepts in Viral Heart Disease: Virology, Immunology and Clinical Management*. Berlin: Springer-Verlag.

Wreghitt T, Craske J and Roome A (1991) Cytomegalovirus infections in transplant recipients. *PHLS Microbiol. Dig.* 8(4): 131.

[This article is reproduced from the 1st edn (1994).]

P

P2, 186 AND RELATED PHAGES (*MYOVIRIDAE*)



Ian B Dodd and **J Barry Egan**, Department of Biochemistry, University of Adelaide, Australia

Introduction

P2 was isolated by G. Bertani in 1951 from the Lisbonne and Carrère strain of *Escherichia coli*. 186 was isolated by F. Jacob and E. Wollman in 1961 from *E. coli* from human feces. HP1 was the first phage to be isolated from *Haemophilus influenzae* Rd.

P2, 186 and HP1 are the best characterized members of a large and widespread family of temperate double-stranded (ds) DNA bacteriophages. P2 can infect *E. coli, Shigella, Serratia, Klebsiella* and *Yersinia* species but the only known host for 186 is *E. coli* strain K12. Many phages serologically related to P2 have been isolated, with such phages common in natural environments. The genomes of P2, HP1 and 186 have now been completely sequenced and related DNA sequences have been obtained for phages PSP3 (*Salmonella*), K139 (*Vibrio cholerae*), ϕCTX (*Pseudomonas aeruginosa*) and S2, a close relative of HP1. The ϕR67 and ϕR86 defective prophages of *E. coli* are P2-related, as is a chromosomal DNA segment from *Haemophilus somnus*.

The available DNA sequences of the P2-like phages indicate that they are not closely related to other temperate and virulent phages. Thus, they continue to be useful alternative model systems for studying phage morphogenesis, DNA replication, the control of gene expression and site-specific recombination. A unique feature of these phages is their ability to serve as helpers for the P4 family of satellite bacteriophages. In addition, the ϕR67 and ϕR86 prophages contain different retrons (reverse transcriptase-producing elements) inserted at the same location in the prophage. The P2 family of phages may also be medically important; ϕCTX carries a gene for a mammalian cytotoxin that is expressed by the prophage.

Taxonomy and Classification

P2 and 186 virions are indistinguishable and have icosahedral heads of 62 nm diameter and 135 × 18 nm inflexible tails with a contractile sheath and multiple tail fibers of 40–50 nm. On the dubious criterion of virion structure they are classed with T4 and P1 in the *Myoviridae*. The HP1, K139 and ϕCTX virions are similar to P2 but their inclusion in the P2 family is based on DNA sequence similarity.

Genome Structure

The genomes of the P2 family are dsDNA of about 30–35 kb with cohesive ends. The P2 genome is 33 574 bp, 186 is 30 624 bp and HP1 is 32 355 bp.

To a first approximation, the genomes of P2, 186 and HP1 can be divided into early-expressed and late-expressed regions (**Fig. 1**). The late region encompasses two-thirds of the genome and contains the genes for construction of virus particles and cell lysis. The early region is concerned primarily with the choice between the lytic and lysogenic modes and with replication of the phage DNA. The majority of the genome is transcribed rightward. The juxtaposition of the early and late regions is the same in all three phages. However, the location of the cohesive end site (*cos*) is different in HP1 (**Fig. 1**).

The P2, 186 and HP1 genomes each contain 42 genes (**Table 1**). P2 and 186 share 32 homologous or known analogous genes whose order on the phage chromosome is completely conserved (**Fig. 1**). HP1 is less closely related, sharing only nine genes with P2 and 186 (**Table 1**).

Molecular Biology

Lytic versus lysogenic development

The temperate lifestyle involves two alternative modes of reproduction. The lytic cycle comprises infection, rapid DNA replication, virion construction, cell lysis and reinfection. In lysogeny, the P2 family

Table 1 P2 and 186 genes and their functions

Gene[a]			
P2	*186*	*HP1*	*Function (comment)*
Q	*orf2*	*orf15*	Capsid portal vertex
–	*W*		Unknown. (Homology to large terminase subunit)
P	*orf12*	*orf16*	Large terminase subunit
O	*V*	*orf17*	Capsid scaffold
N	*T*	*orf18*	Major capsid protein
M	*R*	*orf19*	Small terminase subunit
L	*Q*		Head completion
X	*orf23*		Tail
Y	*orf24*	*hol*[b]	Lysis – holin
K	*P*	*lys*[b]	Lysis – endolysin. (K and P homologous to λ R)
lysA	–		Lysis – timing
lysB	*orf27*		Lysis – timing
orf	*orf28*	*orf*	Unknown
R	*N*		Tail completion
S	*orf31*		Tail completion
orf30	–		Unknown
V	*orf32*		Tail spike
W	*M*		Baseplate? (Homology to T4 baseplate wedge)
J	*L*		Baseplate/tail fiber
I	*orf38*		Tail
H	*K*	*orf31*	Tail fiber. (Partial homologies with many phages)
G	*orf45*		Tail fiber assembly. (Partial homologies with many phages)
fun	–		Confers T5 resistance on lysogen
F_I	*J*	*orf23*[b]	Tail sheath
F_{II}	*I*	*orf24*[b]	Tail tube
E	*H*		Tail. (Programmed translational frameshift)
T	*G*		Tail
U	*F*		Tail
D	*D*		Tail
ogr	*B*		Late promoter activator. (Zinc finger DNA-binding protein)
–	*orf69*		Unknown – not essential
int	*int*	*int*	Integrase
C	*cI*	*cI*	Immunity repressor
cox[b]	*apl*[b]	*cox*[b]	Excision and transcriptional control
–	*cII*		Establishment of lysogeny. (Transcriptional activator)
orf78	–		Unknown. (Overexpression lethal)
–	*fil*		Inhibitor of cell division
–	*dhr*		Inhibitor of host replication – improves burst size
B	–		Replication. (DnaC analogue?)
–	*orf79*		Unknown – not essential
orf80	–		Unknown. (Overexpression lethal)
orf81	–		Unknown
orf82	*orf80*		Unknown. (Overexpression of Orf82 lethal)
–	*orf81*		Unknown – not essential
orf83	*orf83*		Unknown – not essential in 186
–	*orf84*		Unknown – not essential
A	*A*	*rep*	Replicase. (Rolling circle replication)
orf91	–		Unknown
tin	–		Makes lysogen resistant to T-even phages
old	–		Makes lysogen resistant to λ
–	*tum*		Prophage induction. (Antirepressor)
–	*orf97*		Unknown. (Overexpression inhibits 186 infection)

[a] All known P2 and 186 genes are listed, along with HP1 genes of known function.
[b] Analogous gene products show little or no homology.

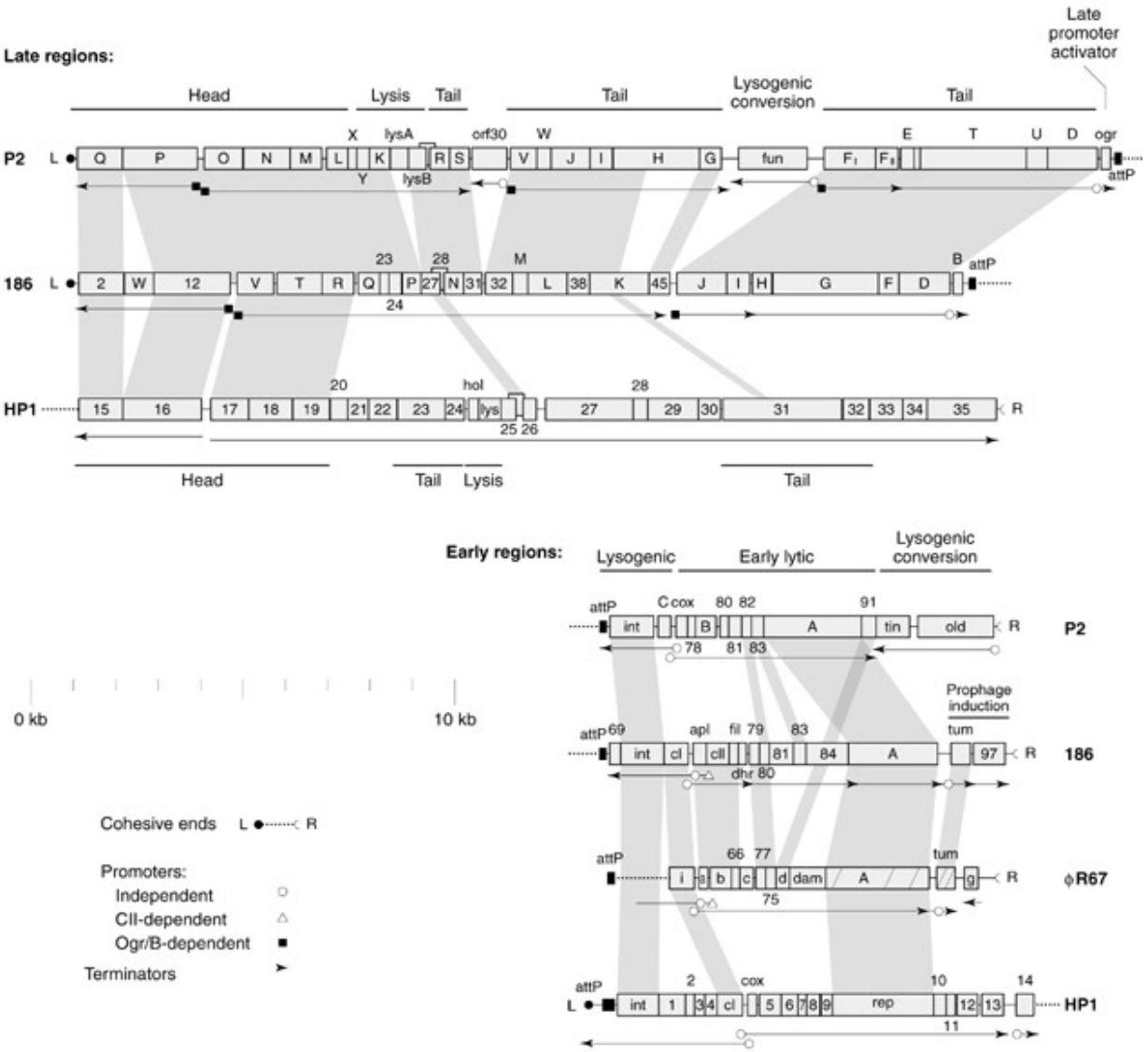


Figure 1 The genomes of P2, 186 and HP1. The late regions (left end of P2/186, right end of HP1) and early regions (right end of P2/186, left end of HP1) are aligned separately. The ϕR67 early region sequence is included. Genes are denoted by gray boxes. RNA transcripts are shown by lines with known or predicted transcriptional signals indicated (see key). Amino acid sequence homology is indicated by shading. A scale is included. Slashes within the ϕR67 *A* and *tum* genes indicate disruptive frameshift mutations. Coordinates are taken from the genome sequences of P2 (Genbank entry AF063097), 186 (Genbank entry B1U3222), HP1 (Genbank entry BHU24159) and the partial sequence of ϕR67 (Genbank entry ECO67RTDM).

prophages are integrated at specific sites on the bacterial chromosome by the Campbell mechanism, the lytic genes are repressed and the phage DNA is replicated passively.

The choice of reproductive mode is controlled by a transcriptional switch that controls whether the lytic or lysogenic genes are expressed and a recombinational switch that controls whether the phage DNA is in the integrated or excised state. These switches are encoded and coordinated in this phage family by a distinctive gene and promoter arrangement (**Fig. 2**). In common with many other temperate phages, there are divergent lysogenic and early lytic operons, with the first gene of the lysogenic operon (*cI* or *C*) encoding the immunity repressor, and the first gene of the lytic operon (*cox* or *apl*) encoding a repressor of lysogenic transcription. However, a unique set of three important features is shared by these phages.

1. The lytic and lysogenic promoters are arranged face-to-face, their transcripts overlapping by 40–70 bases.

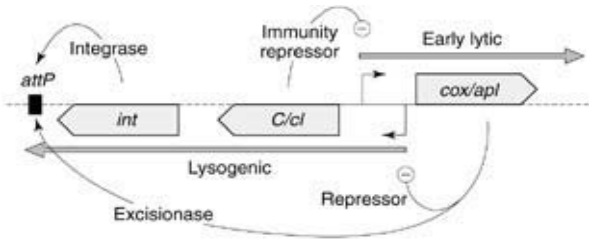


Figure 2 Lytic–lysogenic control features shared by P2, 186 and HP1. Promoters are shown as kinked arrows and their transcripts as thick gray arrows. Genes are indicated as gray boxes and the activities of their products by thin lines.

2. The integrase gene (*int*) is located in the lysogenic operon.
3. The Cox and Apl proteins combine their repressor role with an excisionase function.

Features 2 and 3 simplify the coordination of the transcriptional and recombinational switches in comparison to λ but the face-to-face promoter arrangement leads to some mechanistic complexities in the transcriptional switch.

Lysogeny

Maintenance of lysogeny The CI or C proteins enforce the lysogenic transcriptional state, binding to and repressing the early lytic promoter (**Fig. 3**) and allowing the transcription of the lysogenic operon. 186 CI also binds to sites 350 bp upstream and downstream of the lytic promoter, providing a marginal improvement in repression, and binds at the promoter of the late control gene (see below). The P2, HP1 and φR67 immunity repressors contain recognizable helix-turn-helix DNA-binding (HTH) motifs at their N-termini; a similar location of an HTH-like motif in 186 CI is supported by its general homology with the HP1 and φR67 repressors. 186 CI has been shown to self associate, to bind DNA cooperatively and to recognize two different DNA sequences.

The immunity repressors stimulate their own synthesis in lysogeny by at least one of two indirect mechanisms. First, by their repression of lytic transcription they block the expression of the Cox/Apl proteins, which would otherwise repress lysogenic transcription. Secondly, the P2 and 186 immunity repressors can stimulate transcription from the lysogenic promoter up to 10-fold even in the absence of Cox/Apl, by relieving the lysogenic promoter from inhibition by the convergent lytic promoter. The activity of the strong lytic promoter inhibits lysogenic transcription some 10–20-fold by an uncharacterized mechanism. Negative transcriptional autoregulation

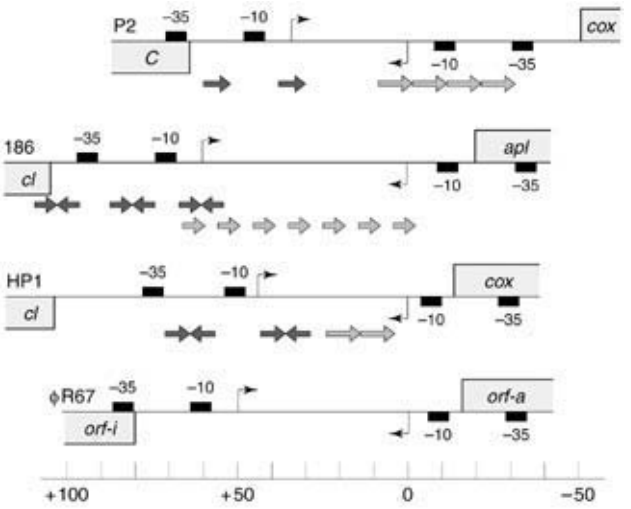


Figure 3 The lytic–lysogenic promoter regions of P2, 186, HP1 and φR67. The known or predicted transcription start points of the face-to-face early lytic and lysogenic promoters are indicated by bent arrows. HP1 has a second, weaker, early lytic promoter 28 bp upstream of the major promoter. The sequences are aligned at their lysogenic promoters to highlight the different spacings of the convergent promoters. The *C/cI* and *cox/apl* genes are shown as gray boxes. Known or predicted DNA recognition sequences for the immunity repressor are depicted as dark gray arrows; Cox/Apl binding sites by light gray arrows.

at high concentrations of the immunity repressor is also apparent in all three phages.

The repression of the lytic operon in lysogeny means that the excisionase (Cox or Apl) is not present and the prophage remains integrated. The *int* gene is transcribed in lysogeny and a 186 prophage produces detectable Int activity. However, a P2 prophage does not produce active Int, due at least in part to post-transcriptional effects. Of unknown significance is the location of *attP* downstream of *int* in the lysogenic operon, which means that the 3′ end of the *int* mRNA changes on integration and may also be affected by Int binding at *att*.

In 186 and HP1 there are additional genes in the lysogenic operon whose expression may confer properties on the lysogen. P2 contains three known lysogenic conversion genes but these are not located in the lysogenic operon and lie instead to the right of the early lytic operon or in the late region. The *old* gene provides resistance of λ infection, *tin* blocks T-even phage infection and *fun* inhibits T5. These genes comprise some 12% of the P2 genome and appear from their high AT content to be recently acquired by P2 from a foreign genome. The HP1 *orf14* gene may be expressed from its own promoter in a lysogen.

Establishment of lysogeny The transition from

lytic to lysogenic development requires establishment of the lysogenic transcriptional state and integration of the phage DNA. The locations of the immunity repressor, integrase and excisionase genes mean that both these requirements can be met simply by an increase in lysogenic transcription and a simultaneous or subsequent decrease in lytic transcription. In 186, like λ, the establishment of lysogeny requires a lytic gene, *cII*, whose product activates transcription of the lysogenic operon from a promoter upstream of the lysogenic promoter. CII binds upstream of this promoter to inverted repeat sequences two turns of the helix apart. φR67 has a *cII* homologue and almost identical CII binding sequences. In contrast, P2 has no *cII* gene and there is no *cII*-like gene apparent in HP1. Presumably, in these latter phages, the choice of lytic versus lysogenic development depends on the relative expression of the two early operons soon after infection. In the absence of phage proteins the strengths of the HP1 lysogenic and lytic promoters are very similar so each choice should occur with a reasonable frequency. However, in P2, like 186, the lysogenic promoter is much weaker than the lytic promoter so it is intriguing that P2 can establish lysogeny without a CII.

No λ-like effects of host physiology or multiplicity of infection on the establishment of lysogeny have been described for P2, 186 or HP1 (except for the SOS system, see below). The Hfl protease mutations that influence λ CII do not affect 186. It has been reported that IHF is needed for the establishment or maintenance of the lysogenic transcriptional state in P2 and binds upstream of the lytic promoter. However, a role for this site is yet to be demonstrated; a similarly located IHF binding site in 186 does not affect phage development.

Prophage induction The transition from lysogeny to lytic development is termed prophage induction. This process represents a major functional division in the P2 family, with P2 and other family members forming non-inducible prophages while 186, HP1 and PSP3 prophages are SOS-inducible. Prophage induction requires two steps: derepression of lytic transcription and excision of the prophage. In P2, the first step is unaffected by SOS induction and the second step is naturally inefficient.

Derepression of the 186 prophage is initiated in response to SOS induction by expression of a phage antirepressor gene, *tum*. The *tum* gene is located to the right of the early lytic operon and is normally repressed by LexA. Tum is a reversible inhibitor of CI DNA binding but does not bind to the DNA itself. φR67 contains a remnant of a *tum*-like gene. SOS induction of HP1 is puzzling, since no *tum* homologue or other LexA-controlled gene is apparent and the CI protein lacks sequences characteristic of RecA-stimulated autoproteolysis.

Derepression of lytic transcription makes Apl/Cox available to catalyze prophage excision. Sufficient Int to carry out the excisive reaction may already be present in the lysogen due to its expression from the lysogenic transcript. However, P2 and HP1 excisive reactions *in vitro* require high levels of Int, so an increase in *int* expression may be needed. No *int*-specific promoters have been detected. The low level of Int production from the P2 prophage is responsible for the inefficient excision of P2.

Repression of the lysogenic promoter is a second role of Apl during 186 prophage induction. Tum inhibition of CI is inefficient and Apl seems to assist the transition to the lytic transcriptional state in the presence of low levels of CI. Whether Cox plays a similar role in P2 and HP1 is not known. All three proteins bind to directly repeated DNA sequences spaced one turn of the DNA helix apart that overlap the RNA polymerase (RNAP) binding region at the lysogenic promoter (**Fig. 3**) and may thus assist derepression during prophage induction by blocking further transcription of the immunity repressor gene. Since the CI and Apl binding sites overlap, Apl may also interfere directly with CI repression of the lytic promoter. 186 Apl is monomeric in solution, binds cooperatively and bends the DNA severely. P2 Cox also appears to bind cooperatively. HP1 Cox is tetrameric in solution. The Cox/Apl proteins each contain an obvious HTH motif but show little similarity outside these motifs.

Site-specific recombination The HP1 and P2 integration and excision reactions are among the better characterized phage recombination systems. The recombination mechanism and its control conform broadly to the λ model but show specific differences in the arrangement of protein binding sites at *attP* (**Fig. 4**). The Int proteins are related to the λ family of integrases and, like λ Int, bind to two distinct DNA sequences. The *attB* sites are simple, comprising a pair of inverted core-type Int binding sequences disposed about the short overlap or strand exchange region. The HP1 and 186 *attB* sites, but not the P2 *attB* site, are located in tRNA genes and the overlap regions are the 7 bp tRNA anticodon loop sequences. The overlap regions in *attP* are flanked by additional arm-type Int binding sites. In HP1 *attP* there are also additional core-type Int binding sequences in the arm regions. The three Ints share strong amino acid similarity and the DNA recognition sequences of the HP1 and 186 Ints are related.

The host DNA-bending factor IHF is required for

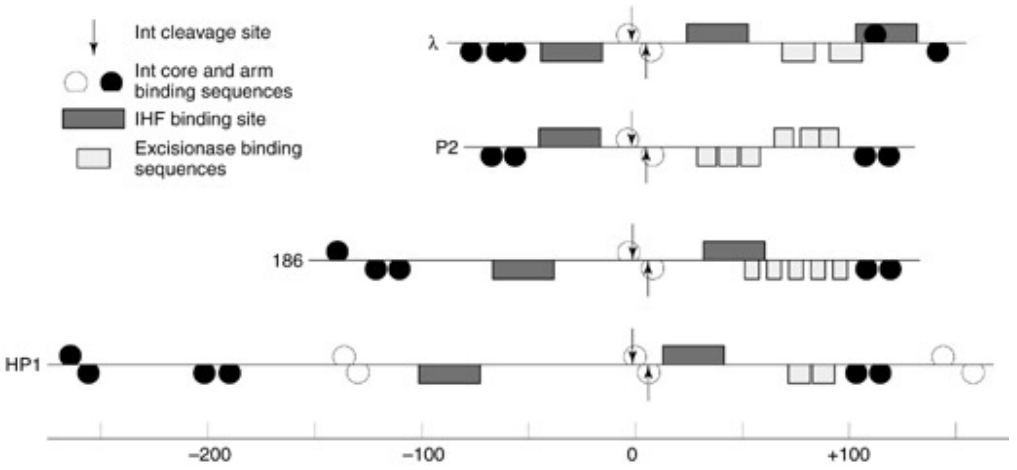


Figure 4 Comparison of the *attP* sites of λ, P2, 186 and HP1. The locations of Int, IHF and excisionase binding sequences are indicated above or below the DNA strands to indicate their orientation. The λ *att* site is shown P′OP and the Fis binding site is omitted. The Int binding sites for 186 and the Int cleavage sites for P2 have not been confirmed.

efficient integration and excision *in vitro* and binds between the exchange site and the Int arm sites in each *attP* arm except for the P2 right arm. Unlike λ, the host protein Fis does not seem to affect P2 or HP1 excision.

In P2, HP1 and 186, as in λ, the excisionase protein binds in one arm to a cluster of direct repeat sequences (**Fig. 4**). The 186 Apl and P2 Cox proteins bind cooperatively and appear to bend the DNA. HP1 Cox binds to *attP* with 4:1 and 8:1 stoichiometries. Like the lambdoid excisionases, Cox and Apl are thought to work by altering the spatial orientation and accessibility of the Int sites in the *att* arm and thus to affect the way that Int bridges between its core and arm sites. As well as stimulating the excisive reaction *in vitro*, the Cox proteins also inhibit the integrative reaction. The Int binding site adjacent to the HP1 Cox sites is needed for integration and inhibits excision *in vitro*; Cox binding interferes with Int binding to this site.

Lytic cycle

Early lytic transcription Upon cell entry and before phage proteins are made, transcription from the early lytic promoter in P2 and 186 is much stronger than lysogenic transcription. The lysogenic promoters are intrinsically weak and are inhibited even further by the active lytic promoter. Any reciprocal inhibition, of the lytic promoter by the lysogenic promoter, does not seem to be significant in these phages. In contrast, the HP1 lysogenic promoter is as strong as the tandem lytic promoters and each inhibits the other some 10-fold.

HP1 Cox repression of the lysogenic promoter may therefore be needed to prevent CI being produced and blocking lytic development but HP1 *cox*$^-$ mutants have not yet been studied in infection. 186 Apl and P2 Cox also repress the lysogenic promoter but mutants in these genes are neither impaired in lytic development after infection nor lysogenize at high frequencies. Presumably, the weakness of lysogenic transcription in the absence of the immunity repressor makes P2 Cox and Apl redundant for lytic development after infection. P2 Cox and 186 Apl, like λ Cro, also negatively regulate early lytic transcription. However, unlike λ Cro, this activity is not essential for lytic development. HP1 Cox does not appear to depress lytic transcription.

The early lytic operons of P2, 186, HP1 and φR67 contain 9–11 genes. The essential replicase gene, *A/rep* (see below), is the only clearly homologous gene in all four phages. The replicase gene in the φR67 prophage is disrupted. Most of the early genes have unknown functions and are not essential. P2 contains a second replication gene, *B*, that is dispensable in some conditions (see below). Both 186 and P2 carry a number of cell-lethal genes (**Table 1**); the 186 genes *dhr* and *fil* inhibit host DNA replication and cell division, respectively.

Early lytic transcription has not been studied in P2 or HP1. In 186, the initial transcript is antiterminated at a Rho-independent termination signal downstream of *dhr* by a mechanism unknown. It is not like the N system of λ, since 186 efficiently infects each class of *nus* mutants. It appears also that antitermination does not require any known 186 protein. The extended transcript is processed by RNAse III, cutting at a site

within the *fil-dhr* region. The *A* gene has no recognizable ribosome binding site and its translation requires translation of the preceding *orf84*, the stop codon of which overlaps the initiation codon of *A*. In most cases early lytic transcription terminates within the start of *A* but we expect that, on those occasions that translation of A is initiated, this termination will be suppressed and RNAP will continue to the end of *A* (**Fig. 1**).

Replication P2 and 186 replication is via a rolling circle (RCR) mechanism similar to ϕX174. Replication is initiated by an A protein-catalyzed single-strand endonucleolytic cut at an origin within the *A* gene and proceeds rightward. The P2 A cleavage site has been identified and similar sequences are conserved in 186 and HP1. The P2 A, 186 A and HP1 Rep proteins show large patches of strong amino acid identity, and share the three amino acid motifs characteristic of RCR proteins. The HP1 *rep* gene is sufficient for minichromosome replication but no further replication studies have been performed.

P2 replication requires also the product of the phage gene *B*, and host functions such as DnaB, DnaE, DnaG and Rep, but not the host initiation functions DnaA and DnaC. P2 B is involved in priming lagging strand synthesis, apparently interacting with DnaB and thus presumably acting as a DnaC analogue. However, B is not required in a *lon* host or in co-infection with P4 or when replicating as a minichromosome, and it would be interesting to know whether a B^- mutant requires DnaC under such circumstances.

Replication of 186 DNA is similar to that of P2, but there is no 186 analogue of P2 B. Accordingly, 186 replication has a requirement for host DnaC. Phage 186 replication also requires, indirectly, host DnaA, which reflects a need for concomitant host initiation – thus 186 replication can occur in an integratively suppressed *dnaA* mutant. Like P2, 186 replication requires host *rep* function and, interestingly, *rep* mutants that block 186 replication but not P2 replication are known. Further mutation in gene *A* can overcome this block, suggesting some interaction between Rep and the A replicase. Phage 186 replication is transiently blocked in a host cell UV irradiated before phage infection, which possibly reflects its dependence on concomitant initiation of host replication, which is itself transiently inhibited by UV.

Late gene transcription The expression of the late genes in P2 and 186 requires an activator of the late promoters, Ogr or B. These proteins are members of a family of small, DNA-binding, zinc-finger transcriptional activators found in P2-like phages and their P4-group satellites. The metal ion is coordinated by four cysteines with the arrangement $C\text{-}X_2\text{-}C\text{-}X_{22}\text{-}C\text{-}X_4\text{-}C$.

The *ogr* and *B* genes are expressed during early lytic development from their own, factor-independent promoters (**Fig. 1**). The 186 *B* gene is directly repressed by the immunity repressor and is transcribed coordinately with the early lytic operon. In contrast, the promoter of the *ogr* gene is weak and not under immunity control. Activation of the late promoters does not occur until the end of early lytic development and is dependent on phage replication. Thus, increased gene dosage of the activator gene or of the late genes seems to be required.

Ogr, B and other members of the Ogr family activate the σ^{70}-dependent phage late promoters by binding to a dyad-like DNA element centered near the -55 position. The Ogr protein interacts with the carboxyl domain (at least) of the α subunit of RNAP and the interacting regions have been mapped on both proteins. Four late promoters have been identified in P2, whereas 186 may be able to transcribe its late genes with only three promoters.

HP1 does not encode an Ogr homologue and no late control protein has yet been identified, although its existence is suggested by the presence of two oppositely oriented 28 bp conserved sequence elements in the region between the divergent late genes *orf16* and *orf17*.

Late gene function: morphogenesis and lysis The construction of active phage particles and cell lysis have been reasonably well characterized in P2. All but two of the 26 late genes of 186 have homologs in P2, whereas HP1 shares only six late genes with P2 and 186.

Capsid/packaging Construction of DNA-filled P2 capsids requires six gene products. The majority of the capsid is formed from the N protein with the aid of an internal capsid scaffold protein, O. N and O are proteolytically processed to shorter forms during capsid maturation. Q forms the portal vertex in the capsid to allow DNA entry. The DNA is packaged from monomeric circles and requires a 125 bp region around *cos*. Cutting and packaging is carried out by the terminase proteins M and P in the presence of proheads and ATP but does not require IHF. Phage 186 encodes an extra protein, W, that is homologous to the central region of P/Orf12 and which may modulate terminase activity. The L protein completes the head. HP1 encodes homologues of all the capsid proteins except L, suggesting that HP1 may require a different protein to complete a P2-like head to suit its nonhomologous tail. Despite the similarity of the terminase proteins, the HP1 *cos* site sequence is unlike

the *cos* sites of P2 and 186 and is cut to give a 7 base rather than a 19 base 5′ extension.

Tail P2 tails are constructed using 15 phage genes and contain six tail fibers attached to a baseplate with a single spike, a tube with contractile sheath and a collar involved in head attachment. Tail construction is not well understood; the functions of a few tail genes are given in **Table 1.** The P2 *E* gene (and probably 186 *H*) produces two proteins by a programmed translational frameshift. The P2 baseplate protein W and 186 M show similarity to a T4 baseplate wedge protein. The C-terminus of the tail fiber protein, presumed to be involved in specifying host attachment during infection, is different in P2 and 186 and shares short motifs with various other phages, including λ, P1, Mu, T2, T3, T7 and HP1. Apart from this short region in HP1 Orf31, the tail genes of HP1 show no significant homology with 186 or P2 and the gene order is different. Nevertheless, the HP1 tail has a similar structure to those of P2 and 186.

Lysis Lysis follows the λ strategy, with a phage holin protein forming holes in the inner membrane to allow a phage lysozymal enzyme to access and degrade the cell wall. The P2 and 186 lysis proteins are homologous, with the endolysin homologous to λ R, but are not similar to the HP1 lysis proteins. The P2 *lysA* and *lysB* genes affect the timing of lysis. Despite the general lack of homology with HP1 in this region, a short gene of unknown function located downstream of the lysis genes is conserved in P2, 186 and HP1.

Diversity in the P2 Family

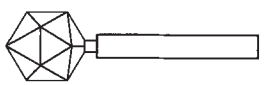
As well as providing a useful contrast with other well-studied temperate phage systems, the P2 family phages display a considerable diversity within their own group. HP1 is a chimera of sequences that are strongly related to P2/186 and sequences that are completely different. Even P2 and 186, although closely related, have evolved remarkably different lifestyles within the temperate phage niche. The P2 prophage takes up almost permanent residence in the host chromosome and makes a large commitment to fortifying this state. In comparison, 186 is nomadic; it devotes little of its genome to protecting its lysogenic home and breaks camp as soon as danger threatens. The near future promises to reveal further diversity as more relatives are characterized.

See also: **Coliphage lambda (*Siphoviridae*); Lysogeny and prophage; Nonoccluded baculoviruses; T4-like phages (*Myoviridae*).**

Further Reading

Bertani LE and Six EW (1988) The P2-like bacteriophages and their parasite P4. In: Calendar R (ed.) *The Bacteriophages*, vol. 2, p. 73. New York: Plenum Press.

P4 PHAGE (SATELLITES)



Gianni Dehò and **Daniela Ghisotti**, Dipartimento di Genetica e di Biologia dei Microrganismi, Università degli Studi di Milano, Milano, Italy

The Satellite Phage and its Alternative Life Styles

P4 is a genetic element at the crossroad of plasmids and viruses. A P4 virion is made of a linear, double-stranded, cohesive-ended DNA molecule 11.6 kb long, encapsidated into a tailed icosahedral protein head (**Fig. 1**). What is peculiar is that all its tail and most capsid proteins are not encoded by the P4 genome but by a helper phage, P2. As a consequence, P4 may undergo lytic cycle only if a helper phage genome is present in the host *Escherichia coli*. Discovered by E. Six in 1963, bacteriophage P4 is the prototype of 'satellite viruses'.

P4 requires all the helper's genes necessary for the construction of the head and the tail and to lyse the host cell (P2 late genes). Notably, a specific P4-encoded function directs the assembly of the head proteins produced by P2 into a small-size head suited to encapsidate the P4 DNA molecule, which is one-third the length of the helper's genome.

In the absence of a helper phage, the P4 genome may be maintained as a freely replicating multicopy plasmid. Moreover, both in the presence and in the

the *cos* sites of P2 and 186 and is cut to give a 7 base rather than a 19 base 5′ extension.

Tail P2 tails are constructed using 15 phage genes and contain six tail fibers attached to a baseplate with a single spike, a tube with contractile sheath and a collar involved in head attachment. Tail construction is not well understood; the functions of a few tail genes are given in **Table 1**. The P2 *E* gene (and probably 186 *H*) produces two proteins by a programmed translational frameshift. The P2 baseplate protein W and 186 M show similarity to a T4 baseplate wedge protein. The C-terminus of the tail fiber protein, presumed to be involved in specifying host attachment during infection, is different in P2 and 186 and shares short motifs with various other phages, including λ, P1, Mu, T2, T3, T7 and HP1. Apart from this short region in HP1 Orf31, the tail genes of HP1 show no significant homology with 186 or P2 and the gene order is different. Nevertheless, the HP1 tail has a similar structure to those of P2 and 186.

Lysis Lysis follows the λ strategy, with a phage holin protein forming holes in the inner membrane to allow a phage lysozymal enzyme to access and degrade the cell wall. The P2 and 186 lysis proteins are homologous, with the endolysin homologous to λ R, but are not similar to the HP1 lysis proteins. The P2 *lysA* and *lysB* genes affect the timing of lysis. Despite the general lack of homology with HP1 in this region, a short gene of unknown function located downstream of the lysis genes is conserved in P2, 186 and HP1.

Diversity in the P2 Family

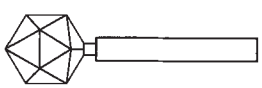
As well as providing a useful contrast with other well-studied temperate phage systems, the P2 family phages display a considerable diversity within their own group. HP1 is a chimera of sequences that are strongly related to P2/186 and sequences that are completely different. Even P2 and 186, although closely related, have evolved remarkably different lifestyles within the temperate phage niche. The P2 prophage takes up almost permanent residence in the host chromosome and makes a large commitment to fortifying this state. In comparison, 186 is nomadic; it devotes little of its genome to protecting its lysogenic home and breaks camp as soon as danger threatens. The near future promises to reveal further diversity as more relatives are characterized.

See also: **Coliphage lambda (*Siphoviridae*); Lysogeny and prophage; Nonoccluded baculoviruses; T4-like phages (*Myoviridae*).**

Further Reading

Bertani LE and Six EW (1988) The P2-like bacteriophages and their parasite P4. In: Calendar R (ed.) *The Bacteriophages*, vol. 2, p. 73. New York: Plenum Press.

P4 PHAGE (SATELLITES)



Gianni Dehò and **Daniela Ghisotti**, Dipartimento di Genetica e di Biologia dei Microrganismi, Università degli Studi di Milano, Milano, Italy

The Satellite Phage and its Alternative Life Styles

P4 is a genetic element at the crossroad of plasmids and viruses. A P4 virion is made of a linear, double-stranded, cohesive-ended DNA molecule 11.6 kb long, encapsidated into a tailed icosahedral protein head (**Fig. 1**). What is peculiar is that all its tail and most capsid proteins are not encoded by the P4 genome but by a helper phage, P2. As a consequence, P4 may undergo lytic cycle only if a helper phage genome is present in the host *Escherichia coli*. Discovered by E. Six in 1963, bacteriophage P4 is the prototype of 'satellite viruses'.

P4 requires all the helper's genes necessary for the construction of the head and the tail and to lyse the host cell (P2 late genes). Notably, a specific P4-encoded function directs the assembly of the head proteins produced by P2 into a small-size head suited to encapsidate the P4 DNA molecule, which is one-third the length of the helper's genome.

In the absence of a helper phage, the P4 genome may be maintained as a freely replicating multicopy plasmid. Moreover, both in the presence and in the

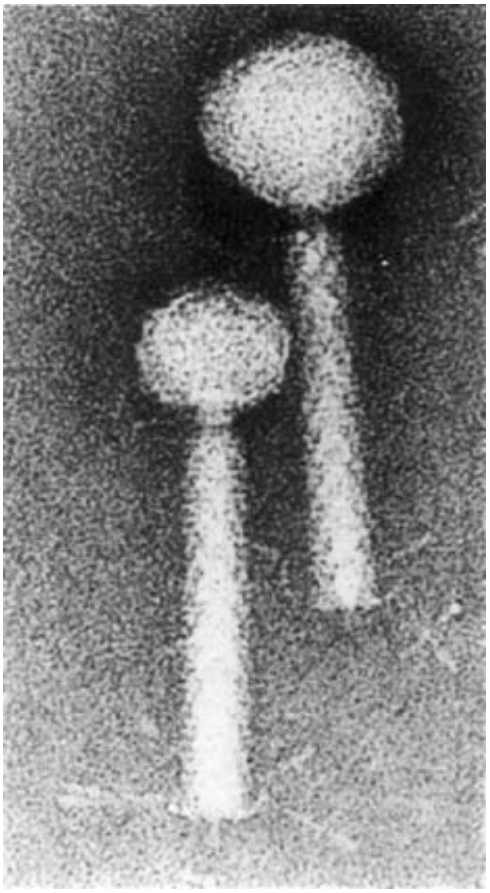


Figure 1 Satellite bacteriophage P4 (left) and its helper P2 (right) virions. Negative stain with phosphotungstate. The tail structures are about 135 nm long; the P4 and P2 heads are, respectively, 45 nm and 62 nm in diameter. Electron micrograph by Robley C. Williams, Unversity of California, Berkeley. (Reproduced with permission from Goldstein R, Lengyel J, Pruss G, Barrett K, Calendar R and Six E (1974) *Curr. Topics Microbiol. Immunol.* 68: 59.)

absence of the helper, P4 may lysogenize the host cell by integrating its genome into the bacterial chromosome and establishing prophage immunity. The alternative life styles of P4 are illustrated in **Fig. 2.**

By no means may P4 be considered a 'defective P2', since the two replicons are unrelated to each other. Rather, P4 may be thought of as a plasmid that evolved the ability to be specifically transduced by a class of bacteriophages, thus acquiring the potential for horizontal propagation.

Like P4, other phages such as the filamentous phages and the temperate phages P1 and N15, may be stably maintained as autonomously replicating plasmids in the bacterial host. These natural phasmids (phage–plasmid) make less definite the boundary between plasmids and viruses. Understanding the life cycle of P4 and other viruses that can establish a permanent interaction with the host cell, such as temperate phages and retroviruses, has been instrumental in developing a different perception of the biologic meaning of viruses. No longer can these genetic elements be considered merely as 'infectious agents' but, rather, as part of a large pool of mobile genetic information that can be exchanged among living cells.

Genome Organization

The genome organization of P4 is shown in **Fig. 3A** and the known genes and functions are detailed in **Table 1.** All the functions required for lysogenic, lytic and plasmid development are located in the right 80% of the genome and include the origin of replication *ori*1, the two main α and *sid* operons, which are transcribed divergently from the *ori*1 site, the prophage integration *att* site, and the *int* (integrase) gene in a monocistronic operon at the left of *att*. The genes in the two constitutively expressed operons at the left of *int* (nonessential region) may be deleted without affecting the lysogenic, lytic or plasmid development.

The α operon, which is transcribed leftwards from the two promoters P_{LE} and P_{LL}, encodes both genes required for P4 lytic and plasmid propagation (α and *cnr*, replication; ϵ, helper prophage derepression), and the prophage immunity determinants required to prevent P4 replication in the lysogenic condition. Thus control of the α operon expression from its two promoters is crucial for the establishment and maintenance of the different developmental phases and involves complex and elegant mechanisms (see below).

The *sid* operon codes for regulatory and morphogenetic proteins involved in the plasmid and/or lytic development: the positive regulator Delta, which can both activate the satellite and *trans*-activate the helper late operons (*bona fide trans*-activation, i.e. the activation of unexpressed genes of one chromosome by the gene product(s) of a different chromosome, was first described in the P2-P4 system); and two proteins with a role in P4 head morphogenesis: Sid, which determines the small size of the P4 capsid, and Psu, a bifunctional protein that helps to stabilize the viral particle (capsid 'decoration' protein) and suppresses transcription termination at Rho-dependent terminators (polarity suppressor). The *sid* operon is transcribed late after infection from its unique P_{sid} promoter that is activated by P4 (Delta) and P2 (Ogr)-encoded positive regulators.

DNA Replication

P4 replicates as a circular DNA molecule in a bi-

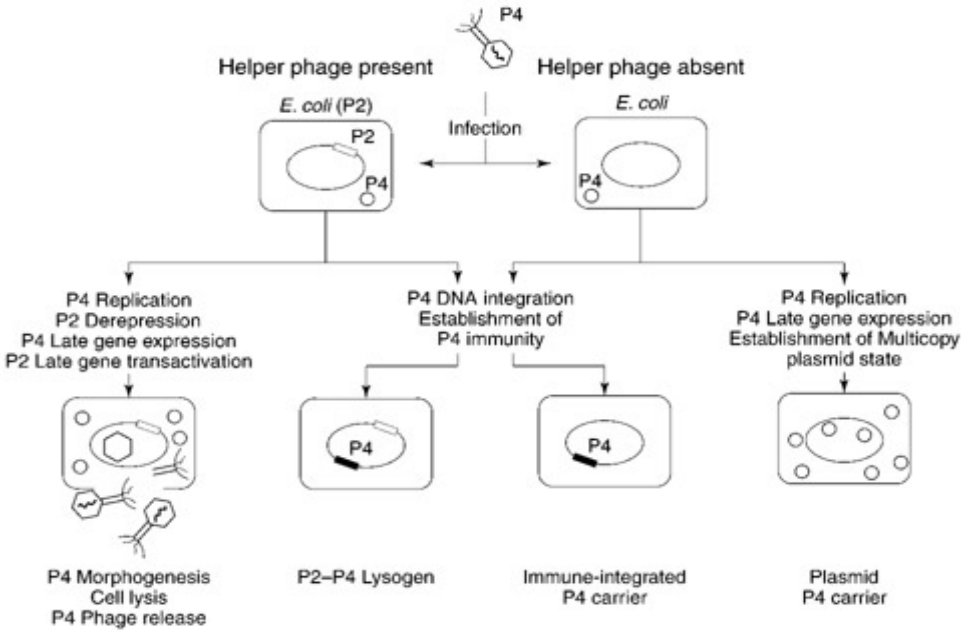


Figure 2 P4 life cycle. See the text for explanation. (Modified from Dehò G, Bertoni G and Polissi A (1992) In: Galli E, Silver S and Witholt B (eds) *Pseudomonas Molecular Biology and Biotechnology*, p. 358. Washington DC: American Society for Microbiology.)

Table 1 Bacteriophage P4 genes and functions

Gene or site	*Gene product and/or function encoded*
cos	19 nt long cohesive ends
gop	Causes host cell killing in the absence of β
β	Inhibits *gop* killing
*c*II	Function unknown. Mutants kill the host cell
int	Integrase
att	Site for integrative recombination
crr	Required in *cis* for DNA replication
α	Essential for replication. Primase, helicase, *ori*1 and *crr* recognition and binding
*ori*2	With *crr* supports α-dependent *ori*1-independent replication
cnr	Controls DNA replication and plasmid copy number
*orf*151	Function unknown
ϵ	Derepression of the P2 helper prophage
kil	Kills the bacterial host if overexpressed
t_{imm}	Rho-dependent transcription termination. Elicits strong transcription termination from P_{LE} when the CI RNA is present
*c*I	Prophage immunity. Encodes the CI RNA
P_{LE}	Constitutive promoter
*orf*199	Function unknown. Its translation prevents transcription termination from P_{LL}
vis	Negative regulator of P_{LL}; stimulates P_{sid}; binds P_{LL}, P_{sid}, and *att*; putative excisionase
P_{LL}	Late promoter. Positively regulated by P4 Delta and P2 Ogr and Cox proteins; negatively regulated by Vis
*ori*1	Origin of DNA replication
P_{sid}	Late promoter. Positively regulated by P4 Delta and P2 Ogr, stimulated by Vis
sid	Small head determination. External procapsid scaffold.
δ	P4 and P2 late promoter activator
psu	Capsid decoration protein and polarity suppression

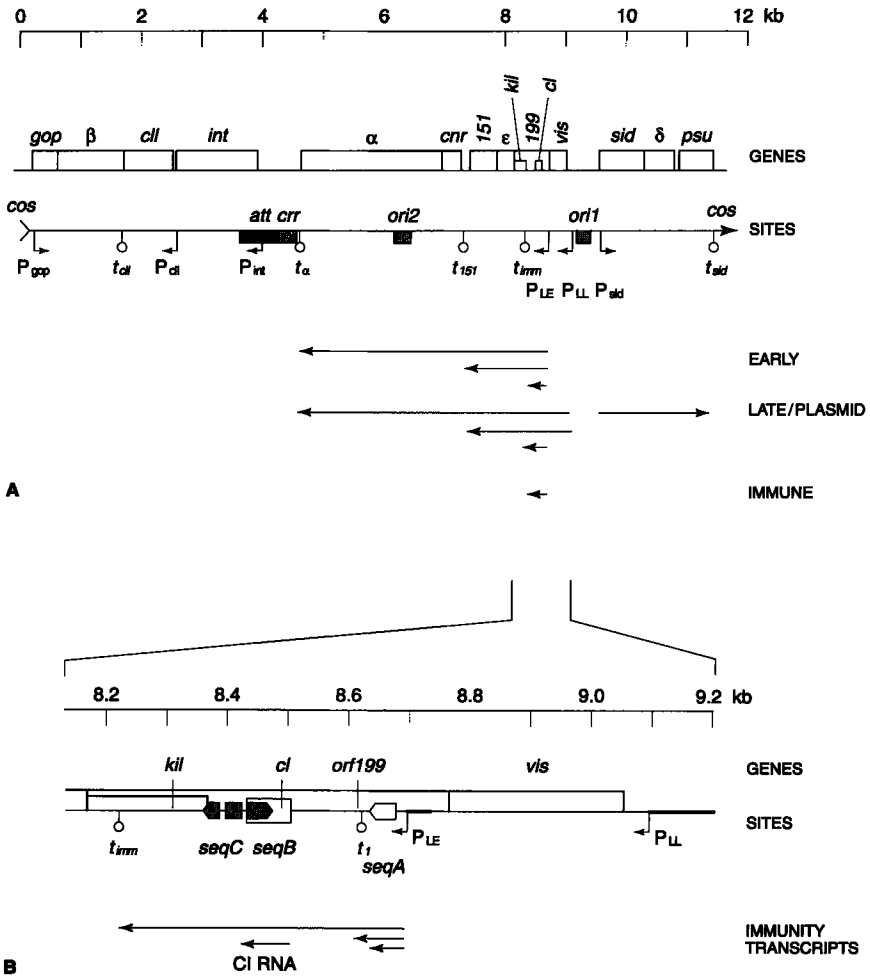


Figure 3 (**A**) Physical and genetic map of P4 and (**B**) P4 immunity region. Coordinates are from the annotated complete nucleotide sequence of P4, GenBank accession number X51522. Transcription start and termination sites are indicated by bent arrows and hanging open circles, respectively. The arrows beneath the map in panel A indicate the early, late and immune phase transcripts from the two main operons. t_1 (**B**) is a weak Rho-independent terminator. For other explanations see **Table 1** and the text. (Modified from Sabbattini P, Forti F, Ghisotti D and Dehò G (1995) *J. Bacteriol.* 177: 1425, and from Briani F, Zangrossi S, Ghisotti D and Dehò G (1996) *Virology* 223: 57.)

directional θ-type manner starting at the *ori*1 site. A second site, *crr*, is essential in *cis* for P4 replication.

P4 replication depends on both host and phage genes. Host-encoded functions are necessary for elongation (DNA polymerase III, SSB proteins) whereas initiation proteins such as DnaA (initiator), DnaB (helicase), DnaC (DnaB complex) and DnaG (primase), as well as the Rep helicase, which is involved in the chromosome replication forks progression, are not required. All such functions are provided by the essential product of the P4 α gene. Alpha is a multifunctional protein: it has primase and helicase activity, binds specifically to octameric repeats located in both *ori*1 and *crr*, and can oligomerize, thus inducing the formation of DNA loops between *ori*1 and *crr*. Finally, Alpha interacts with the P4 encoded Cnr protein, which modulates P4 DNA replication and controls the plasmid copy number. The modular structure of Alpha is shown in **Fig. 4** and a model for P4 replication is proposed in **Fig. 5**.

The presence of both *ori*1 and *crr* in the proper orientation on a miniplasmid is sufficient to allow Alpha-dependent, Cnr-regulated replication. Constructs lacking *ori*1 and carrying both *crr* and the *ori*2 site (a 270 nt region within α with no homology to either *crr* and *ori*1) still replicate in an Alpha-dependent fashion. Thus it appears that P4 is a composite of two replicons. Whether the *ori*2 replicon is a relic of a P4 ancestor or it is still functional under

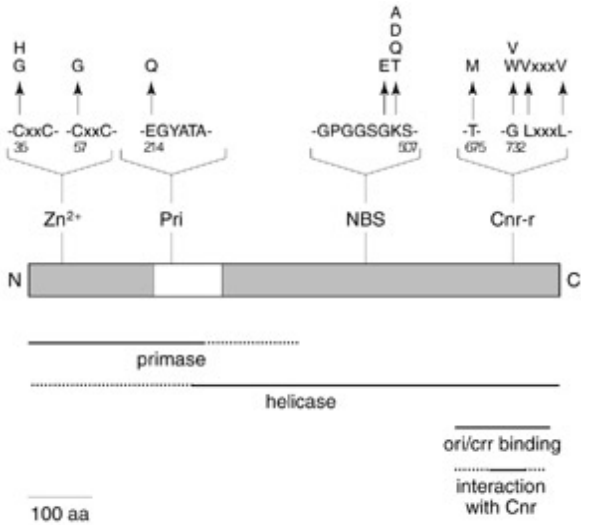


Figure 4 Functional domains of the P4 Alpha protein. The grey bar represents the Alpha protein; its functional domains are indicated by the solid and dotted lines below. Highlighted in white is a 120 amino acid region exhibiting sequence similarity to primases of IncI and IncP plasmids. The amino acid consensus sequences of conserved motifs are reported on top, and the amino acid changes affecting the different activities are listed over the arrows. Pri (primase motif) is conserved among several prokaryotic primases; Zn^{2+}, metal binding motif found in other prokaryotic primases and DNA-repair proteins; Mg^{2+}, potential metal binding site, has similarity with other DNA and RNA polymerases; NBS (type A nucleotide binding site) is found in other helicases of small DNA and RNA viruses; the Cnr-r site, defined by Cnr resistance mutations, interacts with the Cnr protein. (Modified from Ziegelin G, Calendar R, Lurz R and Lanka E (1997) *J. Bacteriol.* 179: 4087.)

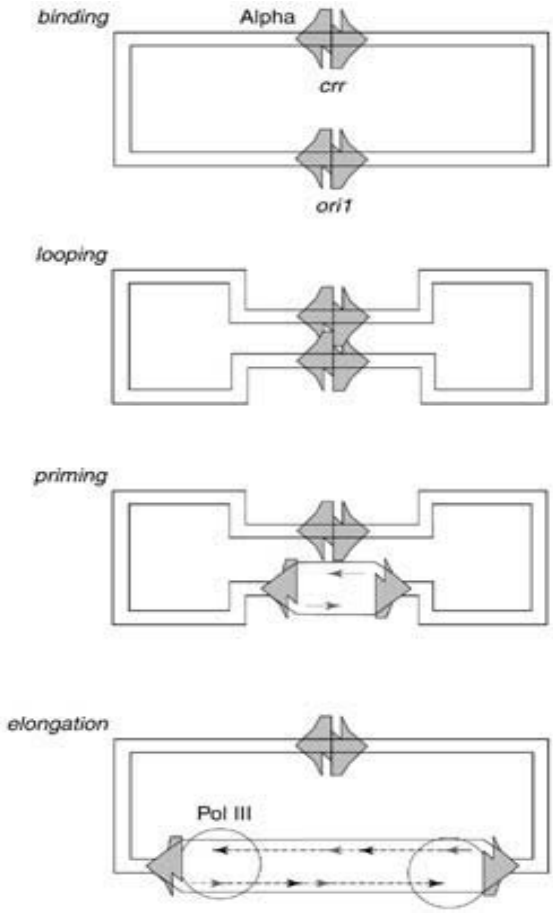


Figure 5 A model for P4 replication. Alpha protein (gray irregular polygon) binds to both *ori*1 and *crr* sites in oligomeric form and may cause looping of P4 DNA (continuous double line) by protein–protein interactions. Alpha is thought to make RNA primers (short, continuous arrow) both at the origin of replication (priming) and at the replicative forks for lagging strand priming (elongation). Dotted arrows: newly synthesized DNA; circles: DNA polymerase III.

some physiological conditions has not yet been ascertained.

Lysogeny

Lysogenization by P4, which may occur in either the presence or absence of the helper, requires integration of the P4 genome in the bacterial chromosome and the establishment of the immune condition which prevents expression of the replication genes.

Integration

Integration of the P4 genome into the host chromosome occurs according to the Campbell model by site-specific recombination between the P4 and the bacterial *att* sites; the latter corresponds to the 3′-end of a gene (*leuX*) encoding a $tRNA^{Leu}$ isoacceptor. Recombination occurs within a 20 nt-long core region identical in both phage and host *att* sites so as to preserve the integrity of the *leuX* gene sequence upon prophage integration, and requires the P4 Int protein. Little is known about excision, that may occur both spontaneously and, at much higher efficiency, upon infection of a P4 lysogen by the helper phage. Vis, the negative regulator of P_{LL}, is coded by the first P4 gene to be expressed upon P2 infection of a P4 lysogen (see below) and can bind to the *attP* region. It has been suggested that Vis may play a role also in P4 prophage excision.

Integration within known or putative tRNA genes appears to be a widespread phenomenon among both prokaryotic and eukaryotic mobile genetic elements such as viruses, plasmids and transposons; this may reflect structural and/or mechanistical features (such as the presence of palindromic sequences, the repetition of sequences and/or structures within a genome, the conservation among species) offered by tRNA

genes that may be exploited for evolution of integration systems.

Immunity

For most known temperate phages, immunity is elicited by a repressor protein which prevents transcription initiation at promoter(s) controlling expression of lytic functions.

The P4 immunity mechanism is unique among the known phages for several aspects: (1) P4 α operon expression is prevented by premature termination of transcription starting at the constitutive promoter P_{LE}; (2) the P4 immunity factor is a short, stable RNA (CI RNA); (3) transcription termination is controlled via RNA–RNA interactions between the CI RNA and two specific target sequences on the nascent transcript; (4) the CI immunity factor is produced by specific processing of the same transcript that it controls.

Uncommitted phase Immediately after infection of a sensitive cell, irrespective of the presence of a helper genome, P4 DNA circularizes through its cohesive ends and enters the early, 'uncommitted' regulatory phase, which will be followed either by the immune or the late-plasmid mode of transcription. In this transient phase the α operon is transcribed from the constitutive promoter P_{LE} and a burst of P4 DNA replication is observed.

Transcription from P_{LE} appears to be termination prone and yields RNA molecules of different length: (1) full-length mRNA 4.1 kb long, which covers the entire operon; (2) 1.3 kb mRNA, which stops at a terminator just upstream of the replication genes *cnr* and α; and (3) a family of transcripts less than 0.5 kb long, which do not extend beyond a strong Rho-dependent terminator, t_{imm} (**Fig. 3A**).

Prophage immunity About 15 min after infection transcripts reading through t_{imm} can no longer be detected. Transcription of P4 lytic and plasmid functions from P_{LE} is turned off by efficient transcription termination at t_{imm} and thus only the immunity region is expressed. This latter transcription pattern of the α operon is only detectable in the immune prophage condition.

Establishment and maintenance of the immunity transcription mode depends on genetic determinants located within a 500 nt region (immunity region, **Fig. 3B**) that includes P_{LE}, the downstream untranslated leader region, and t_{imm}, which is located within *kil*, the first translated gene downstream of P_{LE}.

The untranslated leader region of the P_{LE} transcript is rich in direct and inverted repeats that may allow the formation of multiple, mutually exclusive secondary structures in the leader RNA. A region, called *seqB*, exhibits complementarity with both the *seqA* and *seqC* sites, located upstream and downstream of *seqB*, respectively. *seqB* is internal to *cI*, which encodes the CI RNA (**Figs 3b** and **6**), a small RNA generated by processing of the leader transcript.

Transition from the early transcription pattern, with expression of full-length mRNA, to the immune pattern, where transcription is totally restricted to the immunity region, is concomitant with the appearance of the mature CI RNA. Moreover, the presence in the cell of the CI RNA is sufficient to efficiently cause premature transcription termination at t_{imm} in an infecting phage: thus the CI RNA is the P4 immunity factor.

Most recessive mutations that cause an immunity defect map in *seqB*, whereas *seqA* and *seqC* double mutants are insensitive to P4 immunity. Mutations in both *seqA* and *seqC* that restore complementarity with a *cI* mutation in *seqB* also re-establish efficient transcription termination. Thus the *seqA* and *seqC* sites in the nascent transcript appear to be the targets of the P4 immunity factor, both being required for the establishment and the maintenance of prophage immunity. CI RNA-target interaction appears to occur primarily between complementary regions predicted to be single-stranded by computer analysis (**Fig. 6**).

The mechanism of P4 immunity is not completely understood. The complementary sequences in the immunity region may allow both intra- and intermolecular pairing of RNA molecules. Before the appearance of CI RNA upon P4 infection, a substantial transcription of the entire α operon is allowed, whereas transcription terminates efficiently as soon as the mature CI RNA is produced in the cell. This suggests that intramolecular interactions in the leader sequence allow readthrough, whereas intermolecular interactions between the CI RNA and the nascent leader transcript cause strong termination.

seqC overlaps the ribosome binding site and the start codon of *kil*, the first translated gene in the P_{LE} transcript. This suggests that termination at t_{imm} might be indirectly elicited by the immunity mechanism by preventing *kil* translation; this may allow access of the Rho factor to t_{imm}, thus causing transcription termination. However, a more direct role of the CI RNA and/or the untranslated leader RNA on transcription termination and/or stability of the P_{LE} transcript has not been ruled out.

The CI RNA is processed from the P_{LE} transcripts by RNase P, an endonucleolytic ribozyme also involved in maturation of tRNAs, which specifically generates the 5′-end of the mature molecule; polynucleotide phosphorylase, which degrades RNA

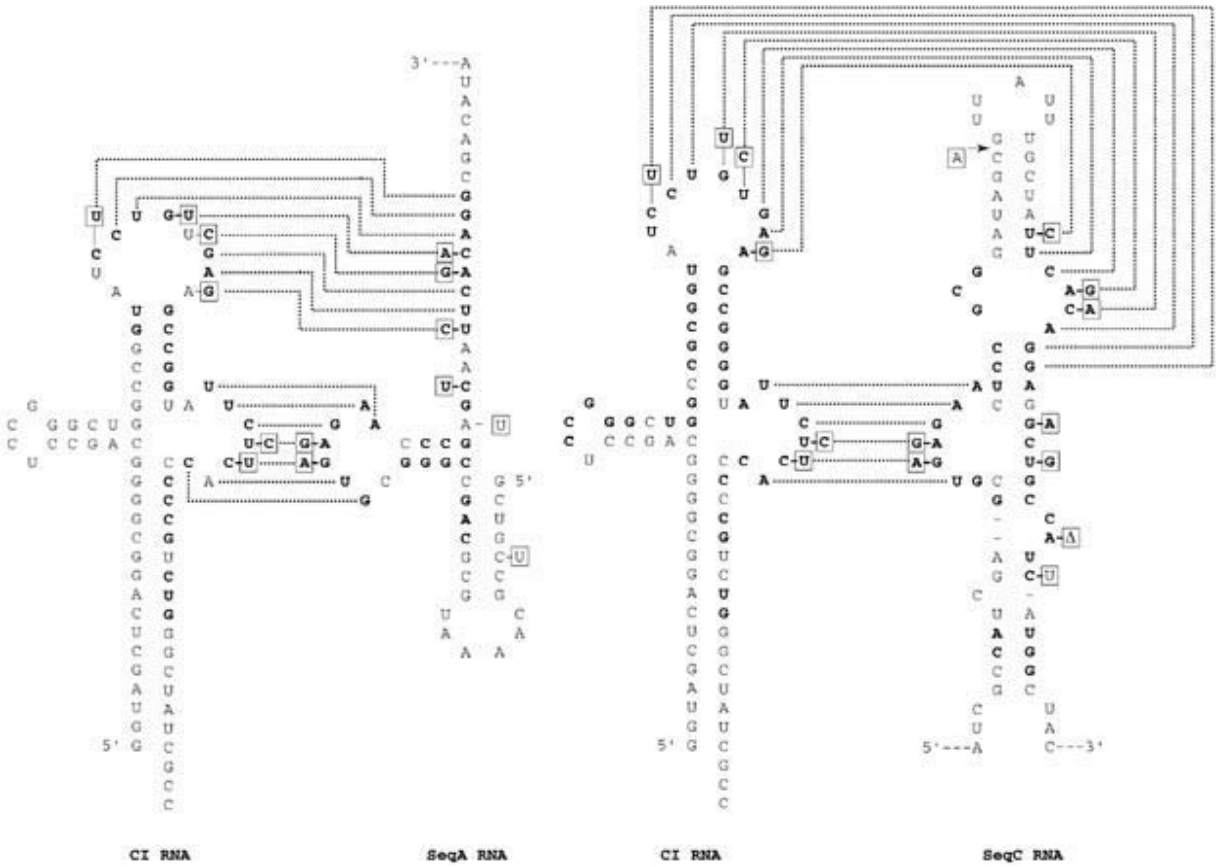


Figure 6 Predicted secondary structure of the CI RNA and possible interactions with *seqA* and *seqC* in the leader transcript of the immunity region. *seqB-seqA* and *seqB-seqC* complementary bases are in boldface type. The complementary bases in single-stranded regions are connected by dotted lines. The base changes in the P4-like retronphage ΦR73 are boxed. Arrow indicates insertion, Δ deletions. (Modified from Sabbattini P, Six E, Zangrossi S, Briani F, Ghisotti D and Dehò G (1996) *Virology* 216: 389.)

exonucleolytically from the 3′-end, is also required for efficient processing of the P4 immunity factor.

Multicopy Plasmid State and Lytic Cycle

The late-plasmid transcription pattern

Both the plasmid state and the late phase of the lytic cycle are characterized by continuous expression of the α and *sid* operons, due to the activation of the positively regulated late promoters P_{sid} and P_{LL}. The pathways leading to their activation, however, are different in the two developmental conditions, and are discussed below.

P_{LL} is located 400 nt upstream of P_{LE} and the transcripts starting at P_{LL} encode two additional genes: *vis* and *orf*199; the latter overlaps *kil* in frame (**Fig. 3B**) and thus Kil is a truncated form of Orf199.

In the late-plasmid transcription mode P_{LE} is still active: the short transcripts characteristic of the lysogenic condition are abundantly produced and premature termination at t_{imm} occurs efficiently. On the contrary, transcription from the upstream promoter P_{LL} is not subject to the strong termination mechanism of P4 immunity. This seems to be due to *vis–orf*199 translation that may impede interactions between the P_{LL} transcripts and the Rho factor and/or the CI RNA, thus preventing transcription termination.

In most temperate phages the choice between lysis and lysogeny depends on the activation of either of two mutually exclusive regulatory patterns. In P4, on the contrary, the late-plasmid and the immune modes of transcription are not mutually exclusive; rather, the late-plasmid mode is superimposed on the immunity control and the choice between lytic-plasmid versus lysogenic development appears to rely on the activation, or lack thereof, of the two late promoters P_{LL} and P_{sid}.

The P4 late promoters and their transcriptional activators

Both P_{LL} and P_{sid}, like the promoters of the late

```
Phage Protein          10        20        30        40        50        60        70        80
                        :         :         :         :         :         :         :         :
P2    Ogr       MFHCPLCQHAAHARTSRYITDTTKERYHQCQNVNCSATFITCESVQRYIVKPGE-VHAVRPHPLPSGQQIMWM
186   B         MFHCPKCHHAAHARTSRYLTENTKERYHQCQNINCSCTFMTMETIERFIVTPGA-IDPAPPHPTVGGQRPLWL
PSP3  Pag       MMHCPLCQNAAHARTSRYLSTETKERYHQCQNINCGCTFITFETLSRFIVKPGT-VDPAPPHPIRNQQQQLWL
 -    NucC      MMHCPLCGHVAHTRSSRYLSESTKERYHQCRNINCSCTFATHESVARVIVKPGDDIVPAQPHPPENQHKQSAAAL
ΦR73  δ         MMRCPFCRHSAHTRTSRYVSDNVKESYLQCQNIYCSATFKTHESICAVIRSPVTEEKPAPASTAPAVVRKVKGCYSSPFNH
P4    δI        MIYCPSCGHVAHTRRAHFMDDGTKIMIAQCRNIYCSATFEASESFFSDSKDSGMEYISGKQRYRDSLTSASCGMKRPKRMLVT
P4    δII       GYCCRRCKGLALSRTSRRLSQEVTERFYVCTDPGCGLVFKTLQTINRFIVRPVTPDELAERLHEKQELPPVRLKTQSYSLRLE

      Consensus M-hCP-C-h-AH-RtSRyls--tKryhQCqNinCs-TF-T-Es--r-Iv-Pg-----a-php----------
```

Figure 7 Amino acid sequence of the late gene *trans*-activating factors of P2- and P4-like phages. NucC is encoded by a cryptic prophage of *Serratia marcescens*. δI and δII are the N and C terminal halves of Delta, respectively. The amino acids used to define the consensus sequence are in bold. Bottom line: letters underlined, amino acids present in all the seven sequences; upper case letters, amino acids present in six sequences; lower case letters; amino acids present in four or five sequences. (Modified from Pountney DL, Tiwari RP and Egan JB (1997) *Protein Sci.* 6: 892.)

(morphogenetic) operons of P2, are positively controlled by the P4 encoded Delta and the P2 encoded Ogr transcriptional activators.

Ogr is the prototype of a class of transcriptional activators that control the late operons of phages related either to P2 or P4. These activators are small proteins (72–81 amino acids) with a zinc-finger-like metal-binding domain. P4 Delta, however, is twice as much the size of the other members of the family, contains two zinc-binding motifs, and appears to be a tandem duplication of the basic module (**Fig. 7**). Both domains of Delta are required for transcriptional activity.

The Ogr-family activators interact with the C-terminal domain of the RNA polymerase α subunit and bind to a consensus sequence centered at about −55 nt from the transcription start point in all the responsive σ^{70}-dependent promoters. The P4 P_{sid} promoter, however, contains a second copy of the consensus sequence in the −18 region (**Fig. 8**) that appears to quench transcription activation by Delta, since mutations in the P_{sid} −18 region increase Delta-dependent promoter activity. This seems to explain why Delta-promoted transcription is less efficient from P_{sid} than from P2 late promoters.

Although P_{LL} and P_{sid} share the positive regulators Delta and Ogr, they differ in three aspects that are relevant for the plasmid and the lytic development: (1) a low basal level of transcription from P_{LL} may be detected early upon infection, due to an overlapping weak promoter P_{LL}*. No basal level of transcription has been detected from P_{sid}; (2) transcription from P_{LL} is repressed by Vis, the product of the first gene downstream of P_{LL}, whereas Vis appears to enhance Delta-dependent transcription from P_{sid}. Accordingly, the Vis binding site for P_{LL} is located immediately downstream of the transcription start, whereas a Vis-binding consensus sequence is located upstream in P_{sid}; (3) P_{LL} can be activated by the helper early gene product Cox, whereas P_{sid} cannot. The Cox-binding region is located −60 to −150 in P_{LL} (**Fig. 8**).

The multicopy plasmid state

Upon infection by wild-type P4, the multicopy plasmid state may be established at low frequency, the lysogenic cycle being preferred in the absence of a helper phage genome. Mutations affecting P4 immunity may increase the frequency of plasmid establishment.

The average number of P4 plasmid genomes per *E. coli* cell is about 30–50, and an integrated copy may also be found, but it is not essential for the plasmid maintenance. The steady level of P4 replication appears to depend on the regulatory loops controlling

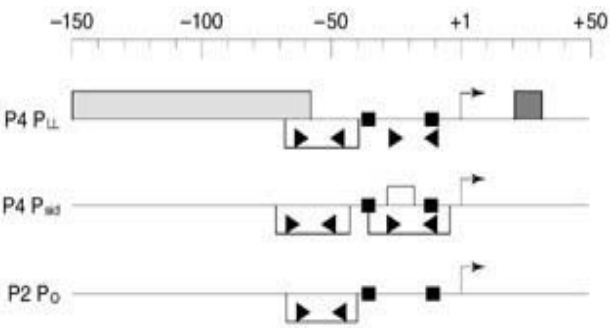


Figure 8 Structure comparison of the P4 and P2 late promoters. The figures on the top scale indicate the nucleotide position from the transcription start point (bent arrow). The short black bars are the −35 (TRGYgt) and −10 (cAaaMT) late promoter consensus sequences; the black and gray boxes are the Vis and Cox binding sites, respectively; the open boxes are the Delta binding sites, and the inverted arrowheads indicate the conserved TGT-ACA dyad elements within the Delta binding sites. The P2 P_O promoter is shown as a representative of the P2 late promoters.

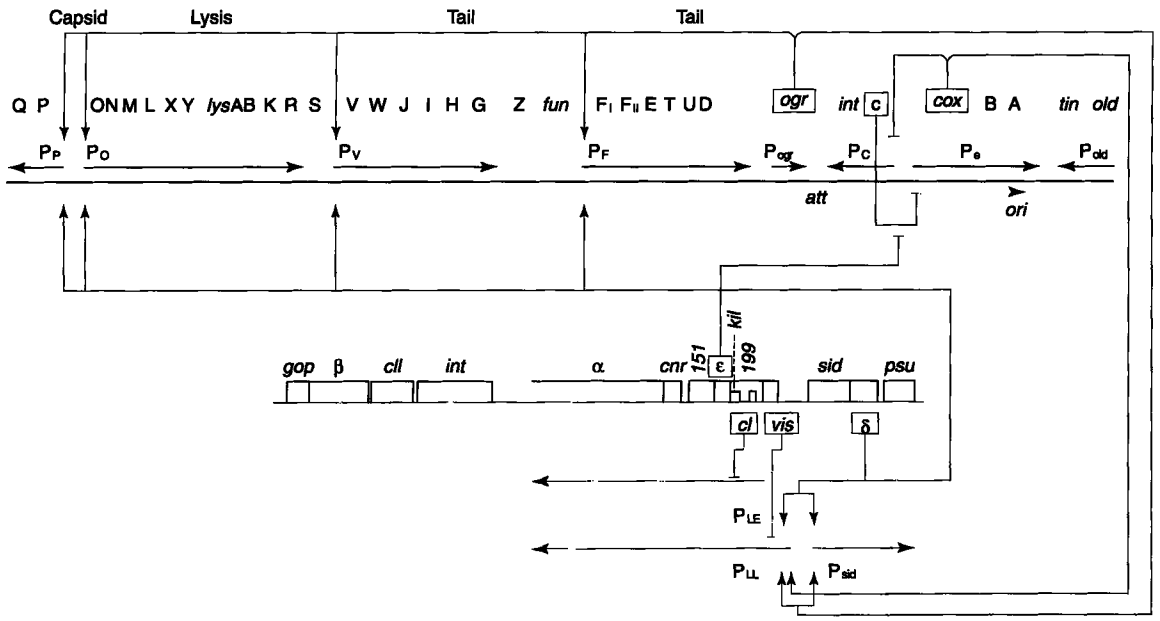


Figure 9 Regulatory loops controlling expression of P4 and P2 late genes. Straight arrows, transcription units. Transcriptional or functional activation and inhibition are indicated by bent lines ending with an arrowhead or with a minus sign, respectively. The P2 map is not drawn to scale. (Modified from Lindqvist B, Dehò G and Calendar R (1993) *Microbiol. Rev.* 57: 683.)

P_{LL}, which is activated by Delta and repressed by Vis (**Fig. 9**). This poses the unsolved problem of how P_{sid} transcription (and, as a consequence, the plasmid regulatory state) is primed upon infection, when Delta is not yet present in the cell. It is possible that the right operon may be transcribed at a very low basal level in a Delta-independent manner. Alternatively, either Vis or other unidentified P4 functions might be involved.

The lytic cycle

The P4 lytic cycle requires efficient exploitation of the helper phage genetic information to obtain the morphogenetic and lysis gene products. This may occur under different scenarios: (1) P4 infecting a repressed P2 lysogen; (2) P4 and P2 co-infecting a nonlysogenic host; (3) a P4 lysogen being infected by P2; (4) a P4-plasmid carrier being infected by P2. In each of these situations, the satellite phage senses the presence of the helper and responds by activating its own functions that will in turn modify the pattern of gene expression of the helper. Reciprocal regulatory interactions developed in this satellite–helper system involve lifting the immunity mechanisms (mutual derepression) and direct activation of the late operons (reciprocal *trans*-activation) of both phages. Moreover, morphopoietic interactions are elicited by P4 in order to assemble a capsid of the correct size. The type and timing of the satellite–helper interactions vary in the different infection conditions and give different outputs, in terms of satellite vs. helper virions produced, that appear to fit the reproductive strategies of the satellite phage.

Satellite–helper regulatory interactions When P4 infects a repressed P2 lysogen and the lytic option is set, the helper prophage immunity is lifted by the essential P4 ε gene product. P2 derepression leads to expression of the helper early genes (*cox* and the replication genes A and B), and to activation of unidirectional P2 replication *in situ*, without excision of the integrated prophage genome. The low P2 excision frequency, together with the interference on P2 growth due to the efficient production of small capsids, appear to be responsible for the fact that mostly a progeny of P4 virions is produced. Thus in an environment of P2 lysogenic bacteria, where P2 replication would be inhibited by the P2 prophage immunity, all the morphogenetic potential is efficiently directed toward the P4 propagation.

The mechanism of P2 derepression is not known, although Epsilon seems more likely to interfere with the repressor protein itself rather than directly activate the P2 early genes. P2 derepression is essential for P4 lytic growth in a P2 lysogen, but the underlying molecular mechanisms are not known. It seems, however, that this step is required for the timely activation of the helper's morphogenetic operons by the P2 Ogr and/or the P4 Delta proteins. Either

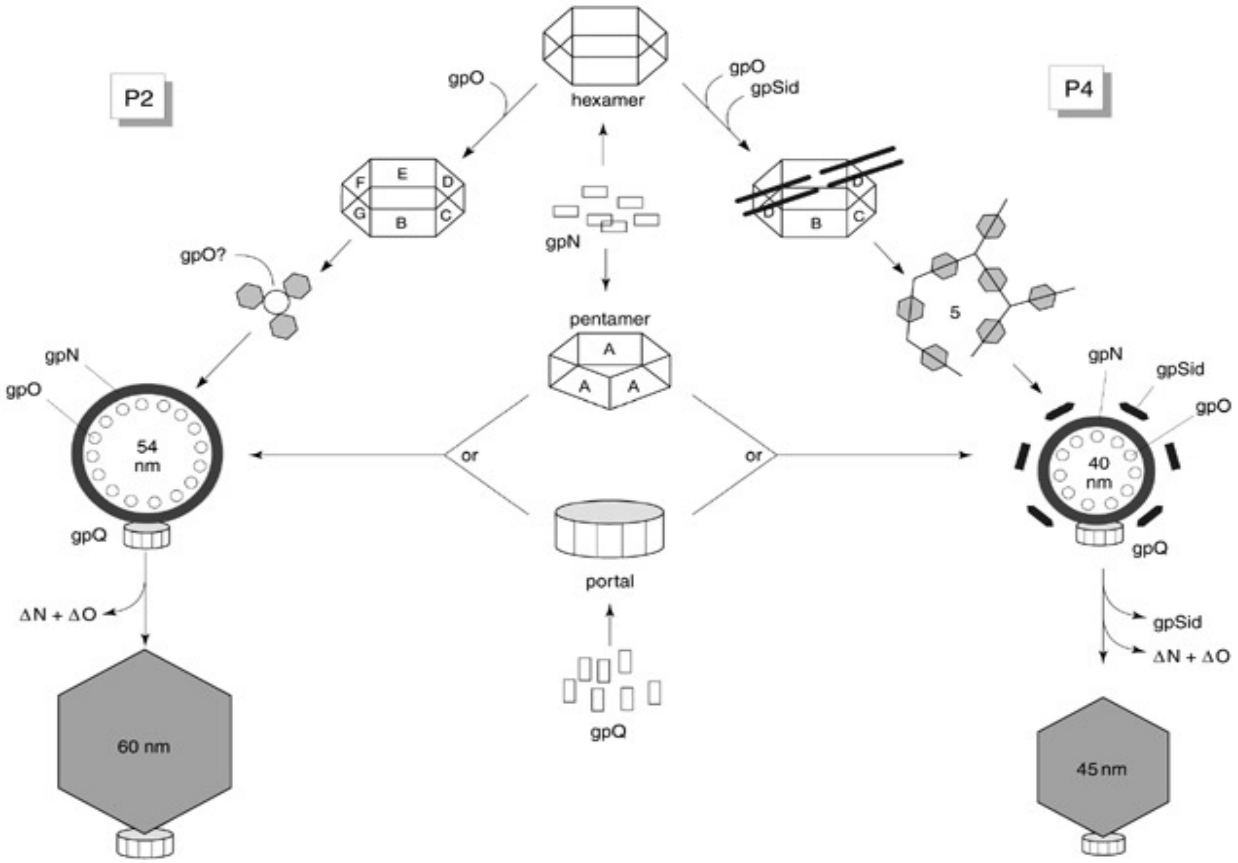


Figure 10 Schematic model for P4 and P2 capsid assembly. The gpN (gp = gene product) precursor (open rectangles) are assumed to form uncommitted hexamers, which will subsequently associate with the scaffolding proteins gpSid (filled bars) and/or gpO. The letters B to G indicate the different quasi-equivalent positions of gpN in the hexameric capsomers. In P4, the gpSid-gpN hetero-oligomers become connected by gpSid bridges into 5-fold rings (about to close in the Figure), with trivalent branching at every icosahedral 3-fold axis. In P2, the speculative function of gpO (in the absence of gpSid) would be to connect three gpN hexamers into a complete icosahedral face with triangulation number $T = 7$. The resulting shell structures will undergo maturation, involving shell expansion, processing of gpN, and removal of the scaffold molecules. (From Marvik OJ, Dokland T, Nøkling R, Jakobsen E, Larsen T and Lindqvist BH (1995) *J. Mol. Biol.* 251: 59.)

transcriptional activator is sufficient to *trans*-activate both P4 and P2 late genes, although in the presence of both Ogr and Delta functional proteins the P4 lytic cycle occurs more timely and efficiently than with a single activator.

When P4 and P2 co-infect a nonlysogenic host, the burst size of P2 is reduced and a progeny of both phages is produced in about the same proportions. Interference on P2 growth is stronger if P4 infects the host earlier than P2. This fits with a balanced spreading of both P4 and P2 in a dynamic competition in a nonlysogenic population. The ϵ derepression activity is dispensable for P4 lytic cycle, although it appears to be required for interference with the helper's growth.

Infection of a P4 lysogenic strain by P2 induces the P4 lytic cycle. The yield of P4 phage, however, is low (ca. one per cell) and interference on P2 growth is not observed. Thus also under such a condition the P4 genome can be rescued, albeit at low efficiency, and survives host cell death. The P4 prophage senses the infection of the helper via the early P2 Cox protein, which activates P_{LL} and thus the expression of the α operon. Cox does not activate P_{sid}, which lacks the Cox binding site and, later on after infection, may be efficiently activated by the P2 Ogr protein.

When P2 infects a cell carrying P4 in the plasmid condition, the Cox function is not required to induce P4 lytic cycle since the P4 late promoters are already activated. In such conditions P4 strongly interferes with P2 growth and mostly P4 virions are produced.

From the above description it is apparent that P4 has developed sophisticated regulatory strategies to exploit P2 and maximize the chances for its own horizontal transmission in bacterial populations that may differ with regard to the presence and the state of the helper phage.

Morphogenesis The helper phage P2 packages its 33 kb genome into 60 nm isometric capsids made up of 60 hexameric and 12 pentameric capsomers. A complex capsid maturation pathway involves proteolytic processing of the capsid proteins as well as structural reorganization of the shell lattice. The capsomers are made of the N-terminal processed products (N*) of the P2 *N* gene. P4 interferes with the P2 morphogenetic pathway by directing the assembly of a 45 nm isometric capsid (made of 30 hexamers and 12 pentamers) more suitable for its own three times smaller genome. A schematic model for P4 and P2 capsid assembly is shown in **Fig. 10.**

The P4 *sid* gene product is sufficient to direct the assembly of P4-sized heads by interacting with the P2 *N* gene product and forming a continuous dodecahedral scaffold on the outside of the procapsid particles. The only other known example of external scaffolding in phage morphogenesis is provided by ΦX174. Maturation of the procapsid involves proteolysis of the P2 *N* gene product to give not only the N* but also a substantial amount of two partially processed forms, h1 and h2. In addition, the Psu protein associates on the top of the hexameric capsomers and stabilizes the phage particle. Psu is the only P4 encoded protein that may be found in the mature P4 virion, but it is not essential for P4 morphogenesis.

The P4 dependence on the helper for the morphogenetic pathway includes DNA maturation and packaging. Circular DNA molecules are the preferred substrate for packaging by both P2 and P4 proheads. A staggered double-strand cut that generates the 19 nt long single-stranded cohesive ends of the mature genome occurs within a 55 nt-long sequence, essential for DNA packaging, that is identical in P2 and P4.

Natural History

Although, to our knowledge, a systematic search for satellite phages has not been performed, another P4-like P2-dependent phage, ΦR73, has been serendipitously identified in a natural isolate of *E. coli*. P4 and ΦR73 exhibit more than 95% sequence identity in the *crr-cos* essential region. Exceptions are gene *δ*, which is only 31% identical and does not show the tandem-duplication structure of P4 *δ* gene, and the immunity region, with 85% identity. ΦR73 immunity system is similar to that of P4, but each phage is insensitive to the immunity of the other prophage. This heteroimmunity is due to six base differences in the single stranded *seqB* region of the CI RNA and to compensatory base substitutions in the target sites *seqA* and *seqC* (**Fig. 6**).

The *int* genes of the two phages diverge considerably (40% identity) and no homology exists between their *att* sites. ΦR73 prophage has been found integrated at the 3′ end of *selC*, which encodes the selenocystyl-tRNA. The nonessential region to the left of the *att* site in ΦR73 appears to be rearranged, relative to P4; interestingly, to the right of *att*, ΦR73 carries a retron, a chromosomal element encoding reverse transcriptase and responsible for the synthesis of a peculiar single-stranded DNA–RNA chimeric molecule called msDNA.

At least three distinct P4-related sequences, named CP4 for 'cryptic P4' phage, have been identified in the completely sequenced *E. coli* K genome, and cross-hybridization of P4 DNA with a large proportion of *E. coli* clinical isolates has been reported. These seemingly P4 related prophage remnants witness the diffusion of P4-like satellite phages and the role they may have played in horizontal gene transfer in natural populations of *E. coli*.

See also: **Coliphage *ϕ*X174 and related phages (*Microviridae*); Enterobacteria phage P1 (*Myoviridae*); Salmonella phage P22 (*Podoviridae*); Lysogeny and prophage; P2, 186 and related phages (*Myoviridae*); Satellite RNAs and Satellite viruses.**

Further Reading

Bertani LE and Six EW (1988) The P2-like phages and their parasite P4. In: Calendar R (ed.) *The Bacteriophages*, vol. 2, p. 73. New York: Plenum Press.

Kahn ML, Zierman R, Dehò G *et al.* (1991) Bacteriophage P2 and P4. *Methods Enzymol.* 204: 264.

Inouye S and Inouye M (1993) The retron: a bacterial retroelement required for the synthesis of msDNA. *Curr. Opin. Genet. Dev.* 3: 713.

Lindqvist BH, Dehò G and Calendar R (1993) Mechanisms of genome propagation and helper exploitation by satellite phage P4. *Microbiol. Rev.* 57: 683.

Ziegelin G and Lanka E (1995) Bacteriophage P4 DNA replication. *FEMS Microbiol. Rev.* 17: 99.

PAPILLOMAVIRUSES – ANIMAL

William C Phelps, Department of Virology, Glaxo Wellcome Inc, North Carolina, USA

Gary L Bream, Lineberry Research Associates, North Carolina, USA

Alison A McBride, National Institute of Allergy and Infectious Diseases, Laboratory of Viral Diseases, National Institutes of Health, Bethesda, Maryland, USA

History

Warts or papillomas have been recognized in animals for centuries. One of the earliest reports was from the stablemaster for the Caliph of Baghdad who first described equine warts in the ninth century AD. Contemporary with the discovery of 'filterable agents' by Ivanowski, Beijerinck, Loeffler, and Frosch at the end of the nineteenth century, Penberthy (1898) demonstrated the infectious transmission of warts in dogs and Ciuffo confirmed a viral etiology for papillomas with the report of the transmission of warts in humans using sterile filtrates. Bovine warts were also recognized to be contagious; however, demonstration of viral transmission did not occur until 1929. Cottontail rabbit papillomavirus (CRPV) or Shope papillomavirus was isolated and described in 1933 by Richard Shope.

Following the observations by Peyton Rous in 1935, that benign rabbit warts could progress to malignant carcinoma, the Shope papillomavirus system became a favorite model for the study of carcinogenic progression in animals. As one of the earliest indications that viruses could be involved in the development of cancer, this model has been instrumental in the study of viral oncology and neoplastic progression, and has contributed to our recognition of cancer as a multistep process. The potential for malignant conversion of virus-induced benign lesions is a hallmark of papillomaviruses (PVs) and was important in the recognition of the role of the human papillomaviruses (HPVs) in cervical cancer.

Up to the 1980s, research on the papillomaviruses had been relatively slow due primarily to the absence of a conventional, permissive cell culture system for virus propagation. Although great difficulties persist even today for *in vitro* replication of PVs, the advent of molecular cloning early in the 1980s greatly expanded the capacity for experimental manipulation of the viral genomes and brought renewed vigor to papillomavirus research.

Taxonomy and Classification

Papillomaviruses belong to the *Papovaviridae* family of DNA viruses that includes mouse polyoma and the monkey virus, SV40. The family name, 'Papova' is derived from the first two letters of the three principal virus groups, PApilloma, POlyoma, and simian VAcuolating virus (SV40). Based on comparative structural and genetic characteristics, these viruses are considered as two discrete subfamilies: *Papillomavirus* and the *Polyomavirus* (polyoma, SV40, JC, BK). The members of the *Papovaviridae* family share a number of properties including a relatively small size, nonenveloped icosahedral capsids, and a double-stranded, covalently closed, supercoiled DNA genome that replicates in the nucleus of infected cells in association with cellular histones.

Classification of papillomaviruses into types is based first upon species specificity, and second, upon the degree of relatedness of the viral genomes. For example, six different PV types that infect cattle have been isolated and described. The bovine papilloma viruses (BPV) types (1–6) are numbered sequentially according to their chronological order of identification. To distinguish individual PV types, the initial classification scheme described in the 1970s was based on measuring the extent of nucleic acid sequence homology using liquid hybridization under stringent conditions of dissociation and reannealing. More recently, with the availability of DNA sequence data on the vast majority of PV genomes, classification of PV types is carried out through comparison of the nucleotide sequence of specific regions of the genome. To be considered a new PV type, the DNA sequence of the E6, E7, and L1 open reading frames should not exceed 90% identity when compared to other known PV sequences. If DNA sequence identity exceeds 90%, the genome is considered a subtype or variant. Papillomaviruses have been isolated and characterized from a number of animals including cattle, sheep, deer, horses, rabbits, dogs, mice, birds and nonhuman primates such as monkeys (**Table 1**). To date, DNA sequence information has become available on about 25 different PV types isolated from among these diverse animal families.

Geographic Distribution

PV infections of animals occur worldwide although virus types can display some geographic variability,

Table 1 Well characterized animal papillomaviruses

Host	*Lesion*	*Designation*
Domestic cattle	Fibropapilloma, papilloma	BPV 1–6
Cottontail rabbit	Papilloma	CRPV
Domestic dog	Oral papilloma	COPV
Domestic sheep	Papilloma, fibropapilloma	OvPV 1, 2
Deer	Fibroma	DPV
European elk	Fibroma	EPV
Reindeer	Fibropapilloma	RPV
Harvest mouse	Papilloma	MmPV
Multimammate rat	Papilloma, keratoacanthoma	MnPV
Chaffinch	Papilloma	FPV
Chimpanzee	Oral	ChPV
Pigmy chimpanzee	Oral	PCPV
Rhesus macaque	Genital	RhPV
Long-tailed macaque	Genital	MfPV
Colobus monkey	Papilloma	CgPV 1, 2

and may be observed to cause periodic, localized outbreaks. For example, endemic CRPV infection is typically confined to the populations of wild cottontail rabbits in the mid-western US. As the wild rabbit host is found throughout the US, it is not clear what exogenous factors may contribute to the geographic exclusivity of enzootic CRPV infection.

BPV-4 causes alimentary tract papillomas and was associated with a high rate of progression to carcinoma in Great Britain. Subsequent studies revealed that bracken fern grown in that region, contained a cocarcinogenic substance that promoted progression of alimentary papillomas to squamous carcinomas and urinary bladder tumors. BPV-1 causes naturally occurring fibropapillomas in cattle. In the US, lesions are typically found on the head, neck, and shoulders whereas in Germany, lesions on cattle are more often seen on the abdomen, legs and back. Although different virus types will preferentially infect different tissues, in this instance, the site variability of BPV-1 infected lesions is likely due to differences in animal husbandry.

Host Range and Virus Propagation

PVs infect a broad range of vertebrate species and are especially prevalent in mammals. Three PV infections have been described in avian species; the genomes of two have been cloned and partially characterized thus far (chaffinch and gray parrot). Papillomatous lesions have also been described in species of amphibians (e.g. newts and salamanders) and reptiles (e.g. snakes, turtles and crocodiles) although a PV etiology has not yet been confirmed.

Infection with specific PVs has been confirmed in approximately 50 different mammalian species. One of the hallmark features of PVs is pronounced host specificity with infection being typically restricted to closely related animals of a given family. For example, CRPV naturally infects the cutaneous epithelium of wild cottontail rabbits, and experimental infection of related jackrabbits or snowshoe rabbits will result in the growth of papillomas and production of infectious particles. In contrast, although experimental infection of domestic rabbits induces growth of papillomas, the lesions are typically nonproductive containing little or no infectious virus. CRPV-induced benign warts on domestic rabbits appear to support normal early viral gene expression and genome replication, but are unable to support late gene expression and virus particle production. In addition, benign lesions of domestic rabbits are roughly three times more likely to progress to carcinoma than comparable lesions in wild rabbits, the natural host. The genetic determinants of the host that are responsible for the variability in pathogenic outcome are thus far, not well defined. Recent studies have suggested a role for polymorphic genes of the major histocompatibility complex that are important for recognition of viral antigens.

There are two subgroups of bovine papillomaviruses; one which causes fibropapillomas and the other epithelial papillomas. BPV types 3, 4 and 6 were isolated from true papillomas whereas types 1, 2 and 5 were isolated from fibropapillomas. BPV types 1 and 2 exhibit a slightly broader host range causing endemic fibropapillomas in cattle and nonproductive (i.e. no infectious virus produced) sarcoids in horses. Experimental infection with BPV types 1 and 2 results in a somewhat broader host range and results in nonproductive fibromas in horses, hamsters, rabbits, pikas and mice. The ability of BPV-1 to infect and transform mouse cells in culture enabled the development of a focus-forming assay that was critical to early work to genetically define viral genes responsible for cellular transformation and genome replication.

Canine oral papillomavirus (COPV) infection is seen sporadically in domestic dogs of all ages. In contrast to BPV-1 and -2, COPV cannot successfully infect mice, hamsters, or guinea pigs in the laboratory; however, COPV has been detected in feral coyotes. During the last ten years, several PVs have been isolated from nonhuman primates including monkeys and chimpanzees. As both the primate PVs and their hosts are examined more closely, it is possible that we may find that certain HPVs will infect certain primates and that certain primate PVs will infect humans.

Another method for the extension of the inherent host range of PVs is through the use of transgenic animals. Recently, both transgenic rabbits and mice have been described harboring CRPV and BPV-1, respectively. These systems provide additional tools to examine a myriad of specific issues regarding tissue-specific expression, cellular transformation, cytogenetic abnormalities associated with carcinogenic progression, and immune recognition and tumor immunity. BPV-1 transgenic mice will develop nonproductive epidermal fibropapillomas after a delay of 8–9 months. The warts occasionally become malignant and are locally invasive. Extrachromosomal BPV-1 DNA is found exclusively in the wart tissue in contrast to the normal skin and tissues that harbor only integrated, transcriptionally silent BPV DNA. CRPV transgenic rabbits develop an extensive population of cutaneous papillomas within 1–3 months, and viral mRNAs are only expressed in skin tumors, not in normal tissues.

Several systems have been developed to propagate the papillomaviruses. Small pieces of epithelial tissue can be infected with virus and implanted in the renal capsule of an immunocompromised mouse; this results in the production of virion particles. Skin from various species can also be grafted onto immunocompromised mice and either infected with virus or transfected with viral DNA. The infected xenograft will form a papilloma-like lesion that, in some cases, can produce virion particles. Artificial skin equivalents (organotypic rafts) can also be grown in tissue culture and this system can also support the viral life-cycle. Although these systems are still far from ideal, they are being used to begin to understand the complete viral life cycle.

Genetics

The emergence of techniques for molecular cloning in the late 1970s permitted viral genomes to be isolated and propagated in the laboratory to provide an unlimited source of homogeneous viral DNA for study. In 1980, Doug Lowy and co-workers showed that BPV-1 virus and cloned viral DNA could morphologically transform established mouse cell lines. The viral DNA was shown to persist extrachromosomally and replicate as a stable episome. As a result of these features, BPV-1 has been the model system of choice for the genetic analysis of the viral transformation, DNA replication, and transcriptional regulatory functions.

The genetic information of all PVs is expressed from a single strand of the double-stranded genome which, by convention with the polyomaviruses, is divided into three regions (**Fig. 1**). The long control region (LCR) is a 1 kb sequence containing many of the viral transcriptional regulatory signals as well as the origin of replication. The early region contains those opening reading frames (ORFs) expressed primarily in nonproductively infected cells (labelled E1–8 in descending order of size). The late region contains the ORFs for the capsid proteins L1 and L2, which are expressed only in productively infected cells and are dispensable for BPV-1 DNA replication and *in vitro* transformation of mouse cells. A polyadenylation site (poly(A)) for the early messages is located between the early and late regions whereas the late transcripts use a second poly(A) site in the LCR. Transcriptional termination within the late region occurs in nonproductively infected cells and may prohibit expression of the late mRNAs in these cells.

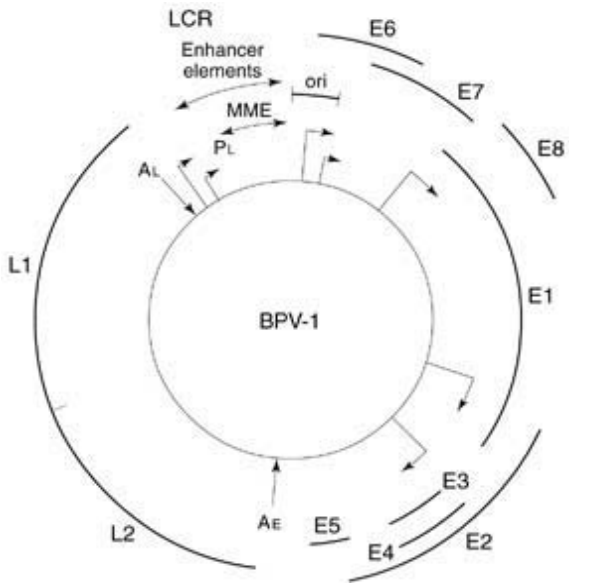


Figure 1 The circular BPV-I genome. Nucleotide numbers are noted within the map. E and L designate the early and late open reading frames, respectively; promoters are indicated by arrows. LCR indicates the long control region. A_E and A_L represent the early and late polyadenylation sites, respectively and MME indicates the minichromosome maintenance element.

Seven transcriptional promoters have been identified in the BPV-1 genome, of which one (the presumptive late promoter) is expressed only in productive warts. The activities of the six early promoters are regulated by host factors and the virally encoded E2 proteins. There are three E2 proteins encoded by BPV-1 (E2TA, E2TR and E8/E2) that are the result of expression from multiple promoters and alternative RNA splicing. All three have sequence-specific DNA binding and dimerization activities whereas the third (E2TA) has, in addition, a transcriptional activating domain. E2TA stimulates viral promoters by binding to multiple 12 bp palindromic sequences in E2-specific

enhancers within the LCR. This activity is modulated by the E8/E2 and E2TR proteins which antagonize the functions of E2TA.

E6 is a zinc binding protein found in the nucleus and nonnuclear membranes. It is capable of transforming certain mouse cells. The E7 protein is unable to tranform cells alone but both E6 and E7 expression are required for the full transformed phenotype. The E6 and E7 proteins of the HPVs are known to bind to the cellular p53 and pRB tumor suppressor proteins but the BPV-1 and CRPV proteins do not have analogous activities. However, BPV-1 E6 protein can bind the cellular focal adhesion protein, paxillin, a clathrin adaptor complex and a calcium binding protein, E6-BP. These interactions may be important for its transforming activity. The E6 protein has also been shown to have transcriptional activating activity in a heterologous assay system.

The BPV-1 E5 protein plays a central role in transformation of established mouse cell lines and, at 44 amino acids, is the smallest known transforming protein. E5 is a membrane protein localized to the Golgi apparatus and plasma membrane and is well conserved among the animal PVs that cause fibropapillomas. Acute expression of E5 results in rapid morphological transformation of mouse cells indicating that E5 is sufficient for transformation and that no secondary cellular event is required. The E5 protein is thought to function by activating the β type receptor for platelet-derived growth factor (PDGF). It is also able to interact with a vacuolar H(+)-ATPase. This appears to block acidification of endosomes which may in turn prolong growth factor receptor stimulation by inhibiting receptor degradation and promoting receptor recycling. E5 is highly expressed in the basal cells of a BPV-1 fibropapilloma and this may result in increased proliferation of these cells. Enhanced replication of basal cells may be important to increase the population of infected cells and to provide a suitable environment for establishment of a productive lesion.

E4 is a cytoplasmic phosphoprotein which is poorly conserved among the PVs. It is thought to be a late-associated protein since it is found in high concentration in the productively infected cells of the wart. Its function in productive infection is unknown and it is not required for viral transformation or DNA replication.

E1 is a nuclear phosphoprotein required for replication of the viral DNA. The E1 protein is an ATP-dependent helicase that specifically binds and unwinds the origin of replication. The E2TA protein is also required for DNA replication and the E1 and E2TA proteins cooperatively bind to the origin. Viral replication also requires host replication factors such as the host DNA polymerase. A second protein translated from the E1 ORF is called the E1M protein. It contains only the N-terminal region of the E1 ORF but its function is unknown. Transient BPV-1 DNA replication requires the E1 and E2TA proteins and the replication origin but long-term episomal maintenance of viral genomes requires an additional *cis* element from the LCR that contains multiple E2 binding sites. This has been designated a minichromosome maintenance element (MME). The E2TA protein and the viral genomes are attached to mitotic chromosomes in dividing cells and this may be important for segregation of viral genomes.

Injection of rabbit skin with CRPV DNA results in papillomas and this system has been used to define which gene functions are required for the formation of warts. These studies have shown that the E1, E2, E6 and E7 genes are required but that the E5 protein is dispensible for papilloma formation.

Evolution

All human and animal PVs sequenced to date have a similar genomic organization with the exception of the B subgroup of bovine papillomaviruses (BPV-3, -4, -6). These viruses have no E6 ORF but instead have an ORF homologous to E5 in the position between the LCR and the E7 gene. This suggests that genomic rearrangements may have occurred during the evolution of the B subgroup of BPVs. The papillomaviruses are evolving very slowly and diversification has occurred over millions of years. It has been estimated that the most variable parts of PV genomes change at a rate of 0.25% per 10 000–20 000 years. For example, the DNA sequence of two different isolates of BPV-1, one isolated in the US and the other in Sweden, differ only by five nucleotides in 8000. The relationship between different papillomaviruses can be established by sequence comparison and represented by a phylogenetic tree, as shown in **Fig. 2.** Within a single species, multiple, minimally related serological papillomavirus types exist. This implies that PVs have evolved in response to pressures from the host immune system, especially since regions of the capsid proteins that are not exposed to the immune system have remained conserved. Papillomas can be infected with more than one viral type, yet there is little or no evidence that recombination occurs among papillomavirus genomes.

Serological Relationships and Variability

Until recently, the study of the serological relationships among papillomaviruses based on antibody

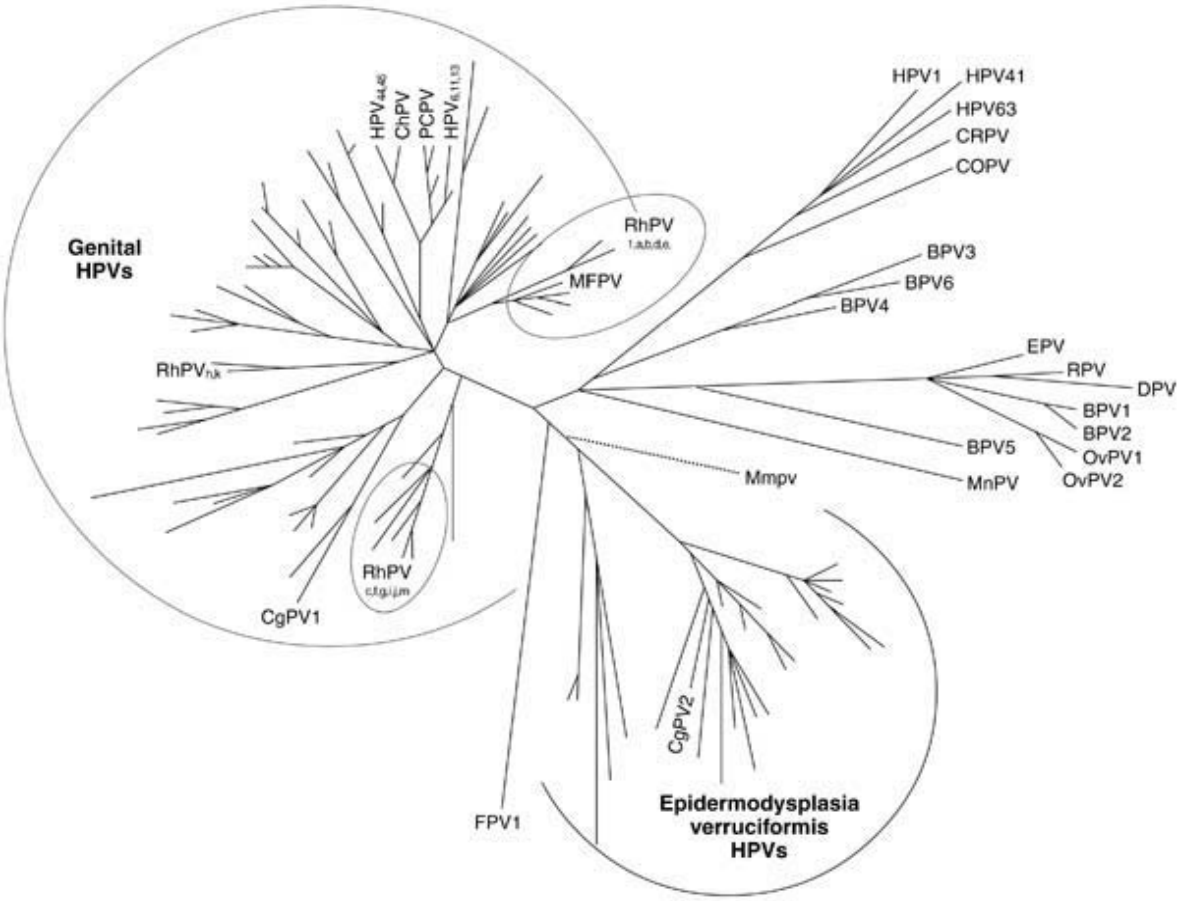


Figure 2 The evolutionary relationship between animal and human papillomaviruses is represented by a phylogenetic tree. (Adapted from Bernard H-U and Chan S-Y (1997) Animal papillomaviruses. In: *Human Papillomavirus*. Los Alamos National Laboratories, *http://hpv-web.lanl.gov/*)

recognition of viral capsids was limited because of the difficulties in obtaining virion particles. However, virus-like particles (VLPs) can be produced by expressing recombinant L1 and L2 proteins and used for antigenicity studies. All types of papillomaviruses (PV) share common, so-called group antigenic determinants. The cross-reactive epitopes are clustered in two regions of the L1 capsid protein. In general, antisera prepared against intact virions of one type are specific to that virus type. However, antisera to detergent-disrupted virus particles reveal internal, genus-specific antigenic determinants that are broadly cross-reactive against the spectrum of PVs. These cross-reactive sera have been used successfully to screen tissue from papilloma-like lesions for the presence of PV antigens.

Transmission and Tissue Tropism

Animal PVs predominantly infect cutaneous epithelium although some show specificity for the mucosal epithelium (e.g. ROPV, COPV, BPV-4). Unlike human PVs, animal PV infections can result in both papillomas and fibropapillomas in which hyperplastic growth is evident in both the infected epithelial cells and the underlying fibroblasts. For example, BPV types 1 and 2 predominantly induce fibropapillomas on the skin of cattle although lesions may also be found on the less keratinized epithelium of the rumen. BPV-3 induces true papillomas on the skin of adult cattle; BPV-5 is associated with 'rice-grain' lesions on the teat whereas BPV-6 causes true papillomas also in association with the teat. BPV-4 is associated with papillomas of the mucosal epithelium of the alimentary tract. Although BPV-4 can be experimentally transmitted to the soft palate, bovine skin is apparently refractory to BPV-4 infection.

BPV infection is readily transmitted in herd animals through direct contact of abraded skin. Natural infection of horses with BPV often occurs after placing the horses in stalls previously housing infected cattle. Some sexual transmission of venereal warts in cattle apparently occurs since such lesions are rare in animals that are artificially inseminated. PV virions

have also been detected in milk suggesting that mammary epithelium may also be susceptible to infection, and represent a potential route for vertical transmission.

CRPV infection is restricted to the cutaneous epithelium of rabbits and lesions can be experimentally induced on the head, neck, back and abdomen. Naturally infection of wild rabbits is most often observed to occur on the head or around the ears suggesting transmission may occur through grooming or fighting, or spread by biting insects such as rabbit ticks. The susceptibility of tissue to virus infection is often stringent. CRPV will only infect hair-bearing skin and is unable to infect epithelium of the tongue, gums, conjunctiva, genitals or mammary glands. Recent evidence suggests that the primary target of experimental CRPV infection may be the epithelial stem cells associated with the hair follicles.

Infection requires direct access to the basal layer of the epithelium that typically occurs through wounding. As a consequence of wounding, a regenerative healing process is initiated which is accompanied by a localized reactivation of cell division. This transient hyperplasia associated with tissue repair may be a critical step in the establishment of stable replicating viral genomes in the nuclei of infected host keratinocytes. Although episomal PV DNA is maintained in the basal cells of the epithelium and in the dermal fibroblasts of BPV-induced fibropapillomas, virus particle production is restricted to the overlying, terminally differentiated cells of the epithelium. It is thought that under certain conditions, a reservoir of latently replicating PV genomes are maintained at a low copy number in the basal cells of the epithelium; and that the latently infected cells can give rise to macroscopic lesions in response to appropriate physical or chemical stimuli.

The factors responsible for the characteristic tissue tropism of animal PVs are not yet fully understood. Tissue specificity could result in part from selective expression of a cell-type specific virus receptor in the basal cells of the epithelium. Recent data suggest that the glycoprotein, α_6 integrin, may serve as a virus receptor for infection. Interestingly, the $\alpha_6\beta_4$ integrin heterodimer has been observed to be selectively expressed in basal keratinocytes where PV infection is thought to occur, and is downregulated in differentiating keratinocytes.

At least on the surface, viral DNA replication does not appear to require tissue-specific factors that are exclusively expressed in epithelial cells. Studies have shown that origin-specific replication of PV DNA is dependent on the expression of two viral proteins, E1 and E2, and the host replicative machinery (e.g. DNA polymerase, topoisomerases, and DNA binding proteins) that are typically found in all actively growing cells. In infected tissues *in vivo*, however, the virus may utilize two different modes of DNA replication: latent replication and vegetative replication. Latent replication in the basal cells of the epithelium refers to the stable maintenance of a small number of copies of the viral genome, which replicate once per cell cycle. Vegetative replication (amplification) occurs exclusively in the more differentiated layers of the epithelium and is thought to be regulated through specific factors expressed (or turned off) during terminal differentiation.

In addition to the host factors involved in virus entry and genome replication, tissue-specific viral gene expression appears to rely heavily on cellular transcription factors. The control of PV transcription is very complex due to the presence of multiple promoters, alternate RNA splicing and polyadenylation, and differential mRNA production. For BPV-1, more than 20 different mRNA species have been described in transformed mouse cells and infected bovine tissue. At least six different promoters are active in productively infected fibropapillomas including the major late promoter (P_L) that is found to be activated in infected keratinocytes. Differential regulation is evident in the stratification of mRNA expression in different layers of BPV-infected warts. The LCR contains a number of binding sites for host transcription factors that act as *cis* regulatory elements in the control of viral gene expression. Clearly, tissue tropism, in part, results from an obligatory and complex pattern of coordinate gene expression that is exclusively found in actively differentiating epidermis.

Pathogenicity

In animals, PV infection causes benign epithelial neoplasms commonly called warts. Individual or clusters of benign papillomas can persist for many months or years or may regress spontaneously. Localized warts do not generally represent a significant threat to the health of the host animal although severe, disseminated outbreaks of warts in wild animals may result in an increased susceptibility to predation or secondary infections.

In a subset of infected animals, benign warts can progress to malignant carcinoma. Carcinogenic progression of benign warts has been relatively well-studied in CRPV and BPV-4 infected lesions. Progression of virus-induced benign tumors to carcinoma is a multifactorial process in which PV infection alone, although necessary, is not sufficient for malignant transformation. Progression is thought to require multiple secondary events in the infected host cells

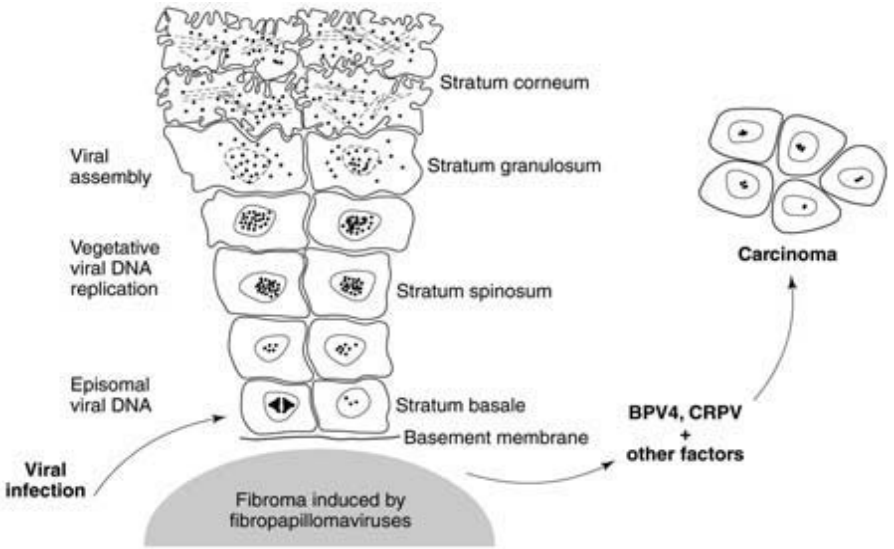


Figure 3 Life cycle of PVs. The diagram shows a model of stratified epithelium. The stages of the viral replicative life cycle that occur in each cell layer are indicated. Fibropapillomaviruses also infect fibroblasts in the dermis and this results in a fibroma. Papillomas infected with certain PV types will often progress to carcinomas.

which can result from the interplay between virus expression, genetic determinants of the host, and external, nutritional or environmental cofactors.

BPV-4 infection is associated with benign papillomas in the alimentary tract of cattle. It was noted some years ago, that an unusually high proportion of cattle in certain regions of Scotland had developed alimentary carcinomas in association with BPV-4 infected benign tumors. Alimentary papillomas were observed to become malignant in areas where cattle grazed on bracken fern which are known to contain various radiomimetic and immunosuppressive agents particularly when grown in specific geographic regions. Therefore, it is only within these specific regions that the consumption of bracken fern by cattle can act synergistically with BPV-4 infection to promote malignant transformation.

CRPV infection of rabbits was reported almost 70 years ago to induce benign cutaneous warts that could progress to malignant carcinomas. In addition to virus infection, several other factors were observed to be important determinants for progression. For example, the genetic background of the host is important; infection of domestic rabbits induces benign warts which will progress to carcinomas at a rate that is roughly three times higher than similar lesions in wild rabbits, the natural host. In addition to host-specific effects, progression to carcinoma is a relatively slow process occurring in both wild and domestic rabbits over the course of 1–2 years. As noted above with BPV-4, cocarcinogenic effects have been observed with CRPV-induced lesions. The topical application of methylchloranthrene will both increase the proportion of benign tumors which will progress and will reduce the time required for progression to as little as 6 months.

Clinical Features of Infection

PV infection is not associated with overt, systemic clinical symptoms. In cattle, exposure often occurs in young calves who will develop protective immunity subsequent to regression of the benign fibropapillomas. Such prior exposure presumably accounts for the relatively low incidence of disease in adult animals.

During natural outbreaks, BPV infected lesions arise in 101–126 days after exposure and can regress spontaneously between 18 and 173 days after warts become visible. At present, little is understood about clinically inapparent or latent infection. Several studies of CRPV and rabbit oral papilloma virus (ROPV) have indicated that viral DNA can be detected in clinically normal epithelium although the pathological consequences are not well understood.

Pathology and Histopathology

BPV-1 associated fibropapillomas in cattle occur either singly or as multiple nodules and may reach several centimeters in size. They may appear sessile or pedunculate and lobate, fungiform or verrucate. Other bovine PVs cause flat or filiform teat papillomas and BPV-4 causes alimentary papillomas that can progress to carcinomas. CRPV-induced papillomas occur as multiple, gray or black, well-keratinized

masses. They range in size from 0.5 to 1 cm in diameter and can reach several centimeters in height resulting in cutaneous 'horns'. The associated carcinoma appears as a raised, fungoid or eroding lesion.

Fibropapillomas show a marked proliferation of the underlying fibroblasts within a dense matrix of collagen. Fibroplasia occurs within the first week after infection whereas hyperplasia of the epithelia may not be visible before 4–6 weeks. This long delay in the appearance of epithelial changes is similar to that observed with the strictly epithelial PVs such as CRPV where it may take 3–8 weeks for a papilloma to appear.

The PV life cycle is exquisitely attuned to the progressive vertical differentiation that occurs during maturation of the epidermis (see **Fig. 3**) and the study of the PVs has been hampered because of the difficulties in reproducing such a system in cell culture. Papillomavirus infection results in a number of changes in the epidermis. The rate of division of the epidermal cells is increased and this results in acanthosis (thickening), papillomatosis (folding) and hyperkeratosis (increase in the horny layer). Vacuolation of cells (koilocytosis) occurs in the stratum spinosum and granulosum and abnormal keratohyalin granules are produced. PVs infect the basal layer of a stratified epithelium. The $\alpha 6\beta 4$ integrin protein, that is expressed exclusively in this cell layer, probably acts as the receptor for virus binding and entry. Damage to the superficial skin or mucosa is presumed necessary to allow access of virus to the basal layer. Within basal cells, the viral genome is thought to be amplified to a low copy number and maintained as an episome. In a bovine fibropapilloma, the E5 protein is expressed in the basal cells and this may increase proliferation of these cells. The basal cells differentiate and move upwards to form the stratum spinosum and granulosum. These cells no longer divide and are committed to terminal differentiation which includes progressive keratinization and enucleation. In the stratum spinosum, expression of the viral E2 proteins is greatly increased and vegetative DNA replication begins. In the HPVs the E7 protein is required at this stage to induce cells to enter S-phase and synthesize cellular replication proteins. However, this disruption of cell cycle control in differentiating cells can cause cells to undergo apoptosis (programmed cell death to eliminate damaged cells) and one function of the E6 protein may be to prevent this. Presumably the animal PV proteins have similar functions. Viral-induced hyperplasia is believed to be induced by the viral early gene products, and results from both increased division of the basal cells and delayed maturation of the committed keratinocytes of the spinous layer (acanthosis). Expression of the viral capsid proteins, L1 and L2, is first detected in cells of the stratum spinosum and virus-specific cytopathic effects are most pronounced in the stratum granulosum. Virions are assembled in the upper differentiated layers of the papilloma and are found throughout the nuclei, frequently organized into paracrystalline arrays.

Immune Response

Both humoral and cell-mediated immune responses can develop in papillomavirus infections. Infection results in benign proliferative lesions that usually regress spontaneously through a cell-mediated immune response. Infected animals demonstrate a typical primary response to antigen, with the development of a full IgG response by 6 weeks. However, bovine and rabbit papillomas continue to grow in the presence of circulating antibodies and only regress following leukocyte infiltration. Once regression of a single wart begins all papillomas begin to regress, indicating that the mechanism of immunity is systemic. The immune system is the major determinant of regression since animals which have had their immune system suppressed by methylprednisolone show almost a 20-fold reduction in regression frequency. This regression leaves the host immune to reinfection with the same PV type and this protection is thought to be due to the production of neutralizing antibodies against the capsid proteins.

Regressing warts contain a mononuclear cell infiltrate. Leukocytes are present near the basement membrane but do not coincide with the areas of reduced cell proliferation, suggesting that soluble lymphokine-like substances are involved. Lymph node cells from regressor and nonregressor rabbits inhibit *in vitro* colony formation by papilloma- and carcinoma-derived cells. However, nonregressor sera can specifically block the inhibitory effect of lymph node cells, suggesting that immunosuppressive humoral factors may contribute to the persistence of tumors. These results also suggest that a cell-mediated response may be the primary factor for regression.

In rabbits, cattle and dogs, papillomavirus neutralizing antibodies (Ab) generated against either the capsid proteins or VLPs protect the animal from reinfection and have been the basis of very successful prophylactic vaccines. When Ab neutralization of inoculated virus is avoided by transplantation of infected skin or by transfection with viral DNA, papillomas occur in animals with persistent warts but not in those where the warts have regressed, again suggesting cell-mediated immunity is the primary factor in rejection. Although neutralizing antibodies

are not sufficient to bring about tumor rejection, vaccination with CRPV-derived tumor cell preparations has been shown in some cases to increase the rate of regression. Regression and immunity to subsequent PV infections may result from a cell-mediated reaction against a tumor associated antigen as opposed to a virus-specific antigen; however, such a cellular determinant has not been identified.

Prevention and Control

Treatment of papillomas in animals is not routinely prescribed. Typically the disease is self-limiting although of variable duration. Unlike HPV infection of humans, there is little economic impact resulting from PV-associated disease in animals. Infection of cattle primarily affects the hide with little or no consequence to the quality or harvest of beef. Benign warts or sarcoids in thoroughbred show horses or race horses can however reduce the commercial value of the animals. In contrast however, models of PV infection in animals have seen extensive use in the evaluation of prophylactic and therapeutic modalities prior to human clinical trials against HPV.

Inoculation with homogenized, autologous wart tissue has been used with some success to stimulate regression of CRPV-induced warts. Aromatic retinoids known to affect epithelial differentiation have also been used to treat CRPV-induced lesions. In these studies, up to 60% of the rabbits treated with retinoids showed complete and permanent regression of well-established tumors. BPV-1 transformed mouse cells have been shown to respond therapeutically to murine β-interferon, reducing or eliminating viral DNA in the transformed cells. Cells cured of BPV DNA are no longer morphologically transformed, and they fail to induce tumors in athymic mice. Various photodynamic therapies have been preclinically evaluated against CRPV-induced benign warts. Hematoporphyrin derivatives (HPD) are preferentially retained in wart tissue over the surrounding normal tissue. When activated by exposure to light, HPD is toxic to cells resulting in localized eradication of warts.

Prophylactic vaccination has been used successfully in cattle with a commercially available, formalin-inactivated homogenate of bovine fibropapillomas that is injected intradermally in two doses. Vaccinated herds show a lower incidence of warts and cows are protected from experimental infection. Vaccination with purified capsid proteins, L1 and L2 has shown encouraging results. In one study, 12 of 13 calves were successfully protected from experimental challenge with BPV-1 following intramuscular vaccination with 5–10 mg of L1 protein expressed in bacteria. More recent work in which the L1 and L2 proteins have been expressed to high levels in insect cells, yeast, or from vaccinia vectors has shown that self-assembly into 'virus-like particles' (VLPs) can occur in the absence of viral DNA. VLPs are currently being evaluated for use in prophylactic vaccination of humans against HPV infection. Vaccination of dogs with VLPs derived from COPV was recently used to determine whether prophylactic modalities would result in the generation of immunity to PV infection at mucosal sites. These experiments in dogs were of particular importance since genital and oral HPV infection afflicts the mucosal epithelium which is somewhat remote from systemic immunity.

Perhaps one of the most interesting vaccine technologies currently under investigation is the use of naked DNA capable of expressing various viral gene products. In a typical PV infection, virus particles and capsid antigens are present only in the more superficial regions of the epithelium that may not be readily accessible to circulating antibodies. A more useful target for therapeutic vaccination may be the early proteins that are expressed in the proliferating cells of the basal layer. DNA vaccination offers an opportunity for expression of early proteins, and for natural proteolytic processing of viral antigens by the immune system, which is thought to be critical to the generation of cell-mediated immunity. Again it has been animal models such as CRPV infection, which has offered proof of principle for the potential applications of this technology. In the future, primate models of PV infection may see substantial use in the preclinical evaluation of both prophylactic and therapeutic treatments for HPV infection.

Future Perspectives

Many questions remain to be answered, including which viral and host factors are responsible for malignant progression and what is the basis for the differences in tissue tropism exhibited by the many viral types. Our understanding of the mechanisms by which viral gene expression is regulated by keratinocyte differentiation is not well understood. More also needs to be learned about the interaction of the virus with the host immune system, especially as it relates to regression. Is there a role for latency in the virus life cycle and what signals might activate latent virus?

The lack of a productive *in vitro* system for passaging virus has severely hindered the study of PVs but in recent years the use of organotypic cell culture techniques, xenografts on immunocompromised animals and methods of introducing viral DNA to the intact epithelium of animals has enabled viral particles to be produced from cloned viral DNA.

These systems are continually improving and should allow a detailed genetic analysis of the complete viral life cycle.

The ability to generate VLPs has revolutionized papillomavirus research. These VLPs are very useful for serological studies and have been the basis of very successful prophylactic vaccines in cattle, dogs and rabbits. These animal systems should also prove useful for developing efficient therapeutic vaccines and for testing antiviral drugs that may be used in treatment of PV infections in humans.

BPV-1 continues to serve as the molecular prototype for the study of papillomavirus DNA replication and transcriptional regulation. The ability of this viral genome to efficiently and stably replicate extrachromosomally will prove to be very useful in understanding the mechanisms by which extrachromosomal elements stably replicate and segregate their DNA. Such studies may also lead to improved gene transfer vectors based on papillomavirus genomes. BPV-1 genomes packaged in heterologous VLPs have been used in neutralization studies because of the ability of BPV-1 genomes to quantitatively transform certain mouse cells. This property of BPV-1 should continue to prove useful for many different studies.

See also: **Immune response: Cell mediated immune response, General features; Latency; Papillomaviruses – human (*Papovaviridae*): General features, Molecular biology; Transformation: Animal viruses; Virus–host cell interactions.**

Further Reading

Howley PM (1995) Papillomavirinae: the viruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al*, *Fields Virology* p. 2045. Philadelphia: Lippincott-Raven.

Myers G, Baker C, Wheeler C *et al* (eds), (1995, 1996, 1997) Human papillomavirus Los Alamos National Laboratories, *http://hpv-web.lanl.gov/*

Lancaster WD and Olson C (1982) Animal papillomaviruses. *Microbiol. Rev.* 46: 191.

Pfister H (ed.) (1990) *Papillomaviruses and Human Cancer*. Boca Raton: CRC Press.

Salzman NP and Howley PM (eds) (1987) The Papovaviridae; The Papillomaviruses Series. *The Viruses*. New York: Plenum Press.

Sundberg JP (1987) Papillomavirus infections in animals. In: Syrjanen K, Giasman L and Koss LG (eds) *Papillomaviruses and Human Disease*. Berlin: Springer Verlag.

PARAINFLUENZA VIRUSES (*PARAMYXOVIRIDAE*)

Contents

Human

Animal

Human

Allen Portner, Department of Virology and Molecular Biology, St Jude Children's Research Hospital, Memphis, USA

Introduction

The human parainfluenza viruses (PIVs) are an important cause of respiratory disease in infants and children. Four types were discovered between 1956 and 1960. PIV-1, PIV-2 and PIV-3 were first isolated from infants and children with lower respiratory tract disease and subsequently shown to be a major cause of croup (types 1–3) and pneumonia and bronchiolitis (type 3). PIV-4 was initially isolated from young adults and has been associated with mild upper respiratory tract disease of children and adults. Other viruses antigenically and structurally related to the human paramyxoviruses have been isolated from animals. Sendai virus, a natural pathogen of mice and not of humans, was the first PIV isolated and is antigenically related to human PIV-1. Simian virus (SV)5, recovered from primary monkey kidney cells, causes croup in dogs and is a subtype of human type 2, and bovine shipping fever virus is a subtype of type 3.

Taxonomy and Classification

The PIVs belong to three genera, *Respirovirus*, *Rubulavirus* and *Morbillivirus*, of the subfamily *Paramyxovirinae* in the family *Paramyxoviridae*. Some other species found in the *Rubulavirus* are

PAPILLOMAVIRUSES – HUMAN (*PAPOVAVIRIDAE*)

Contents

General Features

Gérard Orth, Unité des Papillomavirus, INSERM U. 190, Institut Pasteur, Paris, France

History

The recognition of different clinical types of warts and the suspicion of the venereal transmission of anogenital warts date from as early as the Greco-Roman times. The infectious nature of warts was substantiated in the 1890s. Skin wart transmission to humans by cell-free wart extracts demonstrated their viral etiology (Ciuffo, 1907). Rabbit skin papillomas and derived carcinomas induced by the cottontail rabbit papillomavirus (Shope, 1933) provided the first model of viral carcinogenesis in mammals. This led to such concepts as virus masking in tumors and synergy between viruses and chemical carcinogens in the development of cancers. The study of papillomaviruses has been hampered by the lack of tissue culture systems allowing their propagation. It has long been admitted that all types of warts in humans are due to a single papillomavirus, the human wart virus, and that differences in wart morphology and clinical evolution are determined by local conditions. The plurality of human papillomavirus (HPV) was demonstrated in the late 1970s, as a consequence of advances in DNA technology. It was proposed then that the diversity of papillomavirus-associated lesions reflected distinct biological properties of the different HPV types. The first evidence for the role of HPVs in human cancer was provided by the specific association of HPV type 5 (HPV5) with the skin cancers of epidermodysplasia verruciformis (EV), a rare genetic disease. In the mid-1970s, koilocytes in cervicovaginal smears had been recognized as HPV-infected cells, revealing the frequent HPV infection of sexually active women. The frequent association of morphologic signs of infection and features of cervical dysplasia suggested a role of HPV in the carcinogenesis of the uterine cervix. Specific HPV types (HPV16, HPV18) were found associated with invasive cervical cancer in the early 1980s. More than 100 HPV genotypes have been identified, so far. The high prevalence of sexually transmitted genital HPV infections and the central role played by some HPV types in the development of cervical carcinoma, the second most common cancer in women worldwide, explain the present wide interest in these viruses.

Taxonomy and Classification

Papillomaviruses constitute a genus of the *Papovaviridae* family. They are characterized by a naked icosahedral capsid of 55 nm diameter, composed of 72 pentameric capsomeres (**Fig. 1**). Their genome is a double-stranded, covalently closed, circular DNA molecule of 7500–8000 bp. The genetic information is located on one DNA strand and consists of at least eight open reading frames (ORFs). The viral genome is divided into three parts: (1) a noncoding region, the long regulatory region (LRR) (7–11% of the genome length); (2) the early (E) region (about 50% of the genome length) which encodes proteins capable of interacting with cellular proteins negatively regulating cell division (E6, E7, E5), and proteins involved in the replication and transcription regulation of the viral genome (E1, E2) or expressed late in the viral cycle (E4); and (3) the late (L) region which encodes

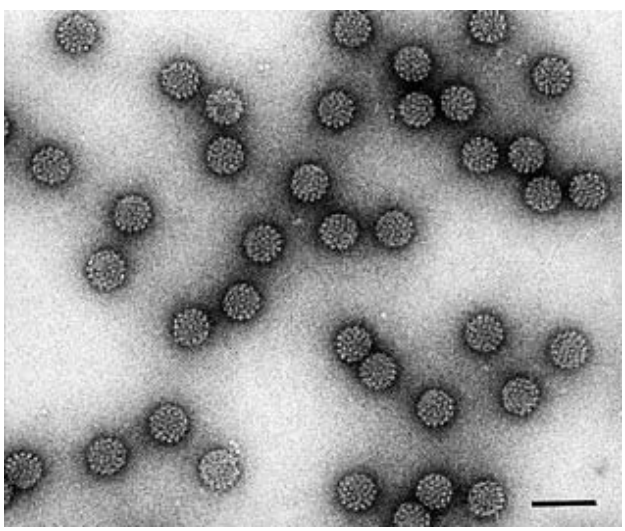


Figure 1 Human papillomavirus type 1 particles purified from plantar warts. Bar = 100 nm.

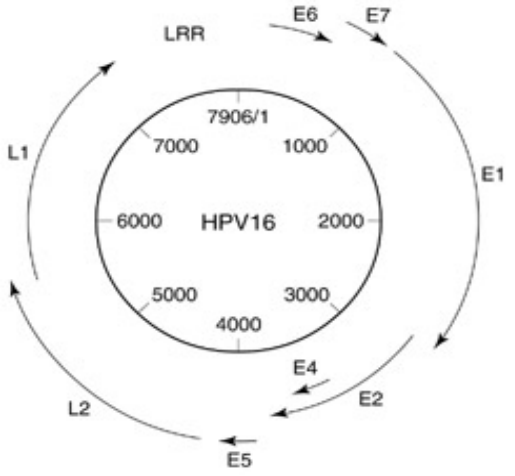


Figure 2 Genetic map of HPV16.

the capsid proteins, the major L1 protein constituting the capsomeres and the minor L2 protein (**Fig. 2**).

HPVs are classified into genotypes. Types were first defined on the basis of a crosshybridization between viral genomes less than 50%, in the most stringent hybridization conditions. According to an almost equivalent recent definition, genotypes should share less than 90% identical nucleotides in the L1 ORF. HPV isolates sharing a DNA sequence identity in the range of 90–98% correspond to subtypes, while isolates with more than 98% DNA sequence identity are considered as variants. Genotypes are designated by a number and subtypes by a letter, following the chronological order of their characterization. To date, at least 80 HPV genotypes have been cloned and the complete nucleotide sequence is available for almost all of them. The most distantly related HPV1 and HPV41 and the closely related HPV6 and HPV11 display 47% and 82% identical nucleotides, respectively.

DNA heteroduplex analysis and sequence comparison have revealed the existence of conserved regions between the viral genomes, mainly localized in the 3′ part of the E1 ORF and in the L1 ORF. These conserved sequences have allowed the design of consensus or degenerate primers for the detection of a broad range of HPV DNA sequences in cutaneous and genital lesions, using polymerase chain reaction (PCR) techniques. By sequencing the PCR amplification products, more than 50 putative novel HPV types have been identified. This indicates that the total number of HPV genotypes largely exceeds 100.

Geographic and Seasonal Distribution

HPV infections have a worldwide distribution, as illustrated by skin warts and genital warts, their well-known clinical manifestations. All around the world, HPV5 and HPV16 are the types most frequently associated with EV skin cancers and cervical carcinomas, respectively. Differences in geographic distribution have been noted for some HPV types. A higher prevalence of cervical cancer associated with HPV45 has been reported in Africa, and with HPV52 or HPV58 in China and Japan. HPV13 and HPV32 cause an oral disease mostly restricted to Central and South America, Alaska and Greenland.

Host Range and Virus Propagation

HPVs are highly host specific and humans are not susceptible to infection by animal papillomaviruses. It has been shown, however, that papillomaviruses bind a widely expressed and evolutionarily conserved cell surface receptor. HPV capsids agglutinate mouse erythrocytes and oncogenic genital HPVs are capable of transforming established rodent cell lines. Routine propagation of HPVs *in vitro* has not yet been achieved. A major reason for this is that HPVs are strictly epitheliotropic and replicate exclusively in terminally differentiating keratinocytes of the benign and premalignant lesions they induce. Upon grafting under the renal capsule of athymic mice, human foreskin chips infected *in vitro* with a particular strain of HPV11 form condylomatous cysts that produce large amounts of infectious virus. Progress in the ability to express late papillomavirus functions *in vitro* has recently been achieved by using keratinocyte cultures on collagen rafts at an air–liquid interface, that is in conditions that allow terminal differentiation of keratinocytes. Viral particles were produced from condyloma explants containing the HPV11 Hershey strain or from a keratinocyte line derived from a cervical intraepithelial neoplasia that harbored episomal HPV31 DNA. Oncogenic genital HPV types 16, 18, 31 and 33 are capable of immortalizing human keratinocytes. In such cell lines, the viral genome is integrated into the cell genome, an event that is not part of the virus life cycle and precludes virus multiplication. It has been shown recently, however, that cell lines harboring viral episomes could be obtained by transfecting cloned HPV18 or HPV31 DNA together with a drug resistance marker into primary keratinocyte cultures, and that viral particles were produced by growing these cells in raft cultures.

Genetics

Nucleotide sequence variability in the LRR and the E6, E7, L1 and L2 ORFs has been studied extensively for the oncogenic HPV5 associated with EV, for HPV

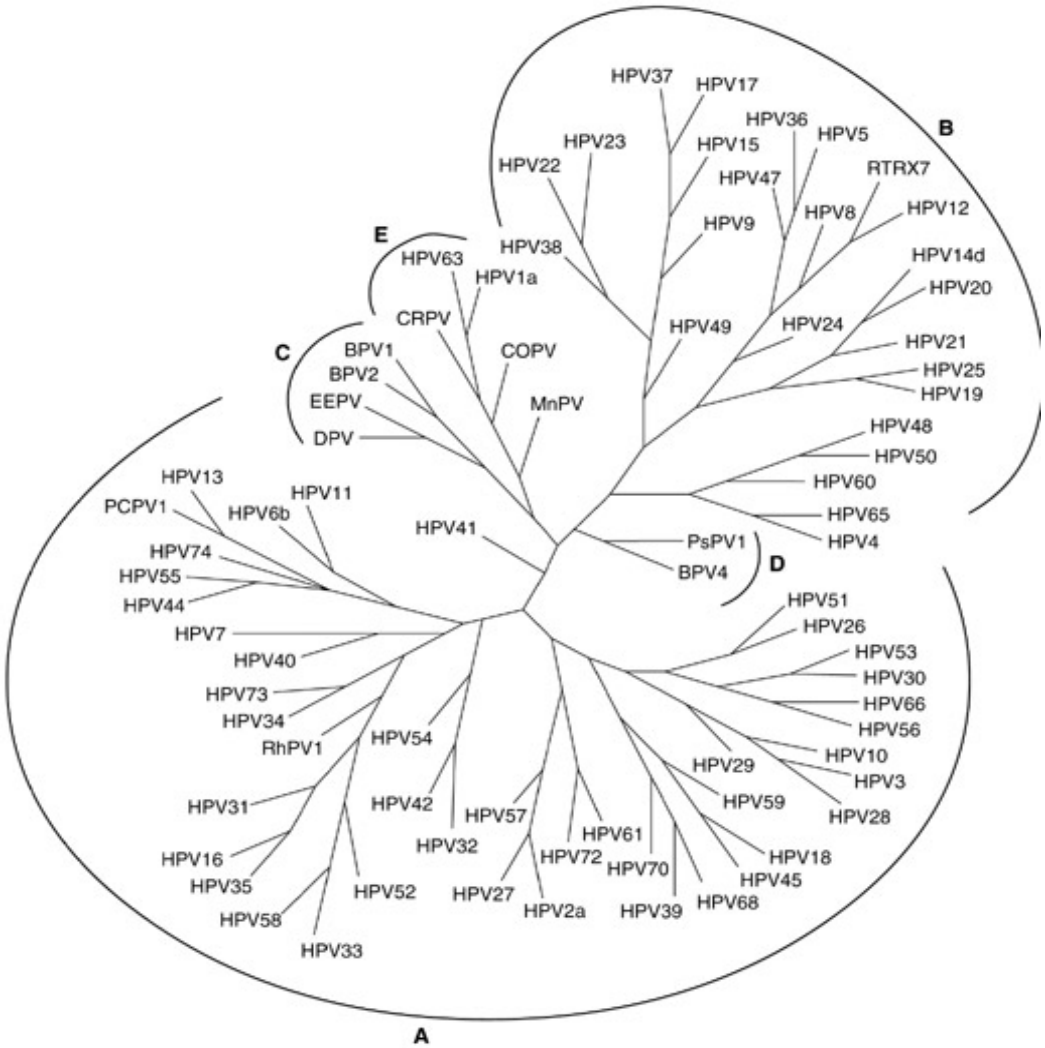


Figure 3 Phylogenetic tree of human and animal papillomaviruses based on the amino acid sequence of the L1 protein. (Adapted from Chan SY, Delius H, Halpan AL and Bernard HU (1995) *J. Virol* **69**: 3074.)

types 6 and 11 associated with condylomata acuminata, and for HPV types 16, 18 and 45 associated with genital cancers. For genital HPVs, the intratype variability does not exceed 2% in the coding region and 5% in the LRR. In contrast, an unusually high degree (about 10%) of intratypic variability has been observed in the LRR and the E6 and L1 genes of HPV5, leading to the identification of three HPV5 subtypes. To what extent this genetic variability affects the biological properties of the viral proteins is still poorly understood. HPV6 variants with short sequence duplications in the regulatory region and HPV5 variants with large deletions within the genes encoding capsid proteins have been identified in Buschke–Loewenstein tumors and EV carcinomas, respectively. It is not known whether such rearrangements play a role in tumor progression.

It has to be stressed that, despite their amazing multiplicity, HPV types, subtypes and variants are stable entities, most probably because HPVs use the cellular machinery for replicating their genome. Furthermore, there is no evidence for inter- or intratypic recombinations.

Evolution

Computer analysis of the sequences of the E6 and L1 genes has distributed the human and animal papillomaviruses into five phylogenetic supergroups and in groups that reflect tissue tropism and pathogenicity (**Fig. 3**). HPVs are distributed into supergroups A, B and E. Supergroup A contains mucosotropic potentially oncogenic genotypes (HPV types 16, 18, 31, 33, etc.) or nononcogenic genotypes (HPV types 6, 11, etc.) and some cutaneous types occasionally infecting mucous membranes (HPV types 2, 3, 7, 10, 28, 57,

etc.). Supergroup B contains 20 HPV genotypes specifically associated with EV, as well as a few other skin genotypes (HPV types 4, 48, 50, 60 and 65), while supergroup E comprises only cutaneous HPV types (HPV types 1 and 63). Phylogenetic grouping of HPV16 DNA variants has allowed identification of African, European, Asian and Asian-American lineages. The greater genetic heterogeneity of African isolates has led to speculation that HPV16 emerged in Africa 200 000 years ago and coevolved with the human races.

Serologic Relationships and Variability

Genital lesions usually contain very low amounts of virions, and purified particles are only available for some cutaneous HPV types (mostly HPV1 from plantar warts) and for the HPV11 Hershey strain. A limitation in the use of bacterial fusion capsid proteins and synthetic peptides as antigens is that only linear epitopes are presented, whereas natural human antibodies and hyperimmune antisera obtained with virions are mostly directed against native conformational epitopes. When produced in various eucaryotic expression systems, the L1 protein is able to self-assemble into virus-like particles (VLPs), with a morphology similar to that of virions. When L2 is coexpressed with L1, it is stably incorporated into the capsids. HPV16 L1/L2 VLPs produced in cells that maintain bovine papillomavirus (BPV) type 1 genome as high copy autonomous replicons are able to incorporate viral DNA. These HPV16 virion pseudotypes are able to transform murine C127 cells, thus providing a means of analyzing antigenic determinants and neutralizing epitopes.

Early studies on cutaneous HPV types 1–5 led to the identification of type-specific and group-specific (type-common) antigens. Monoclonal antibodies raised against VLPs have allowed the identification of both conformational, usually type-specific epitopes, and surface or cryptic linear epitopes that show various levels of cross-reactivity when analyzed by an ELISA test using intact or denatured VLPs.

When the serological relatedness of genital HPV types 6, 11, 16, 18, 31, 33 and 45 was assessed using rabbit antisera and L1/L2 VLPs in a hemagglutination inhibition test, only the closely related HPV types 6 and 11 or HPV types 18 and 45 (more than 85% L1 amino acid identity) exhibited detectable cross-reactions. However, HPV6 and HPV11 were found to have antigenically distinct, strongly immunogenic, conformationally dependent neutralizing epitopes in an athymic mouse xenograft infectivity assay. So far, the existence of distinct serotypes of the different genital HPV genotypes has not been reported but it has been shown that L1 VLPs of the three HPV5 subtypes have distinct antigenic properties.

Type-common epitopes are masked in intact particles. They are detected in sections of formalin-fixed lesions, using antisera raised against disrupted particles of HPV1 or BPV1 or against L1 fusion proteins. Such antibodies are not usually detected in human sera. Type-common epitopes have been mapped to the middle part of the L1 protein, which contains an amino acid sequence remarkably conserved among papillomaviruses.

Epidemiology

It has long been known that HPV infections are widespread from the high prevalence of skin warts among schoolchildren and young adults (about 10%) and among immunosuppressed patients. Epidemiologic studies have proven difficult because of the multiplicity of HPV types and the late recognition of many of them, and because of methodologic problems. HPV detection methods currently used for research purposes can be classified into HPV DNA test methods without amplification (Southern blot hybridization), signal-amplified hybridization assays (hybrid capture test) and DNA amplification methods (PCR-based assays). The PCR-based tests are the more sensitive, allowing the detection of as little as 10–100 viral DNA molecules, and provide a means to screen for the whole spectrum of HPV infections (from latent or inapparent infections to clinical manifestations). Sequencing PCR products allows the identification of HPV DNA variants. Degenerate MY09/MY11 or general $GP5^+/GP6^+$ L1 primer PCR-based tests and the liquid RNA-DNA hybridization hybrid capture test are presently the most widely used methods for epidemiological studies on genital HPV infections. Depending on the probes used to detect PCR products, PCR-based assays allow the detection of a whole broad range of genotypes or of subgroups comprising low-risk or high-risk (cervical cancer-associated) HPV genotypes, or the identification of specific genotypes. The hybrid capture test allows the chemiluminescent detection of low-risk or high-risk HPVs, using two probe cocktails. The seroepidemiological approach is only of recent use and is based on ELISA using L1 or L1/L2 VLPs.

Prevalence studies indicate that genital HPV infections are very common in sexually active young women, and that most of these infections are asymptomatic and transient. HPV DNA has been detected in cervical cell samples from about one-third of normal women in their teens and early twenties, and at tenfold lower rates in women over 50 years. Cytologic signs of overt infection, including koilocy-

totic atypia, are observed in cervicovaginal smears at tenfold lower rates, with the same age dependence. Only a minority of women exposed to genital HPVs will show persistent infection, and only some of these will develop high-grade cervical intraepithelial neoplasia, the immediate precursor of invasive carcinoma. The incidence of genital HPV infections appears to be similar for men and women. Sexual behavior, particularly multiple sex partners, is the major risk factor for HPV infection.

That immune factors play an important role in the control of HPV infections is shown by the high incidence of cutaneous and genital warts and of anogenital intraepithelial neoplasia among patients with genetic (Wiskott–Aldrich syndrome), iatrogenic (allograft recipients) or acquired (AIDS) cell-mediated immune deficiencies. Cervical HPV DNA detection is two- to fourfold more frequent in women infected with human immunodeficiency virus (HIV). Similarly, homosexual male AIDS patients have a high prevalence of anal HPV infection (about 50%). Genetic factors are prominently involved in two HPV-associated diseases affecting the oral mucosa and the skin. Oral focal epithelial hyperplasia, associated with HPV 13 and 32, is highly prevalent in American Indians and Eskimoes but rarely observed in Caucasians. EV is a genetically conditioned abnormal susceptibility to infection by a group of specific related HPV types and to the oncogenic potential of some of them, mostly HPV5. The reservoir of EV HPVs has long been searched for. By using highly sensitive PCR methods, most of these viruses and uncharacterized EV HPV-related types were detected in plucked hairs of about half of healthy volunteers and over 90% of immunosuppressed renal transplant recipients, pointing to the occurrence of widespread latent infections. As for HPV5, it has been shown recently that patients suffering from psoriasis are likely to constitute the reservoir of this oncogenic EV HPV.

The specific association of HPV7 with warts found in butchers and meat handlers remains to be explained.

Transmission and Tissue Tropism

HPVs are very stable viruses which are released from the desquamating superficial keratinocytes of the infected squamous stratified epithelia. Some HPVs infect the epidermis, while others infect the epithelia of the anogenital and oral mucous membranes. A major target for oncogenic genital HPVs is the transformation zone, at the squamocolumnar junction of the uterine cervix, where most cervical precancers and invasive cancers originate. Simple

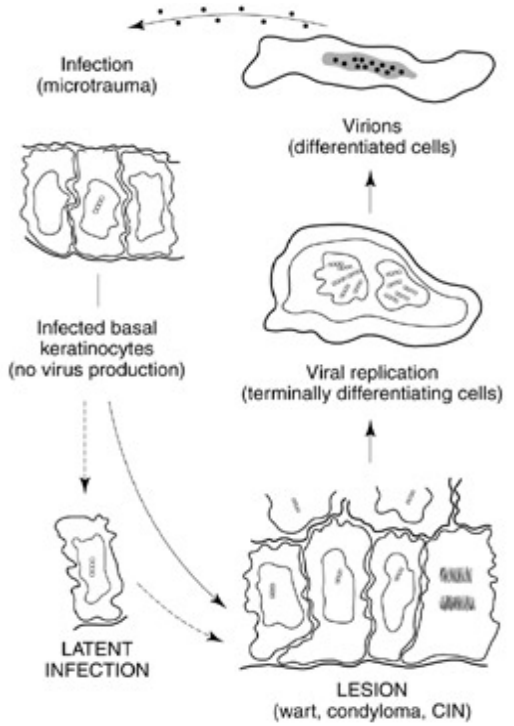


Figure 4 Life cycle of HPVs in keratinocytes.

epithelial cells or reserve cells of the endocervical glandular epithelium are likely to be susceptible to nonproductive HPV infection, which may result in the development of *in situ* or invasive adenocarcinomas. Esophageal, laryngeal, tracheobronchial, nasal, conjunctival and urinary epithelia are uncommon sites of infection with the known HPV types.

Transmission of cutaneous HPVs occurs through direct contact with an infected tissue or through indirect contact with contaminated objects or surfaces (swimming pools). Genital HPVs are sexually transmitted, as shown by partner tracing and epidemiologic studies. Transmission of HPV6 and 11 by passage through an infected birth canal is probably at the origin of laryngeal papillomatosis of young children. *In utero* infection with genital or EV-specific HPVs has been recently considered. Indirect transmission of genital HPVs may occur through contaminated fomites or instruments and by laser plumes.

The first stage of the HPV life cycle is the infection of basal keratinocytes, through minor abrasions (**Fig. 4**). The $\alpha 6$ integrin has been identified as a candidate receptor. Virus entry may result in a latent infection or in the development, within 1–3 months, of a lesion likely to result from the clonal expansion of infected cells. This proliferation is associated with the persistence of episomal viral genomes at low copy number and with the expression of the E1, E2, E5, E6 and E7 viral genes. Vegetative viral DNA replication

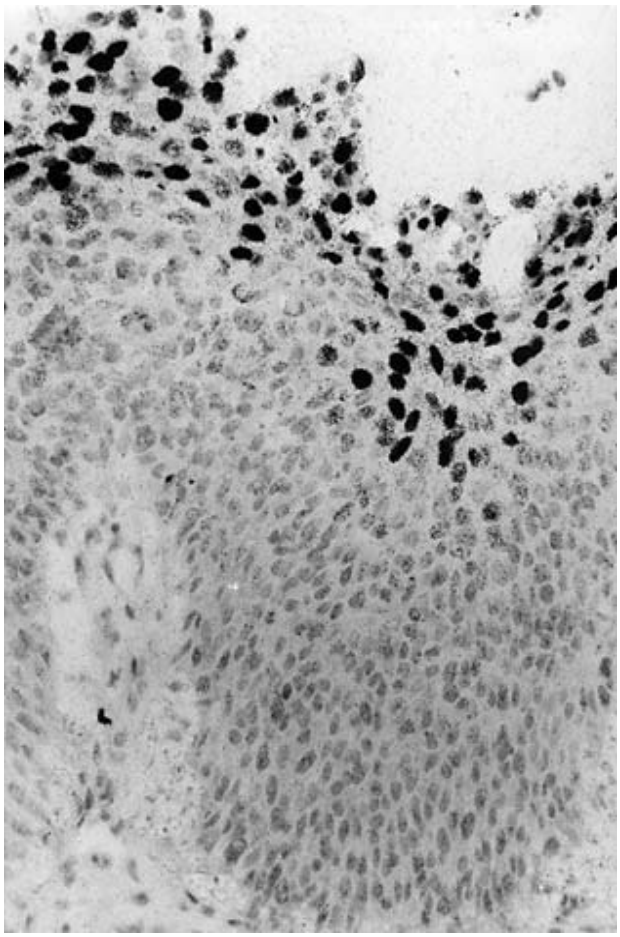


Figure 5 Vegetative replication of HPV16 DNA in the nuclei of superficial cells of a high-grade cervical intraepithelial neoplasia, as detected by *in situ* hybridization.

involving the sustained expression of early viral genes, expression of the E4 nonstructural protein, synthesis of L1 and L2 capsid proteins, and viral particle assembly take place successively in terminally differentiating host cells as they migrate towards the most superficial layers of the lesions (**Fig. 5**).

Pathogenicity

The spectrum of HPV infections includes asymptomatic latent infections, subclinical infections, and various clinical manifestations. HPV genotypes differ in their target tissue and their oncogenic potential, and each of them has a specific pathogenicity. A number of HPV types induce benign, self-limiting proliferations of the skin or the mucous membranes (**Table 1**). Such lesions usually regress, spontaneously or after treatment. Different clinical types of skin warts have been identified, and there is a strong preferential association between a particular HPV type and a clinical type of wart. For instance, myrmecia warts are associated with HPV1, common warts mostly with HPV2 and related types or, in meat handlers, with HPV7, and flat warts with HPV3 and related types. Anogenital warts are usually caused by HPV types 6 and 11. Although these two viruses are found associated with the rare anogenital Buschke–Loewenstein tumors and single cases of carcinoma of the respiratory tract, they are among those genotypes that are associated with low-grade cervical disease and never detected in invasive cervical carcinoma. They are considered as low-risk viruses.

Other HPV types are associated with the development of invasive carcinomas (**Table 1**) and are considered as high-risk viruses. The first example has been provided by the squamous cell carcinomas (SCCs) of the skin observed in about 50% of EV patients. Contrasting with the many HPV types associated with EV benign lesions, only a few of them (mostly HPV5) are found in EV SCCs. Viral genomes are maintained as episomes and transcripts of the E6 and E7 genes are easily detected. The role of HPVs in the etiology of nonmelanoma skin cancers in non-EV patients is still a matter of speculation. If one excepts periungual SCCs, HPV DNA sequences are only rarely detected by Southern blot hybridization in premalignant lesions (actinic keratoses, Bowen's disease) and in basal cell carcinomas (BCCs) and SCCs of the skin from immunosuppressed or immunocompetent patients. The detection limit of this technique is about 0.2 copy of the viral genome per tumor cell, which corresponds to the size of a subgenomic fragment encompassing the LRR and E6 and E7 genes (**Fig. 2**). By using single-step or nested PCR methods and various sets of degenerate primers, numerous known HPV types or putative novel types were detected in up to 90% of the precancerous lesions and invasive carcinomas of immunosuppressed renal allograft recipients and in about one-third of the specimens from immunocompetent patients. Results indicate that EV HPVs and related putative types are the most prevalent, that HPV5 is seldom detected, and that more than one genotype is found in about one-third of the specimens. There are no data available yet on the expression of the viral sequences. It remains to be determined whether PCR detection of HPV sequences identifies passenger viruses or etiologic agents that could act synergistically with ultraviolet radiation, the major known risk factor for skin cancer.

It is now well established that specific, sexually transmitted HPV types play a central role in anogenital carcinogenesis, especially in invasive carcinoma of the uterine cervix, on the basis of virologic, epidemiologic and experimental data. Cervical carcinoma is a progressive disease that begins with a

Table 1 Known pathogenicity of human papillomaviruses

Site	*HPV-associated disease*	*HPV-associated cancer*	*HPV types*
Skin	Wart		**1**, **2**, **3**, **4**, **7**, **10**, 26–29, 41, 48, 60, 63, 65, 75–78
	EV wart and macule		3, **5**, **8**, 9, 12, 14, 15, 17, 19–25, 36–38, 46, 47, 49, 50, etc.
		EV, SCC	**5**, 8, 14, 17, 20, 47
		Periungual SCC	**16**, 34, 35
Anogenital tract	Condyloma acuminatum, papilloma		**6**, **11** 42–44, 54, 55, 74
		Buschke–Löwenstein tumor	**6**, 11
	Intraepithelial neoplasia:		
	Low grade (including flat condylomas)		6, 11, 16, 18, 42–44, 54, 55, 30, 31, 33–35, 39, 40, 45, 51, 52, 56–58, 59, 61, 64, 66–68, 70–74, 79
	High grade (including Bowenoid papules)		**16**, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68
		SCC (mostly cervix)	**16**, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68
		Adenocarcinoma (mostly cervix)	16, **18**
Oral cavity	FEH		**13**, **32**
Tonsil		SCC	**16**, 33
Larynx	Papilloma		**6**, **11**

EV, epidermoplasia verruciformis; FEH, focal epithelial hyperplasia; SCC, squamous cell carcinoma. The most frequent types are indicated in bold type.

noninvasive intraepithelial lesion, designated as squamous intraepithelial lesion (SIL) or cervical intraepithelial neoplasia (CIN). Most low-grade intraepithelial lesions regress; some remain unchanged; 10–15% will progress into high-grade CIN (moderate or severe dysplasia; carcinoma *in situ*). A proportion of high-grade CIN may arise *de novo*. High-grade CIN may evolve into invasive cancer after years or decades. More than 40 known HPV genotypes or putative novel types have been found associated with up to 90% of low-grade CIN. Only some of these HPVs are detected in high-grade CIN and invasive carcinoma. Twelve genotypes were identified in a large worldwide survey of cervical cancers in which 93% of the specimens were found to be HPV positive by a PCR method. HPV16, found in 50% of the cases, HPV18 and HPV45 were the most prevalent and the other genotypes (HPV types 31, 33, 35, 39, 52, 56, 58, 59 and 68) accounted for 1–5% of positive cases. As a rule, HPV16 predominates in SCCs and HPV18 in adenocarcinomas. That HPV was the long sought sexually transmitted infectious cause of cervical neoplasia has been shown by recent epidemiologic studies. Key risk factors for cervical neoplasia (multiple sexual partners, early age at first coitus, etc) were found to be strongly associated with HPV infection. Case–control studies have demonstrated a strong association between the detection of high-risk HPV DNA and high-grade CIN and invasive cervical cancer, with odds ratios usually ranging from 20 to 75. Cohort studies have shown that the risk for progression of a low-grade lesion into a high-grade CIN is closely linked to persistent infection with high-risk HPV types. Experimental studies also strongly suggest that high-risk HPVs play a part in the whole process of cervical carcinogenesis. They point to the central role of the E6 and E7 viral oncoproteins. The E6 and E7 proteins complex and inactivate cellular proteins, such as p53 protein (E6) and pRB protein (E7), that are involved in the negative regulation of the cell cycle and in the control of the cell genome integrity. The development of cervical cancer is a multistep process that remains little understood.

Both immunologic and genetic factors are likely to play a prominent role in the persistence and progression of CIN. Cervical intraepithelial lesions are more frequent and of higher grade, persist at higher rates, and progress more rapidly in HIV-infected women. An immunogenetic control of cervical neoplasia has been suggested by the positive or negative associations of CIN and/or invasive carcinoma with certain HLA class II alleles or haplotypes, some of them showing HPV type-specificity. Among the other factors which may affect the risk of persistence and progression of CIN are hormones (pregnancy, oral contraception),

coinfections (herpes virus, chlamydia), smoking and dietary factors.

Oncogenic genital HPV types, mostly HPV16, are also found in lesions with features of intraepithelial neoplasia (Bowenoid papules, Bowen's disease) and in invasive SCCs of the vulva, penis and anal region. HPV16 and HPV33 are also associated with a high proportion of tonsillar carcinomas. A minority of CIN and cervical carcinomas and a significant number of carcinomas of the external genitalia do not contain detectable HPV sequences and may not be etiologically related to HPV infection. As for HPV sequences detected in 5–25% of carcinomas of the upper aerodigestive tract and urethra, their etiologic role is still unclear.

Clinical Features of Infection

Warts occur predominantly in children over 5 years and in young adults. At least two-thirds of the warts regress spontaneously within 2 years. Palmoplantar myrmecia warts are deep and painful. Common warts are exophytic, hyperkeratotic and located mainly on the hands, fingers and knees. Flat warts are usually multiple, slightly raised, with a flat and smooth surface and are localized mainly on the hands and face. EV is a lifelong disease, characterized by disseminated cutaneous flat wart-like lesions and macules. It may have an autosomal recessive mode of inheritance. Most patients with EV have an impaired cell-mediated immunity. Some benign lesions convert to intraepithelial neoplasia (actinic keratosis, Bowen's disease) and invasive carcinoma in about half of the patients, usually on light-exposed areas, about 20 years after the onset of the disease.

Genital HPV-associated lesions represent the most commonly diagnosed viral sexually transmitted disease. Condylomata acuminata and Bowenoid papules are found mostly in young adults, on the external genitalia and in the anal region. Condylomata acuminata are soft exophytic proliferations, Bowenoid papules are flat or somewhat elevated, often pigmented, usually multiple. In spite of their usual association with HPV16 and their features of intraepithelial neoplasia, they have a rather benign clinical course, in contrast to solitary lesions of genital Bowen's disease affecting individuals over 50 years. Subclinical macules or papules can be detected on the penis and vulva under magnification, after the application of 5% acetic acid. Anal lesions with a variety of morphological appearance and with features of intraepithelial neoplasia are found at the anorectal junction in HIV-infected males. HPV infection of the cervix results mostly in flat aceto-whitening areas corresponding to low-grade CIN, including flat condyloma. Cytologic evidence of HPV infection (SIL) is found in cervicovaginal smears of 2–3% of women in mass screening programs. Infection with genital HPVs are often multiple, and associated diseases are often multicentric. They can affect the entire anogenital region and even the oral mucosa.

Oral focal epithelial hyperplasia, the only disease associated with HPV types that exclusively infect the oral mucosa, is found in children and adults. It is characterized by multiple elevated papules which never undergo malignant transformation. Laryngeal papillomatosis is a rare recurrent disease that can occur at any age. The most frequent site affected is the vocal cords. Trachea, lung, oral and nasal cavities can also be involved. Respiratory papillomatosis may be life-threatening by obstructing airways but rarely progresses to cancer.

Pathology and Histopathology

Benign HPV-induced lesions are characterized by variable degrees of hyperplasia of the different epithelial layers (acanthosis, hyperkeratosis), parakeratosis (retention of nuclei in differentiated superficial cells) and papillomatosis (hypertrophy of dermal papillae). Some features may reflect distinct biological properties of the different HPV types. For instance, the growth pattern is endophytic for HPV types 1 or 4 and exophytic for HPV types 2, 6, 7 or 11. The extent of papillomatosis is prominent for HPV types 1, 2, 4, 6, 7 or 11, much less pronounced for HPV types 3 or 10, and often absent for EV HPVs.

Anogenital lesions with features of intraepithelial neoplasia (IN) are characterized by a disorganized architecture of the squamous epithelium, abnormal mitotic figures and nuclear atypia. Abnormalities restricted to the lower third of the epithelium and well-preserved maturation define low-grade IN (also named mild dysplasia or IN grade I). High-grade IN includes lesions with abnormalities up to two-thirds of the epithelium (moderate dysplasia, IN grade II) or the whole epithelium (severe dysplasia and carcinoma *in situ*, IN grade III). The Bethesda System nomenclature for the evaluation of cervicovaginal smears (Pap smears) has introduced low-grade SIL, which covers CIN I and condyloma, and high-grade SIL, which covers CIN II and CIN III. Features specific to Bowen's disease, especially dyskeratotic cells (individual cell keratinization), are observed in Bowenoid papules and EV lesions undergoing malignant conversion.

HPV replication interferes with the terminal differentiation of the host cells and provokes a cytopathic effect which can manifest in various forms, such as the accumulation of cytoplasmic inclusions

pathognomonic of HPV1-induced myrmecia (containing the E4 viral protein) or the perinuclear cavity that characterizes the koilocytotic cells of genital warts and CIN.

Immune Reponse

The immunobiology of HPV infections is far from being fully understood. Strong immune responses are not usually generated because HPVs most often induce latent infection or chronic disease, and productive infection does not lead to host-cell lysis. A major problem has been the unavailability of native HPV antigens, adequate target cell systems and well-characterized assays.

In natural infection, humoral responses are essentially directed against conformational epitopes borne by intact viral particles. Low levels of antibodies reacting against HPV1 virions are found in patients with skin warts or in nonselected individuals, with a frequency that varies from 10% to 100%, according to the method used, the age group and the type of warts. Antibodies neutralizing HPV11 infectivity assayed in the nude mouse xenograft system have been detected in patients with genital warts or laryngeal papillomas. Owing to the availability of VLPs and the design of sensitive VLP-based ELISA tests, the evaluation of serological responses to HPV infection has recently entered a new era. When using HPV6 or HPV11 L1 VLPs, IgG antibodies have been found in about half of the patients with genital warts. IgG and IgA humoral responses to HPV16 L1/L2 VLPs have been detected in 50–75% of women with HPV16-associated CIN. Antibodies are found in 15–25% of women showing no current signs of HPV infection, probably revealing past infections. Seropositivity to HPV16 L1/L2 VLPs has been shown to be a suitable marker of sexual behavior and a relatively sensitive indicator of persisting cervical HPV16 infection. Antibodies to native HPV16 E6 or E7 oncoproteins are rarely detected in patients with premalignant cervical lesions and are found in about 50% of patients with late-stage invasive HPV16-associated cervical carcinomas. They constitute virus type- and cancer-specific markers but have no prognostic value.

It is most likely that cell-mediated immunity (CMI) plays a major role in the control of HPV infections. A high incidence of cutaneous and anogenital HPV-associated diseases is observed among patients with genetic, acquired or iatrogenic CMI deficiencies. Infiltrates of CD4+ helper and CD8+ cytotoxic T lymphocytes and macrophages are observed in regressing flat warts, genital warts and CIN. This suggests that cytotoxic T lymphocyte (CTL) responses and delayed-type hypersensitivity reactions are important in the regression of HPV-induced lesions. Nonstructural viral proteins are likely to be targets. Helper T lymphocyte and CTL responses against HPV16 E6 and E7 oncoproteins have been demonstrated in murine models. Several HLA-restricted CTL and helper T cell epitopes have been identified in HPV16 E6 and E7 proteins. Occasional memory cytotoxic T cell responses against an HPV16 E7 encoded epitope have been demonstrated in patients with CIN or invasive carcinoma. There is some evidence for a shift from type 1 (IL-2, IFNγ) to type 2 (IL-4, IL-10) cytokine production by helper T cells in patients with extensive genital intraepithelial disease or with invasive cervical carcinoma. Natural killer cells are also likely to be involved in the immunologic surveillance of HPV-associated disease.

HPV-harboring keratinocytes may modify immune responses by secreting various cytokines which could influence local immune responses. Moreover, an altered expression of HLA class I and class II antigens has been observed in a substantial proportion of CIN and cervical carcinomas. Downregulation of HLA class I antigens may provide tumor cells with a means of evading immune surveillance by altering the interaction with cytotoxic CD8+ lymphocytes. Upregulation of HLA class II molecules may turn keratinocytes into nonprofessional antigen-presenting cells which may result in T cell anergy.

Prevention and Control

Education on prevention of sexually transmitted diseases should include appropriate information on HPV infections. Behavior likely to prevent infection (use of condoms, partner examination) should be promoted and the potential consequences of HPV infections should be monitored (routine cervicovaginal smears).

There is at present no vaccine available to prevent HPV infections. The two strategies would be to induce either neutralizing antibodies to prevent virus infection or CMI responses against viral proteins to eliminate HPV harboring keratinocytes. A virus type-specific protection against challenge has been obtained in different animal models (rabbit, cattle, dog) by vaccination with L1 or L1/L2 VLPs. Vaccination against nonstructural proteins has not been found protective but an enhanced rate of regression has been observed in rabbits immunized against the E1, E2 or E6 proteins of the cottontail rabbit papillomavirus and in cattle vaccinated against the E7 or L2 proteins of bovine papillomavirus type 4, indicating that this approach could be used for therapeutic purposes. To date, phase I/II trials of prophylactic HPV vaccines

are currently ongoing or planned. They are based on HPV11 or HPV16 L1 VLPs and on chimeric HPV16 VLPs consisting of the L1 protein plus the E7 protein fused to the L2 protein or constructed by replacing the C-terminal end of the L1 protein by parts of the E7 protein. Therapeutic vaccines are aimed at inducing CTL responses and are based on the use of recombinant vaccinia viruses expressing the HPV16 and HPV18 E6 and E7 proteins, defined HPV16 E6 and E7 peptides or a HPV6 L2-E7 fusion protein. Clinical trials are currently underway to test these vaccines in patients with recurrent late-stage invasive cervical cancer or, as an adjuvant to surgery and radiation therapy, in patients with early-stage cervical cancer, in young women with preinvasive genital disease or in patients with genital warts.

Reliable detection tests of oncogenic genital HPV genotypes have been progressively designed. Ongoing multicentric studies, involving large series of women, are aimed at evaluating their sensitivity, specificity, predictive values and cost-effectiveness in the clinical management of patients or in screening programs.

Since there are no specific anti-HPV therapeutic agents, treatment modalities are mostly aimed at destructing or removing lesions. Conventional treatments involve topical application of caustic agents (salicylic acid, trichloracetic acid, podophyllin), physical destruction (cryotherapy, electrocautery, laser vaporization), surgical removal (including cervical conization) or chemotherapeutic agents (5-fluorouracil, podophyllotoxin). Immune response modifiers could be of value. Interferon has been used successfully for refractory genital warts and, less successfully, in association with surgery, to prevent recurrence of laryngeal papillomatosis.

Future Perspectives

Progress might be expected in the understanding of the intracellular and immunologic mechanisms that control the transition from latent infection to clinically overt infection, the evolution towards regression, and the progression of persistent intraepithelial lesions towards malignancy. Allelic polymorphism or mutations in the genes involved should contribute to the identification of individuals at risk for HPV-associated cancers.

The etiological role of HPVs in cervical cancer should result in new approaches for the early diagnosis and prevention of precursor lesions. An important goal is to determine the proportion of CIN and cervical carcinomas which may be unrelated to HPV infection. It can be anticipated that the association of HPV detection tests to cervicovaginal cytology will permit an increase in the detection rate of high-grade CIN and constitute a means for the adequate management of women with minor cytologic abnormalities. It is also likely that immunoprevention and immunotherapy of HPV-induced genital lesions will be available in the near future. Progress is also expected in the design of therapeutic agents that specifically interfere with viral DNA replication and transcription or with the interactions involving virus-encoded proteins. All these strategies should contribute to the control of invasive carcinoma of the uterine cervix, a major worldwide disease.

Another important achievement will be to understand the biological significance of the frequent PCR detection of HPV DNA sequences in premalignant or malignant tumors at nongenital sites, such as the skin, or in other diseases, such as psoriasis. This may lead to a broadening of the spectrum of HPV-associated diseases.

See also: **Papillomaviruses – human (*Papovaviridae*): Molecular biology; Papillomaviruses – animal; Transformation: Animal viruses; Tumor viruses – human; Pathogenesis: Animal viruses.**

Further Reading

Howley PM (1996) *Papillomavirinae:* the viruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, vol. 2, p. 2045. Philadelphia: Lippincott-Raven.

IARC Working Group on the Evaluation of Carcinogenic Risks to Humans (1995) Human papillomaviruses. *IARC Monogr. Eval. Carcinog. Risks Hum.* 64.

Orth G and Jablonska S (eds) (1997) Papillomaviruses: part one. *Clin. Dermatol.* 15 (2).

Orth G and Jablonska S (eds) (1997) Papillomaviruses: part two. *Clin. Dermatol.* 15 (3).

Shah KV and Howley PM (1996) Papillomaviruses. In Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, vol. 2, p. 2077. Philadelphia: Lippincott-Raven.

zur Hausen H (1996) Papillomavirus infections: a major cause of human cancers. *Biochim. Biophys. Acta* 1288: F55.

Molecular Biology

Paul Lambert, Elsa Flores, Pirkko Heino and **Shiyu Song**, McArdle Laboratory for Cancer Research, University of Wisconsin-Madison, Madison, Wisconsin, USA

Basic Description

Human papillomaviruses (HPVs) infect epithelial cells lining the skin and/or genitalia. Infection leads to the formation of warts, or papillomas. The viral life cycle takes place within these warts and is tied to the differentiation of the infected host epithelial cells. Warts are self-contained benign tumors that by and large do not progress to frank malignancy. A subset of the HPVs that infect the anogenital tract, however, are also associated with malignant tumors, specifically cervical cancer in women and penile cancers in men. This entry describes the basic molecular biology of HPVs, their life cycle, and their contribution to human cancer. General features of HPVs are described in the preceding entry.

Genomic Organization

Papillomaviruses contain a double-stranded circular DNA genome of approximately 8000 bp. Many HPV genomes have been cloned and sequenced; the overall genomic organization among these HPVs is very similar. Ten translational open reading frames (ORFs) are found present among all HPV genomes sequenced. These ORFs are located on a single DNA strand and their relative position is highly conserved among human and animal papillomaviruses. The papillomaviral genome has been divided into three regions, the long control region (LCR), and the early (E) and late (L) coding regions (**Fig. 1**). This division was originally based on the biological properties of bovine papillomavirus type 1 (BPV-1). The LCR contains regulatory sequences that act in *cis* to control the transcription of viral genes and the replication of the viral DNA genome. The early region contains eight ORFs (E1–E8) and was originally defined as the coding portion of BPV-1 that is sufficient to transform rodent cells in tissue culture. The early region is now recognized to contain all of the viral genes expressed in the early 'nonproductive' phase of the viral life cycle (see below). These genes contribute to the regulation of viral transcription and viral DNA replication, and affect the growth of host cells. The late region contains two ORFs which encode the two structural proteins (L1 and L2) that constitute the viral capsid. The late genes are expressed selectively in the late or 'productive' phase of the viral life cycle. In addition there is one gene (E1^E4) residing in the 'early' region of the viral genome that actually is expressed only in the productive phase of the life cycle. By and large, the functions of the individual viral gene products are highly conserved, though apparently important differences in the function and regulation of expression of specific viral genes are seen among the different HPV genotypes.

Papillomaviral Life Cycle

The HPV life cycle is tied to the differentiation of stratified squamous epithelia (**Fig. 2**). The papillomavirus is thought to gain entry to the basal layer of the epithelium at sites of wounding, attaching to the cell via interaction with an integrin, the candidate papillomavirus cell-surface receptor. In this poorly differentiated cell the viral genome establishes itself as a low copy number nuclear episome. A subset of viral genes (the early genes) are selectively expressed at low levels; these genes contribute to the regulated expression of viral genes, viral DNA plasmid replication and cell growth. The initially infected cell is induced to proliferate at a faster rate than the neighboring, uninfected cells, resulting in its clonal expansion and thereby the formation of a papilloma. These events are collectively known as the early or nonproductive stage of the viral life cycle because the infected,

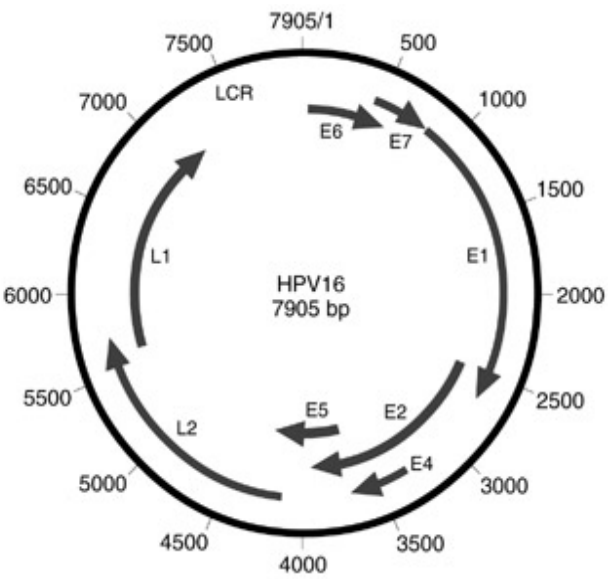


Figure 1 Genomic organization of HPV16. Shown is the circular map of HPV16 double-stranded DNA genome. Early (E) and late (L) translational ORFs are indicated (arrows). The long control region (LCR), also known as the upstream regulatory region, contains transcriptional enhancers and the viral origin of DNA replication.

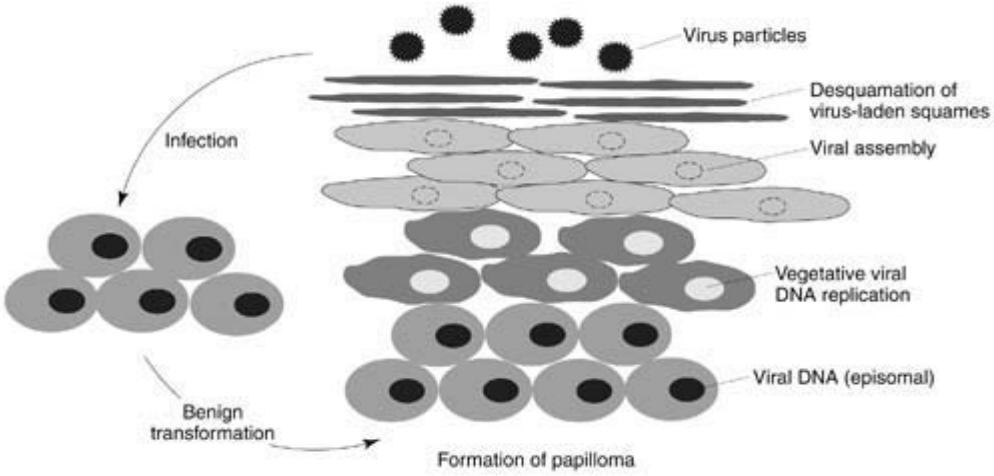


Figure 2 Life cycle of HPVs. The schematic depicts the cross-section of the infected epithelium within a papilloma. The round cells at the bottom represent undifferentiated basal epithelial cells. The flattened cell layers towards the top represent the differentiating suprabasal compartment including the spinous, granular and, most superficially, the corneal layers. Indicated at right are the different events in the viral life cycle occurring in the nonproductively infected undifferentiated and productively infected differentiated cells.

undifferentiated cell population does not produce progeny virus. Following cell division a subset of daughter cells undergo terminal differentiation, as normally occurs in stratified squamous epithelial tissues. As these infected cells progress through the terminal differentiation program, the viral DNA is amplified to high copy number and the full repertoire of viral genes, including the structural genes L1 and L2, are expressed in the spinous and granular layers of the epithelium. Viral assembly occurs in the granular layer. As the squames are sloughed off of the surface of the epithelium, progeny virus particles are released into the environment where they are then available to infect new hosts or new sites on the same host. The papillomaviral life cycle likely allows the virus to evade detection by the host immune system as the higher levels of viral gene expression and progeny virus production occur within the terminally differentiating layers of the stratified squamous epithelia where immune-surveillance is thought to be limiting.

Viral Gene Expression

HPVs have a strict tissue tropism for epithelial cells. This tropism appears to be at the level of viral gene expression. Transcription of papillomaviral genes is dependent upon epithelial specific transcriptional enhancers present in the viral LCR. The regulation of expression of HPV genes within epithelial tissue is complex. Multiple promoters direct transcription of subsets of viral genes. The activities of these promoters are differentially regulated by viral and cellular transcription factors with certain promoters (those directing transcription of early genes) active in both poorly and highly differentiated epithelial cells and others (directing expression of late genes) preferentially active in the highly differentiated cells. Transcripts are differentially spliced, giving rise to mature mRNAs with different coding capacities. These splice events can lead to the fusion of multiple ORFs, thereby generating fusion proteins. One example is the aforementioned E1^E4 gene product which arises from translation of an mRNA in which the E1 ORF is spliced in frame to the E4 ORF. Modulation of gene expression also appears to occur at the level of mRNA stability; mRNA instability elements have been identified in the 3′ untranslated region (UTR) of both the early and late transcripts.

Viral transcription is regulated by viral as well as cellular transcription factors. The viral E2 ORF encodes a family of gene products that possess transcriptional regulatory properties. The full-length E2 gene product, termed the E2 transactivator (E2TA), binds to the viral DNA genome at multiple sites that have the conserved sequence, $ACCN_6GGT$. These sites are primarily located in the LCR. Depending upon the promoter and the amount of E2TA present, binding of E2TA leads to activation or repression of transcription. Based on studies on BPV-1, E2TA-mediated activation results at least in part from the capacity of E2TA to bind cellular transcription factors TFIID and TFIIB. When present at high levels, E2TA can also repress transcription of the HPV promoter positioned directly upstream of the E6

ORF. By binding to DNA binding sites just upstream of this promoter, E2TA is thought to sterically hinder the binding of TBP and SP1 to their cognate sites positioned nearby to the promoter-proximal E2 binding sites. Additional gene products are translated from the E2 ORF through differential splicing or use of internal ATG codons. Originally identified in BPV-1, these E2 proteins act in a dominant-negative manner to inhibit transcriptional transactivation by E2TA. Because they retain the capacity to bind the same $ACCN_6GGT$ motif, they can also repress the promoter that is upstream of E6.

Multiple cellular transcription factors have been shown to bind to the HPV genome primarily within the LCR and to modulate HPV gene expression. These include positively acting (SP1, nuclear factor I (NFI), activator protein 1 (AP-1), AP-2, transcriptional enhancer factor 1 (TEF-1), TEF-2, and enhancer binding factor 1 (PEF-1)) as well as negatively acting (e.g. YY1, Oct-1, c-EBPα and CDP) transcription factors. A subset of the positively acting factors (including cell type-specific members of the NF-1 family) may confer the epithelial specificity in viral gene expression. The negatively acting factors are potentially responsible for the tight regulation of gene expression in the poorly differentiated epithelial cells within the wart, and their reduced abundance in highly differentiated cells may permit heightened expression of viral genes in the productive phase of the life cycle.

Replicative HPV Life Cycle

Papillomaviral DNA replication is dependent on the host DNA replication machinery (DNA polymerase α/primase, DNA polymerase δ/PCNA, replication protein A (RPA) and topoisomerases I and II) and viral proteins, E1 and E2TA. E1 is an ATPase-dependent DNA helicase that is thought to assemble the host DNA replication machinery on the viral genome. E1 binds the viral genome at a specific site positioned at the 3′ terminus of the LCR. E1 also binds to the host DNA polα. E2TA associates with E1 and is thought to help tether E1 to the viral genome through its ability to bind $ACCN_6GGT$ motifs located nearby to the E1 DNA binding site. In basal epithelial cells that are nonproductively infected and harbor the viral genome as a nuclear plasmid, DNA synthesis occurs through Cairns (theta) structures and initiates close to or at the DNA binding site for E1. The viral genomes replicate on average once per cell cycle. Inheritance of the viral genomes to daughter nuclei requires E2TA, which has been postulated to cause the association of viral genomes with host chromatin during mitosis.

In the productively infected epithelial cells of the wart the viral genome is amplified to high copy number for packaging into progeny virions. This amplification appears to occur through a rolling circle mode of replication which is distinct from the theta mode seen in the nonproductively infected basal epithelial cells. Whereas in theta mode of replication reinitiation is required for every round of replication, in rolling circle DNA replication, one initiation event leads to the generation of multiple daughter molecules. Thus, by switching to a rolling circle mode of replication, viral DNA amplification may be facilitated. Little is known as to how this switch in the mode of DNA replication occurs.

Whereas the poorly differentiated basal epithelial cells are mitotically active and produce the necessary factors for DNA replication, the highly differentiated suprabasal epithelial cells are no longer cycling. Thus it is expected that cellular factors necessary for DNA replication are no longer produced in the context of normal, suprabasal stratified squamous epithelia. Because the papillomavirus DNA replication depends on the availability of these factors, the virus must overcome these barriers in the differentiated cells of a wart. One way it may overcome these barriers is by the aforementioned switch in mode of DNA replication. By switching to a rolling circle mode of replication, limiting replicational machinery may be more optimally utilized. The virus may also utilize viral gene products, specifically E6 and E7, to overcome further the unfavorable environment of the highly differentiated suprabasal epithelial cell. E7 protein can bind to and inactivate the retinoblastoma tumor susceptibility locus gene product, pRb, thereby liberating the cellular transcription factor E2F to activate genes necessary for DNA synthesis, including dhfr, DNA polα, PCNA (**Fig. 3**). This activity of E7 may facilitate viral DNA replication. In some cell types, the re-engagement of differentiated cells into a DNA synthesis competent state may lead to the coincident induction of apoptosis. The viral E6 protein can inhibit this apoptosis in part through its ability to bind to and inactivate the p53 tumor suppressor gene product (**Fig. 3**). In addition, E6 may itself affect the proliferative and differentiated character of epithelial cells and thereby independently alter the DNA synthesis competence of the cell. Together E6 and E7 may create a DNA synthesis competent environment in the terminally differentiating layers of the epidermis. This putative role of E6 and E7 is in addition to their likely role in inducing the proliferation of the infected basal epithelial cells that leads to their clonal expansion and consequent formation of the papilloma; and as described below their omni-important role in the association of papillomaviruses with frank cancer.

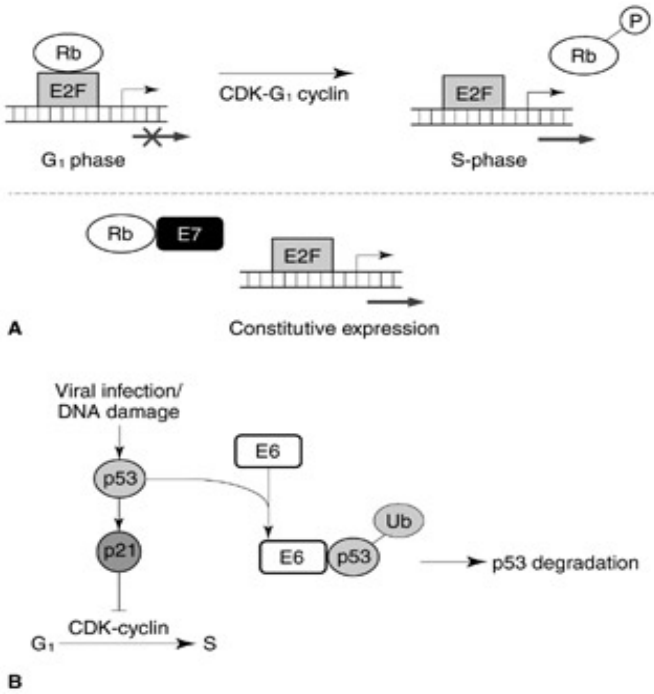


Figure 3 Interactions of high-risk HPV E6 and E7 gene products with cellular tumor suppressors. Shown are macromolecular interactions between the E7 (**A**) and E6 (**B**) gene products and specific cellular tumor suppressors. Note that both E6's interaction with p53 and E7's interaction with pRb leads to increased turnover of the tumor suppressor protein. In the case of E6, the interaction with p53 is mediated by E6-AP, a ubiquitin-ligase that causes the ubiquitination of p53 leading to p53's degradation via the ubiquitin-mediated proteolysis pathway. In the case of E7, its interaction with pRb appears direct through the pocket domain of pRb; this interaction directly interferes with pRb's interaction with E2Fs. The exact mechanism by which E7 induces the degradation of pRb is at present unknown but may involve the ubiquitin mediated pathway as well.

Virus Structure and Assembly

Papillomaviruses (PVs) are nonenveloped icosahedral viruses of about 55 nm in diameter containing the viral genome as circular double-stranded DNA associated with cellular histone proteins. The icosahedral capsids are arranged on a $T = 7$ icosahedral lattice and consist of 72 capsomers ($10T + 2$, $T = 7$), of which 60 are six-coordinated (hexavalent) $10 \times (T - 1)$) and 12 five-coordinated (pentavalent) pentameric capsomers. All capsomers have a fivefold axial symmetry suggesting that they are pentamers of the major capsid protein.

The capsid appears to be composed of 360 copies of the major capsid protein, L1, and 12 copies of the minor capsid protein, L2. The major capsid protein, L1, with a molecular weight of about 55 kDa, is the most highly conserved of the PV proteins. The overall amino acid homology reaches 50% between different PV L1 proteins. The C-terminus of the L1 protein of HPV16 contains two basic sequences which may be responsible for the nuclear localization of the L1 protein. The L1 protein is found only in the nuclei of the terminally differentiated, granular layer of the epithelium. When expressed in heterologous hosts, including bacteria, yeast, insect cells or mammalian cells, L1 protein can self-assemble into virus-like particles (VLPs). VLPs are empty PV capsids consisting of the structural proteins but lacking the viral genome. These VLPs have structural similarity to infectious virus particles and are currently being tested in prophylactic vaccine strategies owing to their ability to induce immunological protection against infection in animal model experiments. The minor capsid protein L2 has a molecular weight of 76–78 kDa. The N-terminal domain of the L2 protein is highly conserved among different PVs. The L2 proteins of fibropapilloma-producing (DPV, BPV-1), cutaneous (CRPV, HPV1a), and mucosal (HPV6, 11, 16) PVs have homologous sequence stretches within each group. This may reflect the tropism of the respective viruses for the skin, the mucosa and fibroblasts. The L2 protein is incorporated into the capsids when L1 and L2 are coexpressed in cells. The exact location of the L2 protein in the capsids is not known; however, it has been speculated that the L2 protein may be located in the center of a pentavalent

capsomer. Immunological studies support the premise that at least the N-terminal portions of L2 are exposed on the surface of the virion. The exact role of L2 protein in the viral life cycle is unclear but it has been suggested to play a role in: (1) correct assembly of the capsid proteins into icosahedral capsids (VLPs composed of L1 only are more heterogeneous in size and shape than capsids where both L1 and L2 are present); (2) selective encapsidation of PV DNA in viral capsids (viral genomes can be packaged in VLPs composed of both L1 and L2 more efficiently than in capsids composed of L1 alone); and (3) infectivity of PV virions. L2 can induce the relocalization of E2, L1 and the viral DNA to promyelocytic leukemia oncogenic domains (PODs). The significance of this subnuclear localization is not yet appreciated; however, it is interesting that components of other viruses (adenoviruses, herpesviruses) can also be found in POD domains.

Association of HPVs with Human Cancers

Certain types of HPVs, such as type 16, 18, 31 and 33, are associated with human anogenital cancers, and are referred as high-risk HPVs. These cancers include cervical cancers in women and penile cancers in men. The advent of Pap smears, which provide early detection of cervical cancer precursor lesions in women, has led to a significant decrease in deaths due to cervical cancer in countries where there is good access to health care. Nevertheless, cervical cancer remains a leading cause of death by cancer among women. More than 95% of cervical cancers are associated with infection by the sexually transmitted, high-risk anogenital PVs. HPV DNA can be detected in these cancers and cells derived from these cancers. The viral DNA is commonly found to be integrated into the cellular genome. The sites of integration appear relatively random with regard to the host genome, but specific with regard to the viral genome. Integration leads to the disruption of the 3′ portion of the viral early region encoding the E1 and E2 genes. In these cervical cancers there is a preferentially increased expression of two viral genes, E6 and E7. This may result in part from the derepression of the viral promoter directing expression of these genes (which can be repressed by high levels of E2 proteins) and in part from increased stability of the E6 and E7 specific mRNAs owing to the disruption of a mRNA instability element in the 3′ UTR of the early region. The presence of transcripts for E6 and E7 in cancer cells suggested these two genes may play an important role in the HPV-associated carcinogenesis. Studies outlined below have clearly proven E6 and E7 to be potent oncogenes that can cause the transformation of cells in tissue culture and induce tumor formation in animal models.

E7 is a nuclear protein of 98 amino acids that is well conserved among different HPVs. E7 protein has several regions that share homology to conserved regions (CRs) in the adenoviral transforming protein E1A. The N-terminal half of E7 protein contains two domains, CR1 and CR2, which are required for E7's transforming and tumorigenic properties. The CR2 domain contains the amino sequence that is necessary for binding pRb, the product of the retinoblastoma tumor susceptibility locus. The CR1 domain appears crucial for E7's ability to induce the degradation of pRb. pRb is a master regulator of cell cycle in the G_1 phase. Normally it is regulated in its activity by phosphorylation by cyclin associated kinases. Hypophosphorylated pRb associates with the cellular transcription factor E2F, blocking the latter's transcriptional transactivation activity. As mentioned earlier, E2F upregulates many genes required for DNA synthesis. Phosphorylation of pRb by cyclin-associated kinases leads to the disruption of the pRb:E2F complex. The active E2F thereby leads to the transition of the cell into S phase. Functional inactivation of pRb leads to unrestrained cell growth and cancer. In humans, mutational loss of pRb causes retinoblastoma in the eye, Wilms' tumor in kidney, and bone tumors. Binding of E7 to pRb results in disassociation of pRb from E2F protein and destabilization and degradation of pRb (**Fig. 3**). Thus the trans-dominant effects of E7 on pRb has been likened to the effects of mutational inactivation of pRb seen in some tumors. Recent evidence indicates that E7 also binds to other cell cycle regulating proteins, such as certain types of cyclin-dependent kinases (CDKs), and CDK inhibitors p21 and p27. These activities of E7 likely also contribute to its capacity to disrupt the control of the cell cycle.

E7 is a potent transforming protein in cell culture. Alone, it can transform established fibroblast cell lines and immortalize primary epithelial cells, as demonstrated in human mammary and cervical epithelial cells. In cooperation with another oncogene, activated H-*ras*, it can efficiently transform baby rat kidney cells. E7 also can abrogate normal cellular responses to DNA damaging agents, including radiation as well as drugs like actinomycin D. This capacity of E7 maps to its ability to bind and inactivate pRb and pRb-like proteins. Direct evidence of the tumorigenic activity of E7 comes from the analysis of E7 transgenic mice. Expression of E7 in the mouse epidermis, driven by human keratin 14 promoter, induces significant skin hyperplasia and tumor formation. E7-induced tumors include skin papillomas, carcinomas and sebaceous epitheliomas.

E7 appears to play primarily a role in the 'promotion' phase of carcinogenesis, reflective of its capacity to induce cell proliferation.

E6, a protein of 151 amino acids, is also a nuclear protein. The characteristic structural feature is the formation of two hypothetical zinc fingers. Each of the fingers contain a motif of two cysteines (Cys-X-X-Cys) at its base. Although the functions of these fingers are not clear, they seem to be necessary structures in the stability of E6 protein and transforming functions of E6. An important function of E6 in tumorigenesis is its binding to and inactivation of p53 (**Fig. 3**). p53 is the tumor suppressor gene most frequently mutated in human cancers. Wild-type p53 can induce cell cycle arrest and/or cell death in response to cell stress including DNA damage. Mutations in p53 contribute to immortalization and transformation of cells in tissue culture. Loss of p53 function in both human and animals causes early development of multiple types of malignant tumors. E6 protein from high-risk HPVs can bind p53 through another cellular protein, E6 associate protein (E6-AP), a 100 kDa ubiquitin-protein ligase. Association of E6, E6-AP and p53 leads to ubiquitination and thereby the subsequent degradation of p53. Analysis of E6 mutants has demonstrated that its ability to bind and inactivate p53 is necessary but not sufficient for E6 to immortalize and transform cells. Though yeast two-hybrid studies have led to the identification of multiple potential cellular targets for E6, including paxillin, E6 binding protein 1, and the human homologue of the drosophila discs large tumor suppressor protein, the p53-independent mechanisms of action of E6 that contribute to its role in cancer have yet to be elucidated.

Oncogenic activities of E6 include, in tissue culture, the immortalization of primary epithelial cells and transformation of baby rat kidney cells in cooperation with activated ras oncogene, and *in vivo*, the induction of tumors in E6 transgenic mice in which expression of E6 is directed from the human keratin 14 promoter. The tumors that arise in these mice are primarily malignant carcinomas. Studies suggest that E6 contributes not only to the 'progression' stage of carcinogenesis, but also earlier stages involved in the formation of benign lesions. The mechanisms of E6 transformation and tumorigenesis may be a combination of many activities on the cells. First, E6 induces cellular genome instability, as indicated by aneuploidy in the E6 transformed cells. Second, E6 can modulate DNA damage responses both in cell culture and in animal studies. Following DNA damage, the cells or tissues expressing E6 show an abrogation of apoptosis, cell cycle arrest, and the induction of p53. Modulation of DNA damage responses may contribute to genetic errors in E6-transformed cells. Third, E6 may modulate differentiation/apoptosis of epithelial cells and/or induce their proliferation.

E6 and E7 act synergistically to induce tumors *in vivo*; transgenic mice expressing both E6 and E7 have increased incidence and earlier onset of tumors compared to the mice transgenic for the individual genes. This synergy can also be detected in tissue culture experiments that score for the immortalization or transformation of cells. In sum, studies to data clearly implicate in carcinogenesis the two viral genes found expressed in cervical cancer.

See also: **Papillomaviruses – human (*Papovaviridae*): General features; JC and BK viruses (*Papovaviridae*); Polyomaviruses – murine (*Papovaviridae*): General features, Molecular biology; Papillomaviruses – animal; Transformation: Animal viruses.**

Further Reading

Howley PM (1996) Papillomavirinae: the viruses and their replication. In: Fields BN, Knipe DN, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 2045. Philadelphia: Lippincott Raven.

Lowy DR and Schiller JT (1998) Papillomaviruses and cervical cancer: pathogenesis and vaccine development. *Monogr. Natl Cancer Inst.* 27.

Munger K (1995) The molecular biology of cervical cancer. [Review.] *J. Cell. Biochem. Suppl.* 23: 55.

Shah K and Howley PM (1996) Papillomviruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 2077. Philadelphia: Lippincott-Raven.

zur Hausen H (1996) Papillomavirus infections – a major cause of human cancers. *Biochim. Biophys. Acta* 1288: F55.

Future Perspectives

Recombinant DNA technology offers new opportunities in the development of PIV vaccines. The HN gene of PIV-3 inserted into a baculovirus vector is expressed on the surface of infected insect cells, is biologically active and cell lysates protect cotton rats. Vaccinia virus recombinants expressing HN and F are also protective in cotton rats, suggesting that live attenuated recombinant viruses may be a feasible approach in the development of vaccines to human PIVs. Finally, cold-adapted mutants and F-altered cleavage-site mutants offer additional strategies for live virus vaccines.

Besides vaccines, antiviral drugs may be designed that curtail viral replication or prevent viral spread. Knowledge of the three-dimensional structure of HN and F, especially those regions involved in viral attachment, penetration and release, will be important in drug design.

See also: **Parainfluenza viruses (*Paramyxoviridae*): Animal; Newcastle disease virus (*Paramyxoviridae*); Respiratory viruses; Sendai virus (*Paramyxoviridae*); Respiratory syncytial virus – human (*Paramyxoviridae*); Vectors: Animal viruses.**

Further Reading

Collins PL, Chanock RM and McIntosh K (1996) Parainfluenza viruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) (1996) *Fields Virology* 3rd edn, p. 1205. Philadelphia: Lippincott-Raven.

Morrison T and Portner A (1991) Structure, function and intracellular processing of the glycoproteins of the *Paramyxoviridae*. In: Kingsbury DW (ed.) *The Paramyxoviruses*. New York: Plenum Press.

Animal

Kailash C Gupta, Department of Immunology/ Microbiology, Rush Medical College, Chicago, Illinois, USA

Introduction

In the past fifty years several animal parainfluenza viruses (PIV) have been identified. Their hosts range from mice to cattle. Like the human parainfluenza viruses, animal parainfluenza viruses primarily cause the upper respiratory tract diseases. Pathogenic parainfluenza viruses for cattle, dogs and sheep have been isolated. The first outbreak of an animal parainfluenza virus disease, the 'shipping fever' caused by bovine PIV-3 (BPIV-3) was recognized in cattle in 1959. Sendai virus, a mouse virus, has been used as a model animal parainfluenza virus for research. Newcastle disease virus (NDV), a virus taxonomically related to parainfluenza viruses, is a scourge of chickens. The term 'parainfluenza' virus has been loosely used to classify the group of paramyxoviruses that are similar to influenza viruses and cause respiratory diseases.

Taxonomy and Classification

Recently the International Committee on the Taxonomy of Viruses (ICTV) has divided family *Paramyxoviridae* into two subfamilies: *Paramyxovirinae* and *Pneumovirinae*. All the known animal parainfluenza viruses fall in two (*Respirovirus* and *Rubulavirus*) of the three genera of the subfamily *Paramyxovirinae* (**Table 1**). The parainfluenza viruses are characterized by the presence of hemagglutinating and neuraminidase activities by virtue of the virus envelope glycoprotein, HN. In contrast to parainfluenza viruses, the third genus (*Morbillivirus*) of *Paramyxovirinae* has the hemagglutinating activity only. This chapter presents the characteristics of *Respirovirus* and *Rubulavirus* genera. The unifying theme of the *Paramyxovirinae* is the similar number of genes and their expression strategy. These viruses belong to the superfamily *Mononegalvirales* that is characterized by single-stranded RNA genome of negative polarity.

Host Range and Virus Propagation

Animal parainfluenza viruses have been isolated from several different animal species ranging from mice to cattle. However, many experimental animals such as hamsters, mice and guinea pigs have been used as hosts. Some parainfluenza viruses, particularly, Sendai virus and BPIV-3 grow well in the amniotic cavity of embryonated chicken eggs. Many viruses have been adapted to grow in primary and established cell cultures of different host origin. For example, simian virus 5 (SV5) grows well in Madin–Darby bovine kidney (MDBK) cells and BPIV-3 in baby hamster kidney (BHK) cells. Cytopathology varies depending on the virus strains and cell types used. For efficient growth, some viruses need trypsin or α-chymotrypsin in the medium for cleavage activation of the virus fusion protein, F.

Properties of Virion and Proteins

Virions are spherical yet pleomorphic in nature and range in size from 150 to 250 nm in diameter. The virions comprise a distinct lipid bilayer envelope with two types of glycoprotein (HN and F) spikes as

Table 1 Some members of the subfamily *Paramyxovirinae*

Genus	*Common name*	*Acronym*	*Genome length (nt)*
Respirovirus	Sendai virus (murine parainfluenza virus type 1)	SNDV	15 384
	Bovine parainfluenza virus type 3	BPIV-3	15 480
	Human parainfluenza virus type 1	HPIV-1	
	Human parainfluenza virus type 3	HPIV-3	15 480
Rubulavirus	Simian virus 5 (Canine parainfluenza virus type 2)	SV5	15 246[a]
	Simian virus 41	SV41	
	Mumps virus		
	Newcastle disease virus (Avian paramyxovirus type 1)	NDV	
	Canine parainfluenza virus 5	CPIV-5	
	Human parainfluenza virus 2	HPIV-2	
Morbillivirus	Dolphin morbillivirus	DMV	
	Equine morbillivirus	EMV	
	Porpoise morbillivirus	PMV	
	Canine distemper virus	CDV	15 616
	Phocid distemper virus	PDV-1	
	Baikal seal morbillivirus	PDV-2	
	Peste-des-pestis-ruminants virus	PPRV	
	Rinderpest virus	RDV	15 881
	Measles virus		15 894

[a] Provided by Dr G. D. Parks.

observed under the electron microscope (**Fig. 1A** and **2**). These glycoproteins are crucial for virus attachment, entry and release. HN binds to sialic acid-containing cell surface molecules. The HN protein possesses both hemagglutinating and neuraminidase activities and the F protein possesses fusion activity. The neuraminidase activity of HN cleaves sialic acid from virions and cell surfaces and prevents virus aggregation during budding. Both glycoproteins traverse the entire lipid bilayer once. HN is a type II integral membrane protein which is anchored in the membrane by its hydrophobic domain at the N-terminus. It occurs as a tetramer consisting of a pair of disulfide-linked dimers. In contrast to HN, the F protein is a type I integral membrane protein in that it is anchored in the membrane by the hydrophobic domain at the C-terminus. The F protein also occurs as an oligomer, but it is not known whether it occurs as a trimer or a tetramer. However, the overwhelming evidence is in favor of a trimer. The F protein is synthesized as a precursor (F_0) which is processed by host proteolytic machinery in the *trans* Golgi network to generate two disulfide-linked fragments, F_1 (large) and F_2 (small). Furin, a protease localized in the *trans* Golgi appears to be the protease responsible for cleavage of F_0. However, in the respiratory tract, a protease secreted by the Clara cells of the bronchial epithelium appears also to be involved in F cleavage. Cleavage of F is important for its fusogenic activity necessary for infection. The N-terminus (20 residues) of F_1 is highly hydrophobic and is termed the fusion peptide domain. Fusion peptide sequences among parainfluenza viruses are highly conserved (70–90% identity). It is hypothesized that the fusion domain is masked by two coiled triple α-helices formed due to heptad repeats; one coiled region is adjacent to the fusion peptide and the other at the C terminus. The fusion domain is somehow exposed by a conformational change during infection.

Encased in the envelope is a single strand of RNase-resistant helical nucleocapsid, a ribonucleoprotein complex consisting of a single molecule of virus RNA genome and about 2500 molecules of the viral nucleocapsid protein (N or NP) (**Figs 1B, 1C** and **2**). NP is the most abundant viral protein. Associated with nucleocapsids are viral polymerase proteins, the phosphoprotein (P) and the large protein (L). P and L together form an RNA-dependent RNA polymerase complex. The functional domains of P and L proteins are not well defined, although several polymerase domains based on sequence similarities have been recognized in the L protein. The characteristic of P protein to be phosphorylated is highly conserved, but the functional role of phosphorylation is yet to be defined. The viral matrix (M) protein underlies the inner surface of the virus envelope. It is a basic protein. It appears to form a bridge between the nucleocapsid and the cytoplasmic tails of the envelope

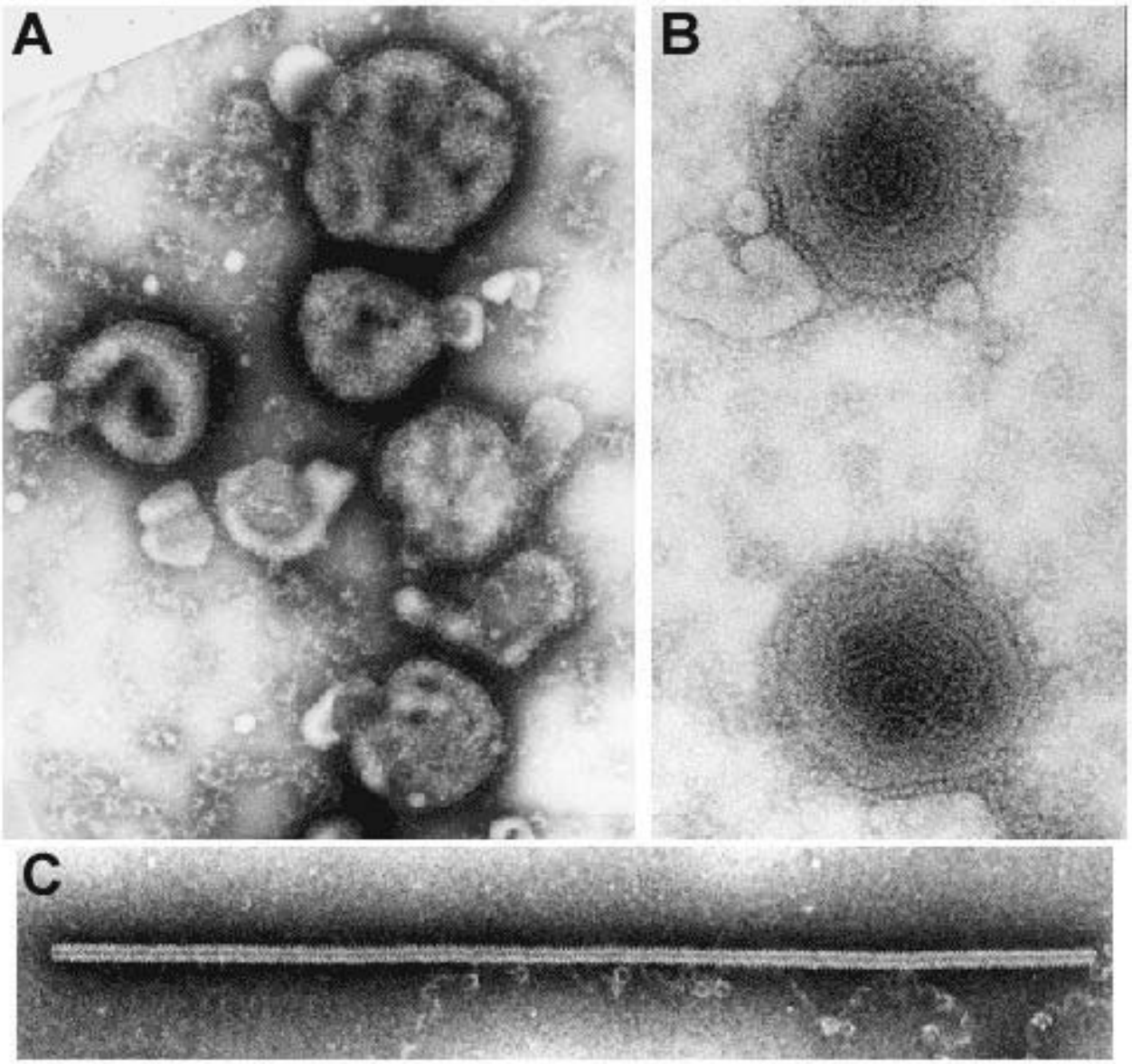


Figure 1 Electron micrographs of Sendai virus and its nucleocapsid. (**A**) Negatively stained virions: spherical and pleomorphic nature is clear. Magnification ×148 000. (**B**) Partially ruptured virions have allowed penetration of the stain into virion: the highly folded nucleocapsid inside the virion and an outer coat of spikes are evident. Magnification ×254 000. (**C**) A single linear nucleocapsid: note the symmetrical helix of NP protein and virus genomic RNA. Magnification ×170 000. (Courtesy of Dr Gopal Murti, St Jude Children's Research Hospital, Memphis.)

proteins. It is involved in virus maturation, assembly and budding.

In addition to the viral structural proteins, certain virus-encoded accessory proteins (V and C) are found in infected cells. Interestingly, both these proteins are encoded in the P gene. Although almost all parainfluenza viruses encode V protein, only those of the *Respirovirus* genus encode the C protein. The C protein is synthesized from a +1 reading frame which overlaps the P and V reading frames. C protein is a basic protein. During transcription, mRNAs from the *P* gene are edited with certain frequency by insertion of one or several G residues at a specific site downstream from the C reading frame. This results in a frameshift in the edited mRNA starting at the insertion site. This strategy of expression allows synthesis of two proteins, P and V, each from a different mRNA. The N-termini of these proteins are identical but the C-termini are different. The C-terminus of V is highly conserved, cysteine-rich and can bind zinc. Both C and V proteins appear to have a role in virus replication. Recently V of Sendai virus

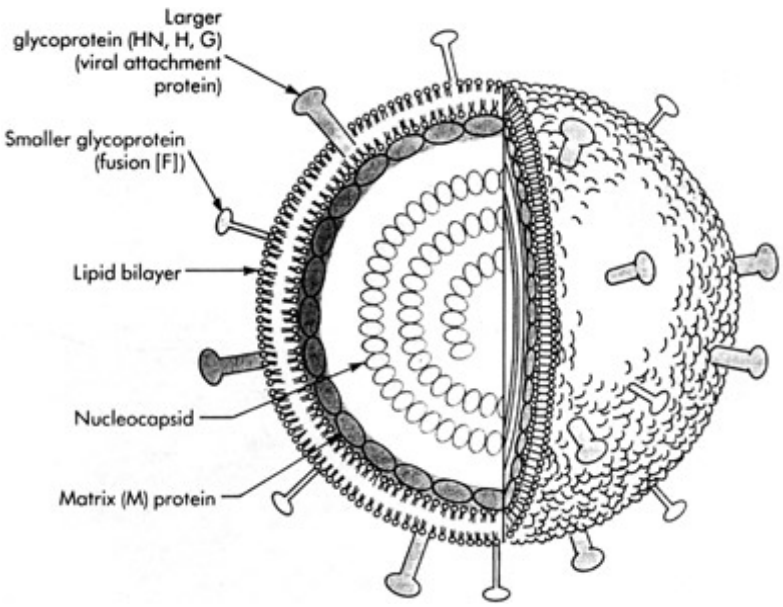


Figure 2 Schematic representation of a paramyxovirus particle. A single helical nucleocapsid is shown inside the virion. The lipid envelope is shown consisting of two types of spikes (HN and F) on the outer surface and the matrix (M) protein lining the inner surface. Not drawn to scale. (Reproduced with permission from Murray PR, Kobayashi GS, Pfaller MA and Rosenthal KS (1994) *Medical Microbiology*, 2nd edn. St Lous: Mosby-Year Book.)

has been shown to be important for pathogenesis. However, more definitive studies for C and V functions are needed.

Genome

The genomes of parainfluenza viruses contain approximately 15 500 nucleotides. Complete nucleotide sequence of several of these viruses have been completed (**Table 1**). A unique feature of their genomes is that the number of nucleotides occur in multiples of six. Approximately 50–60 noncoding nucleotides at each terminus of the genome are important for virus replication and are to a large extent complementary to each other. Apparently, viral polymerase binding and NP binding signals reside in these regions. The gene order in the genome is: *NP-P/C/V-M-F-(SH)-HN(H)-L*, in the 3′ to 5′ direction. The coding sequence of each gene is flanked by noncoding regions. The start and end of each gene is characterized by consensus sequences. Between the end of one and the start of the other is the intergenic region (IR). The IR comprises only three nucleotides in *Respirovirus*, but is of variable length (1–31) in *Rubulavirus*. Some rubulaviruses (e.g. SV5) encode an additional small hydrophobic (SH) protein which is an integral membrane protein. In addition to the fully mature virions, defective interfering (DI) particles are observed when virus is grown at high titers. DI RNAs are essentially internal deletion mutants which retain the terminal sequences essential for their replication. DI RNAs capitalize on the coding capacity of the full-length genome and, therefore, they interfere in the growth of mature virions.

Replication and Virus Assembly

All parainfluenza viruses replicate exclusively in the cytoplasm (**Fig. 3**). They can replicate in enucleated cells. The virus infection cycle begins by its attachment to cell receptors by HN. Various receptors such as sialic acid, gangliosides, and other cell surface proteins are used. Subsequent to attachment, the virus envelope fuses with the plasma membrane releasing the nucleocapsid into the cytoplasm. Recent studies have shown that both F and HN are required for efficient fusion. Parainfluenza viruses can cause cell–cell fusion, generating multinucleated cells (syncytia). Upon entry the nucleocapsid initiates the primary transcription of the virus using associated polymerase complex of P and L proteins. It is the nucleocapsid and not the naked viral RNA that acts as a template for the viral polymerase. The nucleocapsid can reversibly change its configuration (coiling) in response to ionic conditions. It is likely that the pitch of the coiling is modulated during viral RNA synthesis for the polymerase to function. The transcription products are primarily monocistronic mRNAs that are capped, methylated and polyadenylated. However, as described above, the *P* gene is transcribed into alternate mRNA species by cotranscriptional editing. In addition to these mRNA species, a small leader

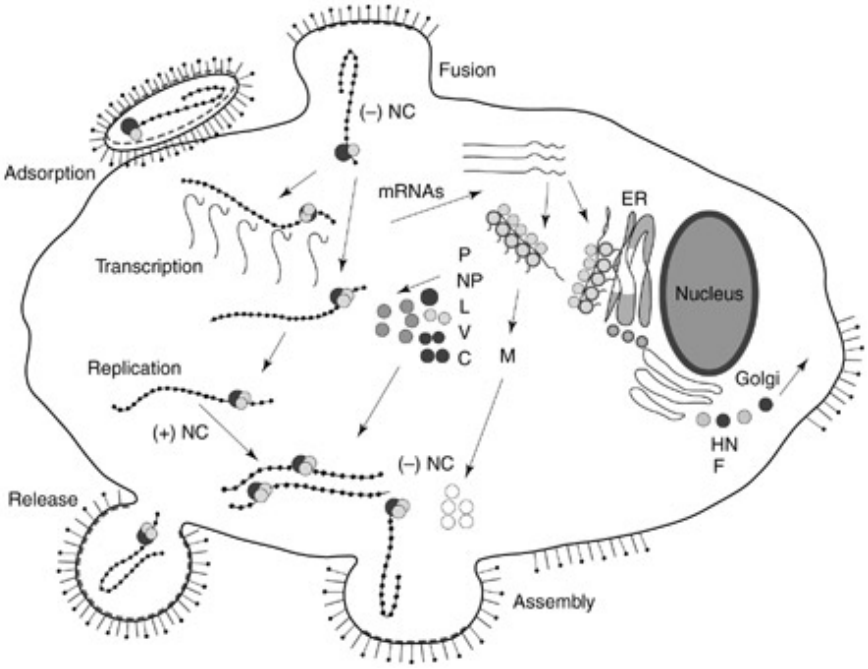


Figure 3 Representation of a paramyxovirus replication. The virus is adsorbed to cell surface by sialic acid containing receptors. Fusion of the virus envelope with the plasma membrane follows the adsorption. Transcription and replication occurs in the cytoplasm. Glycoproteins are transported to plasma membrane through rough endoplasmic reticulum (ER) and Golgi bodies. The M protein helps in virus assembly and perhaps in budding from the plasma membrane.

RNA (1) complementary to the 3′ of the genome is synthesized. The transcription is polar and consequently generates mRNAs in the following gradient: l>NP>P/C/V>M>F>(SH)>HN>L. The transcriptional polarity most likely arises through transcription initiation at the 3′ terminus of the viral genome and transcription termination at the end of each gene. The polymerase then reinitiates with subsequent lower frequency at the start of the next gene. Later in virus infection, when enough virus proteins have been synthesized from these mRNAs, the polymerase replicates the entire genome length RNA (**Fig. 3**). The first step is to synthesize the antigenome nucleocapsid (NC+) which is used as a template to synthesize the progeny of genomic nucleocapsids (NC−). Since more genomic nucleocapsids are synthesized than the antigenomic ones, it is believed that the promoter in the antigenomic nucleocapsid is more efficient than that in the genomic one.

It is assumed that the NP protein in the cytoplasm binds the nascent viral RNA chain initiating the assembly of viral nucleocapsid. It is likely that the accumulation of viral NP protein causes the polymerase from transcriptive mode to replicative mode. Perhaps the binding of NP to nascent RNA causes the polymerase to ignore gene start and end signals allowing genome-length RNA synthesis. Apparently P keeps the NP protein in a solubilized status and transports it to the nucleocapsid assembly site. Association of the polymerase protein complex (P + L) occurs during the nucleocapsid assembly. Subsequently the nucleocapsid is transported to the plasma membrane. It is possible that the M protein helps nucleocapsids to associate with the plasma membrane patches where viral glycoproteins have accumulated. F and HN proteins are processed through endoplasmic reticulum (ER) and Golgi bodies and then transported to the plasma membrane (**Fig. 3**). In polarized epithelial cells, the glycoproteins are transported to the apical surface of the cells. The final assembly and budding of the virus occurs at the plasma membrane.

Genetic Manipulation

Purified nucleocapsids associated with P and L proteins are capable of initiating virus infection. Recently, success has been achieved in constructing nucleocapsids from synthetic RNA and viral proteins *in vitro*. These nucleocapsids contained a reporter gene flanked by 5′ and 3′ termini of the regulatory virus genome sequences. These RNA minigenomes were rescued on transfection of virus-infected cells. Similarly, cDNA minigenomes were rescued on cotransfection with NP, P and L genes. Now infectious virus cDNA clones have been produced. Mutant virus can be derived from these clones. These

experimental approaches make it possible to alter specifically the virus gene or regulatory sequences to define their function more precisely and to create virus strains that could be potentially useful for vaccine development.

Genetics and Evolution

The entire genome sequence of several parainfluenza viruses is known (**Table 1**). Although the gene organization is very similar in these viruses, divergence of individual protein sequence is variable. P protein is the most variable. Interestingly, the V protein encoded in the same gene is relatively conserved at the C-terminus. NP and M are relatively less variable. L protein has four to five regions of significant amino acid conservation. These regions are believed to be important for a number of enzymic activities putatively associated with the L protein.

BPIV-3 is most closely related to human PIV-3 (HPIV-3). Sendai virus is more closely related to HPIV-1. SV5 is more closely related to HPIV-2 and canine PIV-5 (CPIV-5). It is possible that CPIV-5 and SV5 are the same virus. Several strains of NDV from various birds have been isolated. It is likely that the closely related viruses originated from a common stock and diverged due to host adaptation.

Serologic Relationships and Variability

The parainfluenza viruses that have been observed to show strong serological relationship were later found to have strong sequence homologies at the amino acid level. The viruses which show a high degree of antigenic similarities are: Sendai virus and HPIV-1, BPIV-3 and HPIV-3, SV5 and HPIV-2.

Epidemiology

BPIV-3 was isolated from a respiratory disease epidemic in cattle. It is often isolated from bovine respiratory disease, shipping fever, which occurs during or after cattle transport. Interestingly, Sendai virus, a mouse pathogen, was prevalent before 1961 in the pig population of Japan. Sendai virus causes acute epizootic in experimental rodents. NDV has been isolated from both domestic and free-living birds. Several epidemics of NDV infection have occurred in the past killing thousands of chickens. The most recent NDV outbreak was in 1995 in Taiwan. In general, parinfluenza viruses do not show strong host specificity.

Transmission and Tissue Tropism

Almost all parainfluenza viruses primarily infect epithelial cells of the respiratory tract. The mode of transmission is most likely aerosol inhalation. Rarely, these viruses cause viremia and infection of subepithelial cells. However, the presence of virus in the intestinal tract, aborted fetuses and milk indicate that contact transmission is also involved to a lesser extent. Since the receptors of parainfluenza viruses appear to be present on most of the host cells, virus infection to respiratory cells is most likely limited by the presence of cellular protease(s) that cause cleavage activation of the F protein. It has been shown that a pantropic mutant of Sendai virus, whose F is susceptible to cleavage by ubiquitous host proteases, causes systemic infection in mice. Cleavage activation of F allows cell-to-cell spread of the virus.

Pathogenicity

Most of the parainfluenza viruses cause upper respiratory tract diseases, such as cough, croup and bronchiolitis. These conditions could lead to lower respiratory tract infection and pneumonia. In contrast, avian paramyxoviruses (NDV) replicate in the intestinal tract. NDV strains range from avirulent to highly virulent strains. Virulent strains are highly lethal to chicken populations.

Immune Response

Parainfluenza viruses elicit both humoral and cellular immunity. Envelope glycoproteins, HN and F, are primarily responsible for the humoral response whereas NP and M proteins are antigens for cellular response. Mucosal immunoglobulin (Ig) A to HN is responsible for protection from further infection. However, animals become susceptible to reinfection several months after antibody development. The role of cellular immunity is not clear.

Prevention and Control

Live attenuated and killed vaccines have been developed for BPIV-3. Live vaccines are better than the killed ones. Vaccines developed for intranasal use induce effective nasal IgA antibodies. It is very important to keep experimental mice free from Sendai virus. Mice colonies can be freed of virus by maintaining them under pathogen-free conditions after cesarean birth. Several live attenuated and killed vaccines for NDV have been developed. Recent studies with DNA vaccines indicate their considerable potential in effectively controlling spread of NDV.

Future Perspectives

Parainfluenza viruses cause human suffering and

economic losses. A better understanding about their replication and host–virus interactions is essential in order to combat the diseases caused by them. Progress in the basic knowledge of parainfluenza viruses was stymied due to constraints on the manipulation of negative-sense virus genome. With the success of reverse genetics for manipulation of these viruses, a surge of knowledge is expected. This approach will be helpful in the in-depth understanding of the replication strategies of parainfluenza viruses and in designing efficient antivirals and vaccines against them.

See also: **Measles virus (*Paramyxoviridae*); Mumps virus (*Paramyxoviridae*); Newcastle disease virus (*Paramyxoviridae*); Parainfluenza viruses (*Paramyxoviridae*) – Human; Respiratory syncytial virus – human (*Paramyxoviridae*); Rinderpest and distemper viruses (*Paramyxoviridae*); Sendai virus (*Paramyxoviridae*).**

Further Reading

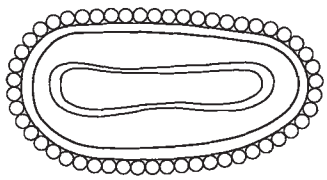
Lamb RA and Kolakofsky D (1996) Paramyxoviridae: the viruses and their replication. In: Field BN, Knipe DM, Howley PM *et al.* (eds) *Fields Virology*, 3rd edn, p. 577. Philadelphia: Lippincott–Raven.

Orvell C, Norrby E (1985) Antigenic structure of paramyxoviruses. In: van Regenmortel MHV, Neurath AR (eds) *Immunochemistry of Viruses, the Basis for Serodiagnosis and Vaccine*, p. 241. Amsterdam: Elsevier Medical.

Pringle CR (1987) Paramyxoviruses and disease. In: Russell WC, Almond JW (eds) SGM Symposium 40, *Molecular Basis of Virus Disease*, p. 51. Cambridge: Cambridge University Press.

Shibuta H (1994) Animal parainfluenza viruses. In: Webster, RG and Granoff A (eds) *Encyclopedia of Virology*, p. 1031. London: Academic Press.

PARAPOXVIRUSES (*POXVIRIDAE*)



Andrew Mercer, University of Otago, Dunedin, New Zealand

David Haig, Moredun Research Institute, Edinburgh, UK

History

There are numerous historical references to diseases of domesticated animals such as sheep and cattle that we would now suspect to be the result of infection by parapoxviruses. These references include Jenner's 'spurious' cowpox which is likely to have been caused by the parapoxvirus, pseudocowpox virus. If a little poetic license is permitted it is easy to believe that when, in 1520, Pope Leo X wrote a letter deploring the actions of Martin Luther and stated that 'we cannot suffer the scabby sheep longer to infect the flock', he was making an analogy which indicates knowledge of the disease caused by orf virus and of its infectious nature.

In the latter part of last century reports appeared in the scientific literature which recognized the distinct identities of the diseases caused by members of this genus. Following an extensive study of contagious pustular dermatitis of sheep, Aynaud produced a report in 1923 which included the observation that the disease could be transmitted by a 'filterable' agent. The isolation of each of the viruses in cell culture was reported in the period from 1957 to 1963. Detailed reports of the transmission of each disease to humans appeared in 1933 (orf virus), 1963 (pseudocowpox virus) and 1967 (bovine papular stomatitis virus). The first molecular analyses of parapoxvirus genomes appeared in 1979 with publication of restriction endonuclease cleavage site maps and reports of G+C contents. These were followed, in 1989, by the first description of the DNA sequence of a region of a parapoxvirus genome.

Taxonomy and Classification

The *Parapoxvirus* genus belongs to the family *Poxviridae*, subfamily *Chordopoxvirinae*. The type species of the genus is orf virus and the other species recognized as members are bovine papular stomatitis virus, pseudocowpox virus and a recently identified member, parapoxvirus of red deer in New Zealand. Synonyms by which these viruses have been known include contagious pustular dermatitis virus and contagious ecthyma virus for orf virus and Milker's nodule virus and paravaccinia virus for pseudocowpox virus. Tentative species of this genus are Auzduk

These systems are continually improving and should allow a detailed genetic analysis of the complete viral life cycle.

The ability to generate VLPs has revolutionized papillomavirus research. These VLPs are very useful for serological studies and have been the basis of very successful prophylactic vaccines in cattle, dogs and rabbits. These animal systems should also prove useful for developing efficient therapeutic vaccines and for testing antiviral drugs that may be used in treatment of PV infections in humans.

BPV-1 continues to serve as the molecular prototype for the study of papillomavirus DNA replication and transcriptional regulation. The ability of this viral genome to efficiently and stably replicate extrachromosomally will prove to be very useful in understanding the mechanisms by which extrachromosomal elements stably replicate and segregate their DNA. Such studies may also lead to improved gene transfer vectors based on papillomavirus genomes. BPV-1 genomes packaged in heterologous VLPs have been used in neutralization studies because of the ability of BPV-1 genomes to quantitatively transform certain mouse cells. This property of BPV-1 should continue to prove useful for many different studies.

See also: **Immune response: Cell mediated immune response, General features; Latency; Papillomaviruses – human (*Papovaviridae*): General features, Molecular biology; Transformation: Animal viruses; Virus–host cell interactions.**

Further Reading

Howley PM (1995) Papillomavirinae: the viruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al*, *Fields Virology* p. 2045. Philadelphia: Lippincott-Raven.

Myers G, Baker C, Wheeler C *et al* (eds), (1995, 1996, 1997) Human papillomavirus Los Alamos National Laboratories, *http://hpv-web.lanl.gov/*

Lancaster WD and Olson C (1982) Animal papillomaviruses. *Microbiol. Rev.* 46: 191.

Pfister H (ed.) (1990) *Papillomaviruses and Human Cancer*. Boca Raton: CRC Press.

Salzman NP and Howley PM (eds) (1987) The Papovaviridae; The Papillomaviruses Series. *The Viruses*. New York: Plenum Press.

Sundberg JP (1987) Papillomavirus infections in animals. In: Syrjanen K, Giasman L and Koss LG (eds) *Papillomaviruses and Human Disease*. Berlin: Springer Verlag.

PARAINFLUENZA VIRUSES (*PARAMYXOVIRIDAE*)

Contents

Human

Animal

Human

Allen Portner, Department of Virology and Molecular Biology, St Jude Children's Research Hospital, Memphis, USA

Introduction

The human parainfluenza viruses (PIVs) are an important cause of respiratory disease in infants and children. Four types were discovered between 1956 and 1960. PIV-1, PIV-2 and PIV-3 were first isolated from infants and children with lower respiratory tract disease and subsequently shown to be a major cause of croup (types 1–3) and pneumonia and bronchiolitis (type 3). PIV-4 was initially isolated from young adults and has been associated with mild upper respiratory tract disease of children and adults. Other viruses antigenically and structurally related to the human paramyxoviruses have been isolated from animals. Sendai virus, a natural pathogen of mice and not of humans, was the first PIV isolated and is antigenically related to human PIV-1. Simian virus (SV)5, recovered from primary monkey kidney cells, causes croup in dogs and is a subtype of human type 2, and bovine shipping fever virus is a subtype of type 3.

Taxonomy and Classification

The PIVs belong to three genera, *Respirovirus*, *Rubulavirus* and *Morbillivirus*, of the subfamily *Paramyxovirinae* in the family *Paramyxoviridae*. Some other species found in the *Rubulavirus* are

mumps virus, which causes disease in humans; Newcastle disease virus (NDV) of chickens; SV5 in dogs; and in the *Respirovirus* genus Sendai virus in mice. The third genus which completes the subfamily is *Morbillivirus* (measles virus, canine distemper virus, rinderpest virus). A second subfamily, *Pneumovirinae*, has two genera, *Pneumovirus* (respiratory syncytial virus) and *Metapneumovirus* (turkey rhinotracheitis virus). The *Paramyxoviridae* family belongs to the order *Mononegavirales*, the distinctive feature of which is a negative-stranded RNA genome and a similar strategy of replication, suggesting that all negative-stranded viruses evolved from an archetypal virus.

Virion Structure, Genome Organization and Protein Composition

The PIVs are roughly spherical, lipoprotein enveloped particles 150–250 nm in diameter with an internal helical nucleocapsid containing the single-stranded RNA genome. Projecting from the surface of the virion are the hemagglutinin–neuraminidase (HN) and fusion (F) glycoproteins. These glycoproteins are anchored to the plasma membrane of the infected cell or the virion envelope by a hydrophobic transmembrane region. In paramyxoviruses the transmembrane domain of HN is located near the N-terminus of the molecule and F near the C-terminus. Extending into the cytoplasm of the infected cell or inside the virion membrane is a short hydrophilic tail region, thought to play a role in viral assembly through its interaction with the matrix (M) protein, which lines the inner surface of the plasma membrane and itself interacts with the nucleocapsid. Inside the lipid bilayer of the viral particle is the RNA nucleocapsid which houses the nonsegmented negative-stranded RNA genome. The genomes of PIV-1, PIV-2 and PIV-3 are approximately 15 000 nucleotides and PIV-4 is certain to fall in this range once the sequencing is complete. The RNA genome serves as a template for transcription of mRNAs specifying six virion structural proteins linked in tandem in the order of 3′-nucleoprotein (NP)–polymerase-associated protein (P/V)–matrix protein (M)–fusion protein (F)–hemagglutinin-neuraminidase (HN)–large protein (L)-5′. The P gene alone is unique in its capacity to express, in addition to the P protein, various other proteins by utilizing internal initiation codons in the same or different reading frames or by RNA editing of the P gene mRNA through insertion of nontemplated G residues. Besides P, these other proteins have been designated V, C, D and X, depending on the PIV. The helical nucleocapsid is 18 nm in diameter, contains approximately 2000 molecules of NP bound to the RNA genome, about 200 P and 20 L molecules. The P protein, which forms a polymerase complex with L, is essential for the enzymatic processes of viral RNA transcription and replication including the 3′ addition of poly(A), modification of the 5′ end, and nucleotide polymerization of viral transcripts.

The approximate molecular weights of the PIV proteins as exemplified by PIV-3 are: NP, 58 000; P, 68 000; M, 40 000; F, 63 000; HN, 72 000; L, 256 000.

Attachment Protein

The process of infection is initiated by the action of the HN glycoprotein, which binds the virion to sialic acid-containing receptors on the host cell surface. The same process is responsible for the hemagglutination of avian and mammalian erythrocytes. HN also causes the enzymatic (neuraminidase) cleavage of sialic acid residues from the carbohydrate moiety of glycoproteins and glycolipids, which functionally serves to prevent the self-aggregation of virus during release from infected cells. Besides attachment, HN provides an unknown function that is either essential or enhances the fusion activity of the F protein. It is believed that HN directly interacts with F, possibly altering its conformation, thereby stimulating the fusion activity of F.

The morphology of HN-based studies of Sendai virus is envisioned as an N-terminal stalk region of approximately 130 amino acids anchoring a large N-glycosylated hydrophilic globular head region to the viral envelope. A small uncharged hydrophobic peptide located near the N-terminus spans the viral envelope, and a small hydrophilic domain is internal to the membrane. The globular head contains the active site for virus attachment, neuraminidase activity and antigenic determinants that induce neutralizing antibodies for PIV-1 and Sendai virus. HN exits on the surface of the virion as disulfide-linked homodimers or tetramers.

Fusion Protein

Following virus attachment, F, the other surface glycoprotein, mediates the fusion of the virion and host cell surface membranes, which allows the nucleocapsid to be deposited in the cell cytoplasm where gene expression begins. F expressed on the surface of infected cells also mediates fusion, allowing the extension of infection to uninfected cells.

All F proteins are synthesized as inactive precursors (F0) which are post-translationally cleaved by a host cell trypsin-like protease to form the biologically active molecule. For the paramyxoviruses in general, the cleavage site is located about 100 amino acids

from the N-terminus of F0. The cleavage site is characterized by a short span of basic amino acids on the amino side of the site and a longer stretch of about 30 hydrophobic residues on the carboxyl side. The number and location of basic amino acids on the amino side of the cleavage site varies with individual PIVs. PIV-1 and Sendai virus have one basic amino acid immediately adjacent to the cleavage site (Arg), whereas PIV-2 and PIV-3 have two (Arg-Lys) and SV5 has five (all Arg). The motif of paired basic amino acids at the cleavage site increases the efficiency of cleavage, host and tissue tropism and pathogenicity, as the dibasic motif is recognized by a ubiquitous protease, whereas the enzymes that cleave at a monobasic site are found in a limited number of tissues. Cleavage of F0 results in two disulfide-linked fragments; the larger one, F1, forms the new hydrophobic N-terminus, which causes membrane fusion. The smaller product, F2, is the original approximately 100 N-terminal residues of F0.

M Protein

The M protein lines the inner surface of the viral envelope and is thought to play a role in virus maturation by interacting with the surface proteins and the nucleocapsid.

Transcription, Translation and Replication

The negative-strand strategy of viral replication involves the synthesis of unique mRNA species of each paramyxovirus gene during infection. After introduction of the infecting nucleocapsid into the host cell, transcription is the first step in gene expression. Transcriptional regulation of mRNA abundance is determined by the gene order; the closer the gene is to the 3′ end of the genome, the more efficient is the transcription. Thus, the abundance of paramyxovirus proteins is determined mainly by the polarity of the genome. Once protein synthesis is underway, replication of the genome begins, which provides additional templates for transcription and replication.

Recent development of 'reverse genetics' systems for SV and PIV-3, in which the RNA genomes of these viruses are expressed from a DNA template, are likely to facilitate our understanding of PIV protein structures and function, virus assembly, regulation of RNA transcription and replication and viral pathogenesis.

Geographic and Seasonal Distribution

All of the PIV types 1–4 have a wide geographic distribution. PIV-1–PIV-3 have been identified in most areas where facilities are available for the study of childhood respiratory tract diseases. PIV-4 has been isolated in fewer areas but this is likely to be due to the difficulty of isolation and it is probably widely distributed.

Host Range and Viral Propagation

The four PIV types were originally isolated from humans: they cause disease in humans and humans are the primary host. Other laboratory animals can serve as experimental hosts: hamsters, guinea pigs and ferrets can be infected with PIV-1, PIV-2 and PIV-3 but these infections are usually asymptomatic. PIV-3 can also infect cotton rats, rhesus and patas monkeys and chimpanzees, but these animals are not good model systems for studying disease caused by PIVs.

Embryonated hens' eggs can support the growth of some strains of PIV-1, PIV-2 and PIV-3, but are much less sensitive than monkey kidney cells for primary isolation. Sendai virus, a murine subtype of PIV-1, is an exception in that it grows exceedingly well in eggs.

All four PIV types grow well in primary monkey or human kidney cells, which are also used in the isolation of virus from clinical samples. LLC-MK2, a rhesus monkey kidney cell line, offers an efficient system for the isolation of PIVs and an experimental tissue culture system. PIV-2 and PIV-1 require trypsin in the medium to cleave the F glycoprotein for cell growth, but not PIV-3 strains. Viral infection of tissue culture can be detected by hemadsorption with guinea pig erythrocytes and the cytopathic effects produced by the viruses.

Genetics and Evolution

The entire genomic sequences of PIV-1, PIV-2 and PIV-3 have been completed. The genomes of other members of the *Paramyxovirus* and *Rubulavirus* genera (Sendai virus, NDV, SV5) have been sequenced as well. In general, the genome organization is remarkably similar, but differences exist in sequence, intergenic regions and nonstructural proteins expressed from the P gene. Sequence and immunological analyses suggest that human PIV-1 and Sendai virus are closely related type 1 PIVs, as is PIV-3 of human and bovine origin. PIV-1 and -3 are more closely related to each other than to PIV-2, SV5, mumps virus and NDV, which in turn show a closer evolutionary relationship to each other. Evolutionary divergence of PIV-3 and PIV-1 is greatest for the P protein (a phenomenon of paramyxovirus P proteins in general),

less for the HN and F glycoproteins, and least for M, NP and L. Similarly, PIV-1 and Sendai virus, and human and bovine PIV-3, show the greatest divergence in the P protein and least for NP.

Serologic Relationships and Variability

Early serologic studies have established that related antigenic determinants are present on PIV-1–PIV-3. Besides these interrelationships, the closest antigenic relationships are exhibited between human PIV-1 and Sendai virus, human PIV-2 and canine PIV-3 (SV5) and human PIV-3 and bovine PIV-3. During primary infection, the antibody response to HN is specific for the infecting serotype, while reinfection is more crossreactive. Recent studies have established that variation occurs in HN and F neutralizing epitopes but these do not involve progressive accumulation of changes with time because changes detected in early isolates may not be detected in later isolates. It appears therefore that random mutation is responsible for the observed heterogeneity which is not under strong immunologic selective pressure.

Epidemiology and Transmission

The PIVs are an important cause of respiratory tract disease in children and infants. Infection with PIV-1, -2 and -3 can result in an inapparent infection or life-threatening lower respiratory tract disease. Most children have been infected with PIV-3 by 4 years of age and PIV-1 and -2 by 5 years. PIV-3 can cause illness during the first month of life, whereas infection is rare with PIV-1 and PIV-2 during the first 4 months of life. Over the past 10 years, epidemics of PIV-3 have occurred in the early spring and PIV-1 and PIV-2 in the fall of odd-numbered years. PIVs can be a problem in hospitals, causing infection in children admitted with other illnesses.

Infection and reinfection is by rapid person-to-person spread, with PIV-3 being the most efficient and PIV-1 and PIV-2 less so.

Pathogenicity

Viral and host properties responsible for determining the severity and localization of human PIV disease are not clear. However, recent studies have identified some factors that may play an important role in the pathogenicity: (1) susceptibility of the F glycoprotein to cleavage by host cell protease, which in turn is determined by the number and alignment of basic amino acids at the cleavage site; (2) whether the host tissues have the cleavage enzymes; and (3) the immune status of the host.

Clinical Features of Infection

The most common type of illness in children is characterized by rhinitis, pharyngitis and bronchitis, often with fever. Cough, hoarseness and fever are common initial symptoms, with fever lasting 2–3 days. When croup develops, children recover in about 24–48 h; in some cases, however, airway obstruction may occur. Bronchiolitis or pneumonia may also develop, in which the fever and cough persist.

Pathology

The mucous membranes of the nose and throat are the initial tissues involved in PIV infection. Some limited involvement of the bronchi may occur with mild disease. In more severe infections with PIV-1 and PIV-2, involvement of the larynx and upper trachea may result in croup; this may extend to the lower trachea and bronchi and result in pneumonia. PIV-3 may also cause severe disease characterized by bronchopneumonia, bronchiolitis or bronchitis.

Immune Response

Pre-existing serum neutralizing antibodies to PIV-3 in children and infants have correlated with resistance to infection and illness; however, resistance is only partial. Upon reinfection, however, a previous immunity results in a milder infectious course.

In adults, resistance to PIV-1 and PIV-2 correlates mainly with local nasal secretory IgA neutralizing antibodies, while in infants these antibodies are of little consequence. Serum antibodies may partially protect infants from infection with PIV-1, PIV-2 and PIV-3, while being ineffective in adults. Infection with PIVs stimulates antibodies to the surface glycoproteins, HN and F. Antibodies to HN neutralize by preventing viral attachment to host cells, while F antibodies may prevent viral penetration and cell-to-cell spread. Cytotoxic T cells may also be an important component in immunity to PIVs in their role in recovery from infection.

Prevention and Control

Currently, no effective vaccines exist for PIVs. Inactivated vaccines have been prepared for PIV-1, PIV-2 and PIV-3 in tissue culture and embryonated eggs but these vaccines have failed to provide protection. The inactivation of the F glycoprotein in these preparations and the failure to stimulate local nasal IgA antibodies may have contributed to the ineffectiveness of these vaccines.

Future Perspectives

Recombinant DNA technology offers new opportunities in the development of PIV vaccines. The HN gene of PIV-3 inserted into a baculovirus vector is expressed on the surface of infected insect cells, is biologically active and cell lysates protect cotton rats. Vaccinia virus recombinants expressing HN and F are also protective in cotton rats, suggesting that live attenuated recombinant viruses may be a feasible approach in the development of vaccines to human PIVs. Finally, cold-adapted mutants and F-altered cleavage-site mutants offer additional strategies for live virus vaccines.

Besides vaccines, antiviral drugs may be designed that curtail viral replication or prevent viral spread. Knowledge of the three-dimensional structure of HN and F, especially those regions involved in viral attachment, penetration and release, will be important in drug design.

See also: **Parainfluenza viruses (*Paramyxoviridae*): Animal; Newcastle disease virus (*Paramyxoviridae*); Respiratory viruses; Sendai virus (*Paramyxoviridae*); Respiratory syncytial virus – human (*Paramyxoviridae*); Vectors: Animal viruses.**

Further Reading

Collins PL, Chanock RM and McIntosh K (1996) Parainfluenza viruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) (1996) *Fields Virology* 3rd edn, p. 1205. Philadelphia: Lippincott-Raven.

Morrison T and Portner A (1991) Structure, function and intracellular processing of the glycoproteins of the *Paramyxoviridae*. In: Kingsbury DW (ed.) *The Paramyxoviruses*. New York: Plenum Press.

Animal

Kailash C Gupta, Department of Immunology/ Microbiology, Rush Medical College, Chicago, Illinois, USA

Introduction

In the past fifty years several animal parainfluenza viruses (PIV) have been identified. Their hosts range from mice to cattle. Like the human parainfluenza viruses, animal parainfluenza viruses primarily cause the upper respiratory tract diseases. Pathogenic parainfluenza viruses for cattle, dogs and sheep have been isolated. The first outbreak of an animal parainfluenza virus disease, the 'shipping fever' caused by bovine PIV-3 (BPIV-3) was recognized in cattle in 1959. Sendai virus, a mouse virus, has been used as a model animal parainfluenza virus for research. Newcastle disease virus (NDV), a virus taxonomically related to parainfluenza viruses, is a scourge of chickens. The term 'parainfluenza' virus has been loosely used to classify the group of paramyxoviruses that are similar to influenza viruses and cause respiratory diseases.

Taxonomy and Classification

Recently the International Committee on the Taxonomy of Viruses (ICTV) has divided family *Paramyxoviridae* into two subfamilies: *Paramyxovirinae* and *Pneumovirinae*. All the known animal parainfluenza viruses fall in two (*Respirovirus* and *Rubulavirus*) of the three genera of the subfamily *Paramyxovirinae* (**Table 1**). The parainfluenza viruses are characterized by the presence of hemagglutinating and neuraminidase activities by virtue of the virus envelope glycoprotein, HN. In contrast to parainfluenza viruses, the third genus (*Morbillivirus*) of *Paramyxovirinae* has the hemagglutinating activity only. This chapter presents the characteristics of *Respirovirus* and *Rubulavirus* genera. The unifying theme of the *Paramyxovirinae* is the similar number of genes and their expression strategy. These viruses belong to the superfamily *Mononegalvirales* that is characterized by single-stranded RNA genome of negative polarity.

Host Range and Virus Propagation

Animal parainfluenza viruses have been isolated from several different animal species ranging from mice to cattle. However, many experimental animals such as hamsters, mice and guinea pigs have been used as hosts. Some parainfluenza viruses, particularly, Sendai virus and BPIV-3 grow well in the amniotic cavity of embryonated chicken eggs. Many viruses have been adapted to grow in primary and established cell cultures of different host origin. For example, simian virus 5 (SV5) grows well in Madin–Darby bovine kidney (MDBK) cells and BPIV-3 in baby hamster kidney (BHK) cells. Cytopathology varies depending on the virus strains and cell types used. For efficient growth, some viruses need trypsin or α-chymotrypsin in the medium for cleavage activation of the virus fusion protein, F.

Properties of Virion and Proteins

Virions are spherical yet pleomorphic in nature and range in size from 150 to 250 nm in diameter. The virions comprise a distinct lipid bilayer envelope with two types of glycoprotein (HN and F) spikes as

economic losses. A better understanding about their replication and host–virus interactions is essential in order to combat the diseases caused by them. Progress in the basic knowledge of parainfluenza viruses was stymied due to constraints on the manipulation of negative-sense virus genome. With the success of reverse genetics for manipulation of these viruses, a surge of knowledge is expected. This approach will be helpful in the in-depth understanding of the replication strategies of parainfluenza viruses and in designing efficient antivirals and vaccines against them.

See also: **Measles virus (*Paramyxoviridae*); Mumps virus (*Paramyxoviridae*); Newcastle disease virus (*Paramyxoviridae*); Parainfluenza viruses (*Paramyxoviridae*) – Human; Respiratory syncytial virus – human (*Paramyxoviridae*); Rinderpest and distemper viruses (*Paramyxoviridae*); Sendai virus (*Paramyxoviridae*).**

Further Reading

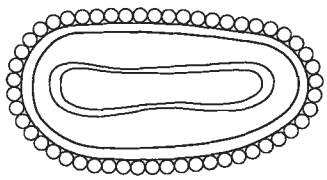
Lamb RA and Kolakofsky D (1996) Paramyxoviridae: the viruses and their replication. In: Field BN, Knipe DM, Howley PM *et al.* (eds) *Fields Virology*, 3rd edn, p. 577. Philadelphia: Lippincott–Raven.

Orvell C, Norrby E (1985) Antigenic structure of paramyxoviruses. In: van Regenmortel MHV, Neurath AR (eds) *Immunochemistry of Viruses, the Basis for Serodiagnosis and Vaccine*, p. 241. Amsterdam: Elsevier Medical.

Pringle CR (1987) Paramyxoviruses and disease. In: Russell WC, Almond JW (eds) SGM Symposium 40, *Molecular Basis of Virus Disease*, p. 51. Cambridge: Cambridge University Press.

Shibuta H (1994) Animal parainfluenza viruses. In: Webster, RG and Granoff A (eds) *Encyclopedia of Virology*, p. 1031. London: Academic Press.

PARAPOXVIRUSES (*POXVIRIDAE*)



Andrew Mercer, University of Otago, Dunedin, New Zealand

David Haig, Moredun Research Institute, Edinburgh, UK

History

There are numerous historical references to diseases of domesticated animals such as sheep and cattle that we would now suspect to be the result of infection by parapoxviruses. These references include Jenner's 'spurious' cowpox which is likely to have been caused by the parapoxvirus, pseudocowpox virus. If a little poetic license is permitted it is easy to believe that when, in 1520, Pope Leo X wrote a letter deploring the actions of Martin Luther and stated that 'we cannot suffer the scabby sheep longer to infect the flock', he was making an analogy which indicates knowledge of the disease caused by orf virus and of its infectious nature.

In the latter part of last century reports appeared in the scientific literature which recognized the distinct identities of the diseases caused by members of this genus. Following an extensive study of contagious pustular dermatitis of sheep, Aynaud produced a report in 1923 which included the observation that the disease could be transmitted by a 'filterable' agent. The isolation of each of the viruses in cell culture was reported in the period from 1957 to 1963. Detailed reports of the transmission of each disease to humans appeared in 1933 (orf virus), 1963 (pseudocowpox virus) and 1967 (bovine papular stomatitis virus). The first molecular analyses of parapoxvirus genomes appeared in 1979 with publication of restriction endonuclease cleavage site maps and reports of G+C contents. These were followed, in 1989, by the first description of the DNA sequence of a region of a parapoxvirus genome.

Taxonomy and Classification

The *Parapoxvirus* genus belongs to the family *Poxviridae*, subfamily *Chordopoxvirinae*. The type species of the genus is orf virus and the other species recognized as members are bovine papular stomatitis virus, pseudocowpox virus and a recently identified member, parapoxvirus of red deer in New Zealand. Synonyms by which these viruses have been known include contagious pustular dermatitis virus and contagious ecthyma virus for orf virus and Milker's nodule virus and paravaccinia virus for pseudocowpox virus. Tentative species of this genus are Auzduk

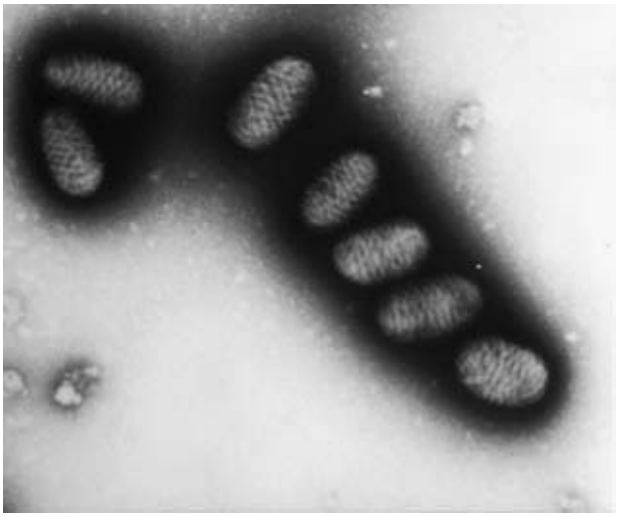


Figure 1 Electron micrograph of a cluster of five negatively stained orf virus particles.

disease virus (camel contagious ecthyma virus), chamois contagious ecthyma virus, sealpox virus and a virus that infects red squirrels.

The three original members of the genus were classified as separate species on the basis of the host animal and/or the pathology of the disease. Likewise, the observation of a parapox-like virus in red deer first suggested that this might represent another species. These separations have been supported by later studies which employed DNA/DNA hybridization, restriction endonuclease profiling or serology.

Properties of the Virion

Parapoxvirus particles are ovoid in shape and measure 220–300 nm × 147–170 nm. In these characteristics they resemble other poxviruses except that parapoxviruses are a little smaller than most other *Chordopoxvirinae* which are also more commonly described as brick-shaped rather than ovoid. A distinctive feature of parapoxvirus virions is their 'ball-of-yarn' appearance when negatively stained specimens are viewed by electron microscopy (**Fig. 1**). This results from a single 10–20 nm wide thread arranged as a spiral coil around the particle. This unique morphology remains the most practical means of confirming a suspected parapoxvirus infection. Such surface tubular elements are seen with other poxviruses but they lack the ordered helical array seen with parapoxviruses. It should be noted that in the case of vaccinia virus it has been suggested that these surface tubules might represent a shrinkage artefact resulting from dehydration during preparation for electron microscopy.

Analysis of thin sections of virions has revealed a lipoprotein bilayer surrounding a biconcave core and two associated lateral bodies. Some particles have an external membranous structure. It seems likely that this is equivalent to the Golgi-derived membrane which forms the outer layer of the extracellular enveloped form of vaccinia virus.

Properties of Viral Proteins

SDS–polyacrylamide gel electrophoresis analyses of orf virus and pseudocowpox virus virions have detected 30 to 40 polypeptides ranging in size from 10 to 220 kDa. Controlled degradation of the virions into core and surface fractions indicated the presence of 10–13 surface polypeptides. Prominent among these were a polypeptide of 39 kDa (orf virus) or 42–45 kDa (pseudocowpox virus) which was suggested to be the subunit of the virion surface tubule protein and a 15 kDa polypeptide. The 39 kDa orf virus polypeptide appears to be a dominant antigen and several independently derived monoclonal antibodies are directed against it.

DNA sequencing of regions of the orf virus genome has allowed the mapping of more than 60 genes. The majority of these are also found in vaccinia virus. Proteins with roles in DNA replication and gene expression are often similar to their vaccinia virus counterparts, with amino acid sequence identities of around 50%. These proteins include DNA polymerase, RNA polymerase subunits, poly(A) polymerase subunits, topoisomerase, dUTPase, RNA helicase and transcription factors. Related viral core and envelope proteins have also been identified. A smaller number of genes do not have counterparts in vaccinia virus and in some cases have no obvious matches in protein sequence data banks. A partial genetic profile of orf virus is listed in **Table 1**.

The genes encoding two major structural proteins of orf virus have been identified. One of these is a 42 kDa protein with strong amino acid sequence similarity to the vaccinia virus protein encoded by gene *F13L*. This vaccinia virus polypeptide is the major, nonglycosylated, 37 kDa protein specific for the extracellular enveloped form of the virus. Cloning and expression of the orf virus gene has shown that both antibody and T cell responses are directed against this protein during infection by orf virus. A second orf virus protein, p10k is a homolog of the vaccinia virus 14 kDa fusion protein encoded by gene *A27L*. This vaccinia virus protein has a surface location and is associated with intracellular mature virus but is required for the formation of extracellular enveloped virus.

Table 1 A partial genetic profile of orf virus.
Each orf virus (strain NZ2) gene is indicated by its location, expressed in kb from the left end of the genome. The orientation of each gene is indicated by an 'R' or 'L' representing transcription toward the right or left genome terminus, respectively. The coordinate given for each gene represents (a) the predicted initiation codon, (b) the termination codon or an internal restriction site. The homologous vaccinia virus genes listed are from strain Copenhagen except for '5k' which is present in strain WR but not in Copenhagen. The listed characteristics are for the vaccinia virus gene products except where no such homolog has been detected. Two genes are located within the inverted terminal repetition (ITR).

Orf virus gene	*Vaccinia virus homologue*	*Characteristic*
3.4L[a]	—	ITR
3.9L[a]	5k	
5.0L[a]	F2L	dUTPase
6.6L[a]	—	Ankyrin repeats
8.1L[a]	F11L	
10.1L[a]	F12L	
11.3L[a]	F13L	Envelope protein
11.6L[a]	—	
15.1R[a]	F17R	Structural phosphoprotein
16.9L[a]	E1L	Poly(A) polymerase subunit
20.0L[a]	E3L	Interferon resistance. PK inhibitor
20.6L[a]	E4L	RNA polymerase subunit
20.7R[a]	E6R	
22.4R[a]	E8R	
23.3R[a]	—	
27.2L[a]	E9L	DNA polymerase
27.2R[a]	E10R	
38.7R	I8R	RNA helicase
48.3R	L1R	Virion protein
53.1Ra	J3R	Poly(A) polymerase subunit
53.1Rb	J4R	RNA polymerase subunit
55.8R	J6R	RNA polymerase subunit
58.5R[b]	H2L	
59.5L[a]	H3L	Virion membrane protein
61.9L[a]	H4L	RNA polymerase associated protein
62.0R[a]	H5R	Late transcription factor-4
62.7R[a]	H6R	Topoisomerase
63.7R[a]	H7R	
66.4R	D1R	mRNA capping enzyme subunit
68.8R	D5R	DNA-dependent ATPase
75.4L	D11L	NPH-I
81.2L[a]	A1L	Late transcription factor-2
81.4L	A2L	Late transcription factor-3
89.4R	A8R	
89.5L[b]	A9L	
93.5R	A11R	Nuclear targeting sequence
103.3R	A24R	RNA polymerase subunit
108.2L[a]	A27L	Fusion protein
108.6L[a]	A28L	
109.6L[a]	A29L	RNA polymerase subunit
109.8L[a]	A30L	
110.8L[a]	A32L	ATPase
110.8R[a]	A33R	Envelope protein
111.2R[a]	A34R	Envelope protein
111.8R[a]	A35R	
129.8R[a]	—	IL-10
133.8R[a]	F10L	S/T protein kinase
135.2R[a]	F9L	
135.9R[a]	—	VEGF
136.5R[a]	—	ITR

Properties of the Genome

The parapoxvirus genome is a single linear double-stranded DNA molecule of about 140 kbp with inverted terminal repeats of about 3.5 kbp in the case of orf virus. The ends of the genome are crosslinked. The G+C content of the genome is high (64%). Restriction endonuclease cleavage site maps have been produced for each of the parapoxviruses except that infecting red deer. These revealed some variability between isolates of the same species, but conserved patterns, consistent with the classification of the genus, were apparent.

DNA sequence derived from numerous points on the orf virus genome, and totalling nearly 30 kbp, has been published. This has revealed a general pattern of genomic organization that is similar to that seen in those orthopoxviruses that have been entirely sequenced. A central region of approximately 90 kbp is highly conserved between the two genera, encoding homologous genes in the same relative order and orientation. Terminal regions show less similarity to other poxviruses and at one end a region of up to 20 kbp appears unlike the DNA sequence reported for any other poxvirus. DNA/DNA hybridization studies suggest that this region may also differ between individual parapoxvirus species.

Passaging of orf virus in cell culture has resulted in genomic rearrangements. A detailed study of one such rearranged isolate showed that 19.3 kbp from the right end of the genome had been duplicated and replaced 6.6 kbp at the left end. This recombination occurred between nonhomologous sequences and caused the deletion of three genes. These alterations to the genome attenuated the growth of the virus in sheep skin. Similar rearrangements have been observed in other isolates.

Physical Properties

Parapoxviruses are resistant to desiccation and in a dried state such as within scab material the viruses retain infectivity for extended periods. Under laboratory conditions infectivity may be maintained over many years. UV light, γ irradiation or heating at 56°C for 1 h cause inactivation of parapoxviruses.

Replication

As with all poxviruses, the replication of parapoxviruses occurs in the cytoplasm of infected cells. Studies in cell culture have shown very similar patterns in the replication of orf virus and pseudocowpox virus. DNA replication begins 4–8 h post-infection (p.i.) and reaches a plateau between 25 and 30 h p.i. The first viral or viral-induced polypeptides appear from 10 h p.i. Viral particles appear 16–24 h p.i. and continue to be produced until at least 40 h p.i. Viral replication is accompanied by inhibition of host DNA and protein synthesis. Some orf virus genes have been shown to be transcribed when infection occurs in the presence of an inhibitor of DNA synthesis and therefore fit the definition of early poxvirus genes. The transition between early and late replication events occurs about 8–10 h p.i. The presence in orf virus of genes with homology to vaccinia virus intermediate genes encoding late gene transactivators suggests that expression of orf virus genes follow a regulated cascade (early – intermediate – late) similar to that reported for vaccinia virus.

Transcription

Transcriptional analyses of 14 orf virus genes have been published. Ten of these genes are expressed early, three late and one at both early and late times. The identification of transcriptional start points of early genes highlighted adjacent sequences which are very similar to sequences shown to act as transcriptional promoters in vaccinia virus. The three late genes are preceded by a TAAAT transcriptional initiation motif typical of vaccinia virus late genes and all the early genes are followed by a TTTTTNT motif which in vaccinia virus induces termination of early transcripts. The 3′ ends of transcripts derived from one early orf virus gene were mapped to a region 24–32 nucleotides (nt) downstream of a TTTTTNT sequence. These data indicate that A/T-rich transcriptional control sequences characterized in vaccinia virus function in very similar ways in parapoxviruses. The common nature of transcriptional regulation was confirmed by analyzing the transcription of three early genes encoded on a 4.4 kbp fragment of orf virus DNA carried by a vaccinia virus recombinant. The vaccinia virus transcriptional machinery recognized the orf virus promoters and specific transcripts were initiated from the same points as were recorded for these genes in RNA from orf virus-infected cells. Analysis of expression of orf virus antigens expressed from vaccinia virus recombinants carrying large multigene fragments of orf virus DNA suggests that orf virus late genes are also faithfully transcribed from their own promoters by vaccinia virus.

Host Range and Virus Propagation

Natural infection by orf virus has been reported in domestic, bighorn and thinhorn sheep, domestic and Rocky Mountain goat, chamois, Himalayan thar, musk-ox, reindeer, steenbok and humans. Experi-

mental inoculations have shown that monkeys are susceptible to orf virus but a wide range of other animals including mouse, rabbit, dog, cat and domesticated chickens are resistant. Bovine papular stomatitis virus and pseudocowpox virus both establish infection in cattle and humans but all other species tested, including sheep, are resistant. The parapoxvirus of red deer induces only very mild lesions on sheep and has not been tested in other species. The parapoxvirus of seals has been reported in a range of seals and sea lions. Parapoxviruses do not produce lesions on the chorioallantoic membrane of developing chick embryo.

The most widely used cell culture systems have been primary ovine or bovine cells derived from sources such as testis, skin biopsy and embryonic kidney, lung and muscle. There have also been reports of orf virus isolates adapted to growth in established cell lines. Yields of infectious orf virus from cell culture tend to be 10- to 100-fold lower than that achieved with vaccinia virus.

Serologic Relationships

Parapoxviruses show extensive antigenic crossreactivity, although monoclonal antibodies have been able to distinguish each of the species. There are also antigens shared with other poxvirus genera but there is no crossprotection between parapoxviruses and either orthopoxviruses or capripoxviruses.

Epidemiology

The parapoxviruses of cattle and sheep are found throughout the world, essentially wherever their host animal occurs. The viruses are maintained in populations by a combination of chronic infection, frequent reinfection and the environmentally resistant nature of the viruses.

Orf virus shed in scab material can remain infective for lengthy periods and infection of naive animals by virus persisting in heavily contaminated areas such as barns, yards and sheep camps is likely to play a major role in maintaining the disease. One study has shown that if the scab material is ground up so as to release the virus then exposure to field conditions quickly results in inactivation of orf virus. Several studies have shown that a productive infection can be established in animals which have recovered from a previous infection. Such reinfections result in lesions that are smaller and resolve more quickly than primary infections. This short-lived immunity is likely to contribute to the persistence of the disease.

In the case of pseudocowpox virus, it is apparent that infection can be spread within dairy herds by contamination of milking machinery and milkers' hands. The introduction of procedures which reduce damage to teats and improve general hygiene at milking time can control the spread of the disease.

Figure 2 Orf virus lesion on a human face.

Clinical Features

Parapoxviruses cause proliferative lesions that are confined to the skin and oral mucosa with no evidence of systemic spread. Infection is initiated in abrasions and generally proceeds through an afebrile, self-limiting lesion that resolves within 3–9 weeks without leaving a scar. Orf virus lesions are most generally seen around the mouth and nares, hence the infection is commonly referred to as scabby mouth or sore mouth. Lesions are also observed on other parts of the body, for example, the coronet, udder or vulva. Following experimental inoculation of scarified skin, lesions progress through erythema, papule, vesicle, pustule and scab before resolving. Very large, proliferative, tumor-like lesions have been observed. It is likely that these are a result of an immune impairment of the host animal.

Lesions around the mouth can interfere with feeding or suckling and especially in young animals result in failure to thrive. Teat lesions can have similar effects through the inhibition of suckling. Lesions on growing deer antler can affect antler growth and severely affect marketability of the product.

It is probable that all parapoxviruses are able to infect humans, although a human case of the parapoxvirus of red deer has not been reported. Transmission to humans occurs readily although there is little evidence of human to human transmission. Progression of the lesions is essentially as seen in sheep and cattle such that the infection is benign and confined to pustular lesions on the skin at the points of infection (**Fig. 2**). More severe progressive disease

can occur in immune compromised individuals. Severe reactions have also been recorded in otherwise normal individuals in cases of burns and in cases of atopic dermatitis. Erythema multiforme reactions in the form of rashes on the backs of the hands and on the legs and ankles are common.

Histopathology

The histological sequence of events in the skin of sheep after orf virus infection is similar in primary and reinfection lesions, in spite of differences in the magnitude of the lesions and the time to resolve. Antibodies to orf virus envelope proteins have been used to detect orf virus antigen in epidermal keratinocytes, particularly those regenerating the damaged skin. Basal keratinocytes at the root of hair follicles can also contain virus. Some infected cells show evidence of a ballooning-like degeneration. There is no evidence that orf virus infects other, nonepithelial cell-types *in vivo*. Orf virus lesions often exhibit epidermal downgrowths (rete formation) into the dermis. This is particularly marked in primary lesions. Another characteristic feature is extensive capillary dilation and proliferation.

Orf virus lesions contain a dense accumulation of immune and inflammatory cells underneath and adjacent to virus-infected cells. These include neutrophils, lymphocytes (T and B cells) and dendritic cells that stain intensely with major histocompatibility complex (MHC) class II antigens. This dense network of dendritic cells is characteristic of orf lesions in sheep. The function of these cells is not known. The accumulating cells increase and decrease in number in parallel with the presence of virus in epidermal cells. The histology of human orf virus lesions is generally similar to that described in sheep. Comparison of orf virus and pseudocowpoxvirus lesions in humans has not detected any histopathological differences.

Immune Response

Parapoxviruses, in common with other poxviruses, stimulate a vigorous immune and inflammatory response in their hosts, and have evolved to replicate in the presence of this response. In sheep experimentally infected with orf virus, studies in the skin and lymph draining into (afferent lymph) and out of (efferent lymph) local lymph nodes have demonstrated that activated $CD4^+$ (helper) and $CD8^+$ (cytotoxic) T cells, B cells and antibodies are generated as part of the sheep acquired immune response to infection. The cytokines generated in lymph in response to virus reinfection are typical of type 1 antiviral cell-mediated immune responses and include interleukin (IL)-1β, IL-2, tumor necrosis factor (TNF)-α, granulocyte–macrophage colony-stimulating factor, interferon (IFN)-α and IFN-γ. Studies of orf virus reinfection in sheep depleted of specific lymphocyte subsets or treated with the immunosuppressant drug cyclosporin A indicated that at least $CD4^+$ T cells and interferons are important components of the host protective response against infection. These studies also indicated that the cutaneous damage sustained during orf virus infection is due in large part to the virus rather than host immune-mediated.

Sheep infected with orf virus mount detectable antibody responses to a small number of viral antigens but there is considerable individual qualitative and quantitative variation in the response. There is no apparent correlation between antibody titers and severity of viral lesions and passive transfer of antibody does not confer protection against virus challenge.

Pathogenic Determinants

In general, parapoxvirus infection is mild and localized with more severe disease only occurring in stressed or otherwise immune impaired individuals. An intriguing feature of parapoxviruses is the ability to repeatedly infect animals despite an apparently typical antiviral immune response to infection. This may in part be a result of the action of viral-encoded immune modulators. Such factors have been characterized in orthopoxviruses and include decoy receptors for IFN-γ, IL-1 and TNF-α, interferon resistance factors, complement-binding proteins and a viral epidermal growth factor. Parapoxviruses have not been analyzed in the same depth but several possible virulence genes of orf virus have recently been identified.

The first of these to be reported was a homolog of mammalian vascular endothelial growth factor (VEGF). The viral VEGF is expressed early and shows 16–27% amino acid sequence identity to mammalian VEGFs. All isolates of orf virus examined have a single copy of the gene but two major forms of the gene have been detected. Each form of the gene is located in the same relative region of the viral genome and each has a similar level of relatedness to mammalian VEGF, but the two genes are unexpectedly different from one another (only 41% amino acid identity). Mammalian VEGF (also known as vascular permeability factor) is a secreted homodimeric glycoprotein that has specific mitogenic effects on endothelial cells and has a primary role in normal and pathological angiogenesis, including the vascularization of solid tumors. Extensive capillary proliferation

and dilatation is a feature of orf virus lesions and it seems likely that the viral VEGF is involved in generating these features. A number of possible roles for the VEGF-like gene in orf virus biology can be imagined. For example, it may act indirectly to enhance proliferation of the epithelium, the growth substrate for orf virus. Alternatively it might induce vascular leakage and thereby promote the extensive scab formation which is typically seen and which acts as a type of 'spore coat', enabling the virus to persist in the environment. The report that human VEGF can inhibit the functional maturation of dendritic cells hints at a possible immune-modulating role for the viral VEGF.

A second potential pathogenic determinant of orf virus is the recently discovered homolog of interleukin-10 (IL-10). The viral IL-10 is an early gene and the predicted protein is very similar to ovine IL-10 (80% amino acid sequence identity). In a murine thymocyte proliferation assay the viral IL-10 showed the same activity as ovine IL-10. IL-10 suppresses cytokine production by T cells via effects on antigen-presenting cells such as macrophages. The orf virus IL-10 may act to suppress the immune response directed at the viral lesion and thereby delay its clearance. It may also have a role in the short-lived nature of immunity to reinfection.

Neither a VEGF or an IL-10-like gene has been reported in any other poxvirus, although homologs of IL-10 have been discovered in Epstein–Barr virus and equine herpesvirus 2. A pathogenic determinant of vaccinia virus which does have a homolog in orf virus, is the vaccinia virus *E3L* gene which plays a part in the resistance of that virus to interferon. This gene encodes a double-stranded RNA binding protein which inhibits the activation of a double-stranded RNA-dependent protein kinase and thereby prevents the phosphorylation of eukaryotic translation initiation factor 2 and the subsequent inhibition of protein synthesis. An orf virus gene encodes a protein which has 31% amino acid sequence identity to vaccinia virus E3L and conserves the double-stranded RNA binding domain.

Prevention and Control

Orf virus vaccines have been available for many years and are widely used to protect lambs against the debilitating effects of natural infection. These vaccines consist of live and essentially nonattenuated virus which is applied to a scratch on the skin of a leg. The ensuing infection does not interfere with feeding and provides significant protection against infection for some months. However, scab derived from vaccination lesions is likely to contaminate the environment and contribute to the perpetuation of the disease. New vaccines that induce protection but do not shed infectious virus are highly desirable. This might be achieved by deleting genes encoding viral virulence determinants or by delivering in an appropriate way the protective antigens of the virus.

Humans may be infected by parapoxviruses following contact with animal lesions or scab. The viruses in scab associated with animal products such as wool or farm equipment can remain infectious for lengthy periods. Care should be taken to avoid contact between any skin wound and potentially contaminated material.

Future Perspectives

Current studies with orf virus have raised the possibility of developing a vaccine able to protect animals against infection by this virus without generating significant amounts of infectious virus. Such a vaccine would also be likely to reduce the frequency of human infections. It is hoped that in the near future the complete DNA sequence of at least one strain of orf virus will be completed. The completed sequence is likely to provide insights into the pathogenesis of parapoxviruses and may well uncover novel virulence genes.

An area of study with parapoxviruses that is likely to see rapid development is the use of orf virus and perhaps other parapoxviruses as vaccine vectors. It is known that recombinant orf viruses can be constructed using approaches similar to those successfully used with other poxviruses. Several features of orf virus may be advantageous in its development as a vector. These include the restricted host range, the acute lesion that remains localized even in immune suppressed individuals and the long history of inoculating sheep with live orf virus.

See also: **Poxviruses (*Poxviridae*): Capripoxviruses, Leporipoviruses and suipoxviruses; Vaccinia virus (*Poxviridae*); Vaccines and immune response.**

Further Reading

Haig DM and Mercer AA (1998) Orf. *Veterinary Research* 29: 311–326.

Mercer AA, Lyttle DJ and Whelan EM (1995) The establishment of a gene map of orf virus reveals a pattern of genomic organisation that is highly conserved among divergent poxviruses. *Virology* 212: 698.

Moss B and Fenner F (1996) *Poxviridae*: the viruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Virology*, 3rd edn, p. 2637. Philadelphia: Lippincott-Raven.

PARTITIVIRUSES – FUNGAL (*PARTITIVIRIDAE*)

Said A Ghabrial, Department of Plant Pathology, University of Kentucky, Lexington, Kentucky, USA

Bradley I Hillman, Department of Plant Pathology, Rutgers, The State University of New Jersey, New Brunswick, New Jersey, USA

History

Viruses that occur naturally and multiply in fungi 'mycoviruses' were first discovered about 35 years ago. Interest in an antiviral activity associated with two *Penicillium* species led to the discovery of dsRNA-containing virus particles in fungi. It is now believed that viruses, mainly dsRNA viruses, are of common occurrence in fungi. The isometric dsRNA viruses with divided genomes, currently classified as belonging to the family *Partitiviridae*, were among the first mycoviruses to be studied and characterized. The main reason for the belated discovery of fungal viruses is that they are mostly associated with symptomless infections of their hosts. Because of similarities in biological properties and genome organization, the International Committee on Taxonomy of Viruses (ICTV) has recently grouped together the fungal partitiviruses (family *Partitiviridae*) and the plant cryptoviruses (family *Cryptoviridae*) into one family (*Partitiviridae*) and the family name *Cryptoviridae* was dropped.

Typically mycoviruses, including those in the family *Partitiviridae*, do not lyse their fungal host cells, and there is no evidence that they have an extracellular phase to their life cycles. For viruses with multiple dsRNA segments, the lack of conventional infectivity assays makes it difficult to ascertain the number of dsRNA segments required for infectivity. To elucidate the genome organization of these viruses thus requires that the complete nucleotide sequence of the dsRNA segments be known. To date, only a single virus, *Atkinsonella hypoxylon* virus 1 (AhV-1), in the genus *Partitivirus* has been completely sequenced.

Taxonomy and Classification

The family *Partitiviridae* includes four genera, *Partitivirus*, *Chrysovirus*, *Alphacryptovirus* and *Betacryptovirus*. Viruses in the first two genera infect fungi, those belonging to the other two genera infect plants. The partitiviruses that infect plants (cryptoviruses) are discussed in a separate chapter. Members and tentative members of the genera *Partitivirus* and *Chrysovirus* are listed in **Table 1**. Because the names of the genus *Partitivirus* and the family *Partitiviridae* have the same root, and to avoid confusion, the term 'partitiviruses' will be used in this article to refer to all members of the family and the term 'fungal partitiviruses' will be used to refer to viruses in the genera *Partitivirus* and *Chrysovirus*. The plant partitiviruses will be referred to as 'cryptoviruses'.

Viruses belonging to the family *Partitiviridae* have bipartite genomes composed of two monocistronic dsRNA segments that are usually similar in size. The complete nucleotide sequence of only one partitivirus (AhV-1) is available; partial sequences for some cryptoviruses including the RNA-dependent RNA polymerase (RDRP) sequence of the beet cryptic cryptovirus (BCV) are also available. The RDRP sequence of AhV-1 was more closely related to BCV than to any other dsRNA virus (or (+) strand RNA virus) justifying the grouping together of the plant cryptoviruses and fungal partitiviruses. The RDRP sequences from these two viruses (AhV-1 and BCV), however, were more closely related to viruses in the family *Totiviridae* than to any of the (+) strand RNA viruses. A phenogram showing the relationships among RDRP sequences of several dsRNA viruses including the two partitiviruses is shown in **Fig. 1**.

Transmission

There are no known natural vectors for viruses in the family *Partitiviridae*. The fungal partitiviruses are transmitted intracellularly during cell division and sporogenesis (serial or vertical transmission), and following cell fusion between genetically compatible host strains (lateral or horizontal transmission). The hosts of viruses in the genera *Partitivirus* and *Chrysovirus* belong to the higher fungi classes, Basidiomycotina and Ascomycotina and their anamorphs. The hyphae are septate and grow by extension of the hyphal tip. Virus transmission during vegetative growth occurs as virus particles which accumulate in the peripheral growth zone (the region of the mycelium where septal pores remain unplugged) are translocated with the flow of protoplasm towards the tip. New growth continues to be virus-infected as long as the septal pores connecting the apical hyphal compartment with virus-containing compartments in the peripheral growth zone remain open.

Transmission through asexual spores is highly

Table 1 Members and tentative members in the genera *Partitivirus* and *Chrysovirus*

Members	*Abbreviation*	*Tentative members*	*Abbreviation*
Partitivirus			
Agaricus bisporus virus 4	AbV-4	Diplocarpon rosae virus	DrV
Aspergillus ochraceous virus	AoV	Penicillium stoloniferum virus F	PsV-F
Atkinsonella hypoxylon virus	AhV	Phialophora radicicola virus 2-2-A	PrV-2–2-A
Gaeumannomyces graminis virus 019/6-A	GgV-019/6-A		
Gaeumannomyces graminis virus T1-A	GgV-T1-A		
Penicillium stoloniferum virus S	PsV-S		
Rhizoctonia solani virus	RsV		
Chrysovirus			
Penicillium brevicompactum virus	PbV	Helminthosporium victoriae 145S virus	Hv145SV
Penicillium chrysogenum virus	PcV		
Penicillium cyaneo-fulvum virus	Pc-fV		

efficient as 90–100% virus transmission into single conidial isolates of the fungal hosts can be achieved. Transmission via spores plays an important role in virus dissemination, as fungi produce many types of propagules and often in great profusion. The presence of virus particles in conidiospores has been demonstrated for a number of fungi including those infected with PsV-S, PsV-F, PcV and PbV (see **Table 1** for abbreviations of virus names). The presence of virus particles in the sexual spores (basidiospores) of the cultivated mushroom *Agaricus bisporus*, possibly including the partitivirus AbV-4, has been demonstrated by electron microscopy in thin sections of basidiospores. Unlike the basidiomycetes, the sexual spores (ascospores) of virus-infected filamentous ascomycetes, including *Gaeumannomyces graminis* (which is a host for several viruses in the genus *Partitivirus* including the type species GgV-019/6-A), are virus-free. There is no evidence that virus infection of fungal spores, whether mitotic or meiotic, has any deleterious effects on their viability.

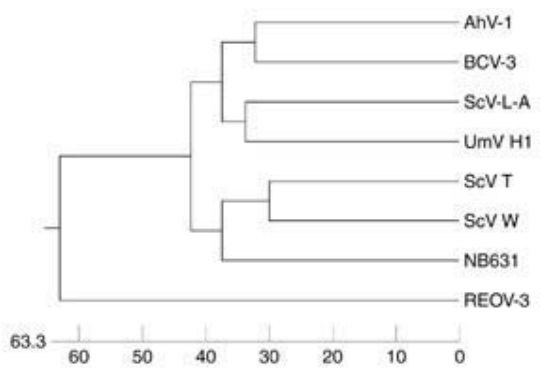


Figure 1 Phenogram summarizing relationships among core RDRP amino acid sequences of several dsRNA viruses including two members of the family *Partitiviridae* (AhV-1 and BCV-3), two members of the family *Totiviridae* (ScV-L-A = Saccharomyces cerevisiae virus L-A and UmVH1 = Ustilago maydis virus H1), and three unclassified nonsegmented dsRNA fungal viruses with genome sizes similar to the largest partitivirus segment (ScVT = Saccharomyces cerevisiae virus T; ScVW = Saccharomyces cerevisiae virus W; NB631 = dsRNA from *Cryphonectria parasitica* isolate NB631). The core polymerase sequence of reovirus-3 (REOV-3) was the outgroup.

Partitiviruses are transmitted intraspecifically from one fungal strain to another via hyphal anastomosis/heterokaryosis. Fungi are known to have a potential for plasmogamy and cytoplasmic exchange during extended periods of their life cycles. Because of vegetative incompatibility, hyphal anastomosis is limited to individuals within a species or within very closely related species. Transmission of several viruses including PsV-S, PsV-F, and PcV, by heterokaryosis has been demonstrated using auxotrophic and colored mutant fungal strains.

Host Range

With the exception of some members of the family *Hypoviridae*, there are no known experimental host ranges for mycoviruses in general including those in the genera *Partitivirus* and *Chrysovirus*. The reason for this is the lack of reliable conventional infectivity assays. The recent availability of infectious RNA transcribed from full-length cDNA to some hypovirus dsRNA has made it possible to extend slightly the host range of hypoviruses. Due to vegetative incompatibility, natural host ranges are restricted to individuals within the species known to be naturally infected with the virus in question. The finding that different fungal species belonging to widely divergent genera may harbor identical or closely related viruses, e.g. the serologically related partitiviruses PsV-S, AoV and DrV which infect *Penicillium stoloniferum*, *Aspergillus foetidus* and *Diplocarpon rosae*, respectively, raises

the question of the presence of vectors or means of transmission other than hyphal anastomosis.

Mixed infections of fungi with two or more unrelated mycoviruses are of common occurrence. Examples of mixed infections involving viruses in the genus *Partitivirus* include GgV-F6-B and GgV-F6-C in *G. graminis* and PsV-S and PsV-F in *P. stoloniferum*.

Virus–Host Relationships

Ultrastructural studies carried out with a number of the fungal partitiviruses including PsV-S, PcV and PbV, indicated that the virus particles accumulate in the cytoplasm of the hyphal compartments and in conidia. Particles often occur as free aggregates or enclosed in single or double-membrane bound vesicles. Whereas the apical hyphal compartments generally contain a small number of particles, virus replication occurs at higher levels in the lower hyphal compartments, particularly in the older plugged compartments which no longer contribute to host growth.

Like other fungal viruses, the partitiviruses generally do not have deleterious effects on their hosts. Because of their intracellular mode of transmission, fungal viruses have probably evolved along with their hosts as persistent subcellular particles. Thus, latency is the rule with mycovirus infections, and viral mutations that lead to pathogenic effects and lysis of host cells are expected to be self-eliminating because of the lack of extracellular mode of transmission.

Particle Properties

The sedimentation coefficients $s_{20,w}$ (in Svedberg units) for members of the genus *Partitivirus* are in the range of 101S to 145S. Buoyant density in CsCl = 1.35–1.36 g cm^{-3}. With some viruses, e.g. PsV-S, purified preparations contain, in addition to the mature virions (termed V1 and V2, respectively, for particles which separately encapsidate the bipartite dsRNA genome of 1.6 and 1.4 kbp, **Fig. 2**), sedimenting and density components that represent empty particles and intermediate components in the replication cycle. The latter include particles encapsidating single stranded (ss) RNAs, presumably the messenger sense strands of V1 and V2 dsRNAs, respectively; and intermediate particles containing both ss- and dsRNA (**Fig. 2**).

Particle Structure and Composition

The fungal partitiviruses have isometric particles, 30–35 nm in diameter, with icosahedral symmetry. The capsids are single-shelled composed of a single major polypeptide. The protein shell consists of 120 subunits of molecular mass in the range of 56×10^3–73×10^3 (for members of the *Partitivirus* genus), or 60 subunits of molecular mass of 125×10^3 (for members of the *Chrysovirus* genus) arranged in $T = 1$ lattices. The virions of members of the genus *Partitivirus* contain two unrelated segments of dsRNA, in the size range 1.3–2.2 kbp, one encoding the capsid polypeptide and the other an unrelated polypeptide, probably the virion-associated RNA polymerase. The two segments are usually of similar size, e.g. the sizes of the two dsRNAs (kbp) of GgV-019/6-A, GgV-T1-A, AbV-4, and RsV-717 are (1.7, 1.8), (2.1, 2.2), (2.0, 2.2) and (2.0 and 2.2), respectively. PsV-F and PrV-2-2-A each contain three dsRNA segments with sizes (kbp) of: 1.5, 1.3, 0.67 and 1.9, 1.8, 1.5, respectively. Sequence analysis of AhV-1 revealed that the three segments have sizes of 2180 (RDRP), 2135 (capsid protein) and 1790 bp. With the exception of the termini, the three segments are unrelated to each other. The absence of any long ORF on either strand of segment 3 (1790 bp) of AhV-1 and its unrelatedness to segments 1 or 2 suggest that it is a satellite segment, not required for virus replication. An unrelated *Partitivirus* of *A. hypoxylon*, AhV-2, contains two dsRNA segments of 2.2 and 1.6 kbp. A comparison of the two serologically closely related viruses GgV-019/6-A and GgV-38-4-A reveals that whereas the former has two dsRNA components, dsRNA1 and dsRNA2, the latter has three segments dsRNA1, 2 and 3. The dsRNA1 from GgV-019/6A and the corresponding one from GgV-38-4-A show a high degree of sequence similarity, as indicated by T1 oligonucleotide fingerprinting analysis and saturation hybridization assays. This is also true for dsRNA 2 from the two viruses. On the other hand, dsRNA3 from GgV-38-4-A has little or no sequence similarity to dsRNA1 or 2 from either virus suggesting that it is probably not required for replication, and may be regarded as satellite dsRNA. Why so many of these viruses contain satellite or defective dsRNA segments is an open question.

The particles of viruses in the genus *Chrysovirus* contain three unrelated dsRNA components with sizes in the range 2.8–3.5 kbp. Thermal denaturation and electron microscopy heteroduplex studies suggest that each dsRNA segment of PcV contains unique sequences. The three segments are separately encapsidated, and each is probably monocistronic. Some virus isolates encapsidate additional segments, which are probably satellite or defective dsRNAs.

Antigenic Properties

The fungal partitiviruses are efficient immunogens. Members and possible members which are serologically related, e.g. PsV-S, DrV and AoV, may be strains of a virus species.

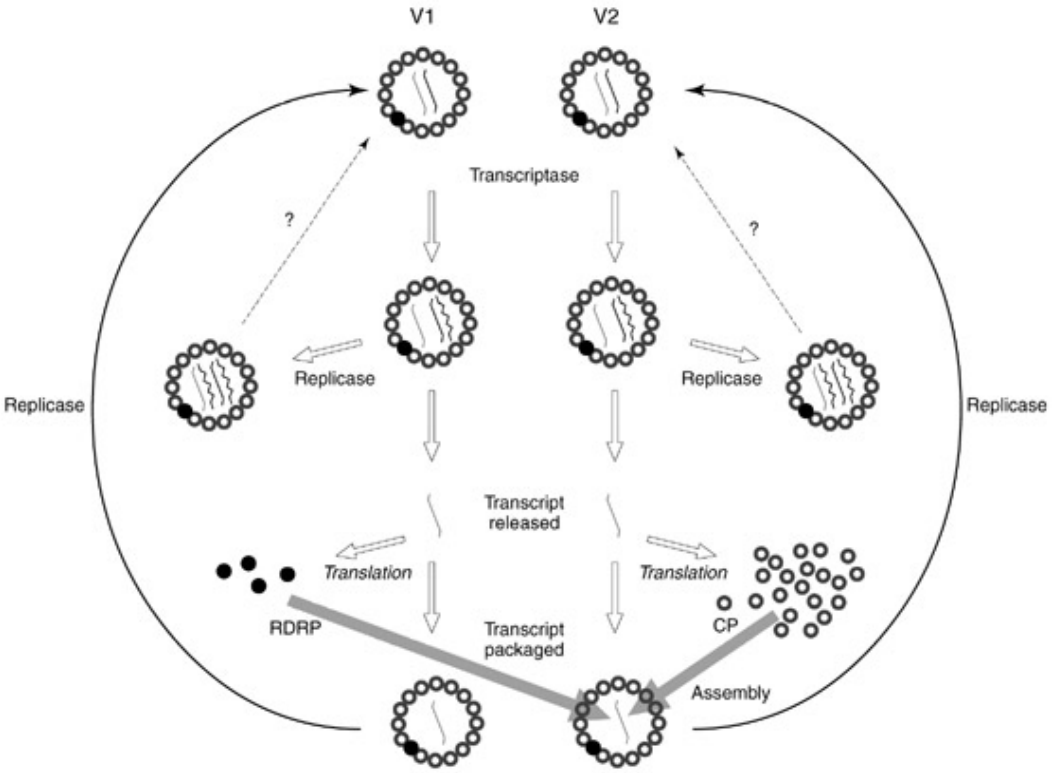


Figure 2 Model for replication of *Penicillium stoloniferum* S virus (PsV-S). The open circles represent capsid protein subunits and the closed circles represent RNA polymerase subunits. Solid lines represent parental RNA strands whereas wavy lines represent newly synthesized progeny RNA strands.

Transcription/Translation

All fungal partitiviruses so far examined have been shown to possess virion-associated RNA polymerase activity. The RNA polymerases associated with GgV 09/6-A and GgV 38-4-A are transcriptases which catalyze the synthesis and release of ssRNA copies of one of the strands of each of the virus template dsRNA molecules. Present evidence favors the idea that the *in vitro* transcription reaction occurs by a semi-conservative mechanism. Thus, the released ssRNA represents a displaced strand of the parental dsRNA and the newly synthesized strand becomes part of the dsRNA duplex.

In a rabbit reticulocyte lysates system, the ssRNA transcripts of GgV-38-4-A dsRNA1, 2 and 3 have each been shown to direct the synthesis of a major polypeptide, with molecular mass 62, 55 and 52 kDa, respectively. The three polypeptides are unrelated, as revealed by peptide mapping. The capsid polypeptide of the virus is encoded by dsRNA2 since the *in vitro* translation product of the ssRNA2 transcript co-migrates with the authentic virus capsid protein. The close serological relationship of GgV-38-4-A and GgV-019/6-A (the type species of the family *Partitiviridae*), coupled with the known sequence similarity of dsRNA2 from the two viruses, indicates that dsRNA2 of GgV-019/6-A also codes for the capsid polypeptide. Because GgV-O19/6-A has only two genome segments and requires the entire coding capacity of dsRNA2 for the capsid polypeptide of molecular mass 60 kDa, dsRNA1 probably encodes the virus RDRP. The dsRNA1 segments from GgV-38-4-A and that from GgV-019/6-A show extensive sequence similarity suggesting that the sequence of the putative RDRP sequence is highly conserved between these two strains. Lack of cross-hybridization of similar-sized dsRNA segments 1 or 2 from the two different partitiviruses infecting *A. hypoxylon*, coupled with the absence of serological cross-reactivity of their particles, supports the idea that the viruses are only distantly related. Relationships of the *A. hypoxylon* viruses to other partitiviruses are unknown.

Virus Replication

Current knowledge on how the fungal partitiviruses replicate their dsRNAs are derived from *in vitro* studies of virion-associated RNA polymerases and the isolation from naturally infected mycelium of particles that represent various stages in the replica-

tion cycle. The fungal partitivirus PsV-S serves as an example (**Fig. 2**). As indicated earlier, purified virus preparations are known to contain several classes of particles: the mature virions V1 and V2 which are composed of the two genomic dsRNA segments separately encapsidated in identical capsids; a small proportion of particles contain ssRNAs corresponding to the (+) strand of the genomic dsRNAs. Furthermore, purified virus preparations still contain a large proportion of a heterogeneous population of more dense particles than the mature virions. These particles represent various stages in the replication cycle which include: (1) particles containing the individual genomic dsRNAs with ssRNA tails of varying lengths; (2) particles with one molecule of dsRNA and one molecule of its ssRNA transcript; and (3) particles with two molecules of dsRNA. In *in vitro* reactions, only the particles with one molecule each of ds- and ssRNA exhibit RNA polymerase activity even though the capsid protein compositions of all three particle types are identical: all contain 120 molecules of the 56 kDa capsid polypeptide and one molecule of another polypeptide, presumably the RDRP. The reasons for lack of RDRP activity *in vitro* by particles containing only dsRNA or ssRNA are not known. The *in vitro* RDRP activity present in the particles with dsRNA + ssRNA is a replicase activity directing the completion of all the intermediate stages of the replication reaction to form particles with two molecules of dsRNA. The replication reaction occurs semiconservatively, as can be demonstrated by density labeling experiments, and probably takes place in two stages. In the first stage, the newly synthesized (+) strand displaces a parental strand of the same polarity and becomes part of the duplex. In the second stage, the displaced parental strand serves as a template for the synthesis of dsRNA. It is believed that the RDRP protein acts both as a transcriptase and a replicase. The transcriptase activity catalyzes the synthesis of (+) strand on parental dsRNA templates followed by the displacement of the parental (+) strand; the replicase activity catalyzes synthesis of (−) strand on the displaced parental (+) strand. There is no evidence for release of newly synthesized dsRNA from particles with two molecules of dsRNA.

See also: **Cryptoviruses (*Cryptoviridae*); Giardiaviruses (*Totiviridae*); Hypoviruses (*Hypoviridae*); Totiviruses (*Totiviridae*): General features, *Ustilago maydis* viruses; Yeast RNA viruses (*Totiviridae*).**

Further Reading

Buck KW (1986) Fungal virology – an overview. In: Buck KW (ed.) *Fungal Virology*. Boca Raton: CRC Press.

Ghabrial SA (1994) New developments in fungal virology. *Adv. Virus Res.* **43**: 303.

Ghabrial SA (1998) Origin, adaptation and evolutionary pathways of fungal viruses. *Virus Genes* 16: 119.

Ghabrial SA, Bozarth RF, Buck KW *et al* (1995) *Partitiviridae*. In: Murphy F *et al* (eds) *Virus Taxonomy: Sixth Report of the International Committee on Taxonomy of Viruses*, p. 580. Wien, New York: Springer-Verlag.

Oh C-S and Hillman BI (1994) Genome organization of a partitivirus from the filamentous ascomycete *Atkinsonella hypoxylon*. *J. Gen. Virol.* **76**: 1461.

PARVOVIRUSES (*PARVOVIRIDAE*)

Contents

General Features

Caroline R Astell, Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, Canada

Taxonomy and Classification

The family *Parvoviridae* includes a group of small, single-stranded viruses that infect a wide range of animals, causing severe disease in many. The family consists of two subfamilies, the vertebrate viruses, members of the *Parvovirinae*, which this article deals

observed with other parvoviruses. These small mRNAs were shown to express a 7.5 kDa protein and a family of three 11 kDa proteins (**Fig. 2**) in transfected and infected cells. The proteins are both proline-rich. The functions of the 7.5 kDa and 11 kDa proteins have not been determined.

Replication

The autonomous viruses (e.g. MVM and B19) replicate in dividing cells. For MVM the normal lab host cell is mouse LA9 fibroblast cells. B19 virus requires fresh peripheral blood cells. The virus replicates in erythroid progenitor cells. A few cultured cells permit very limited replication, hence B19 virus has not been studied as extensively as MVM or AAV2.

AAV2 requires a helper virus for replication – normally adenovirus, which is believed to alter the intracellular environment providing conditions suitable for AAV2 replication. Recent studies indicate that the ORF 6 product from the adenovirus E4 region is required. Cells exposed to chemical carcinogens or UV light also allow replication in the absence of helper virus.

Gene Therapy

One novel aspect of AAV2 replication is that, in the absence of helper virus, the AAV genome can integrate into the host DNA. This occurs predominantly at a relatively specific site on the q arm of human chromosome 19. The integrated DNA often occurs as tandem integrated copies. Superinfection with adenovirus can rescue integrated AAV2 viral genomes.

The integration process requires at least one terminal hairpin and transient expression of the Rep protein. The phenomenon of integration of AAV2 has led to this virus being engineered as a gene therapy vector. The obvious advantages are site-specific integration plus a virus that apparently does not cause disease. Although the virus can only package ~5000 nt of DNA, much larger DNA sequences coupled to an inverted terminal repeat sequence from AAV2 can be transfected into cells using lipid vesicles. Hence, the packaging size limitation of parvoviruses does not appear to be a major problem. More recently, MVM vectors, although nonintegrating. are being developed for special uses (e.g. as anticancer or antiviral (HIV) agents).

Future Perspectives

The role of parvoviruses in gene therapy (as well as anticancer and antiviral agents) underscores the need to elucidate the molecular mechanisms of parvovirus gene expression and replication, in order that vectors can be optimized for their specific uses. Each virus is sufficiently novel that further studies are justified. When a productive cell line is identified for the human B19 parvovirus, molecular studies with this human pathogen will be important.

See also: **Parvoviruses (*Parvoviridae*): General features; Vectors: Animal viruses.**

Further Reading

Anderson LJ and Young NS (eds) (1997) *Monographs in Virology*, vol. 20, p. 153. Basel: Karger.

Astell CR, Liu Q, Harris CE *et al* (1996) Minute virus of mice *cis*-acting sequences required for genome replication and the role of the *trans*-acting viral protein, NS1. *Progr. Nucleic Acid Res. Mol. Biol.* 55: 245.

Berns KI (1996) *Parvoviridae*: The viruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al.*) (eds.), *Fields Virology*, 3rd edn, p. 2173. Philadelphia: Lippincott-Raven.

Ferrari FK, Xiao X, McCarty D and Samulski RJ (1997) New developments in the generation of Ad-free, high titre rAAV gene therapy vectors. *Nature Med.* 3: 1295.

Cats, Dogs and Mink

Colin R. Parrish, James A Baker Institute for Animal Health, College of Veterinary Medicine Cornell University, Ithaca, USA

History

The parvoviruses affecting carnivores have a variety of histories. Feline panleukopenia virus (FPV) has been known as an infectious agent of cats since before 1900, and was shown to be a filterable virus during the 1920s. The disease of cats was originally described by a variety of names (e.g. feline distemper, feline infectious enteritis), and during the 1930s several studies showed that those diseases were caused by the same virus. A similar viral disease of raccoons was described during the 1940s, and it appears that the raccoon parvovirus (RPV) is very similar to or identical with FPV. During the 1960s FPV was shown to be the etiologic agent of feline cerebellar ataxia.

Mink enteritis virus (MEV) was first recognized during the late 1940s as the cause of an outbreak of enteric disease amongst mink in Fort William, Ontario, Canada. Initially called Fort William disease, and later mink viral enteritis, the disease was recognized throughout the USA and Europe during

the next decade. Both FPV and MEV were isolated in tissue culture cells during the 1960s, once the requirement of the viruses for rapidly dividing cells for replication was recognized.

Canine parvovirus (CPV) was first recognized as the cause of new diseases in dogs during 1978, and the virus was isolated from dogs in Europe, the USA, Australia and many other countries throughout the world during that year. Serological studies indicate that CPV was a new virus of dogs that was probably first widespread among dogs in Europe around 1974–1976, and which became globally distributed during 1978.

Canine minute virus or minute virus of canines (MVC) was first identified during the 1960s when it was isolated from the feces of clinically normal dogs. Recent evidence indicates that MVC can cause reproductive disease in dogs, and may also be associated with some cases of canine enteritis. Disease caused by MVC appears to be rare, however, and the limited number of cultured cells permissive for the virus means that the virus is rarely isolated or identified.

Aleutian mink disease, caused by Aleutian mink disease virus (AMDV), was first recognized around 1956 in ranch mink of the Aleutian genotype (see below). It was recognized later that all types of mink could develop Aleutian disease. The virus was identified during 1975, and its identity as a parvovirus confirmed during 1980.

Taxonomy and Classification

FPV, CPV, MEV and AMDV are all classified among the autonomous parvoviruses in the genus *Parvovirus*, within the subfamily *Parvovirinae*, in the family *Parvoviridae*.

CPV, FPV and MEV are host range variants of the feline parvovirus. RPV has not been classified, but available evidence suggests that it is similar to or identical with FPV. MVC is classified as a possible parvovirus. CPV, FPV, MEV and RPV are all very closely related antigenically, and isolates differ by <2% in DNA sequence, while AMDV is <50% similar in DNA sequence to the other viruses. The precise relationship of MVC to the other viruses has not been established, but preliminary DNA sequence analysis indicates that there is not obvious homology between MVC and CPV, and the most similar virus appears to be bovine parvovirus. No serological relationship has been described between MVC and any other parvovirus.

Geographic and Seasonal Distribution

CPV, FPV and the related viruses, as well as AMDV, are distributed as widely as their hosts. Limited studies indicate that in most countries the viruses are endemic, although vaccination has partially controlled reported clinical disease by CPV, FPV and MEV amongst many domesticated animal populations. Serological studies indicate that MVC is widely distributed amongst dogs in the USA. The virus has been isolated in the USA, Germany and Italy but its distribution has otherwise not been extensively studied. A seasonal distribution with greatest incidence of disease in the summer has been described for FPV in cats, most likely related to seasonal breeding patterns. The incidence of disease may be related to the population density of the susceptible host – in endemically infected areas, these are mostly young seronegative kittens or puppies. MEV disease is observed mostly during the summer, again due to the densities of susceptible kits, as mink breed and whelp once per year. Incidence of disease appears to be related to the density, immune status and size of the susceptible population. Acute interstitial pneumonitis caused by AMDV is seen only after infection of newborn mink, and therefore is observed only in spring and summer.

Host Range

CPV, FPV, MEV and RPV

The broad host ranges of these viruses have been defined primarily on the observations of disease. All susceptible hosts appear to be members of the order Carnivora, the various viruses causing disease in many species of large and small cats, most members of the family *Canidae*, including wolf-like canids, Asiatic raccoon dogs (*Nyctereutes procyonoides*), Arctic foxes (*Alopex lagopus*) and probably red foxes (*Vulpes vulpes*), common raccoons (*Procyon lotor*), mink (*Mustela vison*) and ringtail coatis (*Nasua nasua*). Ferrets (*Mustela putorius*) do not appear to be naturally susceptible, but cerebellar disease can be induced by experimental infection of neonatal kits. CPV infects wolf-like canids, as well as South American canids and Asiatic raccoon dogs. As well as cats and raccoons, FPV appears to infect foxes. Although FPV and MEV can both infect mink, only MEV isolates appear to be virulent for mink, and it appears that MEV isolates are differentiated from the other viruses by their ability to cause enteric disease in mink.

MVC

MVC is known only in the domestic dog, and the host range has not been investigated.

AMDV

Disease caused by AMDV has been observed mostly in mink, although ferrets are also susceptible to infection and disease. Other carnivores including raccoons, skunks (*Memphitis memphitis*), and martens (*Martes* spp.) are susceptible to infection, but disease has not been described.

Virus Propagation

CPV, FPV, MEV and RPV

Although these viruses replicate only in a limited number of tissues in the older host animal, this is at least partly due to the dependence of viral replication on cellular DNA replication. These viruses can grow in most primary cells or cell lines of feline origin. Only CPV grows well in canine cells in culture. Some cells may not propagate the viruses (e.g. some variants of the MDCK cell line appear to be not susceptible to CPV).

AMDV

Wild-type AMDV isolates grow poorly or not at all in most tissue culture cells, although certain viral strains have been adapted to feline tissue culture by passaging at lower temperature (~31.8°C). The viral strains propagated in tissue culture often do not replicate in mink. AMDV isolates will replicate in mitogen-stimulated mink lymphocytes (primarily B cells) in culture.

Structure

CPV

The atomic structure of the CPV capsid has been solved by X-ray crystallography (**Fig. 1**). This shows that the capsid shell is assembled from 60 copies of the region of the structural proteins common to VP1 and VP2. The exterior of the capsid is 22.4 nm and 28 nm at its narrowest and widest diameters, respectively.

Features observed on the capsid include a prominent 2.2 nm high by 7 nm wide 'spike' at the threefold axis of rotational symmetry. A hollow cylinder around the fivefold axis apparently allows the DNA genome or the N-terminal sequences of some copies of VP2 to pass through in full (DNA-containing) particles. There is a canyon surrounding the fivefold cylinder and a depression or dimple on the twofold axis. DNA is seen to be associated with the interior of the protein coat – at least 11 bases of single-stranded (ss)DNA with each VP2 equivalent.

Neutralizing epitopes are primarily affected by mutations of residues on the surface of the threefold spike, and mutations which affect hemagglutination of CPV are adjacent to the twofold dimple, indicating that is the site of sialic acid binding.

AMDV

Virus recovered from tissues of persistently infected adult mink is degraded to a number of lower molecular weight forms. Particles prepared from tissue culture or from acutely infected neonatal mink contain intact proteins. Monoclonal antibody analysis shows that there are antigenic epitopes specific for the degraded or intact forms of the proteins.

Genetics

CPV, FPV and the closely related viruses contain ssDNA of about 5200 bases, encapsidating the negative-sense strand (complementary to the mRNA). The AMDV genome is about 4750 bases of ssDNA. The structural proteins (VP1 and VP2) are encoded towards the right-hand end of the genome, while the nonstructural proteins are encoded towards the left-hand end. The CPV/FPV-like viruses are thought to express genes from two promoters (at 4 and 40 genome map units), and alternative splicing gives rise to messages for VP1 and VP2, and two nonstructural proteins (NS1 and NS2) (**Fig. 2**). NS2 shares its N-terminal region with NS1, but has a different C-terminal region derived by alternative mRNA splicing. The NS1 protein plays several important roles in DNA replication, but the function of NS2 is not known for CPV.

Mapping of host range differences between CPV and FPV by genetic recombination analysis shows that the canine host range of CPV is determined by a combination of as few as two surface-exposed amino acid residues within the capsid protein, one of which also determines a CPV-specific antigenic epitope. Feline host range differences are seen between FPV and the original strain of CPV. Those were also due to differences in the capsid protein, and the differences mapped to one region of the capsid structure.

The AMDV genome contains two promoters, and encodes two structural proteins (VP1 and VP2), as well as two or three nonstructural proteins. The structural proteins are derived from the same mRNA by differential use of translation start codons, probably by ribosome scanning. The various NS proteins are derived by differential splicing. There are two polyadenylation sites in the AMDV genome, one in the middle of the genome which is used for some NS protein gene messages, while the poly(A) addition site near the 5′ end of the genome is used for the structural and some of the nonstructural gene messages.

Infectious plasmid clones which contain almost

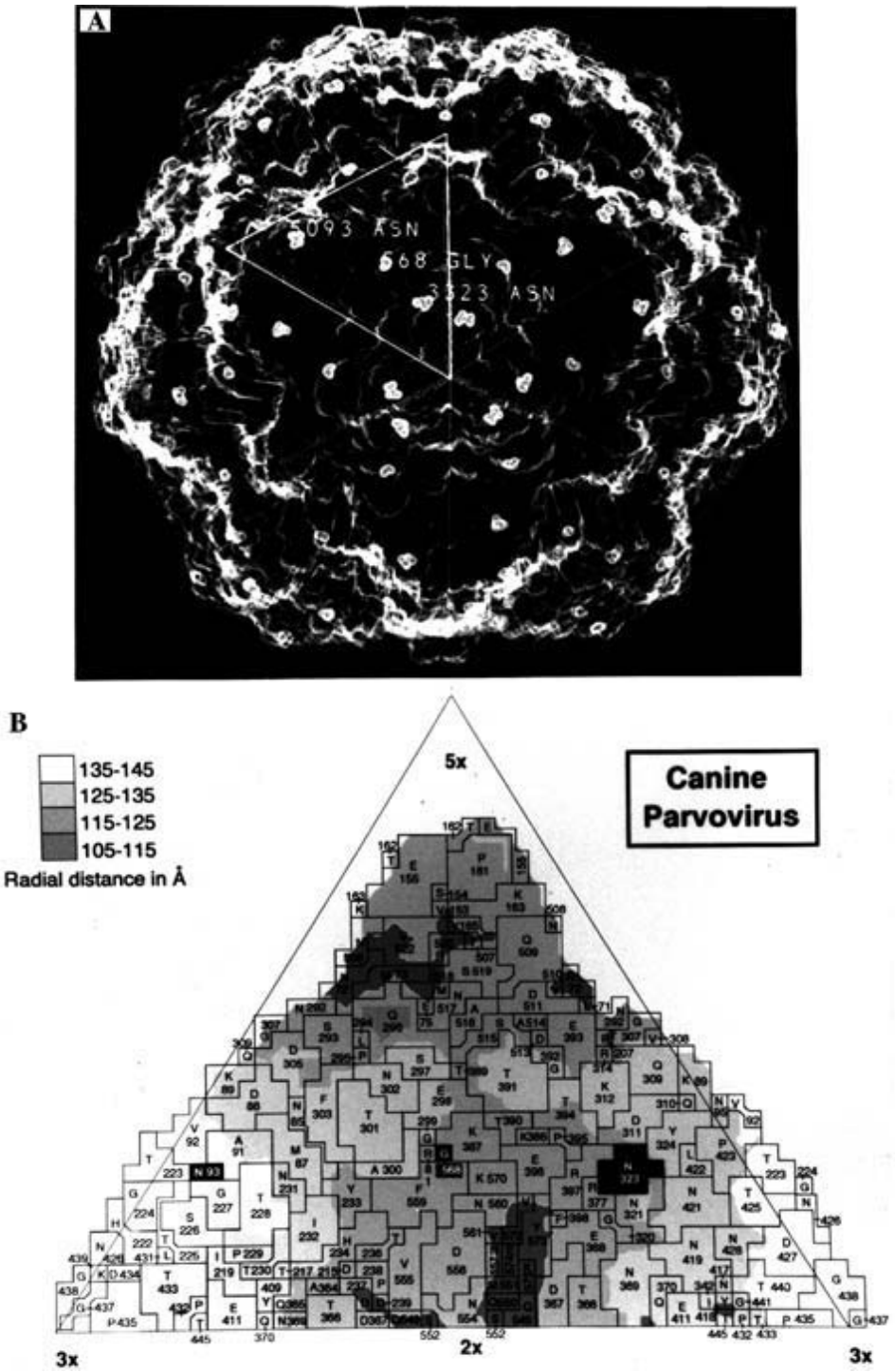


Figure 1 (**A**) The surface-exposed differences between CPV and FPV shown on the structure of the CPV particle which was determined by X-ray crystallography, as described by Tsao J *et al* (1991). (The three-dimensional structure of canine parvovirus and its functional implications. *Science* 251: 1456). The particle model is shown by grid-mesh surfacing, and the view is along the threefold axis of symmetry. The particle is comprised of 60 copies of VP2 (or the very similar VP1). The icosahedral face of the virus indicated by the triangle is comprised of portions of various symmetry-related VP2 molecules. Of the three variant amino acid residues shown on the surface (residues 93, 323 and 375 in the VP2 sequence), residues 93 and 323 are contributed from either the fivefold-related VP2 protein (5093) or the threefold-related protein monomer (3323). (**B**) One icosahedral unit of the virus capsid showing a 'road map' of the surface-exposed amino acids of CPV. Surface-exposed residues which vary between CPV and FPV are indicated by shading. The contours indicate the surface features, including a 'canyon' surrounding the fivefold axis of symmetry and a 'dimple' spanning the twofold axis of the virus. (Figures prepared by Jean-Yves Sgro, Institute for Molecular Virology, University of Wisconsin, Madison, WI, USA.)

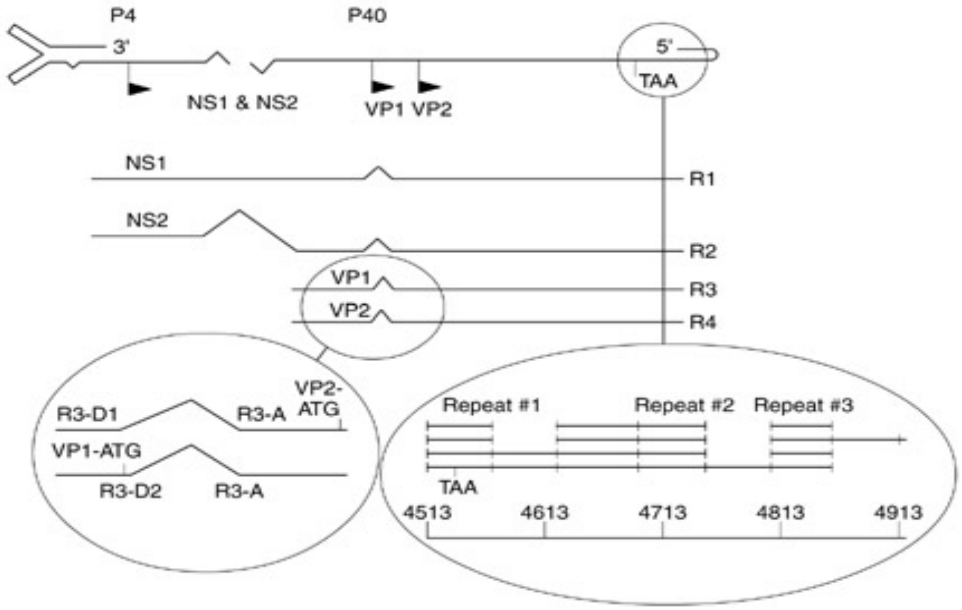


Figure 2 The genetic structure of the genome of CPV and FPV. The ssDNA genome is about 5200 nucleotides in length, and has terminal palindromes of about 150 bases at the 3′ and 5′ ends. Promoters at genomic map units 4 and 40 give rise to messages (R1–R3) for the nonstructural proteins (NS) and capsid proteins (VP), respectively. NS1 and NS2, and VP1 and VP2 are formed by alternative splicing from the same mRNA. Three sequences in the 5′ end of the genome may be present as single or multiple copies.

complete genome sequences have been prepared from CPV, FPV, MEV and AMDV. After transfection of susceptible cells the virus genomes resolve from the plasmids by specific nicking reactions, and infectious viruses are recovered which are indistinguishable from the original viruses.

The DNA sequence of MVC that has been determined indicates that it has a similar genetic organization to the other autonomous parvoviruses.

Evolution

The viruses vary at relatively low rates in nature, and less than 2% of their VP1/VP2 gene sequences over several decades. Sequence analysis shows that the FPV, MEV and RPV isolates are all closely related and comprise one phylogenetic cluster, while CPV isolates form a distinct cluster (**Fig. 3**). The CPV isolates have evolved in a step-wise fashion over time, with a progression of sequences from CPV type 2 to CPV type 2a to CPV type 2b. In contrast, the FPV strains do not show any progressive evolution, and appear to be in evolutionary equilibrium.

Serological Relationships and Variability

By conventional serological techniques (hemagglutination inhibition (HI), serum neutralization), CPV, MEV, FPV and other closely related viruses are difficult to distinguish. Some differences can be revealed by carefully controlled HI and plaque neutralization tests, but the differences are too small to be used for diagnostic purposes.

However, monoclonal antibody analysis readily reveals differences between CPV isolates and the other viruses, there being at least one epitope on all CPV isolates which is not present on any of the other viruses. There is also one (sometimes two) FPV-specific epitope on FPV, MEV and RPV isolates. A virus isolated from an Arctic fox appears very similar to FPV or MEV by DNA sequence analysis and monoclonal antibody typing, and it is quite distinct from CPV isolates.

Monoclonal antibody analysis has revealed variation in antigenic type among MEV isolates collected during the 1970s and 1980s in the USA and Scandinavia. The prevalence of those MEV antigenic variants was not related to the year of isolation, suggesting that these strains are not replacing each other. No difference between the strains was seen in crossprotection studies of animals when vaccinated mink were challenged with the various antigenic types of virus.

Two antigenic variants of CPV have emerged since that virus was observed in 1978. The first variant strain (designated CPV type 2a) emerged around 1979, and by 1981 had replaced the original virus type (designated CPV type 2) in the USA, Denmark, Japan

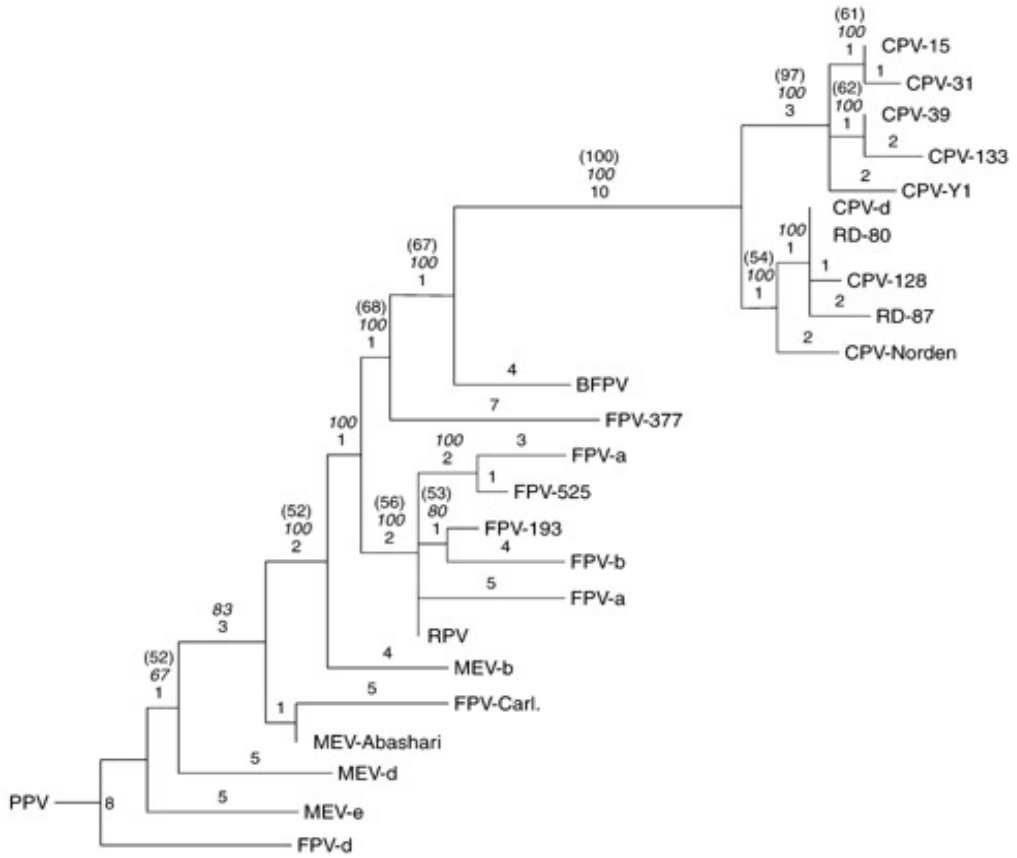


Figure 3 Phylogeny of the DNA sequences of the capsid protein genes of various isolates of dogs and Asiatic raccoon dogs (CPV and RD), cats (FPV), mink (MEV), raccoons (RPV) and Arctic (blue) foxes (BFPV). The numbers shown are the number of nucleotide differences which are present in each branch, the percentage of alternative trees which had that branching order (in italics), and the bootstrap support for that branching order (in parentheses). The tree was rooted using the PPV capsid protein gene sequence as an outgroup. (From Truyen *et al.* 1995 with permission.)

and Australia. A further antigenically variant strain (designated CPV type 2b) emerged around 1984, and by 1988 had become the predominant virus type isolated from dogs in the USA. The CPV-2a and CPV-2b strains each differ from the previous virus type in reactivity of one or two epitopes in the capsid. The variation in virus type was also correlated to the feline host range of the virus. The earliest CPV type (CPV-2) did not replicate in cats, whereas the later strains (CPV-2a and CPV-2b) readily infected cats and caused clinical disease.

Naturally-variant strains of AMDV have been defined antigenically by typing with monoclonal antibodies. The various AMDV isolates differ in virulence for mink. Variation of AMDV has also been shown in the DNA isolated from viruses within AMDV preparations, probably representing mixed virus preparations in tissues used for inoculation. The nucleotide sequences varied by up to 5% between the three principal types of virus sequence (designated types 1, 2 and 3) but the significance of that variation has not been defined.

Epidemiology

CPV, FPV and MEV are shed in high titers in the feces of infected animals, and transmission is via the fecal–oral route. The viruses are highly resistant to inactivation, and may survive in the environment for up to several months. Animals that recover from infection appear to be protected for life from reinfection. Animals born to immune females are protected from infection by maternally derived immunity, and so the susceptible cohort is generally young animals (8–16 weeks of age) with waning maternal immunity. Vaccination is able to protect animals efficiently, but effective immunization is also blocked by maternal immunity.

Spread of the viruses can be very rapid even over long distances; indeed, during 1978, CPV spread

around the world within a few months, even into countries such as Australia and New Zealand with strict quarantine for dogs and cats.

The role of antigenic variation in the epidemiology of these viruses is poorly understood. Antigenic variants of MEV all appear to cocirculate in mink. Variation of CPV has involved the sequential replacement of one viral type by the next, giving rise to the CPV-2a and CPV-2b antigenic types. The selection for the new types is presumably at least in part for the antigenic differences between the viruses, although other epidemiological advantages of the replacing strains may also be present, such as stability of the virus, or increased ability to infect or shed from dogs.

AMDV is shed in the feces, urine and saliva, and as such is readily spread between animals. However, the virus can be eliminated from mink ranch populations by serological testing and subsequent culling of antibody-positive animals, indicating that infected animals do not continuously shed virus.

Pathogenicity

CPV and FPV

Natural differences in virulence of CPV, MEV, RPV and FPV in their normal hosts have not been defined. However, viruses from the various hosts may show differences in virulence when infected into a heterologous host species. For example, although FPV isolates replicate in mink, they do not cause enteric disease, whereas MEV isolates cause severe clinical disease in mink. Attenuated strains of FPV, CPV and MEV have been derived by passage of the viruses in tissue culture, and AMDV and CPV strains which have lost the ability to infect their natural hosts have also been derived by passaging in culture.

The viral factors that result in attenuation of the viruses have not been defined, and the pathobiological basis of the attenuated phenotype is not known. However, some attenuated CPV vaccine viruses are shed at lower titers in the feces, suggesting a reduced replication in the intestinal epithelial cells.

AMDV

The pathogenesis of AMDV disease is at least partly dependent on the genotype of the mink infected. Aleutian mink (named after the Aleutian blue foxes, which have a similar coat color) are most susceptible to clinical disease; these mink suffer from a lysosomal abnormality similar to human Chédiak–Higashi syndrome. However, normal mink also suffer from a similar disease after infection by at least some strains of AMDV. Variation of the virulence of AMDV strains has also been described.

Clinical Features of Infection

Dogs, cats, mink and raccoons affected by CPV, FPV, MEV or RPV respectively suffer from similar enteric diseases. In animals older than about 5 weeks at the time of infection, the first clinical sign is pyrexia between 3 and 5 days after infection, and shortly thereafter virus is shed in the feces. The animals become depressed and lethargic, and may show a panleukopenia (cats) or a relative lymphopenia (dogs). In a proportion of infections (generally fewer than 20%) the animals may develop severe enteritis, with diarrhea and sometimes vomiting. Feces may be mucoid, liquid and flecked with blood. The severity of the disease depends on the condition of the animal – food deprivation prior to infection gives a more severe clinical disease. Animals may become severely dehydrated and die, possibly as a result of the dehydration and also to endotoxemia due to the destruction of the intestinal epithelium. Virus shedding stops once the immune response develops, and surviving animals recover without apparent long-term sequelae. It has been suggested that co-infections with other viral or parasitic agents may give a more severe disease owing to increased replication of crypt epithelium cells of the small intestine.

A number of different syndromes have been described after infection of neonatal or fetal animals. Ataxia may be seen after FPV infection of kittens or ferrets, where the virus replicates in the external germinal cerebellum, resulting in cerebellar hypoplasia. Puppies may develop a multifocal necrosis of the myocardium as a result of CPV infection. The animals die of acute heart failure days to weeks after infection. Many of the clinical signs observed, such as lethargy and dyspnea, are a consequence of the heart failure. Electrocardiographically the pups show a variety of subclinical abnormalities, and death results from ventricular fibrillation.

AMDV

The various clinical syndromes recognized for AMDV infection depend on the age, immune status and genotype of the mink. Infection of mink during pregnancy can result in fetal death or abortion. Kits born to AMDV noninfected dams are susceptible to developing interstitial pneumonia after neonatal AMDV infection, with clinical signs being observed 9–20 days after inoculation. Affected kits suffer from respiratory distress and lethargy, and most die within 24 h of the first observation of clinical signs.

Kits from immune dams or older (immunocompetent) animals develop a chronic disease which, after infection with a highly virulent strain, leads to death of a high proportion of infected animals due to

immune complex-mediated diseases. Affected animals often show few obvious clinical signs until shortly before death due to renal failure or to the rupture of inflamed arteries or enlarged spleens.

AMDV disease is characterized by a hypergammaglobulinemia with circulating immune complexes. The IgG concentration can reach very high levels and in a proportion of affected animals there is a restricted heterogeneity of the IgG produced, suggesting a clonal expansion of some B cell populations. Much of the antibody response is directed against the structural and nonstructural proteins of the virus. In addition, viral replication has been demonstrated in the kidney tubular cells, along with T lymphocytes, indicating that cell-mediated destruction of the kidney tubular cells is probably also occurring.

Pathology and Histopathology

CPV, FPV and MEV diseases

The pathogenesis of these viruses in their respective hosts is determined by the requirement of the parvoviruses for dividing cells for their replication. In fetal or neonatal animals viruses may replicate in a wide variety of tissues. In older animals the viruses replicate primarily in the lymphoid tissues and the intestinal epithelium.

Initial replication after oronasal infection occurs in the tonsils and in the regional or mesenteric lymph nodes, and subsequently in the thymus. Between 4 and 6 days after infection the virus infects the intestinal epithelial cells, and high titers of virus are shed in the feces. The loss of the regenerating epithelial cells results in a flattened attenuated epithelium and shortened intestinal villi. These changes may lead to loss of osmoregulation, resulting in diarrhea. Lymphoid tissues show lymphocytolysis and cellular depletion, followed by tissue regeneration and cellular repopulation in surviving animals. The spleen and bone marrow may be affected with a marked decrease in cellularity and decreases in cells of the myeloid, erythroid and megakaryocytic series. Whether these effects are due to direct viral replication or secondary effects, perhaps to endotoxemia, are unknown at present.

Feline ataxia results from viral replication in the external germinal epithelium of the developing cerebellum of newborn kittens, resulting in cerebellar hypoplasia.

In puppies which develop myocarditis there is a diffuse nonsuppurative myocarditis and often a mononuclear cell infiltration. Myocardial cells often contain Feulgen test-positive amphophilic inclusion bodies. Edema of the lungs is secondary to the acute heart failure.

AMDV

In seronegative newborn mink an interstitial pneumonia results from virus infection of alveolar type II cells of the lung, resulting in decreased surfactant production and causing the observed hyaline membrane disease and respiratory distress. In immune or older animals the virus replicates in various lymphoid tissues, particularly in follicular dendritic cells and macrophages. Probably as a result of infection of such antigen-presenting cells, and also due to the chronic infection and antigen expression by the virus, mink develop very high levels of antibody, and immune complexes are deposited in the kidney tubules and arteries.

Immune Responses

CPV, FPV and MEV

Antibodies appear to play the major role in the active immunity to and recovery from these parvovirus infections. Pups or kittens that acquire antibody from their immune dam are protected against infection until their circulating antiviral antibodies fall to very low levels. Parenterally transferred antibodies will likewise protect dogs against infection by CPV, the antibody acting to prevent systemic replication and spread of the virus. Any role for cellular immunity or secretory antibody is unknown at present. Virus may replicate locally in the gut of passively immunized animals, although at lower levels than in nonimmune animals.

AMDV

Effective protective immunity against AMDV infection, or immunity leading to virus clearance, has not been reported. Although AMDV replication is restricted in mink in the presence of high titers of circulating antiviral antibodies, the virus is not neutralized. The lack of neutralization is due to apparent protection of the virus by a coating of phospholipids or by viral aggregation, and AMDV virus is readily neutralized by antiviral antibody after detergent or organic solvent treatment.

Whereas vaccination with recombinant AMDV capsids had no effect or even enhanced disease, vaccination with the major nonstructural protein NS1 resulted in milder disease, suggesting a role for cell-mediated immunity in the control of the infection in animals.

Prevention and Control of Virus Infection

CPV, FPV and MEV

Vaccination efficiently protects animals against infection by CPV, FPV and MEV. Both inactivated virus and modified live virus vaccines are efficacious, although repeated doses are required to give long-lived protection with inactivated virus vaccines. Maternal immunity will prevent successful vaccination of animals. It appears that wild-type virus from the environment can infect animals with maternal antibodies at an earlier time than the same animals can be successfully vaccinated by parenteral routes. The cause of this 'window' of susceptibility to wild-type virus is not understood.

The control of virus spread between animals is difficult as the viruses are long lived in the environment, and also are shed in high titers in the feces. However, the virus may be inactivated with dilute hypochlorite solutions.

AMDV

No vaccine is available against AMDV, as the immune response is inextricably involved in the pathogenesis of the disease. Control measures involve the identification and culling of infected animals through serological testing, and control of reinfection by quarantine.

Future Perspectives

In future studies the basis of the host range and virulence differences between CPV, FPV and MEV will be defined, and the common features defined, at least in part, in terms of changes in the viral structure. The possible ancestors of CPV amongst the other carnivores should be defined by phylogenetic studies. The restricted but persistent replication of AMDV in mink will be examined, and the roles of the specific immune responses in the restricted replication will be defined. The viral and cellular factors that control replication in mink and in tissue culture cells should also be defined.

See also: **Immune response: Cell mediated immune response, General features; Pathogenesis: Animal viruses; Vaccines and immune response; Virus structure: Atomic structure, Principles of virus structure; Parvoviruses (*Parvoviridae*): General features, Molecular biology.**

Further Reading

Agbandje M, Parrish CR and Rossmann MG (1995) The structure of parvoviruses. *Semin. Virol.* 6: 299.

Bloom ME, Kanno H, Mori S and Wolfinbarger JB (1994) Aleutian mink disease: puzzles and paradigms. *Infect. Agents Dis.* 3: 279.

Corsini J, Afanasiev B, Maxwell IH, Carlson JO (1996) Autonomous parvovirus and densovirus gene vectors. *Advances in Virus Research* 47: 303–51.

Jacoby RO, Ball-Goodrich LJ, Besselsen DG, McKisic MD, Riley LK and Smith AL (1996) Rodent parvovirus infections. *Laboratory Animal Science* 46: 370–80.

Miyamoto T, Taura Y, Une S, Yoshitake M, Nakama S, Watanabe S (1995) Immunological responses to polyvalent canine vaccines in dogs. *Journal of Veterinary Medical Science* 57: 347–9.

Parrish CR (1990) Emergence, natural history, and variation of canine, mink, and feline parvoviruses. *AMDV. Virus Res.* 38: 403.

Parrish CR (1995) Pathogenesis of feline panleukopenia virus and canine parvovirus. *Baillières Clin. Haematol.* 8: 57.

Porter DD (1986) Aleutian disease – persistent parvovirus infection in mink with a maximal but ineffective host immune response. *Progr. Med. Virol.* 33: 42.

Rodents, Pigs, Cattle and Waterfowl

Peter Tijssen, Mohamed Laakel, Zoltán Zádori and **Benoît Hébert**, Institut Armand-Frappier, Université du Québec, Laval, Canada

Introduction

Parvoviruses are among the smallest and sturdiest viruses known and the only viruses with linear single-stranded (ss) DNA genomes. Due to their limited coding capacity and inability to induce cellular replication, the infection by parvoviruses requires rapidly dividing cells which provide proteins necessary for the infection. Consequently, their pathogenicity is restricted to permissive cells of that nature such as fetal tissue, epithelium and transformed cells.

History

Rat virus (RV) was first isolated from 1959, when Kilham and Olivier were searching for a rat papovavirus in rat embryo cell cultures followed rapidly by the isolation of H-1 virus from cell filtrates. The minute virus of mice (MVM) was isolated in 1966 by Crawford from laboratory stocks of mouse adenovirus. More recently, MPV was identified during the

tion cycle. The fungal partitivirus PsV-S serves as an example (**Fig. 2**). As indicated earlier, purified virus preparations are known to contain several classes of particles: the mature virions V1 and V2 which are composed of the two genomic dsRNA segments separately encapsidated in identical capsids; a small proportion of particles contain ssRNAs corresponding to the (+) strand of the genomic dsRNAs. Furthermore, purified virus preparations still contain a large proportion of a heterogeneous population of more dense particles than the mature virions. These particles represent various stages in the replication cycle which include: (1) particles containing the individual genomic dsRNAs with ssRNA tails of varying lengths; (2) particles with one molecule of dsRNA and one molecule of its ssRNA transcript; and (3) particles with two molecules of dsRNA. In *in vitro* reactions, only the particles with one molecule each of ds- and ssRNA exhibit RNA polymerase activity even though the capsid protein compositions of all three particle types are identical: all contain 120 molecules of the 56 kDa capsid polypeptide and one molecule of another polypeptide, presumably the RDRP. The reasons for lack of RDRP activity *in vitro* by particles containing only dsRNA or ssRNA are not known. The *in vitro* RDRP activity present in the particles with dsRNA + ssRNA is a replicase activity directing the completion of all the intermediate stages of the replication reaction to form particles with two molecules of dsRNA. The replication reaction occurs semiconservatively, as can be demonstrated by density labeling experiments, and probably takes place in two stages. In the first stage, the newly synthesized (+) strand displaces a parental strand of the same polarity and becomes part of the duplex. In the second stage, the displaced parental strand serves as a template for the synthesis of dsRNA. It is believed that the RDRP protein acts both as a transcriptase and a replicase. The transcriptase activity catalyzes the synthesis of (+) strand on parental dsRNA templates followed by the displacement of the parental (+) strand; the replicase activity catalyzes synthesis of (−) strand on the displaced parental (+) strand. There is no evidence for release of newly synthesized dsRNA from particles with two molecules of dsRNA.

See also: **Cryptoviruses (*Cryptoviridae*); Giardiaviruses (*Totiviridae*); Hypoviruses (*Hypoviridae*); Totiviruses (*Totiviridae*): General features, *Ustilago maydis* viruses; Yeast RNA viruses (*Totiviridae*).**

Further Reading

Buck KW (1986) Fungal virology – an overview. In: Buck KW (ed.) *Fungal Virology*. Boca Raton: CRC Press.

Ghabrial SA (1994) New developments in fungal virology. *Adv. Virus Res.* **43**: 303.

Ghabrial SA (1998) Origin, adaptation and evolutionary pathways of fungal viruses. *Virus Genes* 16: 119.

Ghabrial SA, Bozarth RF, Buck KW *et al* (1995) *Partitiviridae*. In: Murphy F *et al* (eds) *Virus Taxonomy: Sixth Report of the International Committee on Taxonomy of Viruses*, p. 580. Wien, New York: Springer-Verlag.

Oh C-S and Hillman BI (1994) Genome organization of a partitivirus from the filamentous ascomycete *Atkinsonella hypoxylon*. *J. Gen. Virol.* **76**: 1461.

PARVOVIRUSES (*PARVOVIRIDAE*)

Contents

General Features

Caroline R Astell, Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, Canada

Taxonomy and Classification

The family *Parvoviridae* includes a group of small, single-stranded viruses that infect a wide range of animals, causing severe disease in many. The family consists of two subfamilies, the vertebrate viruses, members of the *Parvovirinae*, which this article deals

with, and the invertebrate viruses, members of the *Densovirinae*. The subfamily *Parvovirinae* consists of three genera: *Parvovirus*, *Erythrovirus* and *Dependovirus*. Although all members of the *Parvoviridae* are similar in structure (single-stranded DNA genome surrounded by a nonenveloped icosahedral protein coat), they differ in detail in host range, pathogenesis and clinical symptoms, as well as in their gene expression and replication (described in *Parvoviruses, Molecular Biology*).

Members of the genus *Parvovirus* are autonomously replicating viruses which require cellular functions associated with the S phase of mitosis in order to replicate. Many *parvoviruses* characterized infect domestic animals: FPV (feline parvovirus or feline panleucopenia virus), CPV (canine parvovirus), BPV (bovine parvovirus), ADV (aleutian mink disease virus), MEV (mink enteritis virus) and PPV (porcine parvovirus), to name a few. These viruses often cause death in fetal or newborn animals, and widespread infection of susceptible animals can have significant financial consequences. There are also many rodent parvoviruses (e.g. MVM, minute virus of mice; H-1, a rat parvovirus; RV, rat parvovirus), and for others the natural hosts are still uncertain (e.g. LuIII, RA1).

Erythrovirus, a relatively new genus, includes human B19 parvovirus plus three distinct primate viruses, SPV (simian parvovirus from cynomolgus monkeys), PTMV (pig-tailed macaque virus) and RMV (rhesus macaque virus). These viruses replicate autonomously in erythroid progenitor cells, causing a transient depression in erythrocytes. In humans with genetic-based hemolytic diseases (e.g. sickle-cell disease, thalassemia, hereditary spherocytosis) that result in reduced red cell half-life, infection by B19 can cause a life-threatening aplastic crisis. This virus can also cross the placenta and infect fetuses, sometimes leading to hydrops fetalis. B19 parvovirus is also responsible for a transient polyarthropathy in ~50% of infected adults.

The third genus, *Dependovirus*, includes several viruses isolated in association with adenoviruses, hence the name adeno-associated viruses or AAV. These viruses include several human serotypes (AAV2, AAV3 and AAV5). AAV5 was isolated from penile cells and, in contrast to all other dependoviruses which require a helper adenovirus (or herpesvirus) for replication, it has been reported to replicate without a helper virus. Other AAV serotypes have been isolated from primates, cattle, dogs and birds.

Geographic and Seasonal Distribution

Parvoviruses have been isolated from infected animals worldwide and new viruses are being reported each year. Seasonal occurrence of parvoviruses has not been well documented; however, in a few studies B19 infections were found to cluster in late spring or early summer. It is difficult to rationalize why this is the case. These viruses are all very stable to inactivation and there are no known (insect or animal) vectors. B19 outbreaks often affect schoolchildren; hence, close association with infected individuals is required for aerosol transmission. With other viruses, seasonal incidence has been noted for FPV, correlating with the age at which kittens lose their maternal protective antibodies. MEV shows a seasonal (summer) occurrence.

Host Range and Virus Propagation

Parvoviruses are noted for their species specificity. MVM infects mice, whereas a closely related virus, H1 (97% sequence identity), infects rats. Within the host, the cells infected are almost always those that are actively dividing (e.g. fetal cells, cells lining the gastrointestinal tract, or cells of the hematopoietic system). In cell culture the species specificity is normally retained. An example of the fastidious nature of these viruses is B19 parvovirus. This virus infects human erythroid progenitor precursors, and in cell culture there is no permissive cell line known, including the erythroleukemia cell lines. Primary isolates of bone marrow cells, fetal liver cells and erythropoietin-stimulated peripheral blood cells will support replication and a few megakaryoblast cells will permit very limited replication. In contrast, the AAV viruses replicate in a number of cell lines when supplied with a helper adenovirus.

Genetics and Evolution

The genomes of the family *Parvoviridae* all consist of single-stranded DNA approximately 4600–6000 nt in length. Members of the genera *Parvovirus* and *Dependovirus* encode both structural and nonstructural proteins on one strand, referred to as the 'positive' or 'plus' strand. (Some members of the *Densovirinae* have their nonstructural and structural proteins encoded on opposite strands.) These viruses vary in their genome packaging strategies. For example, AAV and B19 package both plus and minus strands (in separate particles) in a ratio of 1:1. However, many parvoviruses package predominantly negative strands (e.g. MVM packages 99% minus strands), whereas others (e.g. BPV) package a variable percentage of minus strands from >90% to approximately 70–80%, depending on the host cell. No parvovirus has been characterized that packages predominantly plus strands.

The complete sequence of a number of parvovirus genomes has been reported over the past 18 years. The relatedness of these genomes ranges from >90% to <30%. However, the overall organization of each genome includes terminal inverted repeat sequences (100–300 nt in length) at the ends of the genomes. The remainder of the genome consists of large open reading frames (ORFs) encoding the nonstructural and structural genes. (Note that for viruses that package minus strands, the complement of the genomic DNA encodes the large ORFs.)

Regarding virus evolution, interest has been generated with respect to three closely related viruses, FPV, MEV and CPV. Infection by feline parvovirus has been recognized for more than 100 years. Mink enteritis virus was recognized by the middle of the twentieth century (~1950) and, more recently, CPV (a 'new' dog parvovirus) was recognized in the 1970s. It is believed that CPV evolved from FPV. One hypothesis has suggested that CPV evolved from vaccine strains of FPV, while more recently it has been suggested that the canine virus moved from cats to dogs via an intermediate host such as the fox. Although DNA viruses mutate at a much slower rate than RNA viruses, changes do occur. Studies to map the residues critical to determining the host range of CPV indicate that a very few key amino acid changes in the major capsid protein VP2 are responsible. Similar results have been obtained for MVMp and MVMi, as well as two strains of PPV. MVMp replicates in mouse fibroblasts; MVMi replicates in murine cells of lymphoid origin. This tropism has also been attributed to several amino acid differences in VP2. In each case, the tropism is not exerted at the level of receptor binding but at an, as yet, poorly defined intracellular step in the replication cycle.

Receptors

Over the past decade, a number of cellular receptors for many viruses have been identified. So far, however, few parvovirus receptors have been identified. B19 parvovirus binds to globoside (P-antigen of the P blood group system) on the surface of erythroid progenitor cells. AAV2, which infects a broad range of cell type, uses membrane-associated heparin sulfate proteoglycan as a receptor. Binding of MVM to mouse cells is blocked by neuraminidase, implicating a sialylated membrane protein as the receptor; however, this protein has not been identified.

Epidemiology

Because parvoviruses are very stable to inactivation, they undoubtedly contaminate living areas and can be easily transmitted to other immunologically naive animals. The major routes of transmission are aerosols, oral–fecal, venereal (PPV) and transplacental. Many parvoviruses are endemic in the population, with newborn animals protected by maternal antibodies. Many viruses also appear to exist in a persistent or latent form, causing little illness in adults.

Outbreaks in mink can result in severe disease, especially on mink farms. MEV causes a severe enteritis, while the distinct ADV virus kills young animals as a result of a massive humoral response to both the capsid and noncapsid viral proteins. Most animals die from immune complex glomerulonephritis resulting in kidney failure. PPV, another virus that can have a serious effect on nonimmune animals, causes significant reproductive failure.

B19 virus was identified as the causative agent of erythema infectiosum or fifth disease as recently as the early 1980s. Up to 80% of adults in the population are seropositive. Children are normally exposed during their school years, but if they fail to seroconvert as a child, there is a second wave of seroconversion of parents of school-age children. Immune compromised individuals are at risk of developing a persistent B19 infection.

Pathogenesis

While parvovirus infections are often subclinical, or symptoms are very minor, disease can be significant and death of the host can occur. It has been presumed that disease results from virus replication causing lysis of susceptible cells. Because of the need for actively cycling cells, replication is normally restricted to cells lining the gastrointestinal tract and cells of the hematopoietic system. In fetal animals, many more cells are actively dividing; hence, more types of cells are potentially susceptible. Whether infected cells are killed as a result of cell lysis, toxicity of the major nonstructural protein or induction of apoptosis is as yet unclear. It is generally accepted that the major NS protein of several parvoviruses is very toxic to cells, and recently evidence has been presented that the B19 NS protein is capable of inducing apoptosis.

In one case, disease and death of the infected animals are not the result of virus replication. Aleutian disease in mink causes a relatively mild infection. Death occurs from a massive immune response to both the structural and nonstructural proteins, resulting in immune complex glomerulonephritis and subsequent kidney failure. The characteristic 'slapped cheek' rash observed with B19 infection is also due to immune complexes, as is the associated transient polyarthropathy.

Clinical Features of Several Parvovirus Infections

Feline parvovirus (feline panleukopenia virus)

When FPV is introduced into a susceptible population of cats, all will become infected, although many will have subclinical symptoms. Kittens are especially at risk, with mortality on average ~50%. Perinatal infections can result in cerebellar hypoplasia, and if the infected animal survives it will display a distinctive ataxia that normally lasts for the lifetime of the animal. FPV also causes panleukopenia, a marked depression of all white cells. In addition, enteritis and gastroenteritis are common.

Mink enteritis virus

When MEV is introduced into a susceptible population, the virus spreads rapidly by the fecal–oral route. By 4–7 days postinfection (p.i.) the mink stop eating and develop a severe diarrhea and become dehydrated. Mortality is usually 20–50%. Surviving animals are immune. A vaccine is available, as once a mink ranch has experienced an MEV outbreak, the virus, being so stable, will likely always be present.

Canine parvovirus

CPV infects pups and nonimmune adults. While subclinical infections are common, a severe enteritis or myocarditis can occur and often the animals die. Animals that survive the enteritis recover, with no long-term effects. Puppies are especially susceptible to CPV-induced myocarditis, with a 50% mortality rate. Survivors normally have life-long heart-related problems. There is no treatment for this disease and vaccination of pups is standard in developed countries.

Aleutian disease virus

ADV is distinct from MEV. Both infect mink and are especially troublesome on mink ranches, where thousands of animals are housed in close proximity. Whereas MEV causes severe enteritis within a few days of infection, ADV is associated with persistent, low-level replication. There is a strong immunological response with elevated gammaglobulinemia by 30 days p.i. (3–11 times normal). Infectious immune complexes are readily detected in the serum, and immune complex glomerulonephritis is the cause of death. A recent comparison of both mink parvoviruses in cell culture has shown the MEV virus elicits high levels of viral capsid mRNA and produces large amounts of capsid proteins (80%) compared with NS proteins. In contrast, ADV has a lower level of transcription and 84% of mRNAs encode NS proteins.

Bovine parvovirus

BPV appears to be a common infection in cattle, with 50–90% of animals seropositive. BPV infection causes mild to severe enteritis within 1–2 days. The severity of the disease is increased in animals co-infected with other pathogens. There is some evidence that the virus can cause fetal death.

Adenoassociated virus

Infection with AAV2 continues to be unassociated with any clinical illness. This feature is one of several reasons why AAV vectors designed for use in gene therapy are considered to be preferable.

Human B19 parvovirus

B19 parvovirus is the only pathogenic human parvovirus. Infection is very common, with up to 80% of adults seropositive. This virus is responsible for erythema infectiosum or fifth disease, a relatively minor febrile illness with associated 'slapped cheek' rash. Clinical symptoms are often mistaken for rubella. A small number of infected children may also experience transient joint stiffness. Those who escape infection during their childhood years are again at increased risk as parents of young schoolchildren. In adults, the disease is normally a relatively minor febrile illness; however, as many as 50% (especially women) may develop a mild to severe transient polyarthropathy, normally lasting a few weeks to a few months.

B19 infection of those with an underlying genetic-based hemolytic anemia (e.g. sickle cell disease, thalassemia, hereditary spherocytosis) experience a life-threatening transient aplastic crisis which requires blood transfusion. Also, some fetuses develop hydrops fetalis due to transplacental infections, although the majority of newborns from B19-complicated pregnancies appear to be unaffected in any way. Individuals who are immunocompromised (e.g. human immunodeficiency virus (HIV)-positive, those with hereditary immunodeficiency diseases and acute lymphatic leukemia patients) can also experience aplastic crises and develop a relapsing anemia. These individuals are treated with immune globulin.

It is noteworthy that three additional erythroviruses also replicate in erythroid progenitor cells (all isolated from primates). The best characterized is simian parvovirus (SPV), which replicates in cynomolgus monkeys.

RA1 virus

Around the mid-1980s, RA1 parvovirus was reported as a possible agent of rheumatoid arthritis. It was obtained by multiple passages of synovial tissue

isolate from an individual with rheumatoid arthritis in rodent cells. With time, many have come to suspect RA1 is a rodent virus picked up during passage in rodents. The sequence of RA1 has yet to be published but is reported to be most closely related to BPV.

Detection of Parvovirus Infections

While many parvoviruses elicit distinctive cell morphology in the infected cells, diagnosis of parvoviruses (and many other viral infections) by microscopy has largely given way to immunological and molecular polymerase chain reaction (PCR)-based tests. There are many kits available commercially to detect viral antigens and PCR detection of viral DNA can be extremely sensitive.

Prevention and Control

Vaccines have been available for many parvoviruses that affect domestic animals (e.g. FPV, CPV, PPV) for a number of years. Also, in the mink industry in Denmark, success has been attained by eliminating infected mink. On 'ADV-free' ranches, the litter size is higher and loss due to ADV is significantly reduced.

A vaccine for human B19 virus is being developed. This vaccine will be especially useful for certain groups (e.g. those with hemolytic anemias) that are especially at risk.

Future Perspectives

Over the past decade, many new parvoviruses have been identified. It seems likely that many more will be identified in the next decade due to the use of PCR to detect new viruses. While these new viruses may persist in animals without obvious disease, it is worrisome that they are present as they may be having a subtle yet significant effect.

Note Added

A new human parvovirus has been identified within the past year. This virus is associated with post-transfusion hepatitis of unknown etiology and is referred to as TTV or transfusion transmitted virus.

See also: **Adenoviruses (*Adenoviridae*): General features; Densonucleosis viruses (*Parvoviridae*); Persistent viral infection.**

Further Reading

Anderson LJ and Young NS (1997) Human parvovirus B19. *Monog. Virol.* 20, 153pp.

Okamoto H, Nishizawa T *et al.* (1998) Molecular cloning and characterization of a novel DNA virus (TTV) associated with postransfusion hepatitis of unknown etiology. *Hepatol. Res.* 10: 1–16.

Tijssen P (ed.) (1990) *Handbook of Parvoviruses*, vols I and II. Boca Raton: CRC Press.

Tijssen P and Bergoin M (1995) Densonucleosis viruses constitute an increasingly diversified subfamily among the parvoviruses. *Semin. Virol.* 6: 347.

Molecular Biology

Caroline R. Astell, Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, British Columbia, Canada

Structure of Virus Particles

Members of the family *Parvoviridae* are small non-enveloped DNA viruses with particle sizes of 20–26 nm. These viruses are sufficiently small that the structure of the protein capsid of several viruses has been determined by x-ray diffraction. Canine parvovirus (CPV) was the first structure to be described. CPV contains 60 capsomers of predominantly VP2, the major capsid protein (with minor amounts of VP1 and VP3 structurally related proteins). The symmetry of the icosahedral particles is $T = 1$. The three-dimensional structure of VP2 is similar to other viral capsid proteins which include a central core of eight antiparallel β sheets. Overall the particles have a prominent protrusion at the threefold axis and a cylindrical structure at the fivefold axis which is surrounded by a deep canyon-like depression.

Over the past eight years the structures of feline parvovirus (FPV), minute virus of mice (MVM) and recombinant B19 have been reported, although resolution of the B19 crystals was only at 8 Å (0.8 nm), and provided less detail. These structures are in general very similar to the CPV particles with notable differences (e.g. B19 lacks the protrusion at the threefold axis and instead has a depression at this position). When the structure of CPV was completed, it was predicted that, by analogy with the rhinoviruses, the attachment site for cellular receptors may be within the canyon-like depression surrounding the fivefold axis. However, the receptor for B19 has been shown to bind to the depression at the threefold axis.

The parvoviruses contain a linear single-stranded DNA molecule ranging in size from 4600 to 6000 nucleotides (nt). Many viruses package exclusively the negative-sense strand (complementary to the coding strand) whereas some package predominately minus strands. Adeno-associated viruses (AAV), B19 and a

isolate from an individual with rheumatoid arthritis in rodent cells. With time, many have come to suspect RA1 is a rodent virus picked up during passage in rodents. The sequence of RA1 has yet to be published but is reported to be most closely related to BPV.

Detection of Parvovirus Infections

While many parvoviruses elicit distinctive cell morphology in the infected cells, diagnosis of parvoviruses (and many other viral infections) by microscopy has largely given way to immunological and molecular polymerase chain reaction (PCR)-based tests. There are many kits available commercially to detect viral antigens and PCR detection of viral DNA can be extremely sensitive.

Prevention and Control

Vaccines have been available for many parvoviruses that affect domestic animals (e.g. FPV, CPV, PPV) for a number of years. Also, in the mink industry in Denmark, success has been attained by eliminating infected mink. On 'ADV-free' ranches, the litter size is higher and loss due to ADV is significantly reduced.

A vaccine for human B19 virus is being developed. This vaccine will be especially useful for certain groups (e.g. those with hemolytic anemias) that are especially at risk.

Future Perspectives

Over the past decade, many new parvoviruses have been identified. It seems likely that many more will be identified in the next decade due to the use of PCR to detect new viruses. While these new viruses may persist in animals without obvious disease, it is worrisome that they are present as they may be having a subtle yet significant effect.

Note Added

A new human parvovirus has been identified within the past year. This virus is associated with post-transfusion hepatitis of unknown etiology and is referred to as TTV or transfusion transmitted virus.

See also: **Adenoviruses (*Adenoviridae*): General features; Densonucleosis viruses (*Parvoviridae*); Persistent viral infection.**

Further Reading

Anderson LJ and Young NS (1997) Human parvovirus B19. *Monog. Virol.* 20, 153pp.

Okamoto H, Nishizawa T *et al.* (1998) Molecular cloning and characterization of a novel DNA virus (TTV) associated with postransfusion hepatitis of unknown etiology. *Hepatol. Res.* 10: 1–16.

Tijssen P (ed.) (1990) *Handbook of Parvoviruses*, vols I and II. Boca Raton: CRC Press.

Tijssen P and Bergoin M (1995) Densonucleosis viruses constitute an increasingly diversified subfamily among the parvoviruses. *Semin. Virol.* 6: 347.

Molecular Biology

Caroline R. Astell, Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, British Columbia, Canada

Structure of Virus Particles

Members of the family *Parvoviridae* are small non-enveloped DNA viruses with particle sizes of 20–26 nm. These viruses are sufficiently small that the structure of the protein capsid of several viruses has been determined by x-ray diffraction. Canine parvovirus (CPV) was the first structure to be described. CPV contains 60 capsomers of predominantly VP2, the major capsid protein (with minor amounts of VP1 and VP3 structurally related proteins). The symmetry of the icosahedral particles is $T = 1$. The three-dimensional structure of VP2 is similar to other viral capsid proteins which include a central core of eight antiparallel β sheets. Overall the particles have a prominent protrusion at the threefold axis and a cylindrical structure at the fivefold axis which is surrounded by a deep canyon-like depression.

Over the past eight years the structures of feline parvovirus (FPV), minute virus of mice (MVM) and recombinant B19 have been reported, although resolution of the B19 crystals was only at 8 Å (0.8 nm), and provided less detail. These structures are in general very similar to the CPV particles with notable differences (e.g. B19 lacks the protrusion at the threefold axis and instead has a depression at this position). When the structure of CPV was completed, it was predicted that, by analogy with the rhinoviruses, the attachment site for cellular receptors may be within the canyon-like depression surrounding the fivefold axis. However, the receptor for B19 has been shown to bind to the depression at the threefold axis.

The parvoviruses contain a linear single-stranded DNA molecule ranging in size from 4600 to 6000 nucleotides (nt). Many viruses package exclusively the negative-sense strand (complementary to the coding strand) whereas some package predominately minus strands. Adeno-associated viruses (AAV), B19 and a

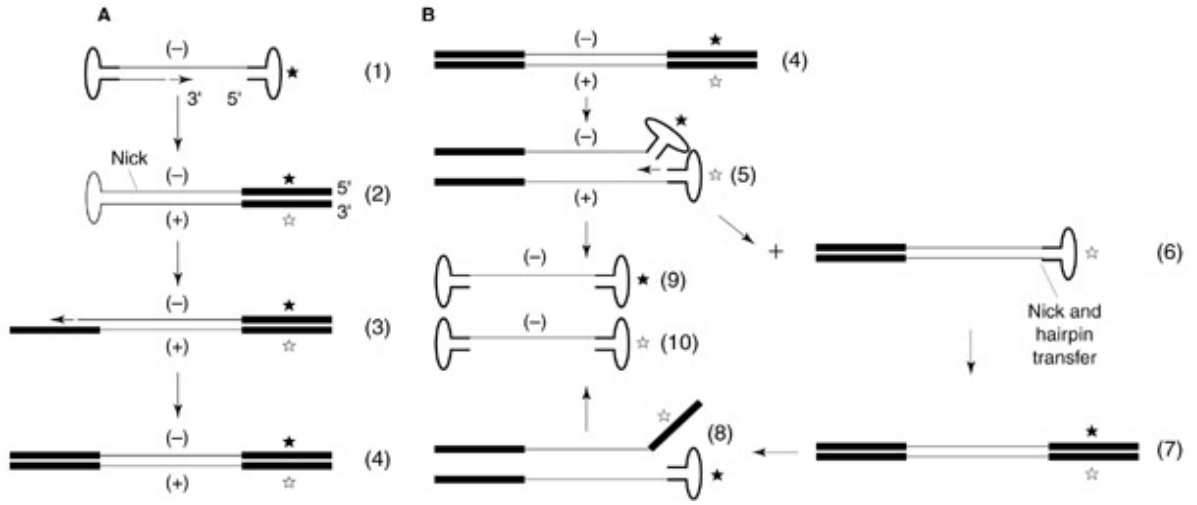


Figure 1 Simplified replication scheme for parvovirus. **A.** Incoming single-stranded genomic DNA (1) is converted into parental RF by strand extension (2). A nick is introduced opposite the site of initiation of strand extension and synthesis of a fully extended, double-stranded monomer RF is achieved (3, 4). **B.** Monomer RF (4) undergoes displacement synthesis (5), displacing a single-stranded progeny strand (9) and generating a covalently closed RF structure (6) which is again converted into a fully extended, double-stranded monomer RF (7) by nicking and hairpin transfer. In diagram (8), displacement synthesis of (7) again generates a single-stranded progeny genome (10). Note that the original genome sequence had a unique sequence denoted by a filled star (1) and the progeny strand (9) has the same sequence. However, repeated hairpin transfer results in two sequences at the end, the filled star (original sequence) (9) and the closely related, inverted complement (10). Hence, in a population of molecules, the hairpin termini exist as two sequences. DNA sequencing studies have shown that two sequences exist at both ends of the AAV genome. MVM, however, has a unique sequence at the left end. The model described is simplified. Both AAV2 and MVM generate dimer RF molecules and resolution of the dimer to monomer length duplex molecules is complex and the mechanism is not fully understood. The result is the left hairpin of MVM DNA contains a unique sequence.

few other viruses package equal amounts of minus and plus strands. No parvovirus packages predominately plus strands.

The structure of the icosahedral capsid results in virus particles which are extremely stable. These particles are resistant to heat inactivation (50° for 30 min) as well as moderate pH ranges (3–11). However, they are inactivated by high pH (>11), formalin, UV radiation, autoclaving, and β-propiolactone.

The DNA Genome

There are two striking features of the parvovirus DNA genome: it is largely a single-stranded DNA molecule and it has terminal hairpin structures at both ends. The ends of the linear genome contain inverted nucleotide sequences (116 to >300 nt in length). This allows the ends to fold back into double-stranded hairpin structures. In addition, within these terminal inverted repeats there are often smaller inverted repeat sequences that result in the termini existing in what has been described as T- or Y-shaped structures.

The viral genomes that have been characterized exist as molecules in which the 5′- and 3 terminal hairpin sequences are unrelated (in sequence) (e.g. MVM,) or they are closely related if not identical (e.g. AAV-2,). The significance of this remains to be determined.

Current models for parvovirus DNA replication propose that incoming parental DNA is converted into a double-stranded molecule using the 3′-OH of the terminal hairpin as a primer. A site-specific nick is introduced opposite the initial site of extension and the parental DNA strand is extended to yield a fully extended (double-stranded) molecule. This process of nicking inboard of the hairpin and strand extension is referred to as 'hairpin transfer' (**Fig. 1A**). Because the terminal sequences are not perfect inverted repeats, hairpin transfer results in two end sequences that are related but the inverted complement of each other. The sequences at the ends of viral DNAs have been sequenced from a population of replicated molecules with replication initiated from a cloned, infectious plasmid DNA (both AAV2 and MVM). Hence, replication began with a single unique sequence at the hairpin ends and two sequences were present in the replicated progeny strands (**Fig. 1B**). These data strongly support the 'hairpin transfer' mechanism for replication of the ends.

Deletion studies have shown that almost all of each hairpin terminus is necessary for efficient replication. Although some nucleotide changes are tolerated, the

overall hairpin structure is necessary. Also, specific binding sites for the viral nonstructural proteins are important. The role of the nonstructural proteins (Rep for AAV2 and NS1 for MVM) is to bind to the hairpin region overlapping the nick site and execute nicking. For MVM it has been established that a cellular DNA binding protein is required in addition to NS1 for nicking to occur. This protein binds distally to the DNA adjacent to NS1. Other cellular proteins involved in replication are presumably most of the host cell DNA replication complex. It is not clear which cellular DNA polymerase is involved.

Studies with MVM have shown that in addition to the hairpin termini, an internal replication sequence (IRS) inboard of the right hairpin is required for efficient replication of recombinant genomes. How the IRS sequence affects replication is unknown, however, it has been suggested that it facilitates initiation of replication at the right hairpin end.

Expression of Viral Polypeptides

The major portion of parvovirus genomes (excluding the hairpin termini) contains two large open reading frames (ORFs). These ORFs are by definition on the plus strand and transcription proceeds in a left to right direction (**Fig. 2**). Using this orientation, the left half of the DNA encodes the NS or Rep proteins while the right half encodes VPs. The pattern of gene expression varies depending on the number of promoters (1–3 promoters).

B19 has a single promoter at map unit 6. Transcription initiates at P6 and proceeds to the opposite end of the DNA. Complex alternate splicing and use of two polyadenylation sites (one in the middle of the genome and one at the right hand end) results in at least nine transcripts which encode NS and VP proteins as well as novel small proteins, the 7.5 and 11 kDa proteins (**Fig. 2**). Of interest is that all nine B19 transcripts contain a common 56 nt leader sequence. Although the B19 transcript map and use of one promoter seemed unusual when it was first discovered, the transcript map for bovine parvovirus is very similar.

MVM is an example of a virus that uses two promoters, P4 for expression of NS proteins and P38 for expression of VPs. All transcripts terminate at the far right of the genome and alternate splicing generates mRNAs that encode two nonstructural proteins (NS1 and NS2) and two VP proteins (VP1 and VP2).

AAV uses three promoters, P5, P19 and P40. The first two initiate transcripts that encode two large and two smaller Rep proteins whereas the P40 promoter generates mRNAs encoding three viral proteins: VP1, VP2 and VP3 (**Fig. 2**).

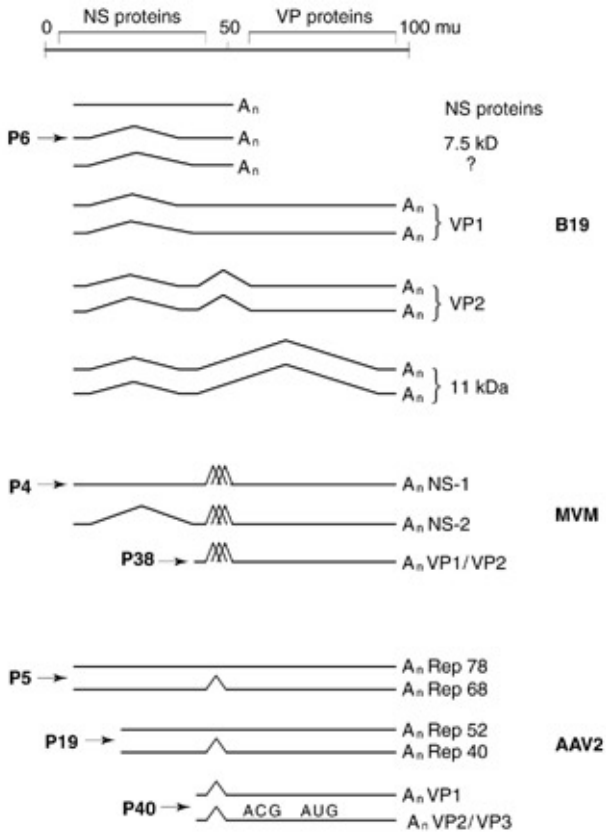


Figure 2 Transcription map of MVM, AAV-2 and B19. Nonstructural proteins (NS) are encoded on the left half of the DNA and structural proteins (VP) on the right half. Exons are represented by horizontal lines joined by brackets (introns). For B19, the nine transcripts that have been mapped are illustrated. All mRNAs contain a 56 nt leader sequence. For MVM the small 'middle' intron can be spliced in three different ways, hence, for each mRNA shown there are three different forms (total of nine mRNAs). All three transcripts coding for the NS-1 protein have the same coding potential as the small intron is 3′ to the stop codon for NS-1. The middle transcript also has three different versions of this small splice and results in three slightly different NS-2 proteins differing in the carboxy terminal residues. Of the three forms of the capsid protein mRNA, one splice allows initiation of synthesis of VP1 ahead of the small splice. The other two RNAs remove this ATG start codon and, thus, VP2 initiation begins to the right of the small splice. For AAV2, there are two mRNAs generated from transcripts initiated at each of the three promoters. The small splice generating the VP1 mRNA has a different splice acceptor from that generating VP2/VP3. VP3 is the major coat protein which initiates at the first AUG codon whereas VP2, a minor coat protein initiates at low frequency at an upstream ACG codon. The promoters for each virus are shown at the left (P6 →, etc.) and the names of the proteins encoded by each mRNA are shown on the right.

The processing of transcripts from all viruses is complex. The selection of intron donor and acceptor sites results in variable amounts of transcripts. For

example, in MVM and AAV, use of alternate splice acceptor sites results in synthesis of low levels of the largest coat protein (VP1) and high levels of the smaller, major coat protein, VP2 and VP3, respectively.

Regulation of gene expression for multiple promoter viruses is controlled by *cis*-acting sequences and transacting factors (both viral and cellular). The major NS proteins of MVM (NS1) upregulates expression of both the P38 and P4 promoters. NS1 binds to $(ACCA)_n$ repeats located upstream of the transcription start sites. An Sp1 motif and TATA box also function in upregulating the P38 promoter and studies have shown that NS1 and SP1 can interact on the absence of the promoter sequence.

Analysis of the DNA sequence near established promoters in many viruses has identified multiple motifs known to bind cellular transactivators. Although many of these motifs are present near parvovirus promoters, in most cases involvement of the cognate transactivator has not been demonstrated. An exception is YY1 which enhances expression of the B19, P6 promoter. It also binds to the +1 region of the AAV2 P5 promoter and allows basal level transcription from this promoter.

For AAV2, the Rep protein stimulates transcription of the P40 promoter, however, the response of the P5 and P19 promoters to Rep varies depending on conditions within the host cell. In the absence of helper virus, Rep protein normally downregulates P5 and P19.

The temporal regulation of early and late gene expression has been studied in MVM and BPV. There is evidence that NS encoding transcripts are expressed earlier than VP encoding transcripts. However, there is not as clear a demarcation between early versus late transcripts, as is seen with some DNA viruses.

Viral Nonstructural and Structural Proteins

Nonstructural proteins (NS, Rep)

The left ORF of parvoviruses encodes nonstructural proteins. MVM makes two NS proteins; a large 80 kDa protein (NS1) is required for replication and gene expression. Alternate splicing also generates a mRNA encoding the NS2 proteins (24 kDa) which exists as three closely related proteins with minor differences in amino acid sequence at the C-terminal as a result of three splicing variations of the small intron near the middle of the genome. The role of NS2 is uncertain. It appears to contribute to viral pathogenesis and also affects folding of viral structural proteins. The AAV2 virus expresses two versions of a large Rep protein (78 and 68 kDa) and two versions of a smaller Rep protein (52 and 40 kDa) depending on the promoter used (P5 or P19) (**Fig. 2**).

The NS1 protein of MVM and two larger Rep proteins of AAV function in gene expression and DNA replication. These proteins have been overexpressed and purified. They contain an intrinsic helicase, ATPase, and site-specific nickase. They also have a transactivation domain, nuclear localization signals and DNA binding domain. The *cis*-acting sequences required for binding the NS proteins have been mapped. Also, the binding sequences within the terminal hairpin structures have been identified. The function of the ATPase is likely associated with the helicase which functions in strand displacement synthesis. Both the large Rep proteins of AAV2 and NS1 of MVM have been shown to be covalently attached to the 5′ end of the genome. This occurs during nicking of the DNA. The attached NS protein is usually removed from packaged viral genomes (presumably by nucleolytic trimming of the 5′ end of the genome). However, particles with NS1 external to the capsid and covalently linked to the 5′ end of the genome have been characterized. Both the large Rep protein and NS1 have also been shown to multimerize and this is likely important for the replication function of these proteins.

As mentioned above, the NS1 protein of MVM interacts with Sp1. Recent reports have identified two other cellular proteins that also appear to interact with the NS1 of MVM and of H1 (a closely related rodent parvovirus). One protein is a novel hnRNP-like protein and it may be involved in regulating mRNA biogenesis.

Structural proteins

Parvovirus capsids are composed of two and in some cases three viral proteins. The coding of these proteins is entirely overlapping. A minor VP1 protein is expressed in addition to a major (shorter) VP2 protein generated from an alternately spliced transcript. MVM has a small amount of a third capsid protein, VP3, which results from proteolytic cleavage of VP2. AAV-2 also has two minor capsid proteins, VP1 and VP2. VP2 is translated from an alternately spliced mRNA using an internal ACG inititating codon at low frequency. The major coat protein, VP3, is expressed from the same spliced mRNA at a downstream AUG codon (**Fig. 2**).

Small proteins

When the transcripts for human B19 parvovirus were characterized it was recognized that there were two classes of small, abundant, spliced RNAs, so far not

observed with other parvoviruses. These small mRNAs were shown to express a 7.5 kDa protein and a family of three 11 kDa proteins (**Fig. 2**) in transfected and infected cells. The proteins are both proline-rich. The functions of the 7.5 kDa and 11 kDa proteins have not been determined.

Replication

The autonomous viruses (e.g. MVM and B19) replicate in dividing cells. For MVM the normal lab host cell is mouse LA9 fibroblast cells. B19 virus requires fresh peripheral blood cells. The virus replicates in erythroid progenitor cells. A few cultured cells permit very limited replication, hence B19 virus has not been studied as extensively as MVM or AAV2.

AAV2 requires a helper virus for replication – normally adenovirus, which is believed to alter the intracellular environment providing conditions suitable for AAV2 replication. Recent studies indicate that the ORF 6 product from the adenovirus E4 region is required. Cells exposed to chemical carcinogens or UV light also allow replication in the absence of helper virus.

Gene Therapy

One novel aspect of AAV2 replication is that, in the absence of helper virus, the AAV genome can integrate into the host DNA. This occurs predominantly at a relatively specific site on the q arm of human chromosome 19. The integrated DNA often occurs as tandem integrated copies. Superinfection with adenovirus can rescue integrated AAV2 viral genomes.

The integration process requires at least one terminal hairpin and transient expression of the Rep protein. The phenomenon of integration of AAV2 has led to this virus being engineered as a gene therapy vector. The obvious advantages are site-specific integration plus a virus that apparently does not cause disease. Although the virus can only package ~5000 nt of DNA, much larger DNA sequences coupled to an inverted terminal repeat sequence from AAV2 can be transfected into cells using lipid vesicles. Hence, the packaging size limitation of parvoviruses does not appear to be a major problem. More recently, MVM vectors, although nonintegrating. are being developed for special uses (e.g. as anticancer or antiviral (HIV) agents).

Future Perspectives

The role of parvoviruses in gene therapy (as well as anticancer and antiviral agents) underscores the need to elucidate the molecular mechanisms of parvovirus gene expression and replication, in order that vectors can be optimized for their specific uses. Each virus is sufficiently novel that further studies are justified. When a productive cell line is identified for the human B19 parvovirus, molecular studies with this human pathogen will be important.

See also: **Parvoviruses (*Parvoviridae*): General features; Vectors: Animal viruses.**

Further Reading

Anderson LJ and Young NS (eds) (1997) *Monographs in Virology*, vol. 20, p. 153. Basel: Karger.

Astell CR, Liu Q, Harris CE *et al* (1996) Minute virus of mice *cis*-acting sequences required for genome replication and the role of the *trans*-acting viral protein, NS1. *Progr. Nucleic Acid Res. Mol. Biol.* 55: 245.

Berns KI (1996) *Parvoviridae*: The viruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al.*) (eds.), *Fields Virology*, 3rd edn, p. 2173. Philadelphia: Lippincott-Raven.

Ferrari FK, Xiao X, McCarty D and Samulski RJ (1997) New developments in the generation of Ad-free, high titre rAAV gene therapy vectors. *Nature Med.* 3: 1295.

Cats, Dogs and Mink

Colin R. Parrish, James A Baker Institute for Animal Health, College of Veterinary Medicine Cornell University, Ithaca, USA

History

The parvoviruses affecting carnivores have a variety of histories. Feline panleukopenia virus (FPV) has been known as an infectious agent of cats since before 1900, and was shown to be a filterable virus during the 1920s. The disease of cats was originally described by a variety of names (e.g. feline distemper, feline infectious enteritis), and during the 1930s several studies showed that those diseases were caused by the same virus. A similar viral disease of raccoons was described during the 1940s, and it appears that the raccoon parvovirus (RPV) is very similar to or identical with FPV. During the 1960s FPV was shown to be the etiologic agent of feline cerebellar ataxia.

Mink enteritis virus (MEV) was first recognized during the late 1940s as the cause of an outbreak of enteric disease amongst mink in Fort William, Ontario, Canada. Initially called Fort William disease, and later mink viral enteritis, the disease was recognized throughout the USA and Europe during

Prevention and Control of Virus Infection

CPV, FPV and MEV

Vaccination efficiently protects animals against infection by CPV, FPV and MEV. Both inactivated virus and modified live virus vaccines are efficacious, although repeated doses are required to give long-lived protection with inactivated virus vaccines. Maternal immunity will prevent successful vaccination of animals. It appears that wild-type virus from the environment can infect animals with maternal antibodies at an earlier time than the same animals can be successfully vaccinated by parenteral routes. The cause of this 'window' of susceptibility to wild-type virus is not understood.

The control of virus spread between animals is difficult as the viruses are long lived in the environment, and also are shed in high titers in the feces. However, the virus may be inactivated with dilute hypochlorite solutions.

AMDV

No vaccine is available against AMDV, as the immune response is inextricably involved in the pathogenesis of the disease. Control measures involve the identification and culling of infected animals through serological testing, and control of reinfection by quarantine.

Future Perspectives

In future studies the basis of the host range and virulence differences between CPV, FPV and MEV will be defined, and the common features defined, at least in part, in terms of changes in the viral structure. The possible ancestors of CPV amongst the other carnivores should be defined by phylogenetic studies. The restricted but persistent replication of AMDV in mink will be examined, and the roles of the specific immune responses in the restricted replication will be defined. The viral and cellular factors that control replication in mink and in tissue culture cells should also be defined.

See also: **Immune response: Cell mediated immune response, General features; Pathogenesis: Animal viruses; Vaccines and immune response; Virus structure: Atomic structure, Principles of virus structure; Parvoviruses (*Parvoviridae*): General features, Molecular biology.**

Further Reading

Agbandje M, Parrish CR and Rossmann MG (1995) The structure of parvoviruses. *Semin. Virol.* 6: 299.

Bloom ME, Kanno H, Mori S and Wolfinbarger JB (1994) Aleutian mink disease: puzzles and paradigms. *Infect. Agents Dis.* 3: 279.

Corsini J, Afanasiev B, Maxwell IH, Carlson JO (1996) Autonomous parvovirus and densovirus gene vectors. *Advances in Virus Research* 47: 303–51.

Jacoby RO, Ball-Goodrich LJ, Besselsen DG, McKisic MD, Riley LK and Smith AL (1996) Rodent parvovirus infections. *Laboratory Animal Science* 46: 370–80.

Miyamoto T, Taura Y, Une S, Yoshitake M, Nakama S, Watanabe S (1995) Immunological responses to polyvalent canine vaccines in dogs. *Journal of Veterinary Medical Science* 57: 347–9.

Parrish CR (1990) Emergence, natural history, and variation of canine, mink, and feline parvoviruses. *AMDV. Virus Res.* 38: 403.

Parrish CR (1995) Pathogenesis of feline panleukopenia virus and canine parvovirus. *Baillières Clin. Haematol.* 8: 57.

Porter DD (1986) Aleutian disease – persistent parvovirus infection in mink with a maximal but ineffective host immune response. *Progr. Med. Virol.* 33: 42.

Rodents, Pigs, Cattle and Waterfowl

Peter Tijssen, Mohamed Laakel, Zoltán Zádori and **Benoît Hébert**, Institut Armand-Frappier, Université du Québec, Laval, Canada

Introduction

Parvoviruses are among the smallest and sturdiest viruses known and the only viruses with linear single-stranded (ss) DNA genomes. Due to their limited coding capacity and inability to induce cellular replication, the infection by parvoviruses requires rapidly dividing cells which provide proteins necessary for the infection. Consequently, their pathogenicity is restricted to permissive cells of that nature such as fetal tissue, epithelium and transformed cells.

History

Rat virus (RV) was first isolated from 1959, when Kilham and Olivier were searching for a rat papovavirus in rat embryo cell cultures followed rapidly by the isolation of H-1 virus from cell filtrates. The minute virus of mice (MVM) was isolated in 1966 by Crawford from laboratory stocks of mouse adenovirus. More recently, MPV was identified during the

mid-1980s due to differences in hemagglutinating properties between MVM and this new agent.

Bovine parvovirus (BPV) was isolated by Abinanti and Warfield in 1961. Originally, this virus was known by its acronym HADEN ('hemadsorbing enteric') which indicated the ability of infected cells to adsorb erythrocytes and the isolation of the virus from gastrointestinal tracts. BPV is endemic and is mainly associated with enteritis and respiratory infections in association with other agents.

Porcine parvovirus (PPV) is a major disease-causing agent in pigs. Although the major syndrome known as SMEDI (stillbirths, mummification, embryonic death and infertility) was described by Dunne and co-workers in the mid-1960s, PPV was isolated and differentiated from the enterovirus agents for the first time in 1975. PPV strains have been isolated from different sources and have also been associated with other syndromes such as respiratory infections, dermatitis, enteritis and rhinitis.

The goose parvovirus (GPV) was first isolated among the waterfowl parvoviruses (WFPs). GPV causes a highly contagious and fatal disease of goslings and muscovy ducklings which emerged in the middle of the 1960s and was known under different names: goose influenza (Hungary), infectious myocarditis (Italy), hepatonephritis ascites (France), viral enteritis (former USSR), goose plaque (The Netherlands) and goose hepatitis (Germany). The working group of the World Poultry Scientific Association named it Derzsy's disease after the late Domokos Derzsy. In 1971, Schettler isolated the causative agent and showed it to be a parvovirus.

Muscovy duck parvovirus (MDPV) and GPV are closely related to each other. MDPV was isolated in France in the late 1980s from an epidemic which was 80% fatal among young muscovy ducks. The third waterfowl parvovirus emerged in Taiwan in 1989 during an epizootic duckling disease. The disease affected all breeds of ducks including their hybrids but geese were spared. The epizootic was diagnosed as a co-outbreak of duck parvovirus (DPV) infection and duck viral hepatitis virus. Inoculating the DPV alone in all duck breeds reproduced the high mortality and symptoms of the disease.

Taxonomy and Classification

All known viruses with a linear ssDNA genome belong to the *Parvoviridae* family which is further divided into two subfamilies: the *Parvovirinae* of vertebrates and the *Densovirinae* of invertebrates. The *Parvovirinae* subfamily comprises three genera: *Parvovirus*, *Erythrovirus* and *Dependovirus*. The three genera in the *Densovirinae* subfamily are: *Densovirus*, *Iteravirus* and *Brevidensovirus*. Phylogenetic analysis of the *Parvovirinae* subfamily reveals different associations which are more biologically relevant in terms of genome organization. For example, while *Parvovirus* members are autonomous, helper viruses can increase their replication substantially. On the other hand, *Dependovirus* members can sometimes replicate autonomously. The parvoviruses discussed here are, in the current taxonomy, all classified in the *Parvovirus* genus. Although the porcine and the rodent parvoviruses are very similar, BPV is quite different, as are the WFPs. The WFPs are genetically closely related to viruses in the *Dependovirus* genus (e.g. AAV) and not to others in the *Parvovirus* genus. Comparison of nonstructural and structural proteins of the WFPs suggests that the AAVs and the WFPs may have evolved from a common ancestral virus.

Properties of the Virion

Parvoviruses are among the most stable viruses. Nonpurified virus has been shown to withstand 2 h at 80°C, although purified viruses are not as stable (e.g. resistant for 1 h at 56°C). Most parvoviruses are also stable within the pH range 2–11. Sonication (without heating) does not affect the virus but it is sensitive to UV irradiation. Resistance to organic solvents, such as butanol and chloroform, and enzymes, such as DNase, RNase and various proteases has been noted. Capsids can also withstand many detergents, although they are dissociated in sodium dodecylsulfate (SDS) solutions. Their compact arrangement of the isometric nonenveloped particles is responsible for these properties.

Parvovirus capsids possess icosahedral symmetry with an approximate external radius of 14 nm and a buoyant density in CsCl of about 1.40 g ml^{-1} (full particles) or 1.30 g ml^{-1} (empty particles). The sedimentation coefficient is approximately 120S for full particles and 60S for empty particles. The capsid contains three to four different proteins (**Table 1**) and a total of 60 protein molecules per virion. The 60 kDa structural proteins found in full, mature particles of BPV, MVM and PPV are proteolytic cleavage products 64–62 kDa proteins. The major structural proteins are produced by a nested set of genes and have identical C-terminal sequences. Although the structural proteins are not glycosylated, they have been shown to be the target for phosphorylation, at least in MVM, and this form appears to be involved in morphogenesis.

The structure of MVM was determined in 1997 whereas structures of empty cpasids of PPV, GPV and MDPV are still being elucidated. The MVM structure

Table 1 Viral gene products (kDa)

Proteins	*MVM, H-I*	*PPV*	*BPV*	*GPV/MDPV*
Nonstructural proteins	NS1 (83)	NS1 (86)	NS1A (83)	REP (65)
	NS2 (21)	NS2 (18)	NS1B (75)	
		NS3 (12)	NS2 (28)	
Capsid proteins	VP1 (86)	VP1 (84)	VP1 (80)	VP1 (81.3)
	VP2 (65)	VP2 (64)	VP2 (72)	VP2 (65)
	VP3 (60)	VP3 (60)	VP3 (62)	VP3 (60)
			VP4 (60)	

Molecular masses of NS2/NS3 of PPV and of GPV REP are deduced from amino acid sequences.

is closely related to the CPV structure and contains an internal antiparallel β-barrel and external threefold spikes. Differences are mostly found on the surface in the twofold depression where a protrusion is located on a dimple-like structure.

Viral Genome and Molecular Biology of Replication

The rodent and porcine parvoviruses encapsidate virtually only the negative-strand (complementary to mRNA) although about 1% of viral DNA has positive polarity. Genome lengths reported for these viruses are 5085/5149 nucleotides (nt) for MVMi/MVMp, 5176 nt for H-1, 5517 nt for BPV, 5106 nt for GPV, 5132 nt for MDPV and 5075/4948 nt for PPV NADL2/Kresse (GenBank accession numbers are listed in **Table 2**). The genomic organizations are almost identical for these viruses. Interestingly, the nonviremic strains MVMp and PPV-NADL2 differ from the viremic strains by a repeated sequence at the 5′ end of the viral genome.

The viral strand is, concomitant with synthesis, excised from the replicative form and encapsidated from the 5′ end in (pro)virions (1.44 g ml^{-1} particles containing only VP1 and VP2). The VP2 are partially converted by proteolysis into VP3 which leads to a decrease in the density to 1.40 g ml^{-1} ('mature' particles). NS1 is still attached to the viral genome to the outside of the capsid and is often removed just before or during infection.

Approximately 10% of BPV virions contain the 5517 nt positive-strand DNA molecule, whereas the remainder contains negative-strand DNA. The genome hairpin termini differ from those of the rodent and porcine viruses in several respects. They lack any sequence homology and are 150 (left) and 121 (right) nucleotides long. The left-hand hairpin (3′ end of negative-strand) has both flip and flop orientations, with a 10-fold excess of flip, whereas the right-hand hairpin contains both orientations in equal amounts.

The WFPs encapsidate the opposite polarity strands in equal amounts. The genome lengths of the GPV and MDPV are 5106 and 5132 nt, respectively, each including long inverted terminal repeats (ITRs) of 444 and 457 nt, respectively. The coding regions of MDPV and GPV do not contain insertions or deletions when compared to each other but differences in length can be attributed to short deletions in the ITRs of GPV and to a 5′ nucleotide deletion in the GPV genome shortly after the first stop codon of the capsid gene.

Table 2 GenBank Accession numbers of parvoviruses of rodents, pigs, cattle and waterfowl

Strain/Isolate	*GenBank Accession number*
MVMi	X02481
MVMp	J02275
MPV-1	U12469
H-1	X01457
RPV-1a	AF036710
PPV NADL-2	L23427
PPV Kresse	U44978
GPV	U25749
MDPV	U22967
BPV	M14363

Accession numbers given are those considered with the least number of errors.

Transcription and Translation

Porcine and rodent parvoviruses have two genes, each coding for several products (**Table 1**). Their genomic organizations are almost identical. The genes are transcribed from two promoters (P4 and P38 for MVM; P4 and P40 for PPV) and the transcripts coterminate near the 5′ end of the negative-strand. The P4 transcripts code for the nonstructural proteins (left half of the transcripts) and P38/P40 codes for the structural proteins. A peculiarity of the transcripts originating from P4 is that both the nonspliced and the spliced transcripts are translated (NS1 and NS2/NS3, respectively). Whereas MVM has different

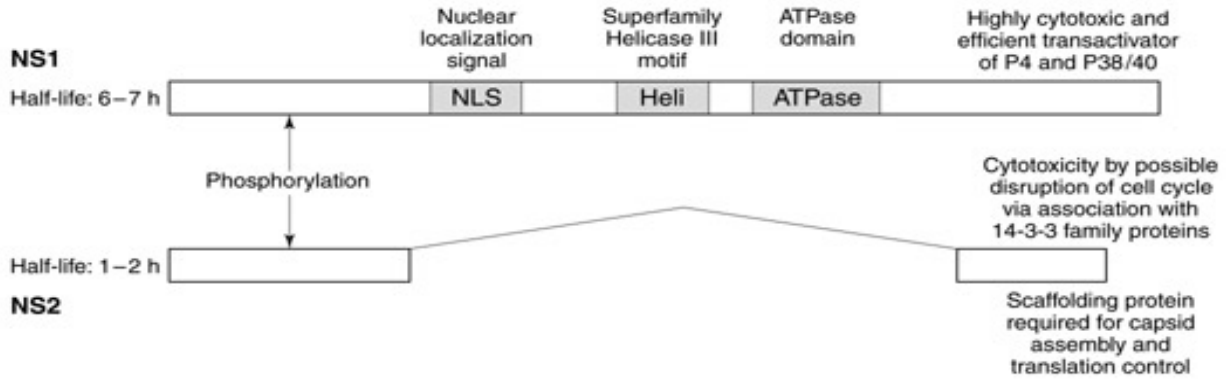


Figure 1 Properties and roles of NS proteins.

forms of NS2, we observed a third transcript of a potential nonstructural protein for PPV, NS3, which has the same N-terminal domain as NS1 and NS2 but the reading frame of the C-terminal domain overlaps the open reading frames (ORFs) of the structural proteins. The role of this protein is unknown. The N-terminal sequences of NS1 and NS2/NS3 are identical whereas the C-terminal sequences differ (overlapping ORFs). The intron removed to generate the NS2/NS3 transcript contains a nucleoside triphosphatase (NTPase) (probably ATPase), a *trans*-activation response element, which upregulates expression from P4, and the P38/P40 promoter elements (**Fig. 1**).

Splicing in parvoviruses is governed often using nonconsensus donor or acceptor sequences. The relative abundance of the spliced transcript (steady-state ratio) has been shown, for MVM, to be dependent on sequences located within the large and small introns. Sequences within the NS2-specific exon itself are required for its inclusion in the mature transcript. As described for MVM, the efficient excision of the upstream large intron (within NS1) from P4-generated mRNAs depends on at least the initial presence of sequences within the downstream small intron.

The start codon for VP1 of the various rodent and porcine parvoviruses is located almost immediately after the stop codons of NS1/NS2. There are small introns at about 40 map units in all P4 transcripts. The donor sites for these small introns are just upstream or just downstream of the VP1 start codon. When the upstream site is used, the start codon is removed and another start codon, almost 800 nt downstream, is used resulting in VP2 (**Fig. 2**).

The strategy used by BPV is different: for example, there are three major ORFs (left, mid and right) instead of two. Analysis of the genome suggests three promoters, P4, P13 and P38. Again, P38 is responsible for the production of capsid proteins. Unlike the rodent and porcine parvoviruses, BPV codes for two large proteins and two smaller proteins. BPV shares

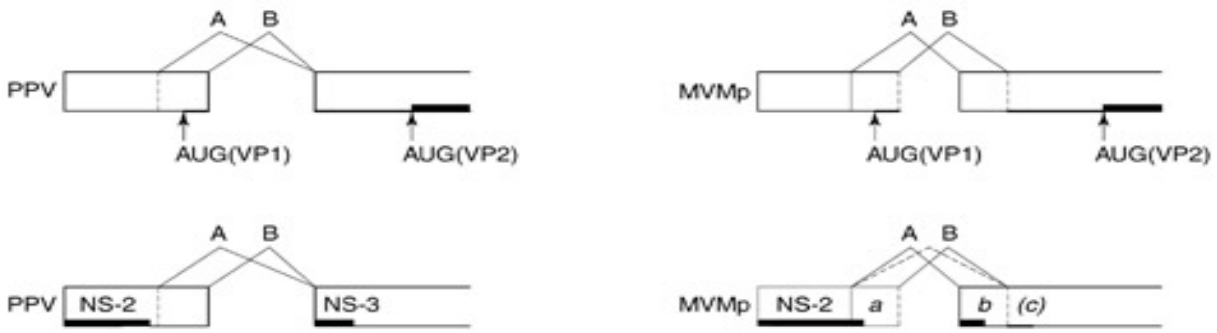


Figure 2 Comparative alternate splicing of NS transcripts in MVM and PPV. MVM has two splicing acceptor sites in the capsid gene ORF, but PPV has only one. Nonetheless, the expression strategy remains unaffected by the divergence in splicing patterns. The strategy of NS2 expression differs considerably between MVM and PPV. The MVM genome is organized to yield a maximum of three different NS2 which differ solely at their C-terminal. Normally, only two forms are detected. Although another (fourth) combination would be possible, it is undetectable due to distance constraints of the splicing machinery. In contrast, PPV NS2 terminates upstream from the VP splicing sites and a separate NS3 is generated by splicing the NS2 donor site to the VP acceptor site. (Adapted from Tijssen P, Bergeron J, Dubuc R and Hébert B *Seminars in Virology* 6(5): 321.)

with all autonomous parvoviruses a glycine-rich region (a stretch of 10–20 G) near the proteolytic cleavage site to generate the smallest capsid protein. This G-run could distort the α-helix structure and make it susceptible to proteolysis. Three BPV NS proteins have been detected (**Table 1**). The 83 and 75 kDa proteins share with NS1 of PPV and rodent parvoviruses the property that they are expressed before the capsid proteins [8 vs 14 h postinfection (p.i.)]. The NS1A and NS1B proteins of BPV may differ in the degree of phosphorylation, although both products can be detected after *in vitro* translation (suggesting primary translation products).

Gel-retardation assays demonstrated that both capsid and NS proteins bind to the left end of the BPV genome and may play different roles in replication and packaging. The right end of the genome does not compete with the left end for these proteins. Cellular proteins, particularly during S-phase, also form a specific complex with the DNA.

Recent work on the genetic analysis of WFP has demonstrated an organization similar to that of the AAVs and, similarly, three promoters, P9, P19 and P41 have been identified. Again, P41 is responsible for the production of capsid proteins whereas P9 and P19 are the transcriptional activators for the REP protein. It is noteworthy that whereas VP1 and VP3 are initiated from conventional AUG codons, VP2 is initiated from an ACG codon. Alternative splicing regulates the synthesis of the various capsid proteins as VP1 is bracketed between two alternative acceptor splicing sites. VP1 is synthesized when the upstream acceptor is used whereas VP2 and VP3 are made when splicing occurs at the downstream acceptor site.

Viral Morphogenesis

Parvoviruses lack the ability of most other DNA viruses to stimulate resting cells to DNA synthesis. The first steps during infection, i.e. absorption, penetration and uncoating in the cell, do not require viral functions but normal cellular processes. Mature (containing VP1, 2 and 3) and immature particles (VP1 and 2) compete equally well for receptors. Internalization through coated pits has been observed. It has been postulated that the virus is transported actively to the nucleus by the signal carried on the first ten amino acids of VP1. Uncoated DNA is not observed in the cytoplasm. The S-phase-dependent event appears to be the synthesis of the complementary strand from the 3′ end of the negative-strand. The resulting duplex can then serve as a template for transcription and expression. It should be noted that for MVMi and MVMp, in A9 cells, the steps up to transcription are equally efficient but that transcription and translation differ significantly. Transcription thus depends on developmentally regulated host-cell factors and is not merely a reflection of the presence of duplex DNA. An attenuation site located ~145 nucleotides downstream from the P4 promoter has been identified. This block could be alleviated by specific cellular factors. Moreover, the promoter for the capsid proteins is activated by NS1.

Parvoviruses interact with host nucleoli. Viral DNA replicates in the host nucleoli and nucleolar protein as well as small nuclear ribonucleoproteins are recompartmentalized. Replicative form DNA is exclusively nuclear-matrix bound early during infection but accumulates later in the soluble fraction. The DNA–matrix interactions are, at least in part mediated by the viral terminal proteins. ssDNA is encapsidated by the (pro)virion, as described above, usually from the 5′ to 3′ direction. This process is probably mediated by NS1 and the virus is ready for a new round of infection after the proteolytic maturation step.

Geographic and Seasonal Distribution

All parvoviruses described here have been isolated worldwide, wherever the host is present. However, some strains may have a wider distribution than others. For example, PPV strains involved in the SMEDI syndrome have been detected throughout the world, whereas strains causing vesicular lesions or enteritis have only been reported sporadically. Interactions of PPV with other porcine viruses are still poorly understood.

It is not known whether similar differences exist for the rodent parvoviruses. Early work showed that many strains differ in serology or hemagglutinating properties from the laboratory strains. A serologic survey from 10 European countries indicated that MVM was among the most prevalent murine viruses in colonies surveyed in the mid- to late 1980s. It has been circulating in mouse colonies for at least 20 years. Retrospective testing indicated that the prevalence of MPV approached 40% in the early 1970s whereas only 7% of tested sera contained anti-MVM antibodies. Similarly, several BPV strains have been compared and serology and hemagglutinating differences have been noted. Caution is warranted since comparisons are often not under identical or standardized conditions. For example, different donors of erythrocytes could be responsible for subtle variations.

Seasonal distributions are related to the age distribution of the host if infection occurs at a certain age. For example, GPV usually infects goslings younger than 1 month, although latent infections

can be established in older animals. Thus, domesticated or wild geese are usually infected in the spring. Similarly, BPV infects calves but latent infections may develop in older animals. The birthing pattern of the host thus contributes to the seasonality of the infections.

Host Range and Viral Propagation

In general, parvoviruses have a narrow host range, probably due to their high dependence on particular host cell functions. Although parvoviruses accumulate mutations at a very slow rate, it has nonetheless been extensively documented that small genomic variations or mutations may lead to phenotypic differences in antigenicity, tissue tropism, host range, virulence or properties such as hemagglutination. It is unknown if these mutations are random or if they appear due to environmental pressures.

The natural host of RV and MVM are rats and mice, respectively, as supported by serology and infection studies. All rodent viruses can induce an osteolytic syndrome after perinatal inoculation of hamsters. This syndrome is characterized by dwarfism, mongoloid features, abnormal teeth and fragile bones because of infection of osteogenic tissues.

About 40 PPV isolates have been obtained from different cell cultures (KBSH from KB cells is the prototype) and it has been suggested that these viruses were introduced with porcine trypsin used in passaging. Field isolates of PPV cluster within four pathogenic groups but, when the region qualified as the allotropic determinant is sequenced, two major groups can be identified. This pattern mainly segregates pathogenic from nonpathogenic isolates whereas other noncharacterized residues or noncoding changes may account for the observed pathologies. Sequencing of the capsid protein gene of isolates originating from Europe and Canada indicate that variations in residues outside the allotropic determinant vary geographically.

Cattle are the natural reservoir of BPV, but goats and possibly horses, may also be a source since many animals are seropositive. Crossreactivity with until now unisolated viruses cannot be ruled out. Canine, monkey and human sera may also be seropositive, by immunofluorescence, but very low titers are obtained when they are assayed for neutralizing activity. Some crossreactivity therefore seems more likely. Domesticated and wild geese as well as Muscovy ducks are susceptible to GPV. Chickens and ducks are resistant.

Parvoviruses are most often propagated in cell culture. GPV is usually replicated in embryonated eggs. The reproduction of parvoviruses in tissue cultures can be dependent on the passage number, temperature or the nature of the strain of the same parvovirus. For instance, lymphotropic and fibrotropic strains have been described for MVM, and restricted temperature ranges for different PPV strains (some only at low temperatures, others at 37°C and still others at 39°C). PPV-NADL2 replicates most efficiently in PFT cells at passage 75–100 and hardly, or not at all, above 200.

Genetics and Evolution

Rodent and porcine parvoviruses are so closely related that they have probably evolved from a common ancestor. Their relatedness with BPV, *Dependovirus* or *Densovirus* is low and decreases in this order, with one noteworthy exception. The amino acid sequence 10–50 of the VP1 capsid protein is also found in the structural protein of many densoviruses (about 90% homology).

Evolution of parvoviruses is evident even in the short time span in which they have been studied. For example, canine parvoviruses evolved, most probably indirectly, from feline parvoviruses. Many of the newly discovered strains with different tropisms may have evolved very recently since only a few (two to three) amino acids are involved in the tropic determinant. It can be speculated that, after a change to a new tropism through one of these amino acids, other mutations may rapidly accumulate until the optimal reproduction rate is obtained.

Parvoviruses have been sequenced to predict the way the genome functions. Subsequently, these predictions were confirmed or rejected by transcription studies and by site-directed mutagenesis of genetic elements (see below). A virulence determinant is also emerging. NS2 nonstructural protein and repetitive elements (at the 5′ end of the negative-strand) seem to be involved. For PPV, we have isolated strains with one, two or four repetitive elements (from mummified embryos almost all have one repetitive element; unpublished results).

Transmission and Epidemiology

Parvoviruses can be transmitted horizontally by the fecal–oral route, respiratory exudates, dust, gloves, clothing and food, provided that the susceptibility of the host and the dosage of the virus are sufficiently high. They can also be transmitted vertically. Toolan noted two types of infection, one that is temporary and produces low levels of antibody which disappear within months and a second type of infection which is latent or permanent leading to high antibody titers, maintained throughout life. The latter type is exemplified by neonatal and latent infections. Similar

patterns can be observed for all parvoviruses discussed in this entry. PPV- or BPV-seronegative herds are sometimes found. In most herds, most animals are seropositive but some asymptomatic long-term virus excretors may be present, mostly because of continuous replication in susceptible gut cells.

Horizontal transmission is most common but vertical transmission has been shown for BPV, GPV and PPV. Vertical transmission of the virus leads to the abortion or resorption of the fetus. A few long-term PPV excretors seem to be less efficient in horizontal transmission in age-segregated herds. However, the resistance of parvoviruses to environmental inactivation is believed to be more important than chronic carriers in the epidemiology by horizontal transmission.

The morbidity and mortality rates depend on the susceptibility of the host and the immune status of the herds. Viral infections can range from completely symptomless to mortality rates that, for GPV, may be close to 100%. Again, the strain of the parvovirus is important.

Tissue Tropism and Pathogenicity

The replication of parvoviruses depends on cellular functions, transiently expressed during late S or early G2 phase of mitosis. Thus, cell division is an essential requirement and parvoviruses infect mitotically active tissues such as those from the fetus, intestinal epithelium and hemopoietic systems. The viruses are considered 'mitolytic', hence their oncosuppressive activities. Additional cellular factors, expressed during differentiation, are also required. Cell division is not the only determinant, and parvoviruses are therefore not truly pantropic. A tropic determinant has been identified in the capsid protein of MVM and in the corresponding region of PPV structural proteins.

The complex set of factors involved in viral pathogenicity is still poorly defined. At a molecular level, much of the current attention is focused on the (until now unknown) role of the viral-encoded NS2 protein and noncoding genetic elements. Both seem to be involved in pathogenesis or virulence. It is not clear yet whether tropism factors can be completely separated from virulence factors. Curiously, RV, H-1 and MVMp are not pathogenic for their native hosts. Only under experimental conditions, such as intracerebral inoculation of rats with virulent RV, does a disease become apparent. RV and H-1 when isolated, were associated with tumors, and MVM has been isolated from oncogenic adenovirus stocks. All rodent parvoviruses have been shown to suppress carcinogenesis, whether spontaneous, virus-induced or carcinogen-induced. In contrast, MVMi, most PPV strains, BPV and GPV are pathogenic. Mixed infections with other pathogens are common and a role for these parvoviruses in certain diseases is emerging.

There is a striking resemblance between MVM and PPV with respect to tropism and pathogenesis. The nonpathogenic PPV-NADL2 and MVMp are nonviremic whereas the pathogenic PPV-NADL8, PPV-Kresse and MVMi are viremic (i.e. high virus titers in blood circulation).

PPV infections are often harmless to the adult animals except for some PPV strains associated with severe dermatitis or enteritis. Nonviremic strains are considered harmless unless inoculated *in utero* prior to immunocompetence where they will cause abortions. Some PPV strains cause embryonic and fetal infections of various degrees through postviremic transplacental infection during the first 70 days of gestation usually without any manifestation of maternal clinic signs since the adult mounts an immune response but the maternal antibodies do not cross the placenta to protect the fetuses. The infection or death of fetuses after immunocompetence is attained around 70 days and is only observed in highly pathogenic isolates such as Kresse and IAF-A54 which are usually associated with necrotic skin lesions in the dams.

The generalized infection of hematopoietic cells, lymphocytes and capillary endothelium leads to bilateral infarcts of the solitary renal papilli for MVMi and fetal infection for PPV. It needs hardly to be emphasized that laboratory diagnosis should distinguish the strains for reliable epidemiological surveys and herd management, instead of mere detection of virus.

Most BPV isolations are from samples from calves with diarrhea, but BPV is increasingly associated with respiratory disease and reproductive failure. Viremia can be established (in leukocytes), and, during the symptomatic phase, different targets become infected (intestinal tissue, brain, heart muscle, adrenal gland, thymus and lymph nodes). Fetal infection after viremia is particularly acute in the first trimester (particularly cerebellum). Active immunity develops in the second half of pregnancy, and a fetus infected in the third trimester usually recovers from the infection.

According to available *in vitro* data, cell tropism relates to the strain properties (capsid amino acids). Different tropisms among variant strains are also a striking feature of other parvoviruses. An example is the sudden appearance of canine parvovirus in the late 1970s probably due to mutations in a feline parvovirus (*See also:* **Parvoviruses (Parvoviridae): Cats, Dogs and Mink**). This flux in parvovirus strains and

properties makes them very important, both in disease prevention and fundamental research.

Clinical Features and Pathology

Except for MVMi, natural infections of rodents are clinically inapparent. Experimental infections of rodents may produce (1) an acute lethal disease in newborn animals, (2) an osteolytic syndrome, (3) cerebellar ataxia or (4) hemorrhagic encephalitis. Successive passaging of H-1 or RV in newborn hamsters can increase the pathogenicity in newborn animals to close to 100% mortality. After 4–10 days, infected animals suddenly become sluggish, gasp for breath and die. Necropsy often reveals hemorrhage of the gut and congestion of the liver. In contrast, no liver damage is observed with the RT strain, but intestines often contain a sanguineous exudate.

Infected newborn rats become apathic just before dying. Intranuclear inclusions are found in cells of almost all organs of the animals.

Hamsters surviving RV or H-1 infections usually develop mongoloid features due to osteolytic activity of the parvoviruses [in rodents, osteogenic activity (osteoblasts, odontoblasts) is high]. Cerebellar ataxia can only be introduced by intracerebral inoculation of hamsters younger than 4 days (and is apparent after a month). The HER agent induces hemorrhagic encephalitis in rats after cyclophosphamide treatment. Upon necropsy, hemorrhage and necrosis are observed in brains or spinal cords of these rats.

The most common feature of an outbreak of PPV reproduction failure is the appearance of mummified fetuses. The litter may contain both mummified and stillborn fetuses. The PPV strain associated with equine abortion has not been reproduced experimentally. Interestingly, this virus also seems to be fetotropic in rabbits. Clinical features of BPV infections, mostly in calves (1–12 months), include enteritis, respiratory disease and conjunctivitis. The severity of the clinical symptoms is usually increased by coinfection with other pathogens and may only be apparent in their presence.

GPV-infected goslings or Muskovy ducklings (at day 1) stop eating after about 3 days, are reluctant to move, remain near a heat source and usually die after a week. Those that survive are usually severely retarded and may become featherless. These animals may also develop a transient leg weakness. When older animals are infected, a symptomless carrier state may be obtained. Birds that succumb in the acute phase after GPV infection have characteristic lesions in the liver and heart muscle. The hyperemic liver contains small grayish-white areas. The apex of the enlarged heart can be rounded off and the myocardium may show a discoloration. Accumulation of fluid in the pericardium and abdominal cavity is often observed.

Immune Response and Prevention

A strong active immunity depends to a large degree on viral multiplication in tissues of the infected organism. The equilibrium between virus produced during latency and the production of circulating antibodies can lead to high antibody titers over long periods. A single injection with cyclophosphamide (immunosuppressive) was shown to be sufficient to convert a latent RV infection into an apparent disease. Toolan observed that infection of pregnant hamsters with H-1 did not yield any antibodies, neither did subsequent inoculations. The reason for this tolerance is not clear.

Passive immunity (maternally acquired antibodies) may protect the fetus against infection in rodents. However, immunoglobulins do not pass through the multilayer placenta (epitheliochorial) in pigs. Production of antibodies in neonatal piglets is the result of active immunity (embryos are immunocompetent from about 70 days on). After birth, pigs can contain high concentrations of PPV antibodies from colostrum while nursing seropositive dams. A similar pattern is observed for BPV; second- and third-trimester fetuses yield an immunoglobulin (Ig)M response that is maximum after about 10 days and is gradually replaced by IgG with maximum titers about 5 months after infection. Calves deprived of colostrum from seropositive cows may develop severe diarrhea upon infection.

Viral strains that do not produce viremia can often be used as live vaccines. The NADL2 strain of PPV, but also others such as the HT strain of PPV (no viremia), can be used. Some workers have been able to induce high antibody titers to PPV with inactivated virus. An early management method for PPV was the back-feeding of fecal material or fetal tissues from PPV-infected sows. Infections with BPV are widespread and are clinically inapparent in adults. BPV vaccines are not yet available nor has the need for vaccination been demonstrated.

Conclusion

Initially, parvoviruses attracted attention since it was felt that these small viruses would be simple and easier to understand than most other viruses. It has become clear, however, that they depend more on cellular functions which are more difficult to study.

Recent knowledge about parvovirus structures has enabled researchers to focus on the role of structurally conserved motifs during infection and their implication in tropism and host range. The extent of knowledge on MVM made it an excellent model especially since more is known about the genetic make-up of the mouse than other vertebrates. Other parvoviruses generate increasing interest due to their role in disease and as models in host–virus relationship and structure–function studies.

See also: **Parvoviruses (*Parvoviridae*): Cats, dogs and mink, General features, Molecular biology.**

Further Reading

Berns K (1990) Parvovirus replication. *Microbiol. Rev.* 54: 316.

Cotmore S and Tattersall P (1987) The autonomously replicating parvoviruses of vertebrates. *Adv. Virus Res.* 33: 91.

Pattison JR (1996) Parvoviruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, Chapter 63. Philadelphia: Lippincott-Raven.

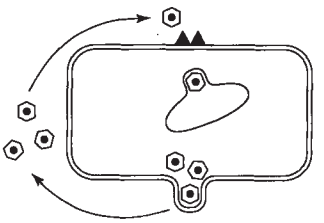
Siegl G (1976) *The Parvoviruses*. Vienna: Springer-Verlag.

Tijssen P (ed.) (1990) *Handbook of Parvoviruses*, vols I and II. Boca Raton, FL: CRC Press.

Parrish, CR (ed.) (1995) Autonomous animal parvoviruses. *Semin. Virol.* 6: (5)

PATHOGENESIS

Contents



Animal Viruses

Kenneth L Tyler, Neurology, University of Colorado Health Sciences Center, Denver, Colorado, USA

Introduction

The term pathogenesis is derived from two Greek words which can be translated to mean, 'the origin of disease'. The study of viral pathogenesis thus concerns itself with understanding the process by which a virus produces disease in the host. It is important to recognize that a virus can enter and replicate in a host, and even induce an immunologic response, without producing overt signs or symptoms of disease. For many viruses, the majority of infections, under normal circumstances, are asymptomatic. Infection may be of short duration and self-limited (acute) or may be long-term (chronic) or even persist for the life of the host. For many viruses, such as influenza, acute infection is followed by viral clearance and subsequent lifelong immunity against reinfection by the same viral strain. In other cases, exemplified by infection with herpes simplex virus, acute infection is followed by persistence of virus in a noninfectious latent state from which there is periodic reactivation and shedding. A third pattern involves acute infection followed by continuous shedding of virus from infected tissues. By definition, in such cases the host immune response fails to completely clear virus. This may result from strategies which allow viruses to successfully evade host immune responses including restricted expression of viral genes, limitation of infection to immunologically privileged sites, rapid evolution of viral antigenic variants, or virus-induced suppression of or interference with host cellular, humoral, or cytokine-mediated immunological defenses.

In the classic model of acute virus infection, injury to cells occurs as a direct result of the replication and release of viral particles. However, it has become increasingly recognized that viruses may also produce disease through a variety of other mechanisms. They can promote the induction of neoplasia (oncogenesis), suppress the immune system and even alter specific cellular functions without killing the target cell.

Viral pathogenesis can be analyzed in terms of a series of interactions between the virus and the host. Although the specific steps in this process may differ for individual viruses and particular hosts, the general outline remains true for most cases. A virus must survive in the environment, enter a susceptible host, multiply to increase its inoculum, and spread from the site of entry to target tissues, where it produces disease as the result of infection and injury to particular organs or populations of cells. Finally, virus must be shed into the environment and transmitted to

Recent knowledge about parvovirus structures has enabled researchers to focus on the role of structurally conserved motifs during infection and their implication in tropism and host range. The extent of knowledge on MVM made it an excellent model especially since more is known about the genetic make-up of the mouse than other vertebrates. Other parvoviruses generate increasing interest due to their role in disease and as models in host–virus relationship and structure–function studies.

See also: **Parvoviruses (*Parvoviridae*): Cats, dogs and mink, General features, Molecular biology.**

Further Reading

Berns K (1990) Parvovirus replication. *Microbiol. Rev.* 54: 316.

Cotmore S and Tattersall P (1987) The autonomously replicating parvoviruses of vertebrates. *Adv. Virus Res.* 33: 91.

Pattison JR (1996) Parvoviruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, Chapter 63. Philadelphia: Lippincott-Raven.

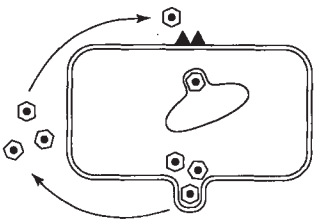
Siegl G (1976) *The Parvoviruses*. Vienna: Springer-Verlag.

Tijssen P (ed.) (1990) *Handbook of Parvoviruses*, vols I and II. Boca Raton, FL: CRC Press.

Parrish, CR (ed.) (1995) Autonomous animal parvoviruses. *Semin. Virol.* 6: (5)

PATHOGENESIS

Contents



Animal Viruses

Kenneth L Tyler, Neurology, University of Colorado Health Sciences Center, Denver, Colorado, USA

Introduction

The term pathogenesis is derived from two Greek words which can be translated to mean, 'the origin of disease'. The study of viral pathogenesis thus concerns itself with understanding the process by which a virus produces disease in the host. It is important to recognize that a virus can enter and replicate in a host, and even induce an immunologic response, without producing overt signs or symptoms of disease. For many viruses, the majority of infections, under normal circumstances, are asymptomatic. Infection may be of short duration and self-limited (acute) or may be long-term (chronic) or even persist for the life of the host. For many viruses, such as influenza, acute infection is followed by viral clearance and subsequent lifelong immunity against reinfection by the same viral strain. In other cases, exemplified by infection with herpes simplex virus, acute infection is followed by persistence of virus in a noninfectious latent state from which there is periodic reactivation and shedding. A third pattern involves acute infection followed by continuous shedding of virus from infected tissues. By definition, in such cases the host immune response fails to completely clear virus. This may result from strategies which allow viruses to successfully evade host immune responses including restricted expression of viral genes, limitation of infection to immunologically privileged sites, rapid evolution of viral antigenic variants, or virus-induced suppression of or interference with host cellular, humoral, or cytokine-mediated immunological defenses.

In the classic model of acute virus infection, injury to cells occurs as a direct result of the replication and release of viral particles. However, it has become increasingly recognized that viruses may also produce disease through a variety of other mechanisms. They can promote the induction of neoplasia (oncogenesis), suppress the immune system and even alter specific cellular functions without killing the target cell.

Viral pathogenesis can be analyzed in terms of a series of interactions between the virus and the host. Although the specific steps in this process may differ for individual viruses and particular hosts, the general outline remains true for most cases. A virus must survive in the environment, enter a susceptible host, multiply to increase its inoculum, and spread from the site of entry to target tissues, where it produces disease as the result of infection and injury to particular organs or populations of cells. Finally, virus must be shed into the environment and transmitted to

a new susceptible host to begin its life cycle anew. Each of these stages is considered separately.

Entry

The most common routes of viral entry are through the skin, respiratory tract, gastrointestinal tract, urogenital tract and conjunctiva.

Skin

Under normal circumstances the skin poses an effective barrier to the entry of viral pathogens. The dead keratinized cells of the outer skin layer (stratum corneum) do not support viral replication. Infection can be initiated when a virus enters the host through breeches in skin contiguity such as cuts, abrasions or wounds. Viruses can also be mechanically transported across the stratum corneum by an insect or animal bite or with human-made implements such as hypodermic needles. The layer of the epidermis below the stratum corneum (stratum Malpighii) contains living cells but is essentially devoid of blood vessels, lymphatics and nerves. Viruses that enter this layer, such as the papillomaviruses, typically induce local pathology (e.g. dermal warts), but only rarely disseminate to produce systemic disease. In the case of papillomaviruses, infection occurs initially in basal cells adjacent to the basement membrane separating the overlying epidermis from the deeper dermal layer. Virus maturation parallels keratinocyte differentiation and proceeds as the cells migrate from the deep epidermal layers toward the stratum corneum. Virion assembly occurs predominantly in keratinocytes in the upper spinous layers of the epidermis and mature virions are found in cells in the granular layers and are shed when the stratum corneum is sloughed.

Deep inoculation through the epidermis may introduce virus into the dermis, with its luxurious supply of vessels, lymphatics and nerves, or even into the underlying subcutaneous tissue and muscle. These tissues often provide fertile ground for viral multiplication and subsequent dissemination. Infection of the dermis may occur when virus is transmitted by a deep bite, as occurs with rabies virus.

The host's dermal barrier is not impervious to invasion. It is penetrated by the openings of the respiratory, alimentary and genitourinary tracts, and modified in areas such as the conjunctiva of the eye.

Respiratory tract

Some viruses that initiate infection via the respiratory tract replicate almost exclusively within the cells of the mucosal surface (e.g. influenza, parainfluenza), whereas others spread systemically after an initial phase of replication in respiratory mucosa (e.g. mumps, measles). Entry of both groups of viruses is typically initiated by means of infected droplet aerosols generated by coughing or sneezing, by nose to hand to nose transmission of infected nasal secretions or by exchange of infected saliva through kissing or sharing of drinking glasses, toothbrushes or other utensils. The fate of inhaled viral droplets is influenced largely by physicochemical factors including particle size, temperature and humidity. Large particles are generally trapped in the nasal turbinates and sinuses, and may initiate upper respiratory infections. Particles smaller than 5 μm can reach the alveolar airspaces to produce lower respiratory infection (pneumonia).

Host defenses against the initiation of viral infection through the respiratory tract are complex. Filtration by the nose reduces the number of particles in certain size categories. Mucociliary clearance further impedes particle entry. Mucus is secreted by goblet cells, and propelled upward in the oropharynx by the coordinated beating of ciliated epithelial cells. Viruses trapped in mucus are efficiently propelled out of the respiratory tract. Viral infection may be facilitated by factors that compromise mucociliary clearance including cigarette smoking, atmospheric pollutants, in-dwelling tubes or certain inherited disorders.

The immune system of the respiratory tract has not been as extensively studied as that of the gastrointestinal tract (see below). The respiratory immune system includes bronchus-associated lymphoid tissue (BALT) and the lymphoid tissue of the tonsils and adenoids. IgA is present in the upper respiratory tract, and its deficiency may increase the risk of developing upper respiratory infections. IgG that is derived from the systemic circulation contributes to antiviral defenses in the lower respiratory tract. Cellular immunity is mediated by intraepithelial lymphocytes and resident alveolar and bronchial macrophages.

Gastrointestinal tract

Viruses that infect the host through the gastrointestinal (GI) tract must be able to survive the acidity of the stomach, the bile salts and proteolytic enzymes of the small intestine, and the mucus coating of the gut. They must also avoid inactivation by secretory IgA and the action of lymphoid cells and macrophages.

Enteric viruses can induce either symptomatic or asymptomatic disease. One determinant of this outcome is the degree of enterocyte injury. Viruses which fail to induce significant gastrointestinal symptoms may still spread to produce systemic infections, as exemplified by poliovirus. Despite the absence of local disease many enteric viruses are shed in high titer and for prolonged periods in feces. The target cells for

enteric viruses include the mature enterocytes of the intestinal villi and the immature dividing cells in the villus crypts. Some viruses including reoviruses, rotaviruses, picornaviruses, retroviruses, astroviruses and toroviruses can also infect M cells, which are specialized epithelial cells overlying collections of small intestinal lymphoid tissue (Peyer's patches). Although most enteric viruses initiate infection in the small intestine there are exceptions to this rule. For example, human immunodeficiency virus (HIV), introduced from infected semen during anal intercourse, may infect dendritic and epithelial cells of the colon and rectum. Rectal and large intestinal infection may also occur in immunocompromised individuals infected with cytomegalovirus (CMV) and herpes simplex virus (HSV). These viruses can also infect the upper gastrointestinal tract producing esophagitis.

Local gastrointestinal symptoms occur when viral infection involves villus epithelial or crypt cells. Destruction of the villus enterocytes results in malabsorption, and the resulting intraluminal osmotic load can induce diarrhea. Infection of the crypt cells, which develop into absorptive cells, ultimately results in loss of absorptive capacity and leads to diarrhea. Another mechanism of diarrhea production may be through the action of viral proteins that act as secretory toxins. For example, the rotavirus NSP4 protein has toxin-like actions that stimulate enterocyte hypersecretion and subsequent diarrhea. Another important consequence of enterocyte infection is the shedding of large amounts of virus in the stool. This fecal shedding is the predominant source of transmission of many enteric viruses with new cycles of infection being initiated by fecal–oral spread.

The first barrier faced by viruses entering the upper alimentary tract is the acidity of the stomach. Viruses must be extremely acid stable to survive this environment. The differing effects of acidity on virions are dramatically illustrated by different species of picornaviruses. For example, rhinoviruses are acid labile and lose infectivity at low pH. Under acidic conditions the viral outer capsid is disrupted, and viral RNA escapes, leaving noninfectious empty capsids. As expected, rhinoviruses do not produce enteric infections. By contrast, other members of the picornavirus family including polio- and coxsackieviruses are resistant to degradation under acidic conditions. They are extremely successful at initiating enteric infections, a fact recognized by their inclusion in the Enterovirus functional group.

Viruses that initiate infection through the enteric route must also resist inactivation by the proteolytic enzymes secreted by gastric and pancreatic cells. In fact, proteolytic digestion actually increases infectivity of several enteric viruses. For example, partial cleavage of the rotavirus spike protein, VP4, by intestinal proteases enhances the direct penetration of virus into enterocytes. In the case of reoviruses, proteolytic cleavage of outer capsid proteins results in the production of infectious subviral particles (ISVPs). ISVPs appear to play a critical role in the initiation of subsequent intestinal infection (see below). Thus, as a general principle, viruses that produce or initiate infection in the intestinal tract are not inactivated by intestinal proteolytic enzymes. In fact, quite the converse appears to be true, as in many cases proteolytic processing of viral capsid proteins seems to trigger conformational changes in the virus particle or expose new functional determinants on specific proteins which in turn facilitate specific events (receptor binding, membrane fusion, cell entry, transcriptional activation) in the viral life cycle.

Bile salts are another important factor in inhibiting viral entry through the intestinal tract. Viral envelopes are particularly susceptible to digestion by bile salts. Before direct ultrastructural visualization of virions was routinely possible, the capacity of bile salts to destroy viral infectivity was taken as prima facie evidence for the presence of a lipid envelope. With the solitary exception of coronaviruses, viruses that initiate infection through the intestinal tract are all nonenveloped.

The enteric immune system provides another important host defense against viral infection. Enteric viral infection typically induces rapid production of intestinal antibody, composed predominantly of secretory IgA. Enteric viruses also induce specific cytotoxic T cells (CTLs) and helper T (Th) cells. These cells form part of the gut associated lymphoid tissue (GALT) system which includes regional collections of lymphoid tissue in the small intestine (Peyer's patches). Cellular immunity (e.g. CD4+ and CD8+ T cells) plays a key role in clearing enteric infection.

The cellular events that underlie the initiation of systemic infection by enteric viruses are becoming better understood. Reoviruses and polioviruses initially bind to the luminal surface of specialized epithelial cells (M cells) which overlie regional aggregates of intestinal lymphoid tissue (Peyer's patches). Virions are then transported in vesicles across the M cell cytoplasm and discharged into the subepithelial lymphoid tissue where primary replication can occur in lymphoid cells and macrophages.

Genitourinary system

A number of human viruses including HIV, herpes simplex and papillomaviruses are venereally transmitted. Small tears or abrasions in the epithelial lining of the rectum, urethra or vagina may occur during

sexual activity and permit entry of virus. Host factors that inhibit viral entry through these routes include cervical mucus, the pH of vaginal secretions, the chemical composition and cleansing action of urine and the presence of secretory IgA.

Conjunctiva

Although viral conjunctivitis is common, and occurs either as an isolated illness or in association with certain systemic infections (e.g. measles), the conjunctiva only rarely serves as a site of entry of viruses into the host. Local infection may be initiated by direct inoculation of virus following ophthalmologic procedures (tonometry, foreign body removal) or in the process of swimming ('swimming pool conjunctivitis'). The offending viruses include adenoviruses and enteroviruses. Most such infections remain localized. Enterovirus 70 (E70) appears to be an important exception to this rule. E70 commonly produces acute hemorrhagic conjunctivitis, and on extremely rare occasions (perhaps 1 in 10 000 cases) spreads from the eye to the nervous system to produce cranial nerve palsies, myelitis or encephalitis.

Spread in the Host

For viruses that produce localized infections, the major steps in pathogenesis are entry into the host and subsequent primary replication in cells and tissues in proximity to the site of infection. In many local infections, virus spread is predominantly by cell-to-cell spread in a contiguous fashion. The brunt of injury is confined to the epithelial layer, although local lymphoid tissues may also be involved. This type of circumscribed infection is typical of uncomplicated upper respiratory diseases caused by coronaviruses, rhinoviruses and influenza, and the acute diarrheal disease induced by rotaviruses. More generalized symptoms (fever, chills, myalgia, malaise, fatigue, anorexia) can accompany these infections, but are generally the result of cytokine production or through the action of other circulating mediators induced as a result of the local infection.

The factors that restrict some viruses to local sites while allowing others to invade the host are poorly understood. They include the direction of viral release from infected cells, the distribution of viral receptors and the effects of differences between core body and epithelial surface temperature.

Release of certain enveloped viruses occurs preferentially from either the luminally facing apical surface (e.g. para- and orthomyxoviruses) or the subepithelially facing basolateral surface (e.g. rhabdoviruses) of infected epithelial cells. The pattern of release is determined by the site in the cell membrane at which viral envelope glycoproteins are inserted. This is in turn influenced by specific amino acid signal sequences within the viral protein. This type of polarized release may be an important factor in either limiting or favoring systemic invasion. For example, release of virus only toward the lumen of the respiratory or GI tract would facilitate local infection of the epithelial surface but would inhibit invasion of deep subepithelial tissues. Conversely, release of virus from the basolateral cell surface would facilitate invasion of the subepithelial mucosa, and the subsequent dissemination of virus through lymphatics, blood vessels, or nerves (see below).

Spread through the bloodstream

Direct entry of virus into the bloodstream without any preceding replication in the host ('passive viremia') is a rare event. It can occur in association with intravenous drug abuse or during transfusion of infected blood or blood products. The bite of an arthropod vector may also allow direct entry of virus into the bloodstream. More commonly, viremia follows an initial phase of virus replication in the host ('active viremia'). Important sites of primary replication include subcutaneous tissue, brown fat, skeletal muscle, endothelial cells and regional lymphatic tissue. Virus enters the bloodstream from these sites ('primary viremia'), and is further disseminated to reticuloendothelial organs (bone marrow, liver, spleen) and endothelial cells. Additional replication is followed by a 'secondary viremia', which is typically of longer duration and higher magnitude than the initial viremia. This sequence of events was originally described for experimental mousepox (ectromelia) infection. Distinct phases of primary and secondary viremia are often difficult to identify in human viral infections.

Virus in the bloodstream may travel free in the plasma or in association with cellular elements. For example, enteroviruses and togaviruses are frequently found free in plasma. HIV, HTLV-1 and the human herpesviruses HHV 6 and HHV 7 infect subsets of T-lymphocytes, Epstein–Barr virus (EBV) infects B lymphocytes and Colorado tick fever virus (CTFV) infects erythrocyte precursors which subsequently mature and enter the circulation. It is important to recognize that plasma and cell-associated viremias need not be mutually exclusive. For example, HIV is found free in plasma as well as in association with CD4+ T-lymphocytes and monocytes. Finding a virus in association with cellular elements may also result from binding or adsorption without associated active replication (e.g. the hemadsorption of influenza to erythrocytes).

Termination of viremia is often abrupt and may coincide with the appearance of neutralizing antibodies. However, a variety of host defenses in addition to antibodies act to clear virus from the bloodstream. The magnitude of viremia is determined by the balance between the entry of virus into the circulation and its subsequent clearance by host defenses. The average time which a viral particle spends in the circulation ('transit time') is generally less than one hour. Larger virus particles and particles coated with antibody or complement are cleared with far greater efficiency than small nonopsonized particles, and may have transit times of only a few minutes. Additional factors that influence clearance and transit time include the net charge of the virion particle and the composition of the viral capsid or envelope. Host factors may also influence the efficiency of clearance. Experimentally, agents such as thorotrast or silica, which decrease the phagocytic capacity of macrophages and other reticuloendothelial phagocytes, enhance the magnitude and duration of viremia induced by some viruses. Differences in the capacity of macrophages from immature animals to clear virus compared to their adult counterparts may be one mechanism contributing to differences in age-related susceptibility to certain viral infections (e.g. herpes simplex). Macrophages derived from different strains of mice vary in the efficiency with which they clear specific viruses (e.g. mouse hepatitis virus, MHV), and this may explain some strain-specific differences in susceptibility to certain viruses. Finally, recent evidence has suggested that different strains of the same virus may show striking differences in their capacity to replicate in macrophages, and that this in turn may be associated with distinct patterns of organ-specific tropism and virulence.

Uptake of virus by phagocytes does not always result in their inactivation. Many viruses including HIV, lentiviruses, and certain toga-, corona-, arena- and reoviruses are capable of replicating in macrophages. The various outcomes possible when viruses infect macrophages are exemplified by the interaction of viruses with hepatic Kupffer cells. Kupffer cells may inactivate virus and limit subsequent spread of infection. Conversely, virus may either actively replicate in or be passively transported through these cells to subsequently enter the bloodstream through the hepatic sinusoids or following biliary excretion. Macrophages contain receptors for the Fc portion of antibody molecules, and in some cases uptake of virus is facilitated by the presence of antiviral antibody [antibody-mediated enhancement' (AME)]. This uptake may either lead to viral inactivation or conversely, may facilitate productive macrophage infection. AME plays an important role in the pathogenesis of dengue and certain other viral infections.

As would be expected, there is a general correlation between the magnitude of viremia generated by blood-borne viruses and their capacity to invade tissues such as the central nervous system (CNS). Conversely, the failure of some attenuated viruses to generate a significant viremia may also account for their lack of invasiveness. For example, certain neurotropic bunyaviruses are fully virulent after direct intracerebral inoculation, but avirulent after peripheral inoculation because they fail to generate sufficient viremia to allow neuroinvasion. It is important to recognize that viremia *per se* does not automatically equate with the capacity to invade tissues from the bloodstream. This has been elegantly demonstrated by certain mutants of Semliki Forest virus (SFV) which have lost the capacity to invade the CNS while retaining the capacity to generate a viremia equivalent in duration and magnitude to that induced by their neuroinvasive wild-type counterparts.

The steps by which blood-borne viruses exit the bloodstream to enter tissues remain poorly understood. In come cases, the viruses appear to directly infect endothelial cells, and then are transported across these cells into the underlying parenchyma. In other cases, viruses may enter tissues inside migrating cells that are capable of emigrating across capillaries (diapedesis). Transendothelial transport of virus inside infected cells has been colorfully referred to as the 'Trojan Horse' mechanism of entry. This type of process may be important in the pathogenesis of lentivirus and HIV infections. Factors that alter vascular permeability (e.g. vasogenic amines) can be shown experimentally to facilitate tissue invasion by certain viruses. This suggests that endothelial permeability may also play a role in determining tissue invasion by blood-borne viruses. Endothelial cells in most organs are joined by tight junctions (zona occludens). However, in certain regions (e.g. the choroid plexus in the brain) capillary endothelial cells lack tight junctions. These areas of fenestrated capillary endothelium may be the site of entry for viruses into perivascular tissue.

Spread through nerves

Many viruses including herpes simplex (HSV), varicella zoster (VZV), rabies and certain strains of poliovirus, reovirus and coronavirus can spread through nerves in the infected host. This pathway of spread is particularly important for viruses that invade the CNS, but theoretically also provides a route for infection of virtually any organ. Neural

spread to sites other than the CNS is exemplified by the spread of rabies virus to salivary glands and VZV and HSV to the skin.

The exact mechanism(s) of neural transport of viruses have not been established, although certain basic principles have emerged. Although spread of many neurotropic viruses along nerves can occur by cell-to-cell spread through nonneural cells (e.g. Schwann cells), the more important mode of viral spread is through the axoplasm of neurons. In the case of enveloped viruses, transport appears to involve predominantly the nucleocapsid rather than the enveloped virion. Neurally spreading viruses appear to all utilize the intraneuronal system of microtubule-associated fast axonal transport. This has been established by studying the kinetics of transport and through the use of selective pharmacologic inhibitors of fast and slow axonal transport. The mechanism by which viruses access axoplasmic transport systems and the form in which they are transported (e.g. free or within vesicles), has not yet been established. Taken as a group, neurally spreading viruses provide examples of spread through motor, sensory and autonomic nerve fibers, and in both the anterograde and retrograde direction. In some cases, individual strains of particular viruses (e.g. HSV) may preferentially travel in only one direction. Similarly, studies with reassortant viruses and viral mutants including rabies virus, pseudorabies virus and reovirus suggest that changes in either the viral envelope or capsid proteins, or in some cases in nonstructural proteins (e.g. HSV), may alter the capacity of viruses to spread through nerves, or even through specific neural pathways.

Viruses that spread within neurons also have the capacity to spread from nerve cell to nerve cell (transneuronal transport). In some cases this appears to occur specifically at synapses (trans-synaptic transport). The factors that influence the release of virus from presynaptic nerve terminals and facilitate their uptake postsynaptically are unknown.

Specific viral proteins play a critical role in determining whether viruses spread through the bloodstream or through nerves in the infected host, and even the specificity of the neural pathways utilized. For example, the principal pathway spread of reovirus type 1 Lang (T1L) from muscle to CNS is through the bloodstream, and for type 3 Dearing (T3D) through nerves. The viral S1 gene, which encodes the outer capsid protein sigma 1, determines this difference. As noted earlier, certain rabies and pseudorabies virus variants with single amino acid substitutions in the envelope glycoproteins, have altered neural spread properties when compared to their wild-type counterparts.

Nonstructural proteins may also influence the efficiency with which viruses infect and spread within the nervous system. However, in most cases this appears to be due to their effect on viral replication rather than due to direct effects on capacity for neural spread. For example, nonneuroinvasive HSV strains often replicate poorly in peripheral sensory ganglia, suggesting that this, rather than an inability to be neurally transported, accounts for their lack of neuroinvasiveness. These nonneuroinvasive strains remain capable of spreading from the site of inoculation, through nerves, to the sensory ganglia, but their spread is arrested at this stage. Genetic studies of recombinant herpesviruses containing portions of the genome derived from both invasive and nonneuroinvasive viruses indicate that the viral DNA polymerase may determine the capacity of certain viruses to replicate in sensory ganglia and subsequently invade the CNS.

Tropism

The capacity of a virus to selectively infect certain populations of cells in particular organs is referred to as 'tropism'. Viral tropism can depend on a variety of viral and host factors, several of which are discussed in detail in the sections which follow.

Receptors

Viruses must bind to target cells prior to initiating infection. Entry may be the result of the interaction of virus with a specific cellular receptor followed by receptor-mediated endocytosis. Alternatively, some viruses are capable of fusing directly with the plasma membrane (e.g. certain alphaviruses), which allows the nucleocapsid to enter the cell cytoplasm through a nonendocytosis-mediated pathway. Viruses that utilize receptor-mediated endocytosis to enter target cells may have receptors that are found on only certain types of cells (e.g. CD4 receptor for HIV), and thus receptor distribution may play an important role in determining viral tropism. In other cases the viral receptors appear to be ubiquitously distributed [e.g. sialic acid receptors for influenza, heparan sulfate glycosaminoglycans for HSV, gangliosides or phospholipids for rhabdoviruses, intercellular adhesion molecule (ICAM) 1 for rhinoviruses], and other factors must account for the specificity in the pattern of viral infection.

A number of principles have emerged from studies of viral receptors. Many of these proteins are membrane glycoproteins. These glycoproteins vary in structure and subserve a variety of different functions in the host. Putative viral receptors include receptors for neurotransmitters, growth factors,

cytokines, complement and laminin. Other viral receptors include integrins, MHC molecules, intercellular adhesion molecules, lymphocyte surface antigens (e.g. CD4, CD46, CD55) and members of the carcinoembryonic antigen (CEA) family. Some receptors have enzymatic or transport functions including aminopeptidase N, the sodium-dependent phosphate transporter, and the cationic amino acid transporter. In many cases, putative viral receptors are glycoproteins whose normal cellular function is unknown. A second major group of viral receptors includes sialic acids, glycosaminoglycans and glycolipids. These compounds are generally ubiquitously distributed across a wide variety of cells and tissues.

It should be emphasized that controversy surrounds some viral receptor assignments. This may result from the fact that viruses can infect cells lacking the putative receptor, indicating that the particular protein is either not the viral receptor or that other mechanisms of entry into the cell (additional receptors, nonreceptor-mediated processes) exist. Many viruses have the capacity to use alternate receptors in different cells, different tissues, and across different animal species. For example, HIV appears to be able to utilize both CD4 and galactosyl ceramide as a receptor. Productive infection subsequently requires the presence of additional 'co-receptor' molecules such as the chemokine receptors including CKR2, CKR3, CKR5 and fusin. Other recently identified putative co-receptors include HVEM, a member of the tumor necrosis factor (TNF) receptor superfamily for HSV, and CD55 (decay accelerating factor) for coxsackievirus A21. Some viruses appear to bind distinct receptors through different proteins or using different regions of the same protein. For example, binding of type 3 reoviruses to sialic acid residues appears to be mediated by a portion of the sigma 1 protein that is distinct from the region utilized for binding to nonsialyated receptors.

In the case of viruses which utilize a widely distributed membrane component such as sialic acid or glycosaminoglycans as a receptor, this may facilitate initial interaction between the viral envelope and the cell membrane ('loose association'), which is then followed by more specific interactions between additional virion proteins and the cell membrane or by virion envelope–cell membrane fusion. Herpesviruses exemplify this pattern. Many members of this family interact via specific envelope glycoproteins (e.g. gB of HSV and HHV 7) with heparan sulfate or related cell surface glycosaminoglycans. Subsequent virus–cell interaction is mediated by a complex process involving a variety of additional envelope glycoproteins.

Different strains of the same virus may use different receptors, and conversely, entirely unrelated viruses may share the same receptor. For example, human rhinoviruses (HRV) 1A, 1B, 2, 49 (the minor group) bind to the low density lipoprotein receptor, whereas HRV14 and all other rhinoviruses (the major group) bind to ICAM-1. Coxsackieviruses B1–6 and adenovirus 2 compete with each other for binding to certain cells, suggesting that they may share a common receptor, despite the fact that they belong to totally unrelated and completely distinct viral families.

It is important to recognize that although the presence of the appropriate viral receptor on a cell may be necessary for infection it is often not sufficient. For example, cultured mouse cells transfected with cDNA encoding the HIV receptor, and expressing the receptor protein, remain insusceptible to HIV infection. Similarly, expression of the human poliovirus receptor in intestinal epithelial cells of mice is not sufficient to allow poliovirus replication in the mouse gut. However, some cell lines that are resistant to infection with viruses including EBV, HIV and polio become fully susceptible when they are made to express the appropriate receptor, indicating that lack of receptor can be the only barrier to susceptibility.

Viral cell attachment proteins

The interaction of a virus with its cellular receptor is typically mediated by one or more cell surface proteins. Among the proteins playing a primary role in cell attachment are envelope glycoproteins (e.g. influenza HA, E2 for togaviruses, G1 for bunyaviruses, SU for retroviruses, gp120 for HIV, G for rhabdoviruses, VP1 for polyomaviruses, penton fiber protein for adenovirus, gp350/220 for EBV). Capsid proteins play a similar role in nonenveloped viruses (sigma 1 for reoviruses, VP4 for rotaviruses, VP1 for polio, large S for HBV). For HSV, more than one envelope glycoprotein may be required for different phases of cell attachment.

High-resolution three-dimensional crystal structures of the influenza HA and of several picornaviruses [HRV14, poliovirus, mengovirus, encephalomyocarditis (EMC), Theiler's] has provided atomic and even subatomic level structural information about viral receptor-binding sites. The sialic acid-binding domain of the influenza HA lies in a small depression near the distal tip of the molecule. The receptor-binding site of picornaviruses typically forms a depression in the virion surface that has been variously described as a canyon (HRV14), a valley (poliovirus) or a pit (mengovirus). Conversely, the receptor-binding site for foot and mouth disease virus (FMDV), a member of the aphthovirus group of

picornaviruses, is located on a prominent outward-facing antigenic loop of the VP1 protein.

For enveloped viruses the close approximation of the viral envelope with the host cell plasma membrane may be followed by fusion of viral and cellular membranes. This interaction may occur independent of the presence of specific viral receptors, or may be dependent on the presence of viral receptors which facilitate the close approximation of the viral envelope and the plasma membrane. Virus proteins with fusion activity may be the same as or distinct from virion cell attachment proteins. Fusogenic activity is typically triggered by a conformational change in the virion fusion protein. This in turn may be triggered by a specific proteolytic cleavage of this protein or by changes occurring consequent to receptor binding. When proteolytic cleavage occurs it may depend on intra- or extracellular host proteases or be dependent on virus encoded proteins or autocatalytic events.

Tissue-specific promoters, enhancers and transcriptional activators

Although the binding of a virus to its receptor may be a necessary initiating event in most viral infections, a number of other host and viral factors influence tropism. Viruses may contain distinct genetic elements, referred to as promoters or enhancers, that may enhance the transcription of certain genes in a cell-, tissue- or even species-specific manner. When mouse embryos are injected with the early region of JC virus (JCV) DNA, pathology is limited to oligodendrocytes within the CNS, despite the presence of viral genome in virtually all cells. The JCV genome contains a region that allows expression of the viral large T antigen only in oligodendrocytes and not other cells. An important role for viral enhancer elements in determining cell-type-specific gene expression has also been described for polyomaviruses, papillomaviruses and hepatitis B virus. It has recently been suggested that a cell-type-specific enhancer region may be contained within the HIV long terminal repeat (LTR), and that differences in this LTR sequence may account for differences between the neurotropism/monocyte-tropism compared to T-lymphoid tropism of some HIV isolates.

Site of entry and pathway of spread

The site of entry of virus into the host may influence its tropism. This has been clearly documented for neurally spreading viruses, whose neural spread is limited by the nature of the pathways available at the site of entry. Variations in the distribution of pathology, infectious virus or viral antigen following different sites or routes of viral inoculation have been clearly demonstrated experimentally with polio, rabies, herpes simplex viruses, reovirus, coronaviruses and the neurotropic (NWS) influenza virus strain. Obviously it has been harder to document this point in human infections, although clinical studies of rabies infection and polio suggest that an identical process occurs in humans. For example, patients who developed paralytic polio after being inadvertently immunized with improperly inactivated lots of poliovirus (the 'Cutter incident') showed a preponderance of paralysis involving the inoculated limb. Similarly, with rabies infection, the site of the bite (e.g. face versus leg) influences prognosis, the initial symptomatology, the incubation period and the probability of subsequent development of clinical disease.

It has also been suggested that the site of viral entry may influence the subsequent tropism of blood-borne as well as neurally spreading viruses. This was initially suggested after clinical observations suggested that local trauma to a muscle (e.g. an injury, an injection, strenuous overexertion) increased the likelihood of this muscle becoming paralyzed during a subsequent attack of paralytic polio ('provoking effect'). This effect could be reproduced experimentally if monkeys were given muscle damaging intramuscular injections followed by intracardiac inoculation of poliovirus type 1. Virus introduced into the bloodstream through the intracardiac route appeared to preferentially induce paralysis in muscles damaged by the preceding intramuscular inoculation. The mechanism of the provoking effect has never been satisfactorily established. It was suggested that local trauma could alter the vascular permeability in the region of the spinal cord innervating the traumatized site, and the increased permeability could result in an increased likelihood that blood-borne poliovirus would localize in that segment of the spinal cord. This phenomenon does not appear to have attracted recent attention, and its existence and mechanism must be considered speculative. Nonetheless, the possibility that local factors can influence the tropism of blood-borne viruses remains intriguing.

Host Factors

It is important to recognize that host factors may play a critical role in determining the outcome and many aspects of the pathogenesis of viral infections. Although a comprehensive discussion of the role of host factors in infection is beyond the scope of this review, several of the more important ones are worthy of emphasis. Among these are age, sex, genetic background, immune status and nutritional state.

The importance of host factors in determining the

outcome of viral infection is dramatically illustrated when populations of individuals are exposed to the same pathogen. Inadvertent experiments of this type have resulted from immunization of large populations with yellow fever virus vaccine contaminated with hepatitis B and with incompletely inactivated lots of poliovirus vaccine. In both cases, those vaccinated showed a wide spectrum of outcomes ranging from no obvious ill effects to severe disease (hepatitis, paralytic polio). These results occurred despite apparent uniformity in the nature of the pathogen, and its dose and route of administration. Epidemics of neurotropic arthropod-borne virus (arbovirus) infection provide a less controlled illustration of the same point. Among infected individuals there is a wide variety of clinical manifestations ranging from asymptomatic seroconversion to lethal encephalitis.

The role of genetic factors in determining the outcome of viral infection has been extensively investigated using inbred strains of mice. Human genes conferring resistance or susceptibility to viral infection have not yet been identified, although one can presume that they will ultimately be shown to exist. By comparing the severity of a particular viral infection in different strains of inbred mice, it can be shown that genetic determinants of viral resistance and/or susceptibility exist for almost all groups of viruses. Genetic factors that determine susceptibility to one virus are typically unique, and differ from those involved with other viruses. In addition, there are clearly multiple mechanisms by which genetic differences lead to differences in viral susceptibility. Among those that have been characterized are differences in immune responses [cytomegalovirus (CMV), murine leukemia viruses], in the expression of viral receptors in target tissues (coronavirus) and in interferon-induced expression of antiviral proteins (influenza virus).

The importance of differences in the age and sex of the host in determining the outcome of viral illness can be seen in a variety of human and animal viral infections. For example, viruses such as varicella, EBV, mumps, polio and hepatitis A typically produce milder infections in children than adults, whereas the opposite is true for viruses such as rotavirus and Rous sarcoma virus (RSV). Studies of experimental viral infection suggest that age-related differences in viral susceptibility have multiple mechanisms. Among those frequently cited are the maturation of the immune system, changes in the nature and distribution of populations of mitotically active cells, or on the state of cellular differentiation. Examples of host factors operating at a cellular level include the requirement of retroviruses that host cells undergo mitosis for integration of proviral DNA into their chromosomes. Other examples include viruses that only replicate efficiently when cells are in specific phases of the cell cycle. This may reflect a requirement for host proteins whose level of activity varies during different phases of the cell cycle such as polymerases or transcription factors.

Differences in the susceptibility of males and females to particular viral infection may be due to differences in the risk of exposure or the mechanism of viral transmission. For example, in the US, HIV infection is far more common in men than women. Similarly, the risk of transmission appears higher when the infected sexual partner is male rather than female. In some cases, sex-related differences in susceptibility cannot be accounted for by obvious epidemiologic factors such as exposure risk or mode of transmission. For example, neurologic complications of mumps infection are two to three times more common in boys than girls. Similarly, following exposure to hepatitis-B-virus-contaminated blood during hemodialysis, men are twice as likely to become chronic HB carriers as women. Striking differences in the nature of and susceptibility to viral infection also occur during pregnancy. These may be related to differences in levels of sex or steroid hormones or to pregnancy-related immunosuppression. Among the infections that are more severe during pregnancy are those caused by polio, hepatitis and herpes simplex viruses. There is also a higher rate of re-activation of latent viruses including polyomaviruses, CMV and herpes simplex viruses.

Exogenously administered hormones, including steroids and thyroid hormone can worsen the course of certain experimental viral infections. Steroids are often believed to exacerbate infections due to viruses such as herpes simplex, although definitive studies on the effects of steroids on human viral infections are lacking.

Transmission and Shedding

The successful propagation of a virus in nature requires that it be able to spread from the infected host to other susceptible individuals. The first step in this process is shedding from the initial host. There are several routes for viral shedding. Enteric viruses are characteristically shed in high titer and for prolonged periods of time in feces. Some respiratory viruses are shed in aerosols generated by coughing or sneezing. Other respiratory viruses are found in saliva or nasal secretions and are transmitted by direct contact with these secretions or by spread from nose to hand to objects which are subsequently contacted by susceptible hosts. Viruses which infect the genital tract are frequently present in genital secretions.

Sexual transmission through infected semen plays an important role in the transmission of HIV and certain other viruses. Virus contained in milk or colostrum may be responsible for initiation of certain perinatal infections including those caused by CMV. Although many viruses are present in urine ('viruria'), this is rarely an important source of viral transmission. An important exception to this rule may be arena- and Hanta-viruses for which rodent urine appears to be a source for shedding virus, and contact with this dried aerosolized urine may initiate human infection.

For certain viruses exposure to virus-infected blood or tissues may be an important source of transmission. Viral infections transmitted by this route include HIV, HTLV, CMV and hepatitis B and C. This route of infection is facilitated by modern medical practices (e.g. transfusion of blood or blood products, surgical procedures including organ transplantation), and certain cultural practices (e.g. tatooing, body piercing, intravenous drug use). In the case of arboviruses, transmission from a viremic host to uninfected individuals by arthropod vectors is an essential part of the natural life cycle of the virus. In this case the likelihood of transmission is affected by the duration and magnitude of viremia and various factors specific to the vector including the degree of viral replication in the vector and its feeding habits and seasonal life cycle. Transmission of infection from blood contaminated material is also an integral part of the life cycle of many viruses that produce hemorrhagic fever syndromes.

See also: **Host genetic resistance; Immune response: Cell mediated immune response, General features; Latency; Nervous system viruses; Persistent viral infection; Viral receptors; Virus–host cell interactions.**

Further Reading

Mims C, Dimmock N, Nash A and Stephen J (1995) *Mims' Pathogenesis of Infectious Disease,* 4th edn. London: Academic Press.

Nathanson N (ed.) (1997) *Viral Pathogenesis.* Philadelphia: Lippincott–Raven.

Nathanson N and Tyler KL (1998) The pathogenesis of viral infections. In: Mahy BWJ and Collier LH (eds) *Virology,* Vol. 1 *Topley and Wilson's Microbiology and Microbial Infections,* 9th edn, London: Arnold.

Tyler KL and Fields BN (1996) Pathogenesis of viral infections. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology,* 3rd edn, p. 173. Philadelphia: Lippincott-Raven.

White DO and Fenner F (1994) *Medical Virology,* 4th edn. San Diego: Academic Press.

Plant Viruses

James N Culver, Center for Agricultural Biotechnology, University of Maryland Biotechnology Institute, College Park, Maryland, USA

Introduction

Viruses depend on their hosts for the cellular machinery needed to complete their life cycles. This level of dependency necessitates precise interactions between virus and host components. For plant viruses, these interactions allow the virus to uncoat its genetic information, express its gene products, replicate its nucleic acid, move cell-to-cell, and spread long distances throughout the plant. Similar types of interactions may also occur between a virus and its vector to permit plant-to-plant movement. Still other types of specific virus–host interactions may result in the activation of host defense responses that arrest disease development. In general, any of these interactions may affect virus pathogenesis.

The ability to identify pathogenesis determinants involved in different virus–host interactions has been greatly expanded as a result of recombinant DNA technologies. Due to the small genome size of many plant viruses molecular approaches to identify viral determinants have proven extremely successful. In particular, the ability to clone and manipulate virus genomes using bacterial plasmid systems that can be used to generate infectious virus nucleic acid has allowed the mapping of multiple virus components involved in disease and resistance responses. In contrast, efforts to identify interacting host genes has, for the most part, been limited to classical genetic approaches. However, the development of screening methods to identify host mutations defective in a specific interaction along with genome sequencing projects for both crop and model plant systems such as *Arabidopsis thaliana* has significantly facilitated our ability to identify interacting host genes.

Other factors, such as the environment or host physiology can also affect virus pathogenesis. However, this article focuses primarily on the interactions between specific virus and host genes that are known to affect the outcome of an infection. The consequences of these interactions range from resistance to severe disease and plant death. What follows are several specific examples of virus–host interactions in which the virus component, host component, or both are known. These interactions have been grouped according to Matthew's definitions of virus–host responses.

Sexual transmission through infected semen plays an important role in the transmission of HIV and certain other viruses. Virus contained in milk or colostrum may be responsible for initiation of certain perinatal infections including those caused by CMV. Although many viruses are present in urine ('viruria'), this is rarely an important source of viral transmission. An important exception to this rule may be arena- and Hanta-viruses for which rodent urine appears to be a source for shedding virus, and contact with this dried aerosolized urine may initiate human infection.

For certain viruses exposure to virus-infected blood or tissues may be an important source of transmission. Viral infections transmitted by this route include HIV, HTLV, CMV and hepatitis B and C. This route of infection is facilitated by modern medical practices (e.g. transfusion of blood or blood products, surgical procedures including organ transplantation), and certain cultural practices (e.g. tatooing, body piercing, intravenous drug use). In the case of arboviruses, transmission from a viremic host to uninfected individuals by arthropod vectors is an essential part of the natural life cycle of the virus. In this case the likelihood of transmission is affected by the duration and magnitude of viremia and various factors specific to the vector including the degree of viral replication in the vector and its feeding habits and seasonal life cycle. Transmission of infection from blood contaminated material is also an integral part of the life cycle of many viruses that produce hemorrhagic fever syndromes.

See also: **Host genetic resistance; Immune response: Cell mediated immune response, General features; Latency; Nervous system viruses; Persistent viral infection; Viral receptors; Virus–host cell interactions.**

Further Reading

Mims C, Dimmock N, Nash A and Stephen J (1995) *Mims' Pathogenesis of Infectious Disease,* 4th edn. London: Academic Press.

Nathanson N (ed.) (1997) *Viral Pathogenesis.* Philadelphia: Lippincott–Raven.

Nathanson N and Tyler KL (1998) The pathogenesis of viral infections. In: Mahy BWJ and Collier LH (eds) *Virology,* Vol. 1 *Topley and Wilson's Microbiology and Microbial Infections,* 9th edn, London: Arnold.

Tyler KL and Fields BN (1996) Pathogenesis of viral infections. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology,* 3rd edn, p. 173. Philadelphia: Lippincott-Raven.

White DO and Fenner F (1994) *Medical Virology,* 4th edn. San Diego: Academic Press.

Plant Viruses

James N Culver, Center for Agricultural Biotechnology, University of Maryland Biotechnology Institute, College Park, Maryland, USA

Introduction

Viruses depend on their hosts for the cellular machinery needed to complete their life cycles. This level of dependency necessitates precise interactions between virus and host components. For plant viruses, these interactions allow the virus to uncoat its genetic information, express its gene products, replicate its nucleic acid, move cell-to-cell, and spread long distances throughout the plant. Similar types of interactions may also occur between a virus and its vector to permit plant-to-plant movement. Still other types of specific virus–host interactions may result in the activation of host defense responses that arrest disease development. In general, any of these interactions may affect virus pathogenesis.

The ability to identify pathogenesis determinants involved in different virus–host interactions has been greatly expanded as a result of recombinant DNA technologies. Due to the small genome size of many plant viruses molecular approaches to identify viral determinants have proven extremely successful. In particular, the ability to clone and manipulate virus genomes using bacterial plasmid systems that can be used to generate infectious virus nucleic acid has allowed the mapping of multiple virus components involved in disease and resistance responses. In contrast, efforts to identify interacting host genes has, for the most part, been limited to classical genetic approaches. However, the development of screening methods to identify host mutations defective in a specific interaction along with genome sequencing projects for both crop and model plant systems such as *Arabidopsis thaliana* has significantly facilitated our ability to identify interacting host genes.

Other factors, such as the environment or host physiology can also affect virus pathogenesis. However, this article focuses primarily on the interactions between specific virus and host genes that are known to affect the outcome of an infection. The consequences of these interactions range from resistance to severe disease and plant death. What follows are several specific examples of virus–host interactions in which the virus component, host component, or both are known. These interactions have been grouped according to Matthew's definitions of virus–host responses.

Immune Interactions

The majority of virus–host combinations result in an immune or nonhost response. In most cases, immunity is likely due to a nonfunctional or missing interaction between virus and host components needed for replication. In practice, this response is observed as the inability of a virus to replicate efficiently in protoplasts.

An example of such an interaction involves the *Tm-1* gene from *Lycopersicon* (tomato) and the replicase gene from tomato mosaic tobamovirus (ToMV). Whole plants and protoplasts from tomato lines that are homozygous for the *Tm-1* gene do not support ToMV RNA or protein synthesis. However, inhibition of virus replication is less effective in cells that are heterozygous for the *Tm-1* gene and absent in cells lacking *Tm-1*. Okada and colleagues have isolated and characterized a ToMV strain, Lta1, that overcomes *Tm-1* resistance. The ability to overcome *Tm-1* resistance was mapped to amino acid alterations in the helicase domain of the Lta1 viral replicase. Further mutagenesis studies suggest that the ability of Lta1 to overcome *Tm-1* resistance is the result of conformational changes in the replicase protein. This indicates that the *Tm-1* gene may encode a host component that must properly associate with the viral replicase before replication can proceed. Two other plant genes that confer a similar type of resistance are the *Ry* gene from *Solanum tuberosum* (potato) and the et^a gene from *Capsicum annuum* (pepper) which direct resistance against potato potyvirus Y (PVY) and tobacco etch potyvirus (TEV), respectively. Both *Ry* and et^a prevent the accumulation of viral RNA in protoplasts and plants. However, the viral components involved in these responses have not been identified.

The induction of host responses may also result in the inhibition of virus replication in the initially infected cell. The *Rx* resistance from potato confers immunity against most strains of potato potexvirus X (PVX). *Rx* resistance usually results in extremely low levels of virus replication in protoplasts and no detectable accumulations of virus in plants. Baulcombe and colleagues have determined that the ability of PVX strain HB to overcome *Rx* resistance is the result of mutations in the virus coat protein. In particular, a single amino acid substitution, Thr to Lys, at position 121 in the coat protein confers the ability to overcome *Rx* resistance. Evidence indicating that *Rx* resistance is the result of a host defense response comes from studies in which protoplasts were co-inoculated with PVX strains that were either avirulent, not capable of overcoming *Rx* resistance, or virulent and able to escape the resistance. Results from these studies demonstrated that in co-infected *Rx* protoplasts there was a significant reduction in the replication and accumulation of both avirulent and virulent PVX strains. Reduction in replication was not observed in similar protoplasts infected with only the virulent PVX strain. Time-course studies using protoplasts also indicated that measurable reductions in virus accumulations occurred only at later time points. This time delay suggests that the accumulation of a threshold level of coat protein is required to activate a resistance response that inhibits further virus replication. These studies demonstrate that plant disease resistance mechanisms can respond rapidly enough to limit virus replication at the single cell level.

Resistance Interactions

Virus localization to initially infected cells can be the result of either nonproductive host interactions that prevent virus movement from initially infected areas or the result of host defense mechanisms that rapidly limit virus spread to tissue surrounding the initial site of infection (**Table 1**). These types of interactions usually produce no visible disease symptoms, or in the case of resistance responses, produce necrotic local lesions at the sites of virus infection as shown in **Fig. 1**.

Single cell or subliminal infections

In general, plant viruses carry genes, such as the 30 kDa protein of tobacco mosaic tobamovirus (TMV), that allow for cell-to-cell movement through intercellular plasmodesma openings. These viral genes, termed movement proteins, have been shown to bind viral nucleic acid and are closely associated with plasmodesmata and cellular networks of microtubules. Movement proteins have also been shown to alter or gate the size of plasmodesmata to allow larger molecules to pass through these intercellular openings. In addition, mutant viruses with defective movement proteins replicate normally in initially infected cells or protoplasts but cannot move to adjacent non-infected cells. These movement-defective viruses can often be complemented by the transgenic expression of a functional virus movement protein. On the host side, genes in pepper and potato have been shown to limit the cell-to-cell movement of PVY in plants but not affect levels of replication in protoplasts. It is clear from these findings that specific interactions between virus movement proteins and host components are required for efficient virus spread and pathogenesis.

Table 1 Virus and host genes involved in resistance

Virus	*Virus gene*	*Host gene*	*Host*	*Level of resistance*
Tomato mosaic tobamovirus	130/180 kDa replicase	*Tm-1*	Tomato	Replication
Potato potexvirus X	Coat protein	*Rx*	Potato	Replication/HR
	Coat Protein	*Nx*	Potato	HR[a]
Tobacco mosaic tobamovirus	Coat Protein	*N'*	Tobacco	HR
		L^1, L^2, L^3	Pepper	HR
		?[b]	Eggplant	HR
	30 kDa cell-to-cell movement protein	*Tm2, Tm2*[b]	Tomato	HR
	Replicase	*N*	Tobacco	HR
Cauliflower mosaic caulimovirus	Gene VI	?	Tobacco/*Datura*	HR
Tomato bushy stunt tombusvirus	p19 and p22	?	Tobacco	HR

[a] Hypersensitive resistance response.
[b] Corresponding resistance gene has not been identified.

Localized infections

Nonproductive virus–host interactions may also result in the limitation of systemic virus movement. At this level of inhibition, the virus is able to replicate and move cell-to-cell normally, but cannot efficiently move through the plant's vascular system (bundle sheath cells to phloem parenchyma to sieve elements). In general, the slow pace of cell-to-cell movement ($\mu m\ h^{-1}$) versus that of long-distance movement ($cm\ h^{-1}$) dictates that viruses incapable of phloem transport cannot establish an infection at the plant's growing apex and are therefore limited to the initially infected tissue. Several different virus proteins have been linked to long distance movement. The coat proteins of TMV and red clover necrotic mosaic dianthovirus are clearly required for long distance movement. Mutations that disrupt the ability of either coat protein to assemble into virus particles interfere with long distance movement. However, it has been shown that replacement of the TMV coat protein open reading frame with that of ondontoglossum ringspot tobamovirus (orchid strain) resulted in a chimeric virus that produced assembled particles but was debilitated in systemic movement in tobacco. This finding suggests that determinants for host specificity in long distance movement also reside on the TMV coat protein. Still other virus proteins, such as the tombusvirus p19 or the potyvirus HC-Pro have been shown to play a role in long distance movement. On the host side, recessive alleles in soybean and tobacco have been shown to limit the systemic movement of cowpea chlorotic mottle bromovirus and TEV, respectively.

Hypersensitive responses

The hypersensitive response (HR) is an active pathogen defense mechanism that occurs in most higher plants. HR induction is dependent on a gene-for-gene recognition event that occurs between plant-encoded resistance gene products and pathogen-encoded molecules termed elicitors (**Table 1**). Recognition results in the induction of a cascade of host defense responses that include: oxidative bursts of H_2O_2, hydrolytic enzymes, callose and lignin precursors, pathogen related proteins etc. These defense responses lead to the rapid localized necrosis of tissue at the site of infection and prevent further virus spread (**Fig. 1**). Interestingly, similar HRs are observed against viruses, bacteria, fungi and nematodes. This implies that specificity for the HR does not involve the defense responses that confine the pathogen, but rather the recognition of the pathogen elicitor. In addition, analysis of cloned plant resistance genes indicate that they share similar characteristics including leucine rich repeats, protein kinase motifs and nucleotide binding sites. Taken together, this information indicates that plants utilize a common defense mechanism against pathogen invasion.

Many different viral proteins have been shown to act as specific elicitors of the HR. For example, the coat protein of TMV elicits HRs encoded by the *N'* gene from *Nicotiana sylvestris* (tobacco; **Fig. 1**), the *L* alleles from *Capsicum* spp. (pepper), and an uncharacterized gene from *Solanum melongena* (eggplant). The involvement of viral process and gene products other than coat protein have been eliminated from these interactions. The structural properties of coat protein required for *N'* gene recognition have been investigated. It has been demonstrated that recognition required a correctly folded coat protein and that amino acid residues along the right face of the coat protein's four helical bundle were critical for HR induction. These findings are consistent with the presence of a receptor binding site that confers *N'* gene recognition. Interestingly, this recognition site is

Figure 1 Hypersensitive resistance response between the *N'* gene of *Nicotiana sylvestris* and the coat protein of tobacco mosaic tobamovirus (TMV). The photograph was taken five days postinoculation and displays necrotic local lesions developing at the sites of TMV infection. This active host defense confines the virus to the area of the lesion and prevents its further spread.

conserved among all tobamoviruses and is even present in TMV strains that overcome N' gene resistance. However, mutations that disrupt the normal aggregating ability of coat protein and lie outside the recognition site can confer N' gene recognition to coat proteins that normally overcome this HR. Thus, strains of TMV that break N' gene resistance have apparently evolved a quaternary configuration that masks this site from host recognition.

The PVX coat protein also acts as an elicitor of *Nx* resistance in potato. It has been shown that the ability of some PVX strains to break *Nx* resistance resides within the coat protein. Specifically, a single coat protein amino acid exchange, Glu78 to Pro, was all that was required to overcome this resistance. It has also been demonstrated that a second site mutation of Ile62 to Val restored host recognition of the Pro78 coat protein. Based on the proposed PVX coat protein structure, the resistance breaking Glu78 mutations may interfere with the structure of a recognition site whereas the second-site Val62 mutation presumably restores the structure of the site. These findings provide another example of how subtle structural changes can result in dramatically different host responses.

Other plant-derived hypersensitive responses have been shown to target virus proteins other than coat protein. It has been shown that the HR conferred by the *N* gene from *Nicotiana glutinosa* is likely elicited by the replicase gene of TMV. Mutations that lead to the induction of the *N* gene HR by TMV-Ob, a strain that normally breaks this resistance, were mapped to the helicase domain of the virus replicase. However, it has not yet been determined if the replicase acts alone to elicit this HR or in combination with other viral components or processes. In addition, the *N* gene is currently the only plant virus resistance gene to be molecularly cloned and characterized. It has been shown that the *N* gene encodes a protein that contains a cytoplasmic domain similar to the *Drosophila* Toll protein, a nucleotide binding site, and a region of leucine rich repeats. The *N* gene product also appears to be cytoplasmic with no discernible membrane spanning domain. This indicates that recognition of the virus occurs within the cell, which is consistent with the location of TMV replication.

In tomato, two genes, *Tm2* and $Tm2^2$, have been identified as conferring an HR-like resistance against TMV. Both genes provide resistance only at the level of the plant and not at the level of protoplasts. TMV mutants that overcome either *Tm2* or $Tm2^2$ resistance have been identified. Meshi and colleagues determined that the *Tm2* resistance-breaking abilities of two independent TMV mutants, Ltb1 and C32, resided with specific amino acid substitutions (Glu52 to Lys, Cys68 to Phe, or Glu133 to Lys) in the virus 30 kDa movement protein. Similarly, Weber and colleagues identified the ability of a ToMV strain capable of breaking $Tm2^2$ resistance also resided with a different set of specific amino acid substitutions (Ser238 to Arg and Lys244 to Glu) in the 30 kDa movement protein. In addition, the transgenic expression of the ToMV 30 kDa movement protein in $Tm2^2$ tomato resulted in a necrotic HR phenotype. This demonstrated that the virus movement protein alone was sufficient to induce this resistance response.

The ability of barley stripe mosaic hordeivirus (BSMV) to induce necrotic local lesions on *Chenopodium amaranticolor* has been investigated. Different strains of BSMV were found to vary in their ability to induce lesions. For example, strain ND18 induced necrotic lesions within three to four days of inoculation whereas the type strain induced chlorotic

lesions at 14 days postinoculation. A genetic analysis of the virus determinants responsible for these lesion phenotypes indicated that they mapped to RNA-γ. RNA-γ is one of three BSMV genomic RNAs and encodes two protein products, γ-a and γ-b. Molecular studies of this RNA revealed that a short open reading frame present in the type strain 5′ leader of RNA-γ was responsible for the attenuated chlorotic local lesions. Translational studies indicated that this leader sequence decreases the production of γ-a protein *in vitro*. Thus, lower levels of γ-a protein may account for the attenuated lesion phenotype. However, a single amino acid substitution, Pro to Leu, in the γ-b protein of RNA-γ was also found to convert lesion phenotype from chlorotic to necrotic, suggesting that factors in addition to γ-a protein accumulation can also affect lesion formation. A third effect on the lesion phenotype of BSMV in *C. amaranticolor* was also found to reside in the composition of the other two BSMV genomic RNAs. For example, the rapid lesion-forming phenotype (4 days versus 14 days) occurred only when the type strain RNA-β was present. Taken together these findings illustrate the ability of multiple virus determinants to influence a host response.

Susceptible Responses

Susceptible host responses are generally associated with the appearance of systemic disease symptoms. This implies that the interactions between virus and host components needed for replication, cell-to-cell movement, and systemic spread are functional. The observed disease responses, which include effects on growth, development and output, are likely the result of numerous factors related to the affects of these interactions on the homeostatic balance of the plant.

Sensitive responses

Disease responses vary dramatically depending on the virus–host combination under investigation. The type of response, for example chlorotic versus necrotic symptoms, is generally dependent on the genetic makeup of both the plant and the infecting virus. However, environmental factors such as the availability of micronutrients, nitrogen, water and light can also have significant effects on the severity of the observed disease. Additionally, the developmental stage of a plant or leaf at the time of infection can also determine the type of disease response. For example, Matthews and colleagues determined that for tobacco leaves infected with TMV the critical leaf size at which mosaic symptoms develop is at or below 1.5 cm in length. Leaves that were developmentally larger when inoculated failed to display the mosaic symptom. This finding likely reflects a requirement for active virus replication at the time of cell differentiation. However, other disease responses, such as vascular necrosis, appear to develop independently of the developmental stage of the plant.

Chlorosis

Chlorosis of green plant tissue is one of the most common disease symptoms associated with virus infection and generally results from a decrease in chlorophyll content due to perturbations in chloroplast structure and function. Chlorosis associated with systemic mosaic disease likely involves the disruption of chloroplast development in the leaf primordium. In some systems the development of a mosaic disease pattern appears to reside with the expression of a single virus gene. For example, gene VI from cauliflower mosaic caulimovirus when expressed transgenically in tobacco has been shown to induce chlorotic mosaic-like symptoms. In another system, detailed mapping of symptom determinants in TMV strains that confer mild mosaic symptoms has shown that specific amino acid substitutions in the viral replicase gene control the severity of the mosaic response. These mutations do not affect the expression of other viral proteins but do reduce the rate of phloem-dependent virus accumulation. However, the rate of TMV accumulation did not strictly correlate with the severity of the mosaic response. Some replicase mutants accumulated poorly but still induced severe mosaic symptoms. Thus, factors other than the rapid early accumulation of virus within the merstematic tissue of the plant appears to be responsible for this mosaic symptom, suggesting that a direct replicase–host interaction may be involved. Similarly, mutations in the replicase genes of cucumber mosaic cucumovirus and barley stripe mosaic virus have also been shown to affect the pathogenicity of these viruses in zucchini and oats, respectively.

The severity of chlorotic symptoms can also be affected by other viral genes. In particular, specific amino acid substitutions in the coat proteins of TMV and cucumber mosaic virus have been shown to enhance the severity of mosaic symptoms (**Fig. 2**). The precise effects of these coat proteins on symptom development have not been determined, however, many of the TMV coat protein mutants that confer severe mosaics also produce a brighter chlorosis on mature leaves compared to that induced by the wild-type virus (**Fig. 2**). This suggests that these coat proteins disrupt the function of developed chloroplasts. In addition, TMV mutants that do not produce coat protein still induce systemic mosaic symptoms that are indistinguishable from the wild-type virus.

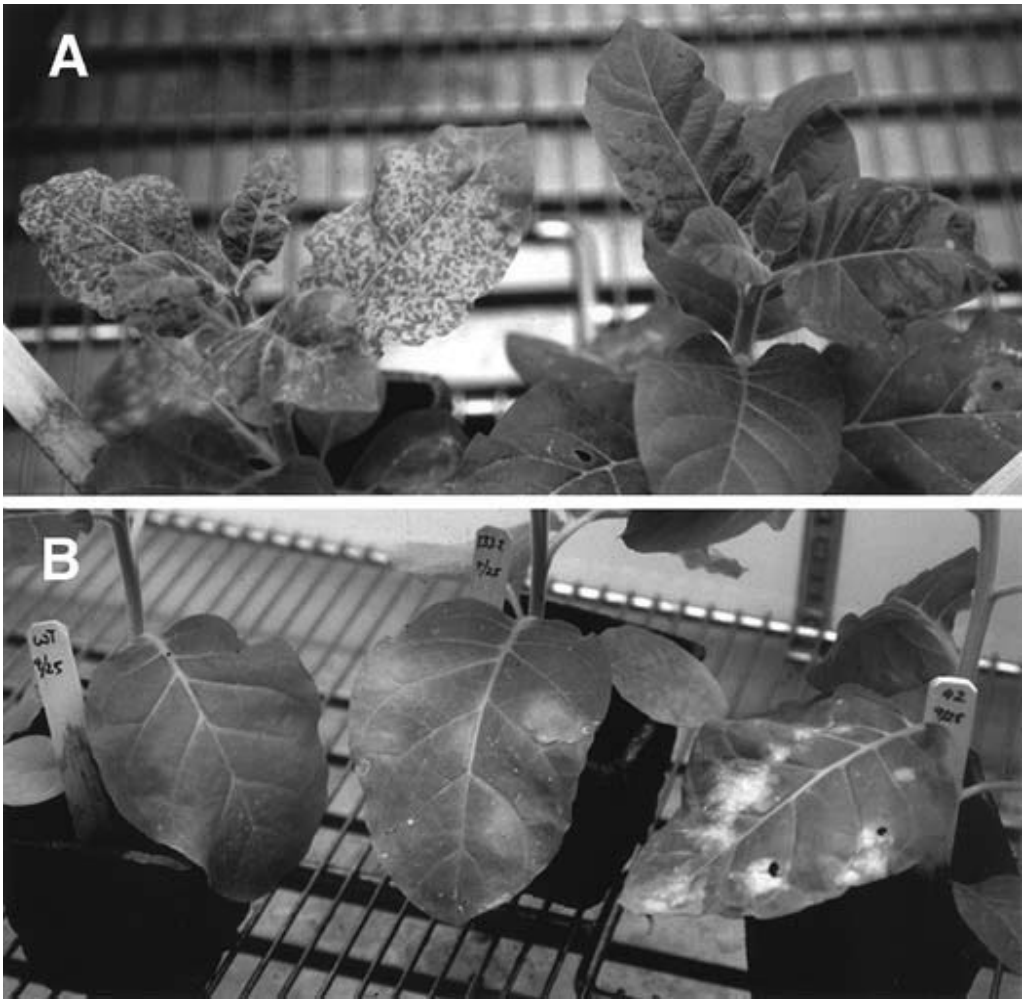


Figure 2 Symptom variations resulting from single amino acid substitutions in the coat protein of tobacco mosaic tobamovirus (TMV). (**A**) Left, Xanthi tobacco systemically infected with a TMV coat protein mutant F67L; right, Xanthi tobacco systemically infected with wild-type TMV. (**B**) Xanthi tobacco leaves inoculated with: left, wild-type TMV showing no symptoms; center, TMV coat protein mutant L133P showing chlorosis at the sites of infection; and right, TMV coat protein mutant T42R showing necrosis at the sites of infection.

Thus, severe mosaic symptoms can be the result of both the developing mosaic and the enhanced chlorosis caused by coat protein. One possible mechanism by which coat protein enhances chlorosis involves the direct accumulation of coat protein within the chloroplasts. Evidence for this comes from the purification of TMV coat protein and virions from chloroplast fractions. In addition, a positive correlation has been found between the level of TMV coat protein associated with photosystem II in chloroplasts and the severity of the observed mosaicism. In another system, the PVY coat protein was targeted to chloroplasts by fusing it to a chloroplast transit peptide. Tobacco plants transformed with the PVY coat protein plus transit peptide produced white mosaic symptoms whereas plants transformed with only the unfused PVY coat protein showed no symptoms. This further suggests that the ability of a coat protein to move into chloroplasts can directly effect the severity of the observed disease. Additional evidence also suggests that coat protein may affect chloroplast function and structure indirectly by interfering in the synthesis or transport of proteins into and out of the chloroplasts. Electron microscopy studies have shown that various coat protein mutants of TMV produce dark staining coat protein bodies (aggregates of unassembled coat protein) within the cytoplasm. The presence of these bodies was correlated with dramatic disruptions in the structures of mature chloroplasts. However, these coat proteins did not accumulate within the chloroplasts, suggesting that their affect on chloroplast structure occurs indirectly from the cytoplasm.

The effects of turnip yellow mosaic tymovirus

(TYMV) replication on chloroplast structure have been well documented. In addition, it has been shown that a single amino acid substitution in the TYMV movement protein was found to confer more severe and uniform systemic chlorotic symptoms. This mutation also correlated with increased virus yields in systemically infected tissue. Alteration of the TYMV movement protein may enhance the ability of the virus to move into the leaf primordium at a time or in a concentration that results in a more uniform and thus more severe systemic infection.

Viral nucleic acid, such as single- or double-stranded RNA, contains a high-degree of secondary structure involved in viral replication, protein translation and virion assembly. Thus, it is not surprising that alterations in disease symptoms have been mapped to RNA segments that do not encode a protein product. One example has come from studies on the satellite RNAs of cucumber mosaic virus (CMV). The induction of chlorosis has been mapped to specific nucleotide sequences present in several satellite RNAs (satRNAs). This region, termed 'chlorosis domain' (nucleotides 97–191 of the B-5 satRNA of CMV) was found to contain a number of specific nucleotides that when altered led to changes in the type of chlorosis (white versus yellow) or the host specificity of the chlorosis (tobacco versus tomato). In addition, the type of induced chlorosis was found to be dependent on the strain of helper virus. This implies that the satellite sequences involved in chlorosis may indirectly affect chloroplast function and symptoms via interactions with specific helper virus components.

Systemic necrosis

Necrosis of systemically infected tissue often results in the most severe form of disease. These necrotic responses can be displayed as either sporadic necrotic areas within a mosaic symptom or widespread necrosis resulting in vascular collapse and plant death. One type of systemic necrosis results from the weak induction of the plant hypersensitive defense response. For TMV, mutants that weakly elicit the *N′* or *Tm2* HRs are not fully localized and can move systemically through the plant. However, as these viruses move they continually trigger the necrotic HR, eventually resulting in plant death. Additionally, a number of different single amino acid substitutions in the coat protein of TMV have been shown to confer necrosis at the site of infection or intermixed patches of necrosis and mosaic in systemically infected tissue (**Fig. 2**). This type of patchy necrosis appears to be independent of any host defense response. Exactly what causes this type of necrosis is not known.

Necrotic responses have also been mapped to the satellite RNAs of CMV. Chemical and enzymatic modifications were used to compare the structural features of CMV satRNAs that vary in their ability to confer necrosis. These studies have identified one helix and two tetraloop regions in the satRNA that correlated with necrosis, suggesting the importance of specific RNA structures in this disease response.

Tolerance

In a tolerant interaction the virus infects the host systemically and can reach high titers, but the infection produces few if any detectable symptoms. A number of host genes that confer tolerance have been described including r_{m1} and r_{m2} in tobacco, conferring tolerance to TMV, and *TTR1* in *A. thaliana*, conferring tolerance to tobacco ringspot nepovirus. Virus components that specifically affect tolerance have not been specifically described, although many components that enhance or attenuate disease symptoms without affecting virus replication and movement have been described (see the above sections).

See also: **Plant resistance to viruses: Natural resistance; Synergism: Plant viruses.**

Further Reading

Baker B, Zambryski P, Staskawicz B and Dinesh-Kumar SP (1997) Signaling in plant–microbe interactions. *Science* 276: 726.

Carrington JC, Kasschau KD, Mahajan SK and Schaad MC (1996) Cell-to-cell and long-distance transport of viruses in plants. *Plant Cell* 8: 1669.

Fraser RSS (1990) The genetics of resistance to plant viruses. *Annu. Rev. Phytopathol.* 28: 179.

Matthews REF (1991) *Plant Virology*. San Diego: Academic Press.

Stintzi A, Heitz T, Prasad V *et al* (1993) Plant 'pathogenesis-related' proteins and their role in defense against pathogens. *Biochimie* 75: 687.

PEA ENATION MOSAIC VIRUS (*LUTEOVIRIDAE*)

Jihad S Skaf, **Steven A Demler** and **Gustaaf A de Zoeten**, Department of Botany and Plant Pathology, Michigan State University, East Lansing, Michigan, USA

Taxonomy and Classification

The genus *Enamovirus* (*Luteoviridae* family) comprises a single member, pea enation mosaic virus (PEMV). PEMV is characterized by a single-stranded, plus-sense, bipartite RNA genome encapsidated in two morphologically distinct isometric particles. Some isolates contain a third, nonessential satellite RNA. The distinguishing features of PEMV include the ultrastructural alterations of infected cells and viruliferous aphids. Also, virus titer in infected plants is much higher compared to related luteoviruses, perhaps because of the true systemic infection that can be established by PEMV. The following text reviews the current state of knowledge concerning PEMV and compares and contrasts PEMV with other viruses. Also highlighted is the symbiotic nature of the PEMV complex that is analogous to that described for helper-dependent, aphid-transmitted virus complexes involving members of the genera *Luteovirus* and *Umbravirus*.

Virus Structure and Composition

Virions of PEMV consist of two isometric nucleoprotein components with estimated $S_{20,w}$ values ranging from 91 to 106S for the top component and 107 to 122S for the bottom component. There is no evidence of empty viral shells in purified PEMV virion preparations. In general, the bottom component is the dominant species, although the amount of top component is highly variable and a variant strain in which the top component dominates has been reported. Particle molecular weights are in the ranges $5.4 \times 10^6 - 5.6 \times 10^6$ for the bottom component and $4.4 \times 10^6 - 4.7 \times 10^6$ for the top component, with an estimated RNA content of *c.* 28–33%. Purified virions display a bimodal diameter distribution of approximately 25 and 28 nm. It has been proposed that the bottom component is composed of 180 subunits arranged in a $T = 3$ icosahedron, whereas the top component is composed of approximately 150 subunits lacking quasi-equivalence. The top component is also less stable (particularly under high salt conditions) than the bottom component, a possible result of the irregularity in virion composition.

Virions of aphid-nontransmissible strains of PEMV contain a single coat protein (CP) of 21 kDa. In contrast, aphid-transmissible strains also contain a second minor protein of 54 kDa. Repeated mechanical inoculation of aphid-transmissible PEMV strains often leads to the elimination of the 54 kDa protein and aphid transmissibility.

Electrophoretic examination of intact virions of aphid-nontransmissible isolates of PEMV reveals two bands, corresponding to the top and bottom components. In contrast, aphid-transmissible isolates display a complex banding pattern attributed to incremental size differences between particles.

Serology

PEMV is moderately immunogenic, with titers of up to 1:1024 attainable by standard methods. Antisera against aphid-transmissible strains of PEMV contain two antibody populations whereas antisera generated against the aphid-nontransmissible strain contain only a single population. There are no reports of serological crossreaction between PEMV and any other virus.

Genome Structure

The genome of PEMV consists of two and sometimes three plus-sense RNAs of molecular weight 1.9×10^6, 1.4×10^6 and 0.23×10^6. The nucleotide sequence of all three species has been determined. The RNAs are not polyadenylated and nonaminoacylatable.

There has been considerable controversy as to the infectivity and particle distribution of the viral RNAs of PEMV. Part of this discrepancy is due to the inability of centrifugation and electrophoretic techniques to adequately separate the bottom and top components to homogeneity, as well as to the uncertain existence and particle affiliation of RNA3 (and its effect on particle density). The favored model suggests that the bottom component encapsidates RNA1 and the top component RNA2, with both components mandatory for wild-type (WT) infection. An alternative proposal argues that RNA1 and RNA3 are encapsidated in the bottom component, with RNA2 encapsidated in both the top and bottom components.

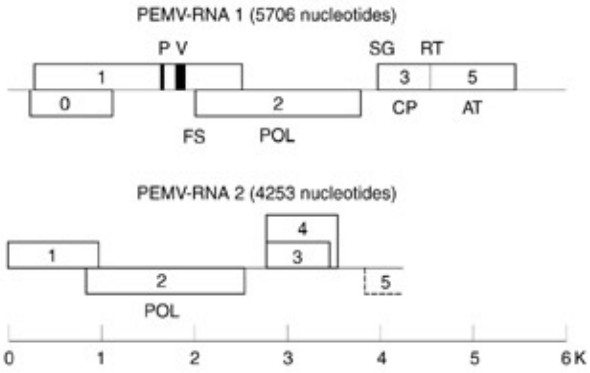


Figure 1 Schematic representation of the genome structure of wild-type PEMV RNAs 1 and 2. Numbered hollow boxes indicate open reading frames. AT, aphid transmission; CP, coat protein; FS, frameshift; POL, polymerase; SG, subgenomic; RT, readthrough; P, conserved 3C-like protease motif; V, genome-linked protein (VPg).

RNA1: the luteovirus component

RNA1 of PEMV (5706 nucleotides) bears strong organizational and sequence similarity to the RNAs of subgroup II luteoviruses encompassing beet western yellows (BWYV), potato leafroll (PLRV) and the NY-RPV isolate of barley yellow dwarf virus (NY-RPV BYDV). RNA1 is composed of five predominant open reading frames (ORF, **Fig. 1**). The first ORF (ORF 0) encodes a protein of 34 kDa of unknown function or relationship to other viruses. ORF 1 encodes an 84 kDa protein and overlaps (out of frame) 90% of ORF 0. *In vitro* translation analysis of RNA1 generated products of 36 and 88 kDa, suggesting that expression of the ORFs 0 and 1 products occurs by independent translational initiation. Sequence analysis has identified a 3C-like protease core sequence within the 84 kDa protein, suggesting that proteolytic processing may be involved in the expression of the viral genome. The central region encompassing this protease motif is conserved in luteoviruses. Similar to other subgroup-II luteoviruses, the genome-linked protein (VPg) of PEMV is encoded by ORF 1 of RNA1 downstream of the putative protease motif (**Fig. 1**). It is not known at this time if this VPg is covalently linked to all three viral RNAs. Protease treatment does not abolish infectivity suggesting that the VPg is not required for the initial translation of viral RNA.

ORF 2 encodes a protein of 67 kDa and overlaps (in a unique frame) the C-terminal 23% of ORF 1. This 67 kDa protein is characterized by RNA polymerase- and helicase-like motifs, and is postulated to represent the core of the viral RNA-dependent RNA polymerase. Sequence comparisons have established a strong relationship between this protein and those of comparable ORFs of subgroup II luteoviruses. *In vitro* translation analysis suggests that this protein is expressed as a fusion of the 84 kDa and 67 kDa protein reading frames by translational frameshift.

ORF 3 encodes the 21 kDa viral CP, and is immediately followed in the same reading frame by ORF 5 (encodes a 33 kDa protein). The amino acid sequence of both the coat and 33 kDa proteins bears strong sequence homology to their counterparts in the 3′-terminal ORFs described in luteoviruses. In addition, the 41-amino acid intergenic region between the opal stop codon of the CP and the first true start codon of the 33 kDa protein contains a proline-rich region also conserved within luteoviruses. The 33 kDa protein is expressed as a translational readthrough fusion with the 21 kDa CP. The resulting 54 kDa minor protein is associated with aphid transmitted isolates of PEMV and is dispensable for infectivity but its presence is mandatory for aphid transmission. In response to a specific selection pressure (mechanical transmission), PEMV suppresses aphid transmissibility via a number of mechanisms including the generation of genotypes lacking the 33 kDa protein or the downregulation of the expression of the translational readthrough protein.

Deletions in the CP ORF do not inhibit the systemic movement of PEMV or alter the accumulation of truncated viral RNA (compared to WT isolates). Pea plants infected with CP-less mutants exhibit wilting and severe necrosis that appear earlier and are more severe than observed in WT infections. Interestingly, the absence of the CP (and hence virions) from cells infected with CP deletion mutants does not affect the formation of replication complexes or the double-membrane bound vesicles characteristic of PEMV infection (see the cytopathology section below). The association of these vesicles, whose production is controlled by RNA1, with viral RNA-dependent RNA-polymerase, viral single stranded- (ss-) and double stranded- (ds)RNA, as well as plasmodesmata and phloem tissue suggests their involvement in virus movement throughout the plant. This CP-independent movement of PEMV is in sharp contrast to the absolute necessity of the CP for the movement of luteoviruses. This contrast may be due to functions provided in *trans* by RNA2 to PEMV complex.

One of the imperfections in the analogy between PEMV and luteoviruses is the lack in PEMV-RNA1 of a 17–19 kDa ORF 4 nested within the luteovirus CP genes. This ORF 4 has been implicated as a possible participant in cell-to-cell luteovirus movement. However, silencing ORF4 of BWYV reduced the level of viral RNA accumulation but did not abolish the systemic movement of the virus.

Neither the coat nor the 33 kDa protein are expressed in *in vitro* translation of RNA1. There is evidence from northern blot analysis of polysomal and total RNA isolation from infected tissue for an 1800 nucleotide RNA specific to the 3′ terminal region of RNA1. These data suggest that expression of these two proteins occurs from a single subgenomic messenger.

RNA1 is perfectly capable of replicating in isolated protoplasts but cannot establish systemic infections in plants on its own regardless of the inoculation method used.

RNA2: the umbravirus component

Unlike all known multicomponent viruses, there is a surprising lack of sequence homology (especially at the 5′ and 3′ termini) between RNA2 and RNA1 of PEMV. The only exception is a region that immediately precedes ORFs hypothesized to be expressed from subgenomic mRNAs (ORFs 3 and 5 of RNA1 and ORFs 3 and 4 of RNA2). The first ORF (ORF 1) of RNA2 can potentially encode a 33 kDa protein of unidentified function. Introducing specific frameshift and or deletion mutations into ORF 1 does not affect the infectivity of RNA2 or PEMV. ORF 1 overlaps (out of frame) the first 37 amino acids of the 65 kDa ORF 2. ORF 2 contains sequence motifs typical of RNA polymerases and bears a strong taxonomic relationship to those of carmo-like viruses encompassing subgroup I luteoviruses. The polymerase encoded by RNA2 is unrelated to the polymerase encoded by RNA1. ORF 2 is followed by two largely overlapping (in unique reading frames) ORFs (ORFs 3 and 4) potentially encoding 26 kDa and 27 kDa proteins. Circumstantial as well as experimental evidence indicate that both cell-to-cell and systemic movement, mechanical transmissibility and the relaxation of phloem limitation of the luteovirus component of PEMV are mediated by ORFs 3 and 4 and by their P26 and P27 products. This evidence includes sequence homology between P27 and other viral movement proteins. In addition, specific changes (both natural and experimental) in ORFs 3 and 4 halt the *in planta* movement of PEMV but not the replication of RNA2 in pea protoplasts. ORFs 3 and 4 are followed by a 293 nucleotide noncoding region leading to a potential fifth ORF (15 kDa) that lacks a conventional stop codon. The pronounced sequence heterogeneity may be indicative of the degenerative nature of this region. The loss of this region in some clones confers no distinctive phenotype in pea or in *Nicotiana benthamiana*.

Serological screening with anti-PEMV antibodies has failed to identify a CP encoded by RNA2.

In vitro translation analysis of RNA2 has demonstrated only a single product of 45 kDa with no evidence of the larger frameshift product postulated above.

RNA2 is mechanically transmissible and can establish a systemic infection in whole plants in the absence of RNA1.

RNA3: the satellite component

In addition to the two genomic components, some isolates of PEMV possess a third, 717 nucleotide RNA (RNA3), with features characteristic of B-type mRNA satellite RNAs (although there is no evidence of translational activity associated with this RNA). RNA3 is not infectious on its own and was long considered an artefact of virus purification. However, recent molecular methods permitted the cloning and further analysis of this RNA. RNA3 has no measurable impact on the symptoms induced by PEMV in pea plants, although its presence results in symptom attenuation in *N. benthamiana*. RNA3 is encapsidated in the CP encoded by RNA1 and replicated by the polymerase encoded by RNA2 (the first 13 and 8 nucleotides at the 5′ and 3′ termini, respectively, are identical between RNAs 2 and 3). In return, RNA3 may have an effect on virion stability which can be advantageous to the two genomic RNAs. This trilateral interaction between RNA3 and the genomic components of PEMV is reminiscent of that described for the satellite RNA of groundnut rosette virus (GRV). Indeed, both satellite RNAs share considerable sequence homology and can be interchangeably replicated and encapsidated by their counterparts' helper luteoviruses. In contrast with the GRV satellite, RNA3 is dispensable for the infectivity of PEMV, although its prevalence and role in natural infections has not been determined.

Host Range

PEMV has a narrow host range, limited mainly to the family Leguminosae, and includes members of the genera *Anthyllis, Astragalus, Cicer, Lathyrus, Lens, Lotus, Lupinus, Medicago, Melilotus, Phaseolus, Pisum, Glycine, Trifolium* and *Vicia*. Nonleguminous hosts include *Gomphrena globosa, N. benthamiana, Nicotiana clevlandii, Nicotiana tabacum* and members of the genus *Chenopodium* (*C. quinoa, C. amaranticolor* and *C. album*) which serve as local lesion hosts.

Transmission

Similar to other luteoviruses, PEMV is transmitted by aphids in a circulative nonpropagative manner. A

notable distinction, however, is the additional capacity of PEMV for effective mechanical transmission. Eight species of aphid have been reported as vectors of PEMV, with the pea aphid *Acyrthosiphon pisum* (Harris) being both the most effective and most significant in the field. There is considerable variability in the literature concerning the dynamics of vector transmission of PEMV, a reflection of variability in both the viral isolate and aphid biotype examined. In general, acquisition periods range from 15 min to 3 h, with a mandatory latent period of approximately 10–18 h, consistent with a circulative transmission pattern. Following the latent period, *A. pisum* was able to transmit PEMV following test feeding probes of less than 60 s, suggesting that inoculation of nonphloem tissues was adequate to transmit the virus. Nymphs are more efficient than adults at virus transmission. Virus particles are retained through moults, and aphids remain viruliferous in excess of 30 days. There is no concrete evidence of viral replication occurring in aphid tissues.

Extended mechanical propagation of PEMV isolates can lead to the loss of vector transmissibility. There are no reports at this time of such aphid-nontransmissible isolates occurring under field conditions, or the reversion of these laboratory strains to aphid transmissibility.

Symptomology

Symptoms attributed to PEMV vary considerably, depending on the viral isolate, the age of the host plant and environmental conditions. Symptom expression in pea seedlings inoculated prior to the unfolding of the first true leaves (*c.* 7–10 days after sowing) is manifested by a downward curling of the uppermost leaves 5–7 days postinoculation. This is followed (7–10 days postinoculation) by a marked vein-clearing and the development of both irregular chlorotic flecks and small irregular translucent lesions (often described as windows) along the leaf surface. From 10 to 14 days after inoculation, the aerial portions of the plant become severely stunted, epinastic and rugose, with continued amplification of foliar symptoms. Apical dominance is often lost, with proliferation of severely distorted axillary buds. In severe cases, top and bud necrosis is also observed. The diagnostic symptom of this virus, the enation, is evident on the lower leaf vein and stipule surfaces as hypertrophic undifferentiated outgrowths. These tend to form late in infection, approximately 2–3 weeks after inoculation. Although infected plants will successfully set pods, their yield, size and quality is significantly reduced. Pod symptoms consist primarily of wart-like protuberances along the outer pod surface, although pod enations have also been observed. In older plants and tolerant varieties, symptoms are often less severe, consisting mainly of the foliar mosaic symptoms with fewer growth abnormalities.

Symptoms on *Chenopodium* species consist of small chlorotic local lesions emerging 3–10 days after inoculation. The reproducibility of *Chenopodium* species as a local lesion host is highly variable and greatly influenced by environmental conditions.

Virus Epidemiology and Control

PEMV was initially described in 1935 by Osborn to occur in *Vicia faba* in New York State. The virus occurs predominantly in northern temperate climates, but has also been reported as far south as Iran and Sicily. Economically, PEMV is considered the most significant viral disease in commercial pea production. In addition, significant disease losses have also been described in commercial chickpea, broadbean and lentil production.

Annual and perennial leguminous host and weed species serve as overwintering reservoirs for both PEMV and its aphid vector. Aphid migration from these overwintering hosts in spring and early summer results in the dissemination of the virus into adjacent areas. Therefore, one method of reducing the incidence of PEMV is to control the aphid vector in both the overwintering host as well as in the secondary crop host. It has also been suggested that control of leguminous weed species and avoidance of perennial legumes, adjacent to production fields may assist in reducing the available virus and vector reservoirs.

Resistance to PEMV in *Pisum sativum* is controlled by a single dominant gene designated *En* derived from USDA PI No. 40295 originating in Iran. Similar tolerance has also been uncovered in *Lens culinaris* accessions PI Nos. 472547 and 472609 originating in India and in Iran, respectively.

PEMV, like several members of the luteovirus group, can serve as a partner in a disease complex with an unrelated aphid-nontransmissible virus. The bean yellow vein banding complex (BYVBV) consists of a helper-dependent association between PEMV (the helper) and BYVBV (the dependent). This affiliation results in an increase in yield loss over that associated with PEMV infection alone. In this complex, BYVBV, which is normally mechanically transmitted, is dependent on PEMV for aphid transmission. Bean leafroll luteovirus also serves as a substitute helper virus in this complex.

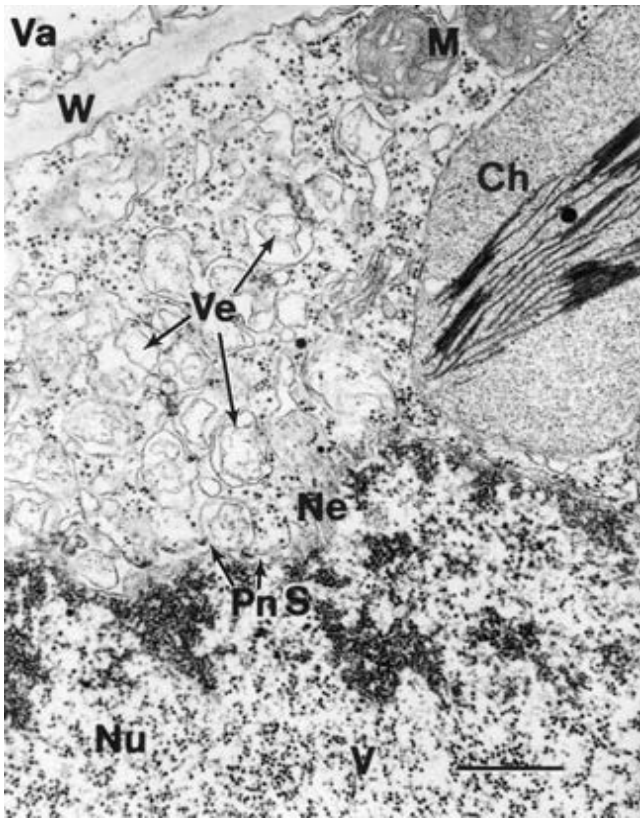


Figure 2 A PEMV-infected pea mesophyll cell. Note the cytoplasmic vesiculation (Ve) representing the replication complex of the virus. The vesicles are formed in the perinuclear space (PnS) of nuclei sustaining virus replication. In many cases, nuclei of infected cells contain assembled virions (V). Ch, chloroplast; M, mitochondrion; Ne, nuclear envelope; Nu, nucleus; Va, vacuole; W, cell wall. Magnification ×31 000; scale bar = 0.5 μm.

Cytopathology

Infections by PEMV are characterized by distinctive ultrastructural alterations of tissues of both plant host and aphid vector (**Fig. 2**). These alterations have strong parallels with those observed in luteovirus infections. Perhaps the most distinguishing characteristic of PEMV infection is the intimate association with the host cell nucleus and with vesicular structures originating from the nucleus. Virions are found throughout the nucleus as well as in the cytoplasm and vacuoles, either scattered or in loosely packed clusters. A proliferation of fibril-containing vesicular structures is evident in infected tissue, including all parenchymatic cell types and particularly in phloem tissues. In time-course studies, these vesicles were shown to originate from the inner nuclear membrane and are deposited into the perinuclear space from which membrane-bound groups of vesicles are extruded into the cytoplasm. *In situ* hybridization studies demonstrated that both the nuclei and fractions enriched for these vesicular structures contained both the negative- and positive-sense strand of PEMV RNA, implicating these structures as the site of viral replication. Supporting this view, PEMV-induced RNA-dependent RNA polymerase activity was also localized to both the vesicular and nuclear fractions. Indeed, isolated healthy pea nuclei were also demonstrated to support replication of PEMV RNA in *in vitro* assays. Combined, these data support the assignment of these membranous vesicles as the replicative complex of PEMV.

In addition to their role in viral replication, these vesicles are also found associated with, or traversing through plasmodesmata of mesophyll, phloem parenchyma, sieve element and fully developed sieve tubes. The systemic mobility of these complexes coupled with their demonstrated polymerase activity suggests a possible role for these structures in the systemic spread of PEMV infection. In addition, transient, electron-dense, dagger-like structures protruding into the cytoplasm of healthy and necrotic companion cells and sieve elements, can often be found associated with plasmodesmata. It is not clear whether this structure is of viral origin or a possible defense reaction of the host. An additional cytopathic feature characteristic of PEMV infection is the presence of elongated feather-like crystalline inclusions in epidermal cells. These anomalous inclusions are always surrounded by membranes and are rich in ribosomes and polyribosomes. They are often associated with the perinuclear space or with the vesicular membranes derived from the perinuclear space. Occasionally these structures are evident in apparently healthy tissue, and may reflect a stress-related response by the host. Their functional significance and origin are currently unknown.

In viruliferous aphids, PEMV virions were identified in the gut lumen, fat bodies, epithelium and muscle cells of the midgut, in hemocytes and in electron-dense viroplasm-like structures hypothesized to be part of the lysosomal apparatus. Electron microscopic analysis of aphid salivary systems demonstrated an association of PEMV virions with the basal lamina and plasma-membrane complex of the accessory salivary gland, establishing a directional membrane-mediated shuttling of virions from the hemocoele to the stylet. In contrast, there was no evidence of similar association of virions of the aphid-nontransmissible isolate of PEMV with these salivary structures. These observations are consistent with similar observations described for luteoviruses.

Future Perspectives

Symbiosis is rarely used to describe interactions between plant viral genomes. One reason is that most of those interactions take place either between distinct and independent viruses in a mixed infection (leading to various degrees of mutualism), or between the components of a virus with a multipartite genome. Extensive analysis of the molecular biology of PEMV suggests that the interactions between its genomic components are actually a combination of both scenarios. Thus, in this curious partnership of the RNAs of PEMV, the vital functions currently lost from each RNA are provided in *trans* by the other component resulting in a highly infectious entity. Now that the sequence and the genomic organization of both viral RNAs is determined, the contribution of various ORFs to the infection cycle of PEMV can be further delineated. The mechanical transmissibility and the high virion yield of PEMV provide additional advantages to the use of PEMV to answer lingering questions related not only to this virus but possibly to related luteoviruses and umbraviruses.

For example, the isolation and further characterization of the VPg will provide important insights into the replication strategies utilized by PEMV.

In addition, the involvement of RNA2 (ORFs 3 and 4 in particular) in spreading the infection of PEMV beyond the phloem tissue raises questions about the mechanism involved and whether RNA2 can indeed change the phloem restriction characteristic of other luteoviruses.

See also: **Luteovirus; Umbraviruses.**

Further Reading

Demler SA, de Zoeten GA, Adam G and Harris KF (1996) Pea enation mosaic virus: properties and aphid transmission. In: Harrison BD and Murant AF (eds) *The Viruses: Plant Viruses. Viruses with Bipartite Genomes and Isometric Particles*, New York: Plenum Press.

Demler SA, Rucker-Feeney DG, Skaf JS and de Zoeten GA (1997) Expression and suppression of circulative aphid transmission in pea enation mosaic virus. *J. Gen. Virol.* 78: 511.

de Zoeten GA and Gaard G (1983) Mechanisms underlying systemic invasion of pea plants by pea enation mosaic virus. *Intervirology* 19: 85.

Harris KF, Bath JE, Thottappilli G and Hooper GR (1975) Fate of pea enation mosaic virus in PEMV-injected pea aphids. *Virology* 65: 148.

Powell CA and de Zoeten GA (1977) Replication of pea enation mosaic virus in isolated pea nuclei. *Proc. Natl. Acad. Sci. USA* 74: 2919.

Skaf JS, Rucker DG, Demler SA, Wobus CE and de Zoeten GA (1997) The coat protein is dispensable for the establishment of systemic infections by pea enation mosaic enamovirus. *Mol. Plant–Microbe Interact.* 10: 929.

PECLUVIRUSES

D. V. R. Reddy and **P. Delfosse**, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, India

M. A. Mayo, Scottish Crop Research Institute (SCRI), Invergowrie, Dundee, Scotland

History

Pecluviruses are viruses so far found in Africa and the Indian Subcontinent that are responsible for the disease called 'clump' in crops of groundnut (= peanut, *Arachis hypogaea*). Clump disease is one of the many biotic and abiotic stress factors that have been shown to contribute to low yields of groundnut. It has been estimated to cause annual losses on a global scale of about 38 million US dollars.

Clump disease was first reported in 1927 in groundnut crops in India. Subsequently, in 1931, a similar disease was reported from West Africa. The economic importance of this disease was recognized following systematic surveys in the Indian subcontinent as well as in parts of West Africa.

Taxonomy and Classification

In taxonomic parlance, pecluviruses comprise the genus *Pecluvirus*. The genus contains two species, *Peanut clump virus* (PCV) and *Indian peanut clump virus* (IPCV). The genus is a relatively recent taxonomic construction and has not been assigned a family. Previously, the two species were considered to

Future Perspectives

Symbiosis is rarely used to describe interactions between plant viral genomes. One reason is that most of those interactions take place either between distinct and independent viruses in a mixed infection (leading to various degrees of mutualism), or between the components of a virus with a multipartite genome. Extensive analysis of the molecular biology of PEMV suggests that the interactions between its genomic components are actually a combination of both scenarios. Thus, in this curious partnership of the RNAs of PEMV, the vital functions currently lost from each RNA are provided in *trans* by the other component resulting in a highly infectious entity. Now that the sequence and the genomic organization of both viral RNAs is determined, the contribution of various ORFs to the infection cycle of PEMV can be further delineated. The mechanical transmissibility and the high virion yield of PEMV provide additional advantages to the use of PEMV to answer lingering questions related not only to this virus but possibly to related luteoviruses and umbraviruses.

For example, the isolation and further characterization of the VPg will provide important insights into the replication strategies utilized by PEMV.

In addition, the involvement of RNA2 (ORFs 3 and 4 in particular) in spreading the infection of PEMV beyond the phloem tissue raises questions about the mechanism involved and whether RNA2 can indeed change the phloem restriction characteristic of other luteoviruses.

See also: **Luteovirus; Umbraviruses.**

Further Reading

Demler SA, de Zoeten GA, Adam G and Harris KF (1996) Pea enation mosaic virus: properties and aphid transmission. In: Harrison BD and Murant AF (eds) *The Viruses: Plant Viruses. Viruses with Bipartite Genomes and Isometric Particles*, New York: Plenum Press.

Demler SA, Rucker-Feeney DG, Skaf JS and de Zoeten GA (1997) Expression and suppression of circulative aphid transmission in pea enation mosaic virus. *J. Gen. Virol.* 78: 511.

de Zoeten GA and Gaard G (1983) Mechanisms underlying systemic invasion of pea plants by pea enation mosaic virus. *Intervirology* 19: 85.

Harris KF, Bath JE, Thottappilli G and Hooper GR (1975) Fate of pea enation mosaic virus in PEMV-injected pea aphids. *Virology* 65: 148.

Powell CA and de Zoeten GA (1977) Replication of pea enation mosaic virus in isolated pea nuclei. *Proc. Natl. Acad. Sci. USA* 74: 2919.

Skaf JS, Rucker DG, Demler SA, Wobus CE and de Zoeten GA (1997) The coat protein is dispensable for the establishment of systemic infections by pea enation mosaic enamovirus. *Mol. Plant–Microbe Interact.* 10: 929.

PECLUVIRUSES

D. V. R. Reddy and **P. Delfosse**, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, India

M. A. Mayo, Scottish Crop Research Institute (SCRI), Invergowrie, Dundee, Scotland

History

Pecluviruses are viruses so far found in Africa and the Indian Subcontinent that are responsible for the disease called 'clump' in crops of groundnut (= peanut, *Arachis hypogaea*). Clump disease is one of the many biotic and abiotic stress factors that have been shown to contribute to low yields of groundnut. It has been estimated to cause annual losses on a global scale of about 38 million US dollars.

Clump disease was first reported in 1927 in groundnut crops in India. Subsequently, in 1931, a similar disease was reported from West Africa. The economic importance of this disease was recognized following systematic surveys in the Indian subcontinent as well as in parts of West Africa.

Taxonomy and Classification

In taxonomic parlance, pecluviruses comprise the genus *Pecluvirus*. The genus contains two species, *Peanut clump virus* (PCV) and *Indian peanut clump virus* (IPCV). The genus is a relatively recent taxonomic construction and has not been assigned a family. Previously, the two species were considered to

be strains of a single species (PCV) that was classified as a furovirus together with other fungus-transmitted viruses with rod-shaped particles. Recent molecular data concerning these viruses have revealed much heterogeneity and several new genera have been created to accommodate the variety of molecular characters.

The current classification does not seem to be very robust because the extent of the sequence variation among the three serotypes of IPCV is as great as is the variation between any serotype and the one isolate of PCV sequenced to date. The only clear feature that distinguishes PCV from IPCV at present is the geographical location in which an isolate is found.

Virion, Genome and Protein Properties

The structural and molecular features that distinguish pecluviruses from other viruses with rod-shaped particles are (1) particles with lengths of about 245 nm and 190 nm and diameters of about 21 nm, (2) genomes that contain a triple gene block that encodes proteins thought to be involved in virus movement, (3) genomes that lack an open reading frame (ORF) expressed by readthrough suppression of the coat protein gene termination codon. IPCV particles are rod shaped, 24 nm in diameter, with two predominant lengths of 249 and 184 nm. PCV particles contain two predominant lengths 245 and 190 nm. Both PCV and IPCV contain a single capsid protein of 24 kD. For more detail see Molecular Biology Section.

Geographic Distribution

In the Asian subcontinent, clump disease has been reported from the Indian States of Andhra Pradesh, Gujarat, Punjab, Rajasthan and Tamil Nadu, and from Pakistan in the states of Sindh and Punjab. In Africa it has been found in Burkina Faso, Niger Republique (Sadore, Maradi and Tarna), Mali, Côte d'Ivoire, Gambia and in Sénégal (Bambey, Kirene, Mbour, N Diongo, Pout, T Kaymor).

Symptoms and Host Range

Diseased plants are conspicuous because of their severe stunting and dark green appearance. They often occur in patches in fields and the disease recurs in more or less the same position in the field in successive peanut crops. Symptoms appear on young leaflets as mottling, mosaic and chlorotic rings. However, when these leaflets mature, they turn dark green with or without faint mottling. Plants infected when relatively young become severely stunted; although the infected plants can produce flowers, any pods that are formed are not well developed. Plants infected at a later stage also become stunted with shortened internodes, and have dark green leaflets. Yield losses up to 60% have been recorded from such late-infected plants.

IPCV and PCV have extremely wide host ranges, which include monocotyledonous and dicotyledonous plants. They include such economically important crops as peanut, wheat, barley, maize, sorghum, pearl millet, finger millet and pigeonpea. The symptoms induced by various IPCV isolates differed in several hosts, namely in *Canavalia ensiformis, Nicotiana clevelandii, N. benthamiana* and the hybrid *N. clevelandii* × *N. glutinosa*. The symptoms induced in *Chenopodium amaranticolor* by various PCV isolates collected from Senegal, Burkina Faso and Niger were shown to differ markedly.

Serological Relationships

Tests conducted with polyclonal antisera using ELISA and immunosorbent electron microscopy have shown that IPCV isolates can be grouped into three serotypes, namely IPCV-Hyderabad (IPCV-H), IPCV-Durgapura (IPCV-D) and IPCV-Ludhiana (IPCV-L). All IPCV isolates are serologically distinct from PCV isolates, and vice versa. By using tests with monoclonal antibodies, a number of PCV isolates have been placed in five distinct groups. None of seven monoclonal antibodies produced against PCV reacted in triple antibody sandwich ELISA with one of the Indian isolates related to IPCV-D.

Transmission

All IPCV and PCV isolates can be transmitted by mechanical sap inoculation with sap from infected plants. However, extracts of infected plants of some hosts, e.g. peanut, were difficult to transmit mechanically. PCV and IPCV can be transmitted through peanut seed. IPCV was also shown to be transmissible in seed of infected plants of pearl millet, finger millet, foxtail millet, wheat and maize. IPCV has been shown to be transmitted by *Polymyxa* sp. However, direct evidence for transmission of PCV by *Polymyxa* has so far not been reported.

Life Cycle

The life cycle of IPCV/PCV and the vector *Polymyxa* sp. is given in **Fig. 1.** Dicotyledonous hosts do not permit multiplication of *Polymyxa* sp. and are therefore considered as fortuitous hosts that will not contribute to perpetuation of virus inoculum. Indeed virus-infected peanut roots or virus-infected peanut seed could not transmit or establish the disease.

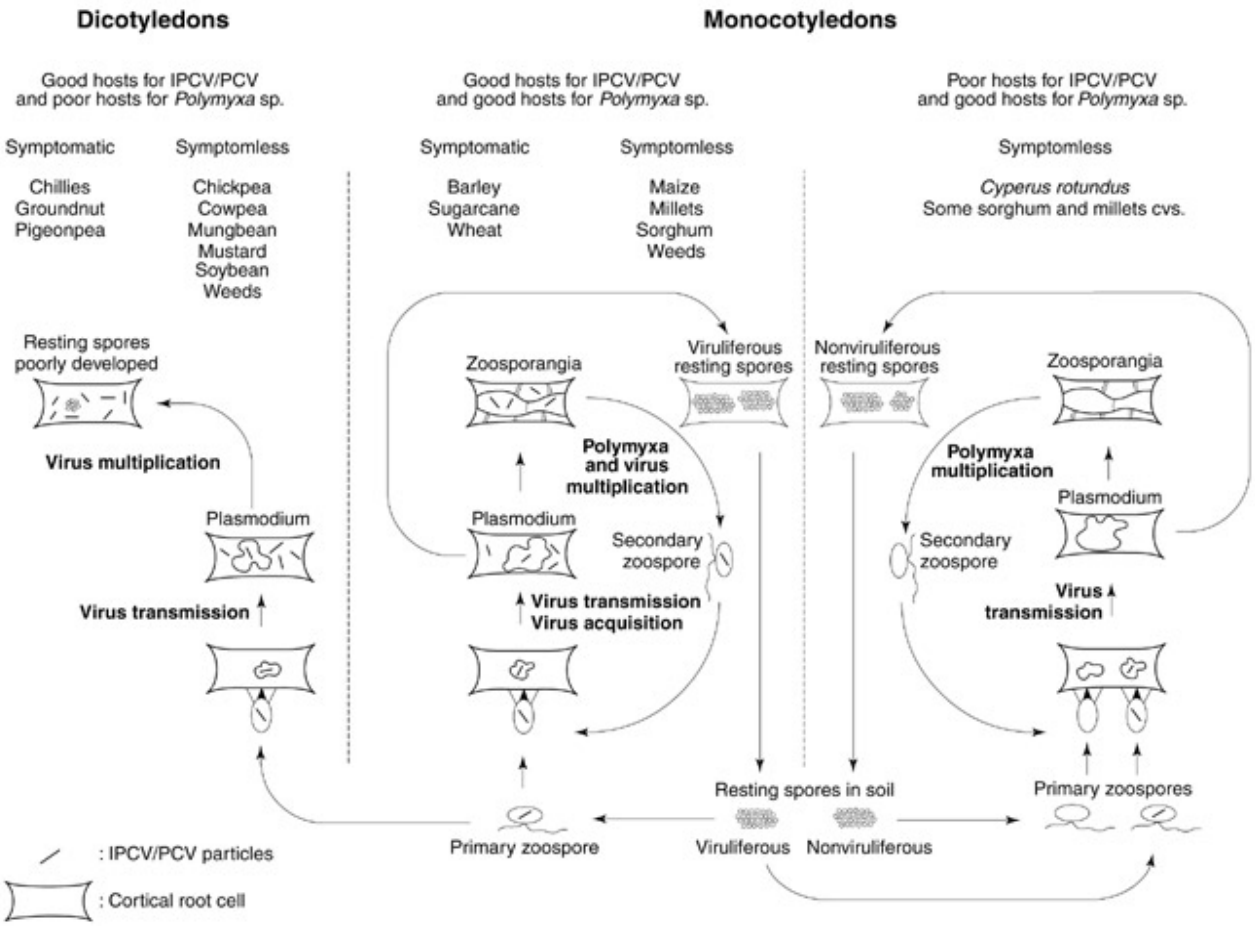


Figure 1 Life cycle of IPCV/PCV and the vector *Polymyxa* sp.

Monocotyledonous hosts were found to be the preferred hosts for multiplication of *Polymyxa* sp. They contribute to building up of *Polymyxa* sp. inoculum potential in the soil. Seed of millets, maize and wheat and rhizomatous grasses such as *Cynodon dactylon* are likely to contribute to the establishment of the disease in new areas by supporting both virus and fungus multiplication. Certain monocotyledonous weeds, e.g. *Cyperus rotundus*, and some sorghum and millet cultivars, although excellent hosts for *Polymyxa* sp., were found to be poor hosts for IPCV. They are likely to contribute to reduction of clump disease.

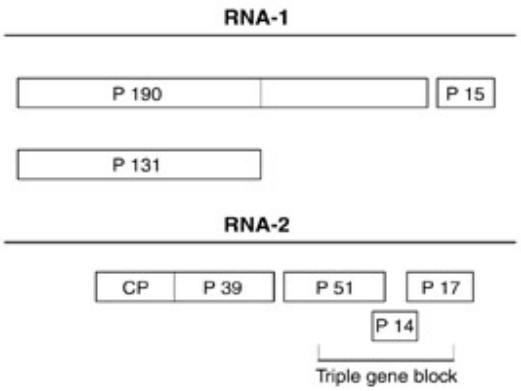


Figure 2 Genome organization of IPCV/PCV.

Molecular Biology

The single-stranded (ss) RNA genome of pecluviruses consists of two components each encoding more than one polypeptide. **Figure 2** illustrates the genome of PCV, that of IPCV is identical in arrangement.

Coding sequences

The 5′-most ORF of RNA-1 encodes a large polypeptide (c. 130 kDa). The ORF is followed immediately by another that is in frame with the termination codon of the 131 kDa polypeptide and which is expressed by readthrough of the UGA termination codon to generate a fusion polypeptide of c. 190 kDa

that contains the c. 130 kDa polypeptide at its 5′ end. A third ORF that encodes a c. 15 kDa polypeptide is downstream of the 190 kDa ORF and separated from it by a noncoding region of about 60 nucleotides. The amino acid sequence of the c. 190 kDa polypeptide contains domains with sequences similar to proteins with methyltransferase, helicase and RNA-dependent RNA polymerase activities (see **Fig. 2**). No function can be inferred from the sequence of the 15 kDa polypeptide.

RNA-2 is more complex. It encodes five polypeptides, each of which is expressed directly, apparently with no readthrough. The 5′-most ORF encodes the coat protein. The following ORF is expressed by leaky scanning by ribosomes of the c. 1 kb leader. The remaining three ORFs form a triple gene block and the encoded polypeptides (c. 51 kDa, c. 14 kDa and c. 17 kDa) are thought to play a role in virus movement within infected plants. No function can be inferred from sequence comparisons involving the 39 kDa polypeptide, although it is thought that the protein may be involved in the transmission of pecluviruses by *Polymyxa* sp.

Terminal noncoding sequences

The terminal noncoding sequences of pecluvirus RNA-1s are about 130 (5′) and 350 (3′) nucleotides in length. Those of RNA-2 are more diverse, between c. 390 and c. 500 nucleotides in length. There is no marked 5′ sequence feature common to all pecluviruses. RNA-1 and RNA-2 have similar 5′ noncoding region (NCR) for six to seven nucleotides and these sequences are shared between pecluvirus species. The 3′ NCR are c. 300 nucleotides in length and the c. 100 terminal nucleotides are almost identical among all pecluvirus RNAs sequenced so far. This sequence similarity has enabled the development of a hybridization probe that consists of the 3′-terminal 700 nucleotides of the sequence of IPCV-H serotype. It can detect any of the currently known IPCV serotypes as well as an isolate of PCV.

Sequence comparisons

The polypeptides encoded by pecluvirus RNA-1 are between 75% (P15) and 95% (readthrough part of P191) identical between species and show significant similarities with other viruses previously classified as furoviruses (e.g. 56% identity with polymerase of soil-borne wheat mosaic virus). The polypeptides encoded by RNA-2 are 39% (P39) to 89% (P14) identical between the species. The coat proteins are c. 60% identical and also show a significant similarity (c. 30% identity) with the coat protein of barley stripe mosaic virus (genus *Hordeivirus*). The triple gene block proteins resemble those of potato mop-top virus (genus *Pomovirus*) more than those of viruses in any other genera.

Control

After confirming the soil-borne nature of clump disease, initial experiments were centered on application of soil biocides and soil solarization. Although both the treatments were effective in reducing disease incidence, they were not economical to adopt and also the biocides that proved effective were known to be hazardous. Resistance to IPCV has so far not been located in cultivated or wild *Arachis* species. Therefore efforts have been concentrated on understanding the epidemiology of clump disease with the hope of devising cultural methods of control. These studies included identification of alternative hosts of the virus and the vector, and of factors that contribute to survival and spread of inoculum. As a result, the following measures have been recommended for controlling IPCV:

- Early planting before the onset of the monsoon and under judicious irrigation;
- Trap cropping with pearl millet, i.e. sowing a pearl millet crop at a high density and then two weeks after germination ploughing the entire crop into the soil and then planting with peanut;
- Avoiding rotation with such highly susceptible cereal hosts as maize or wheat;
- Continuous cropping with dicotyledonous hosts to reduce substantially the inoculum in the soil and thus contribute to low incidence of disease.

The methods recommended are ecofriendly and economical and are applicable even under smallholder farming conditions.

See also: **Furoviruses.**

Further Reading

Herzog E, Guilley H, Manohar SK *et al* (1994) Complete nucleotide sequence of peanut clump virus RNA 1 and relationships with other fungus-transmitted rod-shaped viruses. *J. Gen. Virol.* 75: 3147.

Miller JS, Wesley SV, Naidu RA, Reddy DVR and Mayo MA (1996) The nucleotide sequence of RNA 1 of Indian peanut clump furovirus. *Arch. Virol.* 141: 2301.

Ratna AS, Rao AS, Reddy AS *et al* (1991) Studies on transmission of Indian peanut clump virus disease by *Polymyxa graminis*. *Ann. Appl. Biol.* 118: 71.

Reddy DVR, Nolt BL, Hobbs HA *et al* (1988) Clump virus in India: isolates, host range, transmission and management. In: Cooper JI and Asher MJC (eds) *Viruses with*

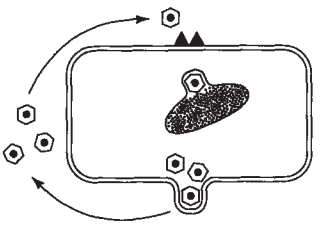
Fungal Vectors p. 239. Association of Applied Biologists, UK.

Sherwood JL and Rush CM (1996) Proceedings of 3rd International Working Group on Plant Viruses with Fungal Vectors. Denver: American Society of Sugar Beet Technologists.

Thouvenel JC, Fauquet C, Fargette D and Fishpool LDC (1988) Peanut clump virus in West Africa. In: Cooper JI and Asher MJC (eds) *Viruses with Fungal Vectors*, p. 247. Association of Applied Biologists, UK.

PERSISTENT VIRAL INFECTION

Maryann T. Puglielli and **Rafi Ahmed**, Emory University Vaccine Center, Atlanta, Georgia, USA



Introduction

Persistent viral infections have been and continue to represent significant health problems to the human population. Millions of people worldwide are chronically infected with pathogens such as human immunodeficiency virus (HIV-1 or -2), hepatitis B virus (HBV) and hepatitis C virus (HCV). **Table 1** illustrates the variety of viruses that can cause persistent infections in humans. This group of viruses is not limited solely to DNA-based types but includes several RNA-based viruses as well. These long-term infections have serious disease consequences with a diverse range including tumor formation, autoimmune diseases such as hemolytic anemia and arthritis, and immune system collapse as seen in acquired immune deficiency syndrome (AIDS). However, it is important to acknowledge that not all persistent viral infections lead to disease. There may be genetic and environmental factors at work; for example nasopharangeal carcinoma is found in unusually high rates in Chinese populations and Burkitts Lymphoma (BL), also an Epstein–Barr virus (EBV) associated cancer, has a high correlation with the incidence of malaria in areas of Africa. Persistent infections are categorized into two main types: chronic and latent infections. Chronic viral infection, as exemplified by HBV, is characterized by active, constant replication of virus progeny throughout the life span of the host. Latent infection occurs when the viral genome is present in host cells in the absence of virion production. An essential component of true latency is the ability for the virus genome to reactivate and reestablish virion production. Otherwise, in the absence of reactivation, latency becomes a dead-end in the life cycle of the virus. Herpes simplex virus (HSV), an alpha herpesvirus, often serves as the prototype for the study of latency and expresses nontranslated transcripts called latency associated transcripts (LATs) whose function remains unknown.

Several viruses, however, incorporate into their life cycle a combined strategy of latency and chronic infection. EBV, a gamma herpesvirus, initially infects epithelium of the oropharynx where viral replication is permissive. At this site B lymphocytes come into contact with EBV and become latently infected. Three different latency transcription programs that express different combinations of viral transcripts have been identified *in vivo*. Group I latency is likely the least visible to anti-EBV cytotoxic T lymphocytes (CTLs) as it involves expression of one viral protein, EBNA 1, which is necessary for genome maintenance in the host cell as an episome. Two small RNAs, EBERs, are also expressed during all forms of EBV latency whose effects on the immune response are discussed later. It has been proposed that this pool of latently-infected B cells come in contact with epithelium of the oropharynx where EBV actively replicates to produce infectious virions in the saliva 15–20% of the time for spread to new hosts. HIV-1 is another well-known example of a virus that combines latency with chronic infection. HIV-1 actively infects and replicates in CD4 T lymphocytes and monocytic macrophages. In some instances during the replication process, the DNA genome intermediate of this retrovirus is incorporated into host chromosomes where it lies dormant depending on the activation state of the cell.

Antiviral Immune Responses

The greatest adversary a virus must face during its replication life cycle is the host's immune response. To understand these selective pressures, it is worth-

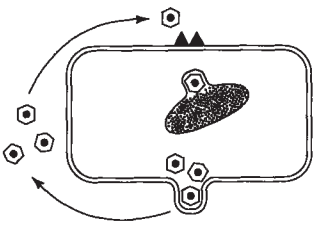
Fungal Vectors p. 239. Association of Applied Biologists, UK.

Sherwood JL and Rush CM (1996) Proceedings of 3rd International Working Group on Plant Viruses with Fungal Vectors. Denver: American Society of Sugar Beet Technologists.

Thouvenel JC, Fauquet C, Fargette D and Fishpool LDC (1988) Peanut clump virus in West Africa. In: Cooper JI and Asher MJC (eds) *Viruses with Fungal Vectors*, p. 247. Association of Applied Biologists, UK.

PERSISTENT VIRAL INFECTION

Maryann T. Puglielli and **Rafi Ahmed**, Emory University Vaccine Center, Atlanta, Georgia, USA



Introduction

Persistent viral infections have been and continue to represent significant health problems to the human population. Millions of people worldwide are chronically infected with pathogens such as human immunodeficiency virus (HIV-1 or -2), hepatitis B virus (HBV) and hepatitis C virus (HCV). **Table 1** illustrates the variety of viruses that can cause persistent infections in humans. This group of viruses is not limited solely to DNA-based types but includes several RNA-based viruses as well. These long-term infections have serious disease consequences with a diverse range including tumor formation, autoimmune diseases such as hemolytic anemia and arthritis, and immune system collapse as seen in acquired immune deficiency syndrome (AIDS). However, it is important to acknowledge that not all persistent viral infections lead to disease. There may be genetic and environmental factors at work; for example nasopharangeal carcinoma is found in unusually high rates in Chinese populations and Burkitts Lymphoma (BL), also an Epstein–Barr virus (EBV) associated cancer, has a high correlation with the incidence of malaria in areas of Africa. Persistent infections are categorized into two main types: chronic and latent infections. Chronic viral infection, as exemplified by HBV, is characterized by active, constant replication of virus progeny throughout the life span of the host. Latent infection occurs when the viral genome is present in host cells in the absence of virion production. An essential component of true latency is the ability for the virus genome to reactivate and reestablish virion production. Otherwise, in the absence of reactivation, latency becomes a dead-end in the life cycle of the virus. Herpes simplex virus (HSV), an alpha herpesvirus, often serves as the prototype for the study of latency and expresses nontranslated transcripts called latency associated transcripts (LATs) whose function remains unknown.

Several viruses, however, incorporate into their life cycle a combined strategy of latency and chronic infection. EBV, a gamma herpesvirus, initially infects epithelium of the oropharynx where viral replication is permissive. At this site B lymphocytes come into contact with EBV and become latently infected. Three different latency transcription programs that express different combinations of viral transcripts have been identified *in vivo*. Group I latency is likely the least visible to anti-EBV cytotoxic T lymphocytes (CTLs) as it involves expression of one viral protein, EBNA 1, which is necessary for genome maintenance in the host cell as an episome. Two small RNAs, EBERs, are also expressed during all forms of EBV latency whose effects on the immune response are discussed later. It has been proposed that this pool of latently-infected B cells come in contact with epithelium of the oropharynx where EBV actively replicates to produce infectious virions in the saliva 15–20% of the time for spread to new hosts. HIV-1 is another well-known example of a virus that combines latency with chronic infection. HIV-1 actively infects and replicates in CD4 T lymphocytes and monocytic macrophages. In some instances during the replication process, the DNA genome intermediate of this retrovirus is incorporated into host chromosomes where it lies dormant depending on the activation state of the cell.

Antiviral Immune Responses

The greatest adversary a virus must face during its replication life cycle is the host's immune response. To understand these selective pressures, it is worth-

Table 1 Viruses that persist in humans[a]

Virus group	*Site of persistence*	*Consequence*
DNA viruses		
Adenovirus	Adenoids, tonsils, lymphocytes	None known
Cytomegalovirus	Kidneys, salivary glands, lymphocytes?, macrophages, stromal cells	Pneumonia, retinitis
Epstein–Barr virus	Pharyngeal epithelial cells, B cells	Infectious mononucleosis, Burkitt's lymphoma, nasopharyngeal carcinoma, non-Hodgkin's lymphoma, oral hairy leukoplakia
Herpes simplex virus 1 and 2	Sensory ganglia neurons	Cold sores, genital herpes, encephalitis, keratitis
Human herpesvirus 6	Lymphocytes	Exanthem subitum
Human herpesvirus 8	PBMC? Endothelial spindle cells?	Kaposi sarcoma
Varicella zoster virus	Sensory ganglia neurons and/or satellite cells	Varicella, zoster
Hepatitis B virus	Hepatocytes	Hepatitis, hepatocellular carcinoma
Hepatitis D virus	Hepatocytes	Exacerbation of chronic HBV infection
Papillomavirus	Epithelial skin cells	Papilloma, carcinomas
Parvovirus B19	Erythroid progenitor cell in bone marrow	Aplastic crisis in hemolytic anemia, chronic bone marrow deficiency
Polyomavirus BK	Kidney	Hemorrhagic cystitis
Polyomavirus JC	Kidney, oligodendrocytes in CNS	Progressive multifocal leukoencephalopathy
RNA viruses		
Hepatitis C virus	Hepatocytes	Hepatitis, hepatocellular carcinoma
Measles virus[b]	Neurons and supporting cells in CNS	Subacute sclerosing panencephalitis, measles inclusion body encephalitis
Rubella virus[b]	CNS	Progressive rubella panencephalitis, insulin-dependent diabetes?, juvenile arthritis?
Human immunodeficiency virus	CD4 T cells, monocytes/macrophages, microglia	AIDS
Human T-cell leukemia virus I	T cells	T-cell leukemia, tropical spastic paraparesis, polymyositis
Human T-cell leukemia virus II	T cells	None known

[a] Reproduced with permission from Ahmed R and Biron CA (1999) Immunity to viruses. In: Paul WE (ed.) *Fundamental Immunology*, 4th edn, Philadelphia: Lippincott-Raven
[b] Measles and rubella viruses typically cause acute infections. However, in rare instances, these viruses have been shown to persist in the CNS.
AIDS, autoimmune deficiency syndrome; CNS, central nervous system; PBMC, peripheral blood mononuclear cells.

while reviewing the components of the antiviral immune response, as shown in **Table 2**. T lymphocytes and antibodies are the two main antigen-dependent defenses against a viral infection. T lymphocytes are composed of two populations, CD8 and CD4. A major function of virus-specific CD8 T cells is to kill an infected cell by one of two mechanisms. (1) Perforin monomers and granzymes (serine proteases) are released onto the target cell surface where pore-like perforin polymers assemble and punch holes in the target cell membrane. This hole punching and the action of granzymes ultimately leads to apoptosis of the infected cell. (2) Upon activation of the CD8 T cell, Fas ligand (FasL) is upregulated on the T cell surface and binds to Fas on the surface of the infected cell. This crosslinking of FasL and Fas also leads to apoptosis of the infected cell. Of these two killing pathways, the dominant mechanism for virus control *in vivo* appears to be perforin-mediated killing due to the faster kinetics of target cell destruction as compared to the Fas/FasL pathway. In addition, some cells do not express Fas and as such would be resistant to Fas/FasL-mediated apoptosis. Of equal importance to direct killing of infected cells is the ability of CD8 T cells to produce and secrete antiviral cytokines such as interferon gamma (IFN-γ) and tumor necrosis factor (TNF). An absolute requirement for the initiation of these antiviral effector responses is the interaction of the T cell receptor (TCR) on the T lymphocyte with a major histocompatability complex (MHC) molecule presenting a processed viral peptide on the surface of the infected cell. CD8 T lymphocytes recognize peptides associated with MHC class I molecules whereas CD4 T lymphocytes recognize viral peptides associated with MHC class II molecules. These target

Table 2 Components of antiviral immunity

Effector system	*Recognition molecule*	*Mechanism of viral control*
Specific immune response		
Antibody	Surface glycoproteins or outer-capsid proteins of virus particles	Neutralization of virus
	Viral glycoproteins expressed on membrane of infected cells	Antibody-complement mediated killing and antibody-dependent cell-mediated cytotoxicity (ADCC) of virally infected cells
CD4+ T cells	Viral peptides (10–20-mers) presented by MHC class II molecules. The peptides can be derived from any viral protein internal or surface. The limiting factor is the ability to bind MHC.	Release of antiviral cytokines (IFN-γ, TNF) and activation of macrophages. Killing of virally infected cells? Optimize antibody responses to virus. Optimize CD8 T cell function.
CD8+ T cells	Viral peptides (9-mers) presented by MHC class I molecules of infected cells. The peptides can be derived from any viral protein, the main limiting factor being the processing and binding affinity of the peptide for MHC molecules.	Killing of virally infected cells. Release of antiviral cytokines (IFN-γ and TNF) and activation of macrophages
Innate immune response		
Natural killer cells	Not known	Release of IFN-γ, participation in ADCC, direct lysis of virus infected cells?
IFN-α/β	Not applicable	Inhibition of virus replication, induction of NK activity

peptides are derived from all proteins of the virus, both internal and external.

CD4 T lymphocytes are known as helper T cells because they enhance CD8 T cell and B cell functions. Like CD8 T cells, CD4 T cells also produce antiviral cytokines. For poorly understood reasons, depending on the viral infection, CD4 T lymphocytes have a varying degree of importance in supporting the CD8 T cell (CTL) response. Acute lymphocytic choriomeningitis virus (LCMV) infection can be controlled initially in the absence of CD4 T cells. However, CTL responses to vesicular stomatitis virus (VSV), influenza and herpes simplex virus (HSV) are much more dependent on these helper cells. In addition, more recent evidence suggests a crucial role for CD4 T cells in maintaining CD8 T cell responses to sustained chronic viral infections such as LCMV and HIV-1. This may be due to interleukin 2 (IL-2) production by CD4 T cells that leads to the survival of CD8 T cells over time or perhaps by the CD4 T cells stimulating the infected cell to increase expression of costimulatory molecules on the infected cell. Clonal expansion and differentiation of virus-specific B cells is oftentimes, but not always, dependent on CD4 T cell function. Likewise, it has been demonstrated that antibody responses to viral infections are greatly compromised in the absence of CD4 T cells.

B lymphocytes, through production of antibodies, are involved in the recognition and elimination of free virus and virus infected cells whereas T lymphocytes are adapted solely for destruction of virally infected cells via the TCR/MHC interactions described above. Antibodies serve to limit viral spread by neutralizing the virus directly by opsonization of viral particles or by tagging a virus-infected cell for destruction by antibody-dependent cell-mediated cytotoxicity (ADCC). Antibodies against viral glycoproteins tend to be more effective at neutralizing and eliciting ADCC than those against more internal viral proteins. Antiviral antibodies can remain in blood serum for years after infection and serve as a first line of defense against secondary infections.

During the time it takes to organize and mount an adaptive, virus-specific immune response, the innate immune response provides the initial line of defense for first exposure to a virus. Natural killer (NK) cells are the main component of this branch of the immune system. These cells produce IFNγ (type 2 interferon) and take part in ADCC. They may also directly lyse infected cells although it is not known how they distinguish virus-positive from virus-negative cells. A second component of the innate immune response is manifest in the ability of virally infected cells to produce antiviral type 1 interferons such as IFN α/β.

Evasion of Host Immune Responses

Given the complexity of an antiviral immune response, there are many ways in which a virus can subvert this defense and persist in the host. **Table 3** provides an outline of these strategies. As described above in EBV group I latency, HSV latency and HIV-1

Table 3 Viral strategies for evading the immune system[a]

Escape mechanism	*Example*[b]
Restricted gene expression; virus remains latent in the cell with minimal to no expression of viral proteins	HSV and VZV in latently infected neurons, EBV in B cells, HIV in resting T cells.
Infection of sites not readily accessible to the immune system	HSV, VZV, measles, and rubella persistence in neurons/CNS. CMV, polyomaviruses BK and JC in the kidney. EBV and CMV in the salivary gland. Papillomaviruses in the epidermis.
Inhibition of NK function	CMV protein UL18 is a MHC class I homologue that inhibits NK activity.
Antigenic variation; virus rapidly evolves and mutates antigenic sites that are critical for recognition by antibody and T cells	Antibody escape variants in lentiviruses. CTL escape variants in HIV, EBV, and HBV. T-cell receptor antagonism by HIV and HBV variants.
Suppression of cell-surface molecules required for T-cell recognition	Suppression of MHC class I molecules by adenoviruses, CMV, HSV, and HIV. Decreased expression of cell adhesion molecules LFA-3 and ICAM-1 by EBV. Suppression of MHC class II molecules by CMV, HIV, and measles.
Interference with antigen processing and presentation	HSV ICP47 protein and CMV U6 protein interfere with TAP to inhibit MHC class I antigen presentation. EBV protein EBNA-1 contains Gly-Ala repeats that confer resistance to proteasome mediated degradation and subsequent MHC class I presentation. CMV protein pp65 phosphorylates the CMV immediate early protein and inhibits its processing/presentation.
Viral defense molecules that interfere with the function of antiviral cytokines and chemokines	Adenovirus proteins E3 and E1B protect infected cells from lysis by TNF. Adenovirus VA RNA, EBV EBER RNA, HIV TAR and TAT RNA, K3L and E3L of vaccinia virus, NS5A of hepatitis C virus, NSP3 of porcine group C rotavirus, and σ3 of reovirus inhibit function of interferon. EBV protein BCRF1 (a homologue of IL-10) blocks synthesis of cytokines such as IL-2 and IFN-γ.
Immunologic tolerance	Clonal deletion/anergy of virus-specific CTLs in HBV carriers, HIV(?).
Cell-to-cell spread by snycytia	SSPE caused by measles.

[a] Reproduced with permission from Ahmed R and Biron CA (1999) Immunity to viruses. In: Paul WE (ed) *Fundamental Immunology,* 4th edn. Philadelphia: Lippincott-Raven.
[b] Only examples of viruses known to persist in humans are cited.
HSV, herpes simplex virus; VZV, varicella zoster virus; EBV, Epstein–Barr virus; CNS, central nervous system; CMV, cytomegalovirus; MHC, major histocompatability complex; NK, natural killer; CTL, cytotoxic T lymphocytes; HIV, human immunodeficiency virus; HBV, hepatitis B virus; TAP, transporter associated with antigen processing; IL, interleukin; IFN, interferon.

latency, viruses can restrict the repertoire of genes they express or even become transcriptionally quiescent. This strategy renders the pathogen effectively invisible to the immune system.

Viruses can also attack areas of the body that are immune privileged. The central nervous system (CNS) is targeted by several viruses among them HIV-1, LCMV, HSV, varicella zoster virus (VZV) and measles. At least two factors favor viral persistence in the CNS: the presence of the blood–brain barrier which limits lymphocyte trafficking through the CNS and the presence of neurons which express little to no MHC class I and no class II molecules therefore preventing T cell recognition. Another organ often targeted is the kidney. Though T cells commonly move through this organ, it is thought that infected epithelial cells are not accessible due to the presence of a continuous basement membrane and vascular endothelium covering them. Similar anatomical blockades are at work in epithelial surfaces of secretory glands (e.g. the salivary gland) and the epidermis with its protective basement membrane.

Mutations often happen during the replication of viral genomes via the error propensity of reverse transcriptase, RNA based RNA polymerases and to a lesser extent, DNA polymerases. Though many of these mutations are nonbeneficial, a small number result in the alteration of a T cell or antibody epitope. This alteration prevents recognition of the mutant antigen and therefore of the virus by antibodies and T cells that would recognize the parental epitope. This 'antigenic drift' phenomenon has been observed in HIV-1, EBV, HCV and HBV infections. However, it should be noted that although these variants have a selective advantage over the parental strain, they do not always become the predominant population in the

host. There have also been cases of TCR antagonism reported with variants arising from HIV-1 and HBV infections. Mutant antigens from these viruses are recognized by CD8 T cells, but instead of delivering an activation signal, an antagonistic signal renders the T cell nonfunctional. TCR antagonism would allow for the continued survival of parental, nonmutated strains of virus by deactivating CTLs that would recognize them.

Antigen presentation is a necessity for the preservation of T cell effector function. Viruses have also tapped into this vital requirement in two ways: (1) by suppressing cell surface molecules needed for T cell recognition such as MHC class I and class II and (2) by interfering with the processing and transportation of viral antigens to the cell surface. Human adenoviruses and human cytomegalovirus (HCMV) have evolved to block terminal glycosylation of viral antigens and thus their proper processing, block the transport of mRNA encoding MHC class I, retain MHC class I molecules in pre-Golgi complexes, and promote rapid degradation of newly synthesized MHC class I molecules. Likewise HCMV, HIV-1 and measles virus have been shown to interfere with MHC class II by blocking IFN-γ-mediated upregulation of MHC class II mRNA transcription. HSV ICP47 gene product is a classic example of attacking antigen transport. It binds to the transporter associated with antigen processing (TAP) and prevents antigen transport to the endoplasmic recticulum. EBV EBNA-1, the only protein expressed during group I latency, has been shown to be resistant to antigen processing in the proteasome due to a series of gly-ala repeats. Though low numbers of EBNA-1 specific CTLs can be found *in vivo*, their presence is due to cross-priming via exogenous processing of EBNA-1. Viruses have also evolved the capacity to interfere with the expression of adhesion molecules that are necessary for prolonged T cell contact with target cells and optimal activation. EBV infection has been demonstrated to decrease expression of ICAM-1 and LFA-3 allowing some BL cell lines to escape CTL killing.

Lastly, cytokines and chemokines are important messengers for the coordination and orchestration of the immune response. TNF is targeted for example by poxvirus T2 protein that acts as a TNF receptor homologue. Secretion of this protein from infected cells results in the quenching of free TNF from the bloodstream. EBV BCRF-1, a homologue of IL-10, can block the synthesis of IFN-γ and IL-2. Adenovirus VA RNA, HIV TAR RNA, and EBV EBER RNA can block the antiviral function of IFN-α/β. There are several additional examples one could add to this list of well-defined anticytokine viral proteins as well as many more viral proteins with suspected, though unconfirmed, homologies to chemokines and their receptors.

Challenges for the Future

Virology and immunology are but two sides of the same coin. Viruses have done well to identify for immunologists important components of the immune response to these pathogens. The mechanisms described here for evading the host's immune response are diverse. Unfortunately, persistent viruses commonly employ several of these tricks as exemplified here. However, the role that these strategies play in persistence *in vivo* is still unclear. Most of the experiments that led to the discovery of these anti-immune response mechanisms were done *in vitro*. To date, antigenic shift, MHC class I suppression, and cytokine interference have not been demonstrated to directly affect persistence of viruses *in vivo*.

Prophylactic vaccines have proven to be very effective against acute infections such as polio, measles, mumps, rubella and smallpox. There is also a very effective prophylactic vaccine for HBV, a normally chronic infection. This vaccine, based on HBV surface antigen, does not work therapeutically for individuals with established HBV infections. The lack of an effective treatment to eliminate pre-existing persistent infections represents a significant gap in the physician's arsenal of weapons against viral infections. Treatment strategies targeting antigen presentation by dendritic cells may also prove helpful. Candidate vaccines, drugs, or combination treatments for the elimination of established chronic viral infections will need to be as sophisticated as the viruses themselves by allowing for countermeasures to T cell suppression, cytokine function suppression and latency. Until treatments for established persistent viral infections exist, these pathogens will continue to present a significant problem for human health worldwide.

See also: **Adenoviruses (*Adenoviridae*): Animal viruses; General features; Malignant transformation and oncology; Molecular biology; Cytomegaloviruses (*Herpesviridae*): Animal cytomegaloviruses; General features (human); Molecular biology (human); Murine cytomegaloviruses; Epstein-Barr virus (*Herpesviridae*): General features; Molecular biology; Herpes simplex viruses (*Herpesviridae*): General features; Molecular biology; Human immunodeficiency viruses (*Retroviridae*): Anti-retroviral agents; General features; Molecular biology; Immune response: Cell**

mediated Immune response; General features; Latency; Transplantation and virus infections.

Further Reading

Ahmed R and Gray D (1996) Immunological memory and protective immunity: understanding their relation. *Science* 272: 54.

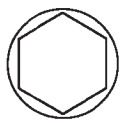
Ahmed R, Morrison LA and Knipe DM (1996) Persistence of Viruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 219. Philadelphia, Lippincott-Raven.

Ahmed R and Biron CA (1999) Immunity to viruses. In: Paul WE (ed.) *Fundamental Immunology*, 4th edn, p. 1295. Philadelphia: Lippincott-Raven.

Ploegh HL (1998) Viral strategies of immune evasion. *Science* 280: 248.

Peste des Petits Ruminants *see* **Rinderpest and Distemper Viruses**

PHAGE ϕ6 (CYSTOVIRIDAE)



Dennis H. Bamford, Institute of Biotechnology, University of Helsinki, Biocenter, Finland

Discovery and Classification

Investigation of bacteriophages of phytopathogenic pseudomonads led to the discovery of a virus (ϕ6) which was extremely sensitive to organic solvents and detergents. The first reports in 1973 described two unique features: this spherical virus had a lipid envelope and the genome was composed of double-stranded RNA (dsRNA). Phage ϕ6 was, until very recently, the only member of the family *Cystoviridae*, genus *Cystovirus*. It appears now that ϕ6-like viruses can be isolated from sources such as degrading plant material. The host range of this virulent phage is restricted to pseudomonads. The largest number of sensitive strains is found among *glysinea*, *phaseolicola*, *syringae*, *tabaci* and *viridiflava* species.

Virus Particle

The overall diameter of the virus is about 85 nm. The spike protein extends from the membrane surface and the membrane is approximately 6 nm thick. The nucleocapsid has a diameter of 58 nm and the polymerase complex about 45 nm (**Fig. 1**). The mass determined for the virion is about 99 000 kDa and that for the nucleocapsid 40 000 kDa. The sedimentation values for these particles are 406S and 298S respectively. The density of the virion in Cs_2SO_4 is 1.22 g ml^{-1} and the corresponding value for the nucleocapsid is 1.33 g ml^{-1}. The virion is composed of approximately 70% protein, 20% lipid and 10% RNA. The spike protein P3 is associated with the integral membrane protein P6 to form an adsorption fusion complex. There are three additional hydrophobic membrane-associated proteins (P9, P10, P13). Between the membrane and the nucleocapsid, in close contact with the nucleocapsid surface protein P8, is a lytic enzyme (P5). Inside the shell of P8 is the icosahedral polymerase complex composed of four protein species (P1, P2, P4, P7). P1 forms the structural dodecahedral framework upon which the rest of the polymerase complex proteins are attached. P2 contains an RNA-dependent RNA polymerase active site. P4, a potent unspecific nucleoside triphosphate phosphohydrolase, provides the energy for RNA packaging. P7 is necessary for stable RNA

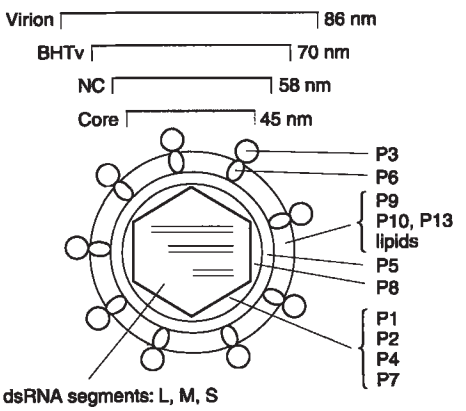


Figure 1 ϕ6 virion structure; for details see the text. BHTv, butylated hydroxy toluene treated virus which is devoid of the spike protein P3. (Courtesy of Dr Jarmo Juuti, Biocenter, University of Helsinki.)

mediated Immune response; General features; Latency; Transplantation and virus infections.

Further Reading

Ahmed R and Gray D (1996) Immunological memory and protective immunity: understanding their relation. *Science* 272: 54.

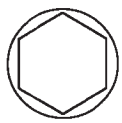
Ahmed R, Morrison LA and Knipe DM (1996) Persistence of Viruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 219. Philadelphia, Lippincott-Raven.

Ahmed R and Biron CA (1999) Immunity to viruses. In: Paul WE (ed.) *Fundamental Immunology*, 4th edn, p. 1295. Philadelphia: Lippincott-Raven.

Ploegh HL (1998) Viral strategies of immune evasion. *Science* 280: 248.

Peste des Petits Ruminants *see* **Rinderpest and Distemper Viruses**

PHAGE ϕ6 (CYSTOVIRIDAE)



Dennis H. Bamford, Institute of Biotechnology, University of Helsinki, Biocenter, Finland

Discovery and Classification

Investigation of bacteriophages of phytopathogenic pseudomonads led to the discovery of a virus (ϕ6) which was extremely sensitive to organic solvents and detergents. The first reports in 1973 described two unique features: this spherical virus had a lipid envelope and the genome was composed of double-stranded RNA (dsRNA). Phage ϕ6 was, until very recently, the only member of the family *Cystoviridae*, genus *Cystovirus*. It appears now that ϕ6-like viruses can be isolated from sources such as degrading plant material. The host range of this virulent phage is restricted to pseudomonads. The largest number of sensitive strains is found among *glysinea*, *phaseolicola*, *syringae*, *tabaci* and *viridiflava* species.

Virus Particle

The overall diameter of the virus is about 85 nm. The spike protein extends from the membrane surface and the membrane is approximately 6 nm thick. The nucleocapsid has a diameter of 58 nm and the polymerase complex about 45 nm (**Fig. 1**). The mass determined for the virion is about 99 000 kDa and that for the nucleocapsid 40 000 kDa. The sedimentation values for these particles are 406S and 298S respectively. The density of the virion in Cs_2SO_4 is 1.22 g ml^{-1} and the corresponding value for the nucleocapsid is 1.33 g ml^{-1}. The virion is composed of approximately 70% protein, 20% lipid and 10% RNA. The spike protein P3 is associated with the integral membrane protein P6 to form an adsorption fusion complex. There are three additional hydrophobic membrane-associated proteins (P9, P10, P13). Between the membrane and the nucleocapsid, in close contact with the nucleocapsid surface protein P8, is a lytic enzyme (P5). Inside the shell of P8 is the icosahedral polymerase complex composed of four protein species (P1, P2, P4, P7). P1 forms the structural dodecahedral framework upon which the rest of the polymerase complex proteins are attached. P2 contains an RNA-dependent RNA polymerase active site. P4, a potent unspecific nucleoside triphosphate phosphohydrolase, provides the energy for RNA packaging. P7 is necessary for stable RNA

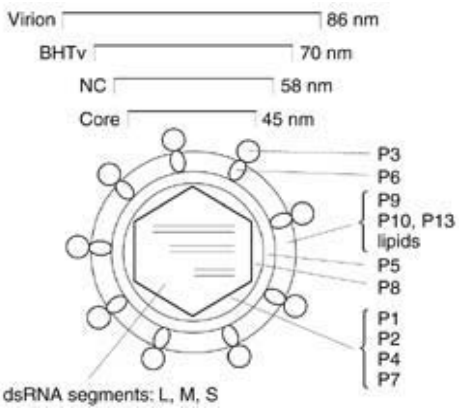


Figure 1 ϕ6 virion structure; for details see the text. BHTv, butylated hydroxy toluene treated virus which is devoid of the spike protein P3. (Courtesy of Dr Jarmo Juuti, Biocenter, University of Helsinki.)

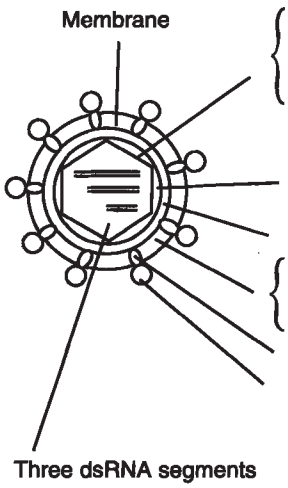


Table 1 Protein functions and proposed stochiometry

Protein	Molecular weight (kDa)	Molecules/virion (estimated)	Functions
P1	85.0	120	Core skeleton
P2	74.8	12	RNA polymerase
P4	35.0	120	Packaging NTPase
P7	17.2	60	Needed for packaging (+)-strand synthesis fidelity
P8	15.9	600	Nucleocapsid coat
P5	24.0	120	Membrane penetration lytic enzyme
P9	9.5	1200	Vesicle formation
P10	4.3	ND	Lysis factor
P13	7.6	ND	?
P6	17.2	60	Membrane fusion
P3	69.2	60	Cell attachment
P12	20.3	NA	Membrane assembly
P14	6.8	NA	?

NA, not applicable; ND, not determined.

encapsidation. The polymerase complex encloses three polycistronic genome segments, small (S, 2948 bp), medium (M, 4061 bp) and large (L, 6374 bp). The membrane phospholipids (8% cardiolipin, 35% phosphatidylethanolamine, and 57% phosphatidylglycerol) are derived from the host pool. The amount of lipid in the virus is enough to cover about half of the necessary surface area, the rest being protein.

Table 1 summarizes the protein functions and proposed stoichiometry.

Genome and Genetics

Phage ϕ6 has a high mutation rate. Temperature-sensitive mutants are found at a frequency of about 0.5%. In order to establish a genetic system for ϕ6 a nonsense suppressor host system was developed using a *Pseudomonas pseudoalcaligenes* strain. Nonsense, thermosensitive, missense or deletion mutants are available for all the genes. The mutants could be placed in three linkage groups consistent with the observation of three genomic dsRNA segments. All the genome segments are polycistronic. The nonsense mutants were assigned to the genes through analysis of phage-directed protein synthesis in the nonsuppressor host.

The entire genome is cloned and sequenced and the genes localized by the N-terminal analysis of the viral proteins (**Fig. 2**). The genes are organized so that the L segment codes for the viral polymerase complex proteins P1, P2, P4 and P7, as well as a small protein P14 of unknown function. The M segment codes for the attachment–fusion complex proteins P3 and P6, and two small membrane-associated proteins P10 and P13. The S segment codes for the nucleocapsid surface protein P8, the major membrane protein P9 and the

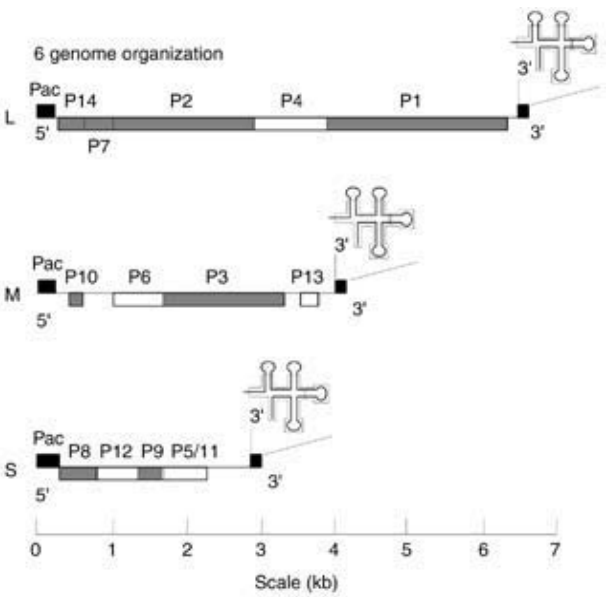


Figure 2 Map of the ϕ6 genome. S, M, L indicate the small, medium and large dsRNA genome segments respectively. The gene number corresponds to the protein number it encodes. (Courtesy of Mikko Frilander, Biocenter, University of Helsinki.)

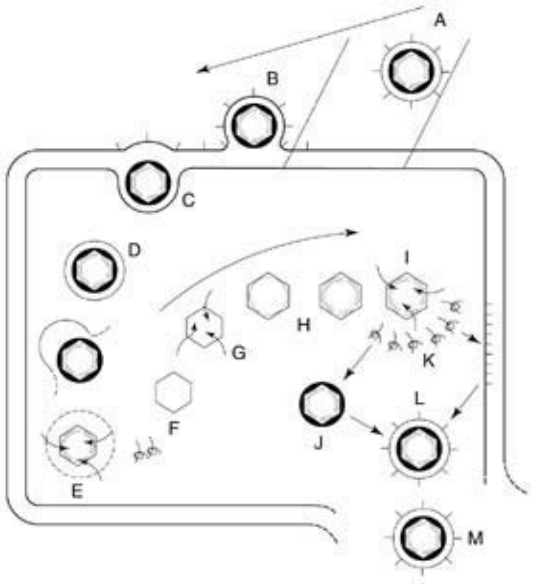


Figure 3 ϕ6 life cycle: (A) adsorption to host pilus; (B) fusion of the viral envelope with bacterial OM; (C) local digestion of cell wall peptidoglycan; (D) penetration of the NC through the CM; (E) NC uncoating and early transcription; (F) synthesis of early proteins and assembly of procapsids; (G) packaging of viral single-stranded RNAs; (H) dsRNA synthesis; (I) late transcription; (J) synthesis and assembly of NC shell protein; (K) synthesis of viral envelope proteins on the CM; (L) intracellular translocation of envelopes on the NCs; (M) release of progeny phage by cell lysis. (From the thesis of Dr Vesa Olkkonen, Department of Genetics, University of Helsinki, with permission.)

lytic enzyme P5, as well as the nonstructural protein P12 needed in membrane assembly.

Each genome segment has noncoding regions of a few hundred base pairs at each end. There is a high degree of similarity between the ends of the three segments. The 3′ ends are identical for 17 bp and the similarity gradually diminishes and disappears at about position 80. At the 5′ end there is an identical block of 18 bp with no further similarity. However, the second base in the L segment differs from that in the M and S segments. The 5′ end contains the packaging (pac) site and the plus-strand synthesis start signal, whereas the 3′ end encodes the functions needed for the minus-strand synthesis initiation.

Life Cycle

Figure 3 depicts the life cycle of ϕ6. Protein P3 mediates the adsorption of the ϕ6 particle to the receptor, the host pilus. It is suggested that the pilus retraction brings the virus particle into contact with the host outer membrane (OM). Protein P6 mediates the fusion of the virus membrane with the OM, exposing the peptidoglycan layer to the virion-associated lytic enzyme (P5). The peptidoglycan is locally digested, allowing the nucleocapsid (NC) to face the cytoplasmic membrane (CM). The NC penetrates the CM via a mechanism which seems to be analogous to endocytosis and driven by acidification of the NC surface protein P8. The formed vesicle and the NC surface proteins (P8) are disassembled upon entry into the cell cytoplasm. The viral transcriptase starts the early transcription via a semiconservative mechanism. Approximately equal amounts of full-length transcripts of all the genome segments are synthesized. However, only the L message is actively translated. This leads to the assembly of polymerase complexes (procapsids) inside the cell. These particles package one of each type of messenger molecule. The packaging is followed by minus-strand synthesis (replication), leading to the dsRNA genomic segments inside the procapsid. These particles direct the late type of transcription where the majority of the transcripts originate from the M and S genome segments. The M and S messages are then translated, leading to the synthesis of the late proteins. The synthesis of the NC shell protein P8 leads to its assembly on to the polymerase complex, silencing its transcription activity. With the aid of the assembly factor P12, the NC is enveloped inside the cell with a virus-specific membrane derived from the

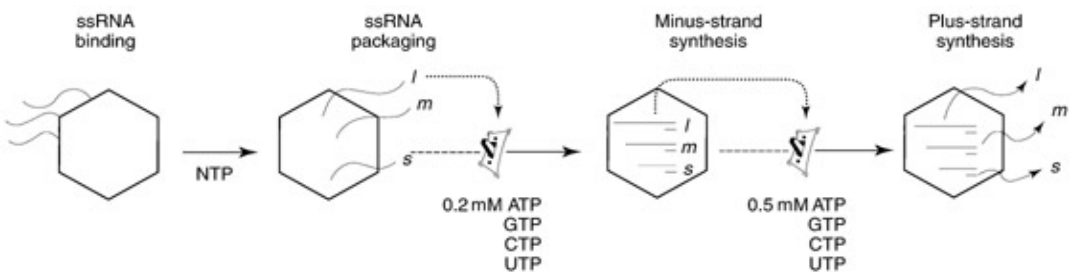


Figure 4 *In vitro* replication cycle of the purified procapsid. *s*, *m* and *l* indicate the single-stranded (ss) genome segments. The *l*-segment acts as a switch to turn on the next step.

host CM. The P3 spikes are assembled on the particle and the mature viruses are released by cell lysis. The cell lysis is caused by the phage-specific lytic enzyme P5 accompanied by the membrane protein P10.

In Vitro Assembly

Several recent technical developments in the $\phi 6$ system have generated an *in vitro* assembly pathway. The expression of the polymerase complex genes in *Escherichia coli* leads to the formation of empty procapsids, which are competent for *in vitro* packaging of the plus-strand transcripts. The packaged particles can replicate the single-stranded RNAs to the double-stranded form. Purified NC shell protein P8 can be assembled on to these particles. The nucleocapsids formed this way can infect $\phi 6$ host cells with partially removed outer membrane. Although these cells do not divide any more, they support one life cycle of the virus, leading to the formation of infective enveloped virus particles. The current focus is on assembling a functional polymerase complex from its four constituent proteins. This will accomplish the *in vitro* reconstitution of an infective complex dsRNA particle using purified structural components (**Fig. 4**).

These techniques are powerful in elucidating, in more detail, the assembly, packaging and replication signals and mechanisms operating in this virus. The nucleocapsid infection system allows the *in vivo* testing of the new constructs obtained. The availability of structural information of the virus and its subassemblies together with the assembly information will extend our understanding of the biology of dsRNA viruses.

See also: **Reoviruses (*Reoviridae*): General features, Molecular biology, Plant reoviruses; Totiviruses (*Totiviridae*): General features, *Ustilago maydis* viruses.**

Further Reading

Bamford DH and Wickner RB (1994) Assembly of double stranded RNA viruses: bacteriophage $\phi 6$ and yeast virus L-A. *Semin. Virol.* 5: 61.

Bamford DH, Ojala PM, Frilander M, Walin L and Bamford JKH (1995) Isolation, purification and function of the assembly intermediates and subviral particles of bacteriophages PRD1 and $\phi 6$. *Methods Mol. Genet.* 6: 455.

Butcher SJ, Dokland T, Ojala PM, Bamford DH and Fuller SD (1997) Intermediates in the assembly pathway of the double-stranded RNA virus $\phi 6$. *EMBO J.* 14: 4477.

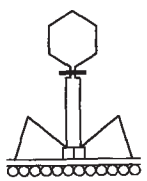
Mindich L (1988) Bacteriophage $\phi 6$: a unique virus having a lipid-containing membrane and a genome composed of three dsRNA segments. *Adv. Virus Res.* 35: 137.

Mindich L (1996) Heterologous recombination in the segmented RNA genome of bacteriophage $\phi 6$. *Semin. Virol.* 7: 389.

Mindich L and Bamford DH (1988) Lipid-containing bacteriophages. In: Calendar R (ed.) *The Bacteriophages*, p. 475. New York: Plenum Press.

Qiao X, Qiao J and Mindich L (1997) Stoichiometric packaging of the three genomic segments of double stranded RNA bacteriophage $\phi 6$. *Proc. Natl Acad. Sci. USA* 94: 4074.

PHAGE ECOLOGY, EVOLUTION AND SPECIATION



Allan M Campbell, Stanford University, Stanford, California, USA

Ecology

Where are phages found in nature, and what impact do they have on their surroundings? Phages infecting most of the major groups of bacteria and archaea have been identified and observed in the diverse natural habitats of their hosts. Meaningful quantitation of phage densities is complicated by the fact that plaque counts enumerate only those phage plating on a specific host (generally with unknown efficiency), whereas electron microscopic counts may include many inactive particles. Concentrations of phage-like particles can be as high as 10^8 per ml in natural aquatic habitats (both fresh and salt water) and 10^{10} per ml in sewage. Plaque counts on specific hosts are generally orders of magnitude lower. A concentration of 10^8 active phage particles per ml could have a substantial effect on bacterial densities and the recycling of bacterial biomass.

Compared with plasmids, phages generally have

host CM. The P3 spikes are assembled on the particle and the mature viruses are released by cell lysis. The cell lysis is caused by the phage-specific lytic enzyme P5 accompanied by the membrane protein P10.

In Vitro Assembly

Several recent technical developments in the $\phi 6$ system have generated an *in vitro* assembly pathway. The expression of the polymerase complex genes in *Escherichia coli* leads to the formation of empty procapsids, which are competent for *in vitro* packaging of the plus-strand transcripts. The packaged particles can replicate the single-stranded RNAs to the double-stranded form. Purified NC shell protein P8 can be assembled on to these particles. The nucleocapsids formed this way can infect $\phi 6$ host cells with partially removed outer membrane. Although these cells do not divide any more, they support one life cycle of the virus, leading to the formation of infective enveloped virus particles. The current focus is on assembling a functional polymerase complex from its four constituent proteins. This will accomplish the *in vitro* reconstitution of an infective complex dsRNA particle using purified structural components (**Fig. 4**).

These techniques are powerful in elucidating, in more detail, the assembly, packaging and replication signals and mechanisms operating in this virus. The nucleocapsid infection system allows the *in vivo* testing of the new constructs obtained. The availability of structural information of the virus and its subassemblies together with the assembly information will extend our understanding of the biology of dsRNA viruses.

See also: **Reoviruses (*Reoviridae*): General features, Molecular biology, Plant reoviruses; Totiviruses (*Totiviridae*): General features, *Ustilago maydis* viruses.**

Further Reading

Bamford DH and Wickner RB (1994) Assembly of double stranded RNA viruses: bacteriophage $\phi 6$ and yeast virus L-A. *Semin. Virol.* 5: 61.

Bamford DH, Ojala PM, Frilander M, Walin L and Bamford JKH (1995) Isolation, purification and function of the assembly intermediates and subviral particles of bacteriophages PRD1 and $\phi 6$. *Methods Mol. Genet.* 6: 455.

Butcher SJ, Dokland T, Ojala PM, Bamford DH and Fuller SD (1997) Intermediates in the assembly pathway of the double-stranded RNA virus $\phi 6$. *EMBO J.* 14: 4477.

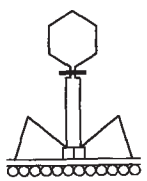
Mindich L (1988) Bacteriophage $\phi 6$: a unique virus having a lipid-containing membrane and a genome composed of three dsRNA segments. *Adv. Virus Res.* 35: 137.

Mindich L (1996) Heterologous recombination in the segmented RNA genome of bacteriophage $\phi 6$. *Semin. Virol.* 7: 389.

Mindich L and Bamford DH (1988) Lipid-containing bacteriophages. In: Calendar R (ed.) *The Bacteriophages*, p. 475. New York: Plenum Press.

Qiao X, Qiao J and Mindich L (1997) Stoichiometric packaging of the three genomic segments of double stranded RNA bacteriophage $\phi 6$. *Proc. Natl Acad. Sci. USA* 94: 4074.

PHAGE ECOLOGY, EVOLUTION AND SPECIATION



Allan M Campbell, Stanford University, Stanford, California, USA

Ecology

Where are phages found in nature, and what impact do they have on their surroundings? Phages infecting most of the major groups of bacteria and archaea have been identified and observed in the diverse natural habitats of their hosts. Meaningful quantitation of phage densities is complicated by the fact that plaque counts enumerate only those phage plating on a specific host (generally with unknown efficiency), whereas electron microscopic counts may include many inactive particles. Concentrations of phage-like particles can be as high as 10^8 per ml in natural aquatic habitats (both fresh and salt water) and 10^{10} per ml in sewage. Plaque counts on specific hosts are generally orders of magnitude lower. A concentration of 10^8 active phage particles per ml could have a substantial effect on bacterial densities and the recycling of bacterial biomass.

Compared with plasmids, phages generally have

rather narrow host ranges, frequently being restricted to one bacterial genus or to a few strains within that genus.

Temperate phages have been identified for many hosts, and molecular probes show that natural bacteria frequently retain remnants of prophages acquired in past infections. For example, the K-12 strain of *Escherichia coli* harbors (in addition to λ itself) four λ-related defective prophages, and most natural isolates of *Escherichia* and related genera contain λ-related sequences. In K-12, λ and its defective relatives comprise about 3% of the bacterial genome.

Prophages sometimes affect the phenotype of their hosts. The ϵ-prophages of *Salmonella* encode enzymes that modify the polysaccharide of the cell envelope, changing the antigenic specificity and rendering the bacteria resistant to infection by other phages of the same type. A number of phages affect the ability of pathogenic bacteria to cause disease. Sometimes a phage gene is directly involved in pathogenesis, as in diphtheria, where the toxin is encoded by a phage. Other phages have ancillary effects, as with bacteriophage λ, which bears genes that increase the serum resistance (and therefore potential pathogenicity) of *E. coli* hosts.

Evolution

Phages can be classified into numerous groups. Of the common coliphages T2, T3, λ, P1, f1, Mu-1, MS-2 and ϕX174, each has several known relatives. Within a group, different phage types can be distributed nonuniformly among local habitats and geographical areas, e.g. the ubiquitous F-specific isometric RNA coliphages are present in sewage from most parts of the world, generally at concentrations of about 10^3 PFU ml^{-1}, but different serotypes are more common in India than in Japan and in the feces of humans versus those of domestic animals. No good molecular phylogeny relates the various groups to one another. Hierarchical taxonomic schemes have been proposed (usually based on type of nucleic acid and virion morphology) but none has gained wide acceptance or carries any claim to phylogenetic significance. As with many animal virus groups, an independent origin of the different groups from host nucleic acid is as plausible as derivation from one ancestral virus. Within a group, a molecular phylogeny can of course be constructed for any one gene, but the phylogenies for different genes are not congruent, indicating frequent natural recombination. It is questionable whether the members of a group are better considered as different species rather than as members of the same species drawn from a common gene pool (see below, under Speciation).

At least for the larger DNA phages, current thinking generally assumes a chimeric origin, with different segments of the phage genome derived from different parts of the host genome, or from different hosts, or from plasmids, transposons or other phages. Even for the phylogeny of the major groups, the pattern of ancestry may therefore be reticulate rather than arboreal. Within a group, the conservation of gene order suggests descent from a common ancestor of that group.

Population Biology

Whatever the evolutionary origin of a given phage, one may ask how the phage population maintains itself over long periods of time. Mathematical analysis and chemostat experiments agree that a virulent phage can persist in permanent equilibrium with its host. Depending on the values of such parameters as adsorption rate, latent period, burst size and host growth rate, the equilibrium may either be stable or unstable with respect to undamped oscillations in phage and host numbers. Theory also indicates that the ability to impart a selective advantage to the host should be an important factor in the survival of temperate phage. The possible advantage might be felt by either the individual bacterium or the bacterial population.

Advantage to the individual in its natural surroundings should be intermittent, otherwise the phage genes imparting the advantage should by now have been incorporated into the host genome. Laboratory situations can be contrived where a prophage gene promotes bacterial survival; for example, some λ-related phages and prophages contain a gene *nmpC* which encodes an outer membrane protein able to substitute for the host OmpC protein; similarly, the recombination genes of lambdoid prophages can replace the *recBCD* genes of the host to impart resistance to DNA damage. In the appropriate bacterial mutant and environment, these phage genes can thus confer a selective advantage on the host. It is frequently assumed that some of the phage genes affecting bacterial pathogenesis are likewise beneficial to the host, although the exact advantage of being pathogenic remains elusive.

At the populational level, phage can transduce genes from one cell to another. Sequence comparisons of homologous DNA regions from natural isolates of related bacteria indicate that recombinational substitution of DNA from other strains is common. Whether this recombination benefits the bacteria remains to be established, as does the magnitude of the phages' contribution to the natural process.

Speciation

In sexual eucaryotes, a species is generally defined as a group of individuals that share a common gene pool because of interbreeding. Until recently, the concept was generally considered inapplicable to either bacteria or viruses. However, molecular data have encouraged reconsideration. The basic fact (cited above) that, within a group, trees based on different genes are not congruent rules out the textbook expectation for asexual organisms related to one another by purely clonal propagation without genetic exchange. The different lambdoid phages appear related to one another by numerous exchange events, suggesting that the entire group is best viewed as a single species.

Classical speciation entails the establishment of barriers to interbreeding so that the gene pools of distinct species cannot mix. The phage equivalent of that process is undefined. If each phage group has an independent origin, classical speciation may not exist. What can be asked is whether, or to what degree, the gene pools of various phage groups are isolated from one another (and from the gene pools of the hosts they infect). When an answer is sought through sequence comparisons, the question overlaps that of viral origins (discussed above, under Evolution).

There are some well-documented cases of homologies between viral genes and their host counterparts. For example, whereas the λ replication protein gp*P* interacts with the host *dnaB* helicase, in at least one lambdoid phage (P22) the N-terminus of the analogous protein is fused to a C-terminal *dnaB*-related segment, and replication does not require *dnaB*. Similarly, homologies have been detected between genes of T4, P2 and λ whose products contribute to attachment of phage to the cell surface or which are closely linked to genes for attachment proteins. In both cases, the genes are clearly of a common origin but not a very recent one. Within a group, homologous genes are also highly diverged in some cases but much more similar in others. Thus some past exchanges have occurred between distantly related phages and between phage and host. If such exchanges were frequent and recent, the concept of distinct species with separate gene pools would have little use. The examples known at present can be reasonably viewed as rare lateral transfers across species boundaries.

See also: **Coliphage lambda (*Siphoviridae*); Phage Homologous Recombination; Phage taxonomy and classification; Phage toxins and disease.**

Further Reading

Campbell A (1988) Phage evolution and speciation. In: Calendar R (ed.) *The Bacteriophages*, p. 1. New York: Plenum Press.

Goyal SM, Gerba CP and Bitton G (eds) (1987) *Phage Ecology*. New York: John Wiley & Sons.

PHAGE PRD1 (*TECTIVIRIDAE*)

Dennis H Bamford, Institute of Biotechnology, Biocenter, University of Helsinki, Finland

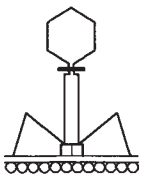
Discovery and Classification

Bacteriophage PRD1 belongs to a group of very closely related phages isolated from different parts of the world. Other members of this group are PR3, PR4, PR5, LI7 and PR772. These viruses are lytic, infecting a vast variety of Gram-negative cells harboring a P-, N- or W-type conjugative plasmid. Among the hosts are *Escherichia coli, Salmonella typhimurium* and *Pseudomonas aeruginosa*. PRD1 is the type virus of the family *Tectiviridae*, genus *Tectivirus*. This virus family also includes members infecting Gram-positive hosts. PRD1 and PR4 are the most thoroughly studied phages in this group. Since these viruses are almost identical, the description of this phage system uses information from both of them. The discovery (1973–1979) was associated with studies of antibiotic-resistant plasmids. In further studies it was observed that they have an internal lipid membrane and a linear double-stranded DNA (dsDNA) genome with 5′-covalently-linked terminal proteins.

Properties of the Virion

The viral particle (**Fig. 1**) has an outer diameter of approximately 65 nm. The outer protein layer sur-

PHAGE HOMOLOGOUS RECOMBINATION



Kenneth N Kreuzer, Department of Microbiology, Duke University Medical Center, Durham, North Carolina, USA

Introduction

For about half a century, bacteriophages have provided very productive systems for analyzing recombination mechanisms, and fundamental advances continue to emerge from these model systems. As described below, every phage that has been studied in detail uses multiple pathways of homologous recombination catalyzed by different combinations of host- and/or phage-encoded proteins.

A typical consequence of genetic recombination is the generation of a progeny genome with a combination of genetic markers different from either parental genome. However, recombination also plays central roles in the life cycles of many bacteriophages, even when considering solo infections in which reassortment of genetic material is not possible. For example, homologous recombination is important in phage DNA replication, repair of damaged phage DNA, and the maintenance of phage genomic structure (e.g. recombination of terminal redundancies). This entry summarizes homologous recombination in a few well-studied phage systems, considering the process of recombination in the context of the phage life cycle. Mechanistic aspects of recombination are also considered, and a closing section attempts to draw parallels. Recent reviews in the Further Reading list should be consulted for more comprehensive descriptions of homologous recombination and for literature citations. Bacteriophages also provide extremely productive systems for analyzing mechanisms of site-specific and transpositional recombination, but these will not be considered here.

Single-stranded DNA Phages

Unlike many other phage systems, recombination is not very important in the life cycle of the single-stranded DNA (ssDNA) phages (e.g. ϕX174, S13, f1). Indeed, ssDNA phage recombination is so infrequent that it is difficult to detect in the absence of agents (e.g. UV) that stimulate the process.

In spite of its low frequency, genetic analyses of ssDNA phage recombination revealed basic characteristics that are shared by many phage systems. For example, a variety of experiments established that the products of ssDNA phage recombination are usually nonreciprocal, eliminating simple break–rejoin models. The preponderance of nonreciprocal products in phage recombination forces recombination models that include one or more of the following features: (1) information from one parental molecule is destroyed during the reaction; (2) mismatch repair of heteroduplex DNA produces unequal recovery of alleles; (3) DNA replication copies information from one homologue so that two products of a single event carry the same allele.

A second characteristic of ssDNA phage recombination shared by many other phage systems is the existence of multiple recombination pathways. A majority of the recombination in ssDNA phages requires the host-encoded *recA* gene product. This is a well-studied bacterial recombination protein which catalyzes the invasion of a single-stranded region of one DNA into a duplex homologue. In most bacterial recombination, the host RecBCD enzyme is responsible for generating the ssDNA for RecA-promoted strand exchange. Surprisingly, however, RecA-dependent recombination of ϕX174 is not significantly reduced by host *recBC* mutations. Perhaps RecA catalyzes strand invasion using the infecting viral ssDNA of one phage and the duplex replicative form of a coinfecting phage.

An alternative means of generating ssDNA is apparently available near the phage replication origin, which acts as a strong hotspot for RecA-dependent recombination. This finding led to a model in which initiator protein-induced nicking of the origin in duplex replicative form DNA sometimes creates a single-stranded tail that participates in RecA-promoted strand invasion, leading to recombination events that are localized to the origin region. The recombination hotspot activity of the replication origin provides one interesting linkage between the processes of phage DNA replication and recombination.

While most ssDNA phage recombination requires RecA, a significant fraction does not. The mechanism of RecA-independent recombination is uncertain, but could involve the annealing of two complementary single strands. RecA-independent recombination is further reduced by about an order of magnitude in a *recB*$^-$ mutant host, implying a requirement for the RecBCD enzyme. Again, this is a surprising result, because all other known modes of RecBCD-promoted recombination require the RecA protein. The genome

of ϕX174 contains a χ site (5′-GCTGGTGG-3′), which is the activation sequence for RecBCD, but it is not known whether this χ site is necessary for RecA-independent recombination.

The RecA-independent recombination of ϕX174 is abolished in phage gene *A* mutant infections, which are blocked for all DNA replication after the initial conversion of infecting viral DNA to a duplex circle. This result strongly suggests that RecA-independent recombination requires phage DNA replication, and provides a second interesting linkage between DNA replication and recombination. How could DNA replication provoke RecA-independent recombination? If ssDNA annealing is central to this mode of recombination, then replication presumably generates ssDNA. Perhaps breakage of a replicative intermediate provides a duplex end that serves as an entry site for the RecBCD enzyme, which can actively denature a duplex to produce recombinogenic ssDNA (see below). Alternatively, perhaps replication generates ssDNA regions on the lagging strand or occasional ssDNA products that have not undergone complementary strand synthesis. In any case, if two complementary single strands are necessary for RecA-independent recombination, one of the participating molecules could once again be the viral strand of one infecting phage (which has not yet been converted into a duplex circle).

Phage λ

Recombination in phage λ has been analyzed in exquisite detail, using combinations of genetic, physical and biochemical approaches. λ recombination is intertwined with phage DNA replication and packaging, and therefore the roles of phage and host recombination proteins in all three processes must be considered.

The packaging of phage λ DNA into infectious particles involves double-strand DNA cleavage at the *cos* site by the terminase enzyme complex. DNA cleavage requires a multimeric DNA substrate, either in the linear or circular form, and cannot occur with monomeric circles. In wild-type λ infections, phage DNA replication proceeds by both the bidirectional (θ) and rolling-circle (σ) modes, the latter producing the linear multimers that are substrates for DNA packaging. In contrast, when the infecting phage is mutant in a gene called *gam*, the rolling-circle mode is blocked by the host RecBCD enzyme. The major function of the Gam protein in wild-type infections is to protect the rolling-circle intermediates from the action of the host RecBCD enzyme, which can degrade linear DNA. Consistent with this function, the Gam protein has been shown to bind to RecBCD enzyme and block all known activities of the enzyme *in vitro*. In spite of the fact that *gam*$^-$ mutants cannot produce linear multimers via rolling-circle replication, such mutants are normally viable. This is due to the fact that recombination between circular monomers can produce circular multimeric DNA and thereby provide a suitable substrate for DNA packaging. Thus, recombination is essential for growth of phage λ *gam*$^-$ mutants, and host- and/or phage-encoded recombination proteins can participate in recombination during the λ infective cycle (see below).

In addition to Gam, phage λ encodes several other proteins involved in homologous recombination, most notably the products of the *red* genes. The *red* genes encode two proteins, an exonuclease (Red α) that can generate single-stranded 3′ tails at duplex breaks and an ssDNA-binding protein (Red β) that can facilitate renaturation of complementary ssDNA *in vitro*. The Red proteins play a major role in phage λ recombination (see below). Red may also play a direct role in λ DNA replication, because *red* mutations reduce the rate of phage DNA replication. It has been proposed that the Red system participates in the rolling-circle mode of λ DNA replication by catalyzing a break–copy mechanism of recombination, much like that used by phage T4 (see below). Nevertheless, *red*$^-$ mutant infections produce concatemeric DNA, presumably via rolling-circle replication, and therefore the Red system is not solely responsible for generating rolling circles. The mechanisms that generate λ rolling circles, either in the presence or absence of Red function, are not understood.

The influence of phage and host recombination proteins on phage λ DNA metabolism can thus be summarized as follows. In λ$^+$ infections, rolling-circle replication generates linear DNA multimers that are the normal substrate for DNA packaging, and the λ Red pathway may be responsible for generating some of the rolling circles. In λ *gam*$^-$ infections, rolling-circle replication is prevented by the host RecBCD enzyme. In this case, recombination by phage- and/or host-directed pathways is essential for survival, because multimeric circular DNA must be produced by recombination to allow DNA packaging.

Phage λ recombination can be directed by several different pathways depending on the genotype of the phage and the host. A single mutation in the host *recA* gene has little effect on the frequency of recombination during a λ infection, but a mutation in the phage *red* genes reduces recombination by several fold. A combination of both mutations essentially abolishes

recombination, except in the genetic interval that is subject to site-specific recombination by the phage-encoded Int system. The simple interpretation is that a majority of λ recombination is catalyzed by the Red pathway, and a minority by one or more host pathways that require RecA protein. An important complexity, however, is that the RecA protein is apparently utilized, in conjunction with the Red proteins, during infection of wild-type *Escherichia coli*. This can be inferred because the qualitative nature of *red*$^+$ λ recombination changes dramatically when the host RecA protein is mutationally inactivated (even though the frequency of recombination does not change much; also see below).

The host recombination pathways that are active during λ growth are difficult to study when the phage Red system is active, because the Red system predominates. However, these pathways can be readily analyzed by inactivating Red. As mentioned above, all Red-independent λ recombination requires the host RecA protein (except for site-specific recombination by Int). In addition, RecBCD is normally required for most λ *red*$^-$ (*gam*$^+$) recombination, because phage recombination is 3- to 10-fold lower in a *recBC*$^-$ host than in a wild-type host. There are several important factors to consider with respect to RecBCD pathway recombination during λ *red*$^-$ infection. First, RecBCD contributes to λ recombination in spite of the production of Gam protein, perhaps during the time when Gam has not yet reached high levels. Nevertheless, the production of Gam clearly reduces RecBCD activity in the λ infection, because λ *red*$^-$ recombination increases several fold (to nearly wild-type levels) if Gam is mutationally inactivated. Second, during infection by such a *red*$^-$ *gam*$^-$ double mutant of λ, active RecBCD enzyme prevents rolling-circle replication but, together with RecA protein, allows sufficient recombination to generate packagable DNA in the form of multimeric circles. Third, wild-type λ contains no χ sites, which act as hotspots for RecBCD-promoted recombination. Mutants of λ containing χ sites utilize the RecBCD pathway more efficiently, and χ-less wild-type λ presumably recombine by the RecBCD pathway using pseudo-χ sites that deviate slightly from the consensus sequence.

Phage λ can also utilize the host RecF and RecE pathways when these pathways are activated by the appropriate host mutations (*sbcBC* and *sbcA*, respectively). One interesting feature of λ's use of the RecF pathway relates to the protein requirements. The host genes *recO*, *recR* and *recF* are not required for λ recombination by the RecF pathway but are necessary for host conjugal recombination by the same pathway. The solution to this puzzle was recently uncovered. λ encodes a protein, Orf, that substitutes for the three host functions, and a mutation that inactivates the *orf* gene results in a requirement for the three host genes. Recombination of λ by the RecE pathway of *E. coli* closely resembles recombination by the λ Red pathway, because the RecE pathway is dependent on the Red-like functions of a cryptic prophage in the genome of some *E. coli* strains.

By using appropriate combinations of host and phage mutations, it is possible to limit λ recombination to any one of the above-mentioned pathways (Red, RecBCD, RecF, RecE). This, together with other advantages of λ, has allowed the λ infective cycle to become a fertile testing ground for models of recombination. The mechanistic details of the two major pathways of λ recombination (Red and RecBCD) will be considered here, and reviews in the Further Reading list should also be consulted for mechanistic descriptions of these and the RecE and RecF pathways.

The Red pathway of λ recombination appears to be primarily, if not exclusively, triggered by double-stranded breaks (DSBs). In the absence of DNA replication, Red-promoted recombination is focused on sites of double-strand breakage, including the natural DSBs created by terminase cleavage at *cos* and engineered DSBs generated by restriction enzyme cleavage. When replication is allowed, Red-promoted recombination occurs throughout the genome. Nonetheless, DSBs appear to be responsible for initiating Red-promoted recombination even in the presence of replication, because such recombination is prevented by production of a protein that blocks double-strand ends. Presumably, replication generates randomly located DSBs, including the tips of rolling-circle intermediates.

Stahl and his colleagues have presented evidence that Red-promoted recombination can occur by at least two mechanisms, depending on whether the host RecA protein is available. In both mechanisms, the 5′ exonuclease activity of the Red α protein (λ exonuclease) generates a single-stranded 3′ tail after production of a DSB by one of the routes listed above. In the presence of RecA, this tail invades homologous duplex DNA to establish a recombination intermediate. Subsequent steps are not entirely clear, and may depend on whether DNA replication can occur (break–copy recombination, as mentioned above). In the absence of RecA, a large fraction of Red-promoted recombination appears to involve the annealing of complementary single strands by the Redβ protein.

The general features of RecBCD-promoted λ recombination now seem clear, based on a convergence of genetic and enzymological approaches. As

with Red-promoted recombination, DSBs are important for activation of recombination, but RecBCD-promoted recombination does not occur at the site of the break. Genetic analyses demonstrated that RecBCD activates recombination whenever at least one of the participating DNAs contains a χ site and a DSB. As mentioned above, wild-type λ contains no χ sites, but recombination is thought to be activated by sequences that closely resemble the χ consensus. In most genetic analyses of RecBCD-promoted λ recombination, a single χ site is introduced into the λ genome, a *gam* mutation is used to allow maximal RecBCD activity, and the natural DSB is provided by terminase-promoted cleavage at the *cos* site. After *cos* cleavage, terminase remains bound to one of the two newly formed DNA ends, resulting in only a single unblocked DNA end (the right end on the standard λ genetic map). With this configuration, RecBCD greatly enhances recombination near and beyond a properly oriented χ site, regardless of the distance between the *cos*-promoted break and the χ site. The localization of recombination suggests that the RecBCD enzyme requires a duplex break to enter, but that it does not stimulate recombination until it encounters a χ site. The genetic information between the right end of the genome and the χ site is lost during the process of recombination, arguing that the RecBCD enzyme destroys DNA prior to its encounter with a χ site. The genetic results just described have recently been duplicated and analyzed at a biochemical level. In an *in vitro* system with purified proteins, RecBCD degrades the DNA between the free end and the χ site, and stimulates (RecA-promoted) recombination at and beyond the χ site. Passage through a χ site grossly alters the properties of the RecBCD enzyme, converting it from a degradative nuclease to a recombinative helicase. The mechanism of this alteration is not known, but has been hypothesized to involve the loss of the RecD subunit of the enzyme.

Phage T7

Unlike the cases described above, phage T7 is largely independent of host recombination functions. Mutations in the host *recA* or *recBC* genes do not affect T7 recombination, even when the infecting T7 carries mutations that reduce recombination. Phage T7 also induces its own replication proteins, making T7 DNA metabolism independent of that of the host.

One of the central themes of T7 DNA metabolism is the close coupling between replication and recombination. An electron microscopic analysis of DNA from T7-infected cells indicated that the largest number of joint molecules (presumptive recombination intermediates) are present at the time of maximal DNA replication. Furthermore, a mutation in the gene that encodes the viral DNA polymerase (gene *5*) or helicase/primase (gene *4*) abolishes DNA replication and greatly reduces recombination. The close coupling of replication and recombination can be explained in at least three ways: (1) replication increases the DNA copy number, leading to more recombination; (2) the products of genes *4* and *5* play important roles in both processes independently; and/or (3) some replicative intermediate is a substrate for recombination.

Although DNA copy number may affect recombination frequency, the first explanation alone is not sufficient to explain the coupling of T7 replication and recombination. In the first place, a mutation in gene *1* (encodes T7 RNA polymerase) or 2 (encodes inhibitor of host RNA polymerase) greatly reduces replication but does not affect recombination. Likewise, the presence of a host DNA gyrase inhibitor (nalidixic acid) or the absence of DNA ligase reduces replication but not recombination. Second, a sensitive direct physical assay of infecting viral DNA failed to detect recombinant products during infections that were deficient in gene *4* or *5*. Third, active plasmid replication apparently stimulates T7-promoted plasmid–phage recombination, even when the plasmid copy number increases only slightly.

There is very good reason to believe that T7 recombination and replication require common proteins. In general, most models for genetic recombination include at least one replicative step and therefore implicate DNA polymerase and associated proteins in recombination. More specifically, the gene *4* helicase and the gene 2.5-encoded ssDNA-binding protein both promote important steps during *in vitro* replication and recombination reactions (see below). An *in vitro* system using crude extracts from T7-infected cells shows a tight coupling between recombination and replication. This *in vitro* system is capable of very extensive DNA replication and recombination, with about 20% of progeny DNA being recombinant. The coupling is particularly evident when a substrate DNA contains a double-strand gap. The break is efficiently repaired, using homologous intact DNA as template for the DNA synthesis necessary to cross the gap.

It also seems very likely that T7 DNA replication generates recombinogenic structures. The frequency of marker rescue from UV-damaged phage T7 genomes is greatest for markers near the primary replication origin, which is also the region of the genome that preferentially replicates after UV treatment. Thus, partially replicated DNA molecules may be preferential substrates for recombination, perhaps

because they contain single-stranded regions or because they are prone to break.

One unusual recombination event is critical for maintaining the genome of phage T7 and is probably directly coupled to DNA replication. The T7 genome is packaged in phage particles as a linear DNA molecule. Because DNA polymerases replicate DNA in the 5′ to 3′ direction and cannot initiate chains *de novo*, the 3′ ends of the T7 viral genome should remain single-stranded after replication from the internal origin. Without a remedy, the ends of the genome would be progressively lost with subsequent rounds of DNA replication. T7 solves this potential problem with 160 bp terminal repeats in its genome. Replication of the infecting viral DNA presumably creates daughter molecules that each contain one single-stranded end (the 3′ end of the parental strand). Because the opposite ends of the genome carry the same sequence, the single-stranded 3′ ends in the two daughters will be complementary, and thus single strand annealing can produce a dimeric molecule with one copy of the repeat in the middle. Subsequent rounds of replication followed by end annealing can produce longer multimers, again with only one copy of the repeat forming the junction between any two genomes. The regeneration of viral genomes prior to DNA packaging therefore requires the duplication of the terminal repeat, which apparently occurs by means of a special replication reaction.

The generation of dimers and higher multimers is a form of recombination from a physical viewpoint. However, from a genetic viewpoint, this recombination event does not lead to recombinant progeny. Even if dimerization is biparental, subsequent cleavage at the concatemeric joint resegregates the markers of the two parents from each other. This formation of dimer T7 DNA molecules forms an interesting model for a presumed ssDNA annealing recombination reaction. Unfortunately, the gene products required for dimer formation have not been directly determined. The ssDNA-binding protein of T7 (product of gene 2.5) mediates a similar annealing reaction *in vitro*, arguing that this protein may be critical for dimer formation *in vivo*. Although the reaction is thought to be normally coupled to DNA replication, a strand-specific exonuclease (see below) could also generate complementary single strands at the genome ends and perhaps obviate the need for DNA replication.

In addition to the T7 helicase/primase, DNA polymerase and ssDNA-binding protein, two T7-encoded nucleases are important in phage recombination. Out of all T7 genes, mutations in gene 6 cause the most dramatic reductions in phage recombination (greater than 100-fold). The gene 6 protein is therefore necessary for virtually all T7 recombination. Even in the absence of other T7 replication/recombination proteins (products of genes 3, 4 and 5), gene 6 protein apparently provides a minor pathway of recombination. Biparental joint DNA molecules resembling Holliday junctions can be detected from cells infected by a multiple $1.3^-\ 2^-\ 3^-\ 4^-\ 5^-$ mutant but not a $1.3^-\ 2^-\ 3^-\ 4^-\ 5^-\ 6^-$ mutant.

In spite of the importance of the gene 6 protein, its precise role in T7 recombination has not been elucidated. Gene 6 encodes the major T7 exonuclease, which generates 3′ single-stranded tails at duplex DNA ends, and also has RNaseH activity. The generation of ssDNA is presumed to be the key to recombination promoted by the gene 6 exonuclease. Perhaps the high level of recombination that is coupled to DNA replication is caused by occasional breakage of the replication fork, followed by gene 6 exonuclease processing of the broken DNA. In any case, once ssDNA regions are created, the T7 ssDNA-binding protein (gene 2.5 product) presumably facilitates an annealing reaction to generate joint molecules.

Recent *in vitro* experiments indicate that a more complete recombination reaction can be achieved using the gene 4 helicase. When a single-stranded circular DNA was incubated with a homologous linear duplex that contained a short single-stranded region at one end, the gene 2.5 protein promoted annealing of the complementary single-stranded regions and the gene 4 helicase then catalyzed a branch migration reaction into and through the duplex region. Thus, a complete strand exchange reaction occurred, generating duplex circular and ssDNA linear products.

The second T7-encoded recombination nuclease is the product of gene 3, T7 endonuclease I. Endonuclease I plays a key role in recombination, namely the resolution of Holliday junctions. An early study indicated that branched dimeric DNA molecules were detected from infections by $2^-\ 3^-\ 4^-$ triple mutants, but hybrid monomers were found with a $2^-\ 4^-$ double mutant. More recently, the endonuclease was shown to cleave branched DNA molecules *in vitro*, and cloned gene 3 protein was shown to substitute, during phage T4 infections, for T4 gene 49 protein (T4 endonuclease VII; resolves DNA branches during T4 DNA packaging; see below).

Pulling together the demonstrated functions of these various T7-encoded proteins, a reasonable (though untested) model for T7 recombination can be formulated as follows. The initiating lesions would be DNA ends, generated by replication fork breakage, random genomic breaks (e.g. nuclease induced), DNA damage (e.g. chemical or radiation induced) and/or

natural genome ends. Gene 6 exonuclease would resect the ends to create single-stranded regions, and the T7 ssDNA-binding protein would then promote annealing of two complementary strands. Subsequent events could include branch migration into duplex regions by the gene *4* helicase and/or *de novo* replication events in which invading 3′ ends are used to trigger localized or extensive DNA synthesis. Finally, endonuclease I would resolve any remaining branches to produce simple linear DNA products.

Phage T4

Viral genetic recombination was first demonstrated using infections with T4 and the closely related T-even phages. T4 recombination has since been extensively studied and has provided key advances in our understanding of recombination mechanisms, for example, the earliest evidence for DSB-directed recombination. An advantage of the T4 system is that the phage encodes its own recombination proteins, most of which have been purified and analyzed biochemically. Furthermore, recombination is extremely active in T4, with the average progeny DNA having experienced between 10 and 20 recombinational exchanges during the infective cycle.

Phage mutations that reduce recombination have profound effects on phage growth, and therefore recombination is very important in the T4 life cycle. As in the case of phages λ and T7, the importance of T4 recombination reflects the intimate connections between phage recombination, DNA replication and packaging. Infecting T4 phage DNA is a terminally redundant linear molecule, and recombination of the genomic ends is required to generate multimeric DNA forms that are necessary for packaging. During a solo infection, recombination at the ends could generate a circular form if recombination precedes replication or a linear dimer if recombination follows replication. Other DNA forms are possible during a coinfection by two or more phages. Because packaging creates circularly permuted genomes, the end of one phage DNA may be homologous to a middle of another coinfecting phage DNA, and therefore Y forms can be generated. As replication produces multiple genome copies during the course of the infection, all T4 DNA within a single infected cell becomes enmeshed in a large, complex concatemeric structure with numerous recombinational joints.

Mutations that reduce T4 recombination also abolish one of the two major modes of T4 DNA replication. At early times of infection, T4 replication origins direct genomic replication regardless of the presence or absence of recombination proteins. However, the origins become repressed as the infection proceeds, and recombination-dependent DNA synthesis becomes the only significant replication mode at late times. T4 mutations that reduce recombination thereby cause a 'DNA-arrest' phenotype. Mosig and her colleagues presented a model for recombination-dependent DNA synthesis in which recombination intermediates are converted into replication forks. In this model, a single-stranded 3′ end invades a duplex homologue, forming a Y-structure with a D-loop at the point of invasion. The single-stranded ends may arise from the incomplete replication of genomic ends (see above), or by exonuclease action, perhaps directed by the T4-encoded gp46/47 nuclease. After formation of the recombination intermediate, the invading 3′ end is proposed to serve as primer for leading-strand DNA replication, and Okazaki fragment synthesis on the lagging-strand completes the replication fork. Note that the two strands of the invaded duplex serve as the template strands for DNA replication.

A variety of results support this general model for T4 recombination-dependent replication. *In vivo* experiments have provided the following evidence: (1) both recombination and late replication are reduced or blocked by mutations in phage genes *uvsX*, *uvsY*, *46*, *47* and *59*; (2) the ends of infecting T4 DNA are highly recombinogenic, and the characteristics of the recombinants are consistent with replication often initiating at the recombination junction; (3) electron microscopy of early replicating DNA has revealed the proposed Y form intermediates; (4) recombination-dependent replication does not require specific DNA sequences (replication origins), but does require homologous DNA sequences to allow formation of recombination junctions; and, (5) an artificial DSB introduced into one DNA can trigger replication of homologous DNA during T4 infection.

T4 recombination-dependent replication has been reproduced *in vitro*. The properties of the *in vitro* system provide strong support for the general model described above, and also reveal important mechanistic details of the reaction. The *in vitro* system utilizes a linear ssDNA fragment and a linear or circular duplex homologue. Strand invasion is catalyzed by the phage-encoded UvsX protein, a functional analogue of the *E. coli* RecA protein, together with the T4 ssDNA-binding protein gp32 and the phage-encoded UvsY protein. UvsY is a prototype for a class of proteins that facilitate the loading of a strand-exchange protein on to ssDNA coated with ssDNA-binding protein. In the presence of appropriate T4 replication proteins, the invading 3′ single-stranded end primes DNA replication, using the duplex homologue as template. In the most complete

reactions, the product of T4 gene *59* facilitates the binding of the T4 helicase/primase complex to the invaded duplex DNA. Thus, gp59 apparently assists leading-strand replication by providing the replication-fork helicase and assists lagging-strand replication by providing the primase for Okazaki fragment synthesis.

Genetic analyses indicate that T4 recombination proceeds by multiple pathways which are dependent only on phage-encoded proteins. The mechanistic details of these recombination pathways are not understood, except that one pathway is identical to the recombination-dependent replication pathway described above. Surprisingly, mutations that inactivate the T4-encoded strand exchange protein (UvsX) reduce phage recombination by only a few fold, regardless of the presence or absence of the host RecA protein. Thus, a significant portion of T4 recombination is independent of any known strand exchange protein. Based on electron microscopic analysis of DNA from replication-blocked coinfections, Broker and his colleagues proposed that the annealing of complementary ssDNA accounts for some fraction of T4 recombination, perhaps explaining recombination in the absence of UvsX. The generation of ssDNA in the absence of replication required the function of gp46/47, which is thought to be an exonuclease, and the ssDNA annealing was apparently promoted by the T4 ssDNA-binding protein, gp32. Although ssDNA annealing has not been demonstrated to contribute to recombination during replication-allowed infections, it would provide a suitable explanation for the large amount of recombination in the apparent absence of *bona fide* strand exchange proteins. Alternatively or in addition, perhaps a T4-encoded helicase is able to drive branch migration into duplex regions after an initial ssDNA annealing reaction, as described above for the phage T7 system.

Many of the recombination proteins encoded by phage T4 provide important prototypes for biochemical analyses of recombination. As implied above, the T4 UvsX protein catalyzes strand-invasion reactions *in vitro,* assisted by the phage-encoded UvsY protein and gp32. In addition, the gene *59* protein loads helicase/primase on to recombination intermediates and the gene *49* product (endonuclease VII) is the best-studied enzyme that resolves Holliday junctions and other structural aberrations in DNA.

Recombination in phage T4 is very closely coupled to DNA replication. As described above, a major mode of T4 DNA replication is triggered from recombination junctions. In addition, T4 DNA replication stimulates recombination by at least two distinct mechanisms. First, the ends of an infecting phage DNA are activated for recombination by DNA replication, presumably because replication leaves 3′ single-stranded termini in the parental DNA. Second, T4 replication origins activate the major hotspots for genetic recombination (following UV damage) by an unknown mechanism.

The linkage of T4 replication and recombination can be analyzed by measuring the integration of a plasmid into the phage genome. The integration of a plasmid that contains a T4 DNA insert but no replication origin strictly requires the UvsX and UvsY proteins. However, the addition of a phage replication origin to the plasmid causes a large stimulation in the recombination that occurs in the absence of UvsX or UvsY (also independent of RecA). Thus, plasmid replication apparently activates a recombination pathway that is independent of strand exchange proteins. Similar to the cases discussed above, replication of the plasmid may generate recombinogenic structures (e.g. ssDNA or DSBs). A clue to the mechanism of this recombination is provided by an analysis of protein requirements. A mutation in either gene *46* or *32* essentially abolishes recombination between the origin-containing plasmid and the phage genome. Thus, generation of ssDNA regions by the gp46/47 exonuclease and ssDNA annealing by gp32 are likely steps in this recombination pathway. This model is very reminiscent of that suggested by the electron microscopic analyses of phage DNA recombination (see above).

DSB-promoted recombination was first inferred in the phage T4 system, based on the facts that the ends of the infecting T4 genome are essentially DSBs and are very active in recombination. Thus, it is appropriate that T4 now provides one of the best-developed systems for studying the mechanism of DSB-promoted recombination. Two self-splicing group I introns of phage T4 encode endonucleases that trigger the highly efficient process of intron mobility. In each case, the endonuclease specifically recognizes an intronless copy of the cognate gene and induces a DSB in that DNA. DSB repair, using the intron-containing DNA as template, results in a unidirectional gene conversion event that essentially transfers a copy of the intron into the intronless gene. The characteristics of this DSB repair event are very closely related to those of recombination-dependent DNA synthesis. Indeed, recent experiments suggest that these two processes are just two manifestations of the same biological reaction.

Drawing parallels

Very diverse mechanisms are clearly responsible for generating the substrates for recombination in differ-

ent phage systems. Specific DNA signals such as packaging sites, genome ends, replication origins and χ sites have all been shown to stimulate recombination, and the action of each of these signals is unique to a particular phage system. Nonetheless, these varied DNA signals may generate just one or a small number of DNA structures that trigger recombination, for example ssDNA tails and/or DSBs. These DNA signals generally stimulate recombination locally or at least in restricted regions of the genome. What then stimulates the background level of recombination that occurs throughout a phage genome? In the case of replication-allowed infections of phage λ, most Red-promoted recombination apparently depends on randomly located DSBs, which are produced by an unknown mechanism that involves replication. Indeed, DSBs may be the major cause of recombination during infections by many phages. Random DNA nicks in phage genomes may also contribute to background recombination, as judged by the fact that the ssDNA phage replication origins are sites of nicking and stimulate local recombination. The pathway of recombination from a DNA nick could involve either local DNA replication (e.g. as in the Meselson–Radding recombination model) or exonucleolytic generation of an ssDNA gap.

Moving beyond the initial stages of generating recombinogenic structures, the formation of the joint (biparental) molecule is the essence of recombination. Much attention has focused on DNA pairing mediated by strand exchange proteins, which involves the invasion of a single strand into a duplex homologue. There is no doubt that this mode of pairing plays a large role in recombination. Mutations in the *E. coli recA* gene abolish most recombination in the bacterial host and during ssDNA phage infections, and *uvsX* mutations in T4 eliminate 70–80% of phage–phage recombination. Numerous biochemical studies have now been performed on both RecA- and UvsX-promoted strand-invasion reactions, revealing a highly detailed view of a central step in genetic recombination. This is not a complete picture, however. Every phage system described above also appears to generate at least some recombinants without the help of a RecA-like protein. In most or all cases, this mode of recombination is activated by replication. A common explanation for the dependence on replication is that replication generates ssDNA regions, perhaps at the sites of DSBs, that serve as substrates for recombination. Judging from the involvement of λ Red β protein and T4 gp32, protein-promoted annealing of two ssDNA regions seems likely to be a critical step, perhaps followed by helicase-mediated branch migration into duplex regions. Naively, ssDNA annealing might be thought of as a pathway peculiar to phage systems, where the genome copy number can be very high. However, recent studies of DSB repair in repeated genes of eukaryotes have led to virtually identical models involving protein-promoted annealing of complementary ssDNA from adjoining copies of the repeated sequence.

The activation of recombination by replication is common to all phage systems that have been studied in detail. Replication may generate recombinogenic DSBs, although the mechanism for generating such breaks is not known. Clearly, phages are very tolerant of DSBs, presumably in part because phages maintain a high genome copy number for most of the infective cycle. Programmed double-strand cleavage of the genomes of many phages occurs during DNA packaging, and the maintenance of genome structure requires efficient DSB repair of some type in the next infective cycle. DSB-promoted recombination was first detected in phage systems, and is now believed to play a dominant role in a wide variety of recombination pathways. Using bacteriophage systems such as λ and T4, the detailed molecular steps in DSB-promoted recombination are currently under intense investigation.

See also: ***Bacillus subtilis* phages; Bacterial identification – use of phages; Phage ecology, evolution and speciation; Phages as cloning vehicles; Filamentous phages (*Inoviridae*); History of virology: Bacteriophages; Host-controlled modification and restriction; Coliphage lambda (*Siphoviridae*); Lysogeny and prophage; Mu-like phages (*Myoviridae*); Enterobacteria phage N4 (*Podoviridae*); Enterobacteria phage P1 (*Myoviridae*); P2, 186 and related phages (*Myoviridae*); Salmonella phage P22 (*Podoviridae*); Phage PRD1 (*Tectiviridae*); Propagation of viruses: Bacteria; SPO1 phage (*Myoviridae*); T1-like phages (*Siphoviridae*); T4-like phages (*Myoviridae*); T5-like phages (*Siphoviridae*); T7-like phages (*Podoviridae*); Bacillus phage ϕ29 (*Podoviridae*); Coliphage ϕX174 and related phages (*Microviridae*).**

Further Reading

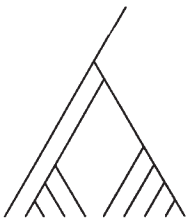
Camerini-Otero RD and Hsieh P (1995) Homologous recombination proteins in prokaryotes and eukaryotes. *Annu. Rev. Genet.* 29: 509.

Karam J (1994) *The Molecular Biology of Bacteriophage T4*. Washington, DC: American Society for Microbiology.

Kobayashi I (1992) Mechanisms for gene conversion and homologous recombination: the double-strand break

repair model and the successive half crossing-over model. *Adv. Biophys.* 28: 81.
Kornberg A and Baker TA (1992) *DNA Replication* 2nd Edn. WH Freeman: New York.
Kowalczykowski SC, Dixon DA, Eggleston AK, Lauder SD and Rehrauer WM (1994) Biochemistry of homologous recombination in *Escherichia coli*. *Microbiol. Rev.* 58: 401.
Kucherlapati R and Smith GR (eds) (1992) *Genetic Recombination*. Washington, DC: American Society for Microbiology.

PHAGE TAXONOMY AND CLASSIFICATION



Jack Maniloff, Department of Microbiology and Immunology, University of Rochester, Medical Center, Rochester, New York, USA

Hans-Wolfgang Ackermann, Département de Biologie Médicale, Université Laval, Faculté de Médecine, Cité Universitaire, Québec, Canada

Audrey Jarvis, New Zealand Dairy Research Institute, Palmerston North, New Zealand

Introduction

In 1915 and 1917, Twort in London and d'Hérelle in Paris independently described agents which destroyed bacteria. D'Hérelle named his agent bacteriophage ('eater of bacteria'), which became shortened to phage, and began studies on the nature of phages which led to modern virology.

D'Hérelle believed there was only one species of phage and that strain diversity was due to extensive variation within this single species. Subsequent workers showed that, whereas different phage strains did indeed vary in their properties (e.g. antigenic specificity, and resistance to inactivation by physical and chemical agents), the properties of any one strain were fairly constant during repeated subculturing and cloning. This demonstrated that there were different phages and, as more phages were isolated, the need for their systematic classification arose.

As with animal and plant viruses, early efforts at phage taxonomy concentrated on groupings based on properties such as antigenic relationships, plaque morphology, particle size by ultrafiltration and heat resistance. Modern viral taxonomy began in 1962 when Lwoff, Horne and Tournier proposed a classification system based on properties of the virion and its nucleic acid. Procedures for viral taxonomy and nomenclature became organized in 1966 with establishment of the International Committee for Taxonomy of Viruses (ICTV). Since then, the ICTV has developed an internationally agreed nomenclature and taxonomy for viruses, through regular meetings and communications among virologists. A complete ICTV Report on virus taxonomy is published every few years: at present, there are 13 families and one unassigned genus of bacterial viruses (**Table 1**). 'Bacterial virus' is used here as a generic term for viruses infecting hosts in the domains *Bacteria* and *Archaea*.

The ICTV Universal System of Virus Taxonomy

Virus classification is based on structural, physicochemical and replication properties. Some of these criteria have been used since the earliest days of virology (e.g. antigenic relationships) and others have been developed only recently (e.g. nucleic acid sequencing). The taxa in virology are order, family, genus and species. Each taxonomic level is defined by a specific subset of properties. For example, virion morphology and size, and genome type and strandedness are high-level properties and define taxa at the order and/or family level, whereas nucleotide sequence and protein properties are low-level properties and define taxa at the genus and/or species level.

In practice, a virus strain (i.e. the progeny virus from a single isolation) is usually classified from the 'top down'. Classification generally begins with data on virion morphology and the nature of the genome to determine the order and/or family. Additional data are then used to determine the genus, and finally whether the strain is a new species. However, in

Speciation

In sexual eucaryotes, a species is generally defined as a group of individuals that share a common gene pool because of interbreeding. Until recently, the concept was generally considered inapplicable to either bacteria or viruses. However, molecular data have encouraged reconsideration. The basic fact (cited above) that, within a group, trees based on different genes are not congruent rules out the textbook expectation for asexual organisms related to one another by purely clonal propagation without genetic exchange. The different lambdoid phages appear related to one another by numerous exchange events, suggesting that the entire group is best viewed as a single species.

Classical speciation entails the establishment of barriers to interbreeding so that the gene pools of distinct species cannot mix. The phage equivalent of that process is undefined. If each phage group has an independent origin, classical speciation may not exist. What can be asked is whether, or to what degree, the gene pools of various phage groups are isolated from one another (and from the gene pools of the hosts they infect). When an answer is sought through sequence comparisons, the question overlaps that of viral origins (discussed above, under Evolution).

There are some well-documented cases of homologies between viral genes and their host counterparts. For example, whereas the λ replication protein gp*P* interacts with the host *dnaB* helicase, in at least one lambdoid phage (P22) the N-terminus of the analogous protein is fused to a C-terminal *dnaB*-related segment, and replication does not require *dnaB*. Similarly, homologies have been detected between genes of T4, P2 and λ whose products contribute to attachment of phage to the cell surface or which are closely linked to genes for attachment proteins. In both cases, the genes are clearly of a common origin but not a very recent one. Within a group, homologous genes are also highly diverged in some cases but much more similar in others. Thus some past exchanges have occurred between distantly related phages and between phage and host. If such exchanges were frequent and recent, the concept of distinct species with separate gene pools would have little use. The examples known at present can be reasonably viewed as rare lateral transfers across species boundaries.

See also: **Coliphage lambda (*Siphoviridae*); Phage Homologous Recombination; Phage taxonomy and classification; Phage toxins and disease.**

Further Reading

Campbell A (1988) Phage evolution and speciation. In: Calendar R (ed.) *The Bacteriophages*, p. 1. New York: Plenum Press.

Goyal SM, Gerba CP and Bitton G (eds) (1987) *Phage Ecology*. New York: John Wiley & Sons.

PHAGE PRD1 (*TECTIVIRIDAE*)

Dennis H Bamford, Institute of Biotechnology, Biocenter, University of Helsinki, Finland

Discovery and Classification

Bacteriophage PRD1 belongs to a group of very closely related phages isolated from different parts of the world. Other members of this group are PR3, PR4, PR5, LI7 and PR772. These viruses are lytic, infecting a vast variety of Gram-negative cells harboring a P-, N- or W-type conjugative plasmid. Among the hosts are *Escherichia coli, Salmonella typhimurium* and *Pseudomonas aeruginosa*. PRD1 is the type virus of the family *Tectiviridae*, genus *Tectivirus*. This virus family also includes members infecting Gram-positive hosts. PRD1 and PR4 are the most thoroughly studied phages in this group. Since these viruses are almost identical, the description of this phage system uses information from both of them. The discovery (1973–1979) was associated with studies of antibiotic-resistant plasmids. In further studies it was observed that they have an internal lipid membrane and a linear double-stranded DNA (dsDNA) genome with 5′-covalently-linked terminal proteins.

Properties of the Virion

The viral particle (**Fig. 1**) has an outer diameter of approximately 65 nm. The outer protein layer sur-

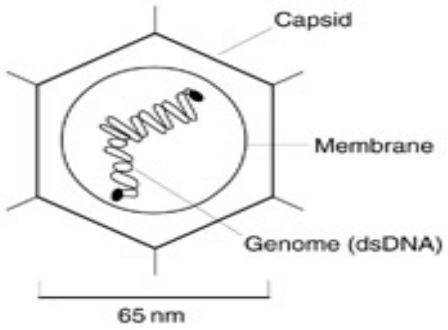


Figure 1 Major structural components of the PRD1 particle.

rounds the viral membrane. The viral genome resides inside this membrane vesicle. The virion is composed of approximately 70% protein, 15% DNA and 15% lipid. The outer protein layer is composed of the major coat protein P3 and the minor coat protein P5, as well as a small protein P30. P3 is a homotrimer. Protein P5 contains a $(Gly\text{-}X\text{-}Y)_6$ motif which is found in collagens and is cleavable with collagenase. Proteins P31, P5 and P2 together form adsorption spikes extending from the fivefold symmetry vertices. There are several proteins associated with the DNA injection (P11, P14, P16, P18) and DNA packaging (P9, P20, P22). In addition to these proteins, the membrane contains several small integral membrane proteins with, as yet, no described function. Protein P8 is covalently linked to the 5′-terminal nucleotides of the linear dsDNA genome. The membrane composition is approximately half protein, half lipid. The membrane phospholipids are composed of approximately 56% phosphatidylethanolamine, 37% phosphatidylglycerol, 5% cardiolipin and 2% neutral lipids. The fatty acid composition of the phospholipids is identical to that of the host.

Properties of the Genome and Genetics

Isolated nonsense mutants form 19 genetic complementation groups. Most of these are assigned to a virion protein. At least eight gene products are known to be nonstructural. The amber mutants can be complemented with cloned genomic fragments and thus the genes are located on the physical genome map determined from the restriction enzyme fragments.

Sequencing of the entire genome revealed 110 bp inverted terminal repeats at both ends of the 14 925 bp linear dsDNA (**Fig. 2**). The early genes for DNA replication and for transcription control are located at both ends of the genome. The genome terminal protein gene VIII and DNA polymerase gene I are at the left, and two genes coding for DNA-binding proteins at the right (genes XII and XIX). The latter two are so far the only genes transcribed from left to right. Gene XV codes for the phage lytic enzyme, the synthesis of which begins before that of the late gene products. The late genes are located in the 'upper' genome strand. Genes X, XVII and XXXII code for nonstructural assembly factors, whereas the rest of the late genes code for structural proteins. A gene designated with a roman numeral (for example I) produces an identified protein with the same arabic number (P1), whereas localized genes with as yet no identified gene product are designated with lower case letters (for example, gp o).

Life Cycle

The PRD1 life cycle is illustrated in **Fig. 3.** The phage adsorbs to the plasmid-encoded receptor. In optimal conditions, up to 50 phage particles can attach to one cell. The phage membrane forms a tube-like structure during the DNA injection event. The early transcrip-

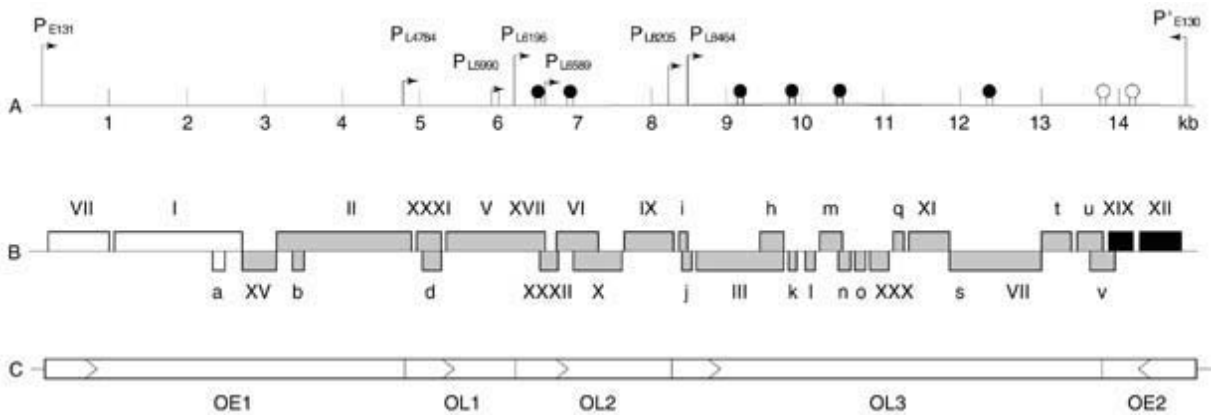
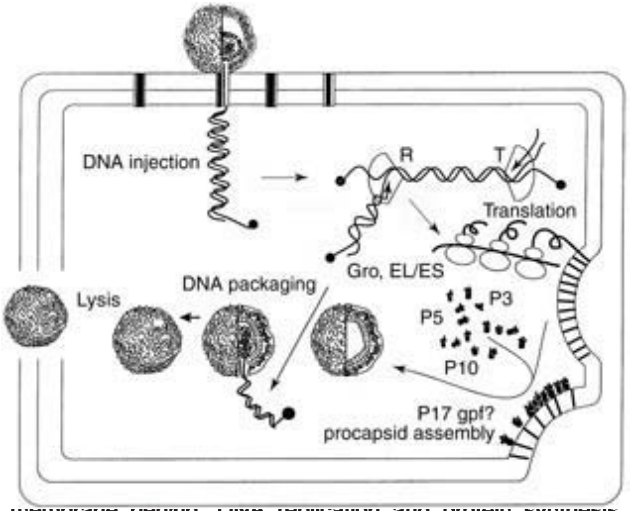


Figure 2 PRD1 genome. Genes with identified protein products are designated with roman numerals. Other localized genes are designated with lower case letters. For details see the text. The promoters (**A**) and operons (**B**) and their reading directions are indicated. (Courtesy of Dr Marika Grahn, University of Helsinki).



membrane behind. DNA replication and protein synthesis begins. The major capsid proteins are formed as soluble multimers, whereas membrane proteins are associated with the host membrane. Upon translocation of the membrane and its associated proteins, empty particles are formed. DNA packaging results in the formation of mature virions. Mature viral particles are released after cell lysis. R, replication; T, transcription.

tion leads to the synthesis of the genome terminal protein (P8) and the DNA polymerase (P1), which can initiate the replication from each genome end. The initial reaction is the formation of a phosphodiester bond between Tyr190 in the terminal protein and dGMP destined to become the third 5′-terminal nucleotide of the synthesized strand. Replication initiation from both ends leads to two identical molecules. However, initiation from only one end produces one displaced single-stranded DNA (ssDNA) molecule in addition to the complete dsDNA genome. The displaced ssDNA molecule can either initiate replication using paired inverted terminal repeats, or pair with another single strand with opposite polarity. The late protein synthesis leads to accumulation of coat protein (P3 and P5) multimers in the cytoplasm as well as to insertion of membrane proteins into the host plasma membrane.

Empty virus particles are formed in a process that is absolutely dependent at least on the major coat protein multimers and two assembly factors P10 and P17. In this membrane translocation event, the virus-specific membrane is removed from the host plasma membrane and placed inside the virus protein coat. The empty virus particles are located in the nucleoplasm area where DNA packaging takes place. The mature virus particles are preferentially located in the cell periphery prior to cell lysis. Cell lysis liberates several hundred virus particles, 10–15% of which are empty DNA-less particles.

Future Perspectives

Bacteriophage PRD1 is the only known protein-primed DNA-replication system operating in *E. coli*. An *in vitro* replication system with purified components produces full-length PRD1 DNA allowing a detailed study of the DNA synthesis. The DNA replication mechanism, trimeric coat protein arrangement and the presence of spikes are features sheared with adenovirus. The atomic resolution structure of the PRD1 coat protein trimer also reveals similarities with the adenovirus. This functional relationship will be under detailed study. The PRD1 membrane system is also potentially valuable in understanding membrane biogenesis and translocation.

See also: **Phage ϕ6 (*Cystoviridae*); Bacillus phage ϕ29 (*Podoviridae*).**

Further Reading

Bamford DH, Caldentey J and Bamford JKH (1995) Bacteriophage PRD1: a broad host range dsDNA tectivirus with an internal membrane. *Adv. Virus Res.* 45: 281.

Bamford DH, Ojala PM, Frilander M, Walin L and Bamford JKH (1995) Isolation, purification, and function of assembly intermediates and subviral particles of bacteriophages PRD1 and ϕ6. *Methods Mol. Genet.* 6: 455.

Butcher SJ, Bamford DH and Fuller SD (1995) DNA packaging orders the membrane of bacteriophage PRD1. *EMBO J.* 14: 6078.

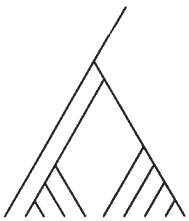
Caldentey J, Bamford JKH and Bamford DH (1990) Structure and assembly of bacteriophage PRD1, an *Escherichia coli* virus with a membrane. *J. Struct. Biol.* 104: 44.

Mindich L and Bamford DH (1988) Lipid-containing bacteriophages. In: Calendar R (ed.) *The Bacteriophages*, vol. 2, p. 475. New York: Plenum Press.

Salas M (1991) Protein-priming of DNA replication. *Annu. Rev. Biochem.* 60: 39.

repair model and the successive half crossing-over model. *Adv. Biophys.* 28: 81.
Kornberg A and Baker TA (1992) *DNA Replication* 2nd Edn. WH Freeman: New York.
Kowalczykowski SC, Dixon DA, Eggleston AK, Lauder SD and Rehrauer WM (1994) Biochemistry of homologous recombination in *Escherichia coli*. *Microbiol. Rev.* 58: 401.
Kucherlapati R and Smith GR (eds) (1992) *Genetic Recombination*. Washington, DC: American Society for Microbiology.

PHAGE TAXONOMY AND CLASSIFICATION



Jack Maniloff, Department of Microbiology and Immunology, University of Rochester, Medical Center, Rochester, New York, USA

Hans-Wolfgang Ackermann, Département de Biologie Médicale, Université Laval, Faculté de Médecine, Cité Universitaire, Québec, Canada

Audrey Jarvis, New Zealand Dairy Research Institute, Palmerston North, New Zealand

Introduction

In 1915 and 1917, Twort in London and d'Hérelle in Paris independently described agents which destroyed bacteria. D'Hérelle named his agent bacteriophage ('eater of bacteria'), which became shortened to phage, and began studies on the nature of phages which led to modern virology.

D'Hérelle believed there was only one species of phage and that strain diversity was due to extensive variation within this single species. Subsequent workers showed that, whereas different phage strains did indeed vary in their properties (e.g. antigenic specificity, and resistance to inactivation by physical and chemical agents), the properties of any one strain were fairly constant during repeated subculturing and cloning. This demonstrated that there were different phages and, as more phages were isolated, the need for their systematic classification arose.

As with animal and plant viruses, early efforts at phage taxonomy concentrated on groupings based on properties such as antigenic relationships, plaque morphology, particle size by ultrafiltration and heat resistance. Modern viral taxonomy began in 1962 when Lwoff, Horne and Tournier proposed a classification system based on properties of the virion and its nucleic acid. Procedures for viral taxonomy and nomenclature became organized in 1966 with establishment of the International Committee for Taxonomy of Viruses (ICTV). Since then, the ICTV has developed an internationally agreed nomenclature and taxonomy for viruses, through regular meetings and communications among virologists. A complete ICTV Report on virus taxonomy is published every few years: at present, there are 13 families and one unassigned genus of bacterial viruses (**Table 1**). 'Bacterial virus' is used here as a generic term for viruses infecting hosts in the domains *Bacteria* and *Archaea*.

The ICTV Universal System of Virus Taxonomy

Virus classification is based on structural, physicochemical and replication properties. Some of these criteria have been used since the earliest days of virology (e.g. antigenic relationships) and others have been developed only recently (e.g. nucleic acid sequencing). The taxa in virology are order, family, genus and species. Each taxonomic level is defined by a specific subset of properties. For example, virion morphology and size, and genome type and strandedness are high-level properties and define taxa at the order and/or family level, whereas nucleotide sequence and protein properties are low-level properties and define taxa at the genus and/or species level.

In practice, a virus strain (i.e. the progeny virus from a single isolation) is usually classified from the 'top down'. Classification generally begins with data on virion morphology and the nature of the genome to determine the order and/or family. Additional data are then used to determine the genus, and finally whether the strain is a new species. However, in

Table 1 Taxonomy of viruses of *Bacteria* and *Archaea*

Order	*Family*	*Genus*	*Type species*	*Host (Domain: taxa)*
Double-stranded DNA viruses				
Caudovirales	*Myoviridae*	'T4-like viruses'	Coliphage T4	*Bacteria:* entero- and related bacteria
		'P1-like viruses'	Coliphage P1	*Bacteria:* entero- and related bacteria
		'P2-like viruses'	Coliphage P2	*Bacteria:* entero- and related bacteria
		'Mu-like viruses'	Coliphage Mu	*Bacteria:* enterobacteria
		'SPO1-like viruses'	Bacillus phage SPO1	*Bacteria: Bacillus*
		'ΦH-like viruses'	Halobacterium phage ϕH	*Archaea: Halobacterium*
	Siphoviridae	'λ-like viruses'	Coliphage λ	*Bacteria:* enterobacteria
		'T1-like viruses'	Coliphage T1	*Bacteria:* enterobacteria
		'T5-like viruses'	Coliphage T5	*Bacteria:* enterobacteria, *Vibrio*
		'L5-like viruses'	Mycobacterium phage L5	*Bacteria:* mycobacteria
		'c2-like viruses'	Lactococcus phage c2	*Bacteria: Lactococcus*
		'ψM1-like viruses'	Methanobacterium phage ψM1	*Archaea: Methanobacterium*
	Podoviridae	'T7-like viruses'	Coliphage T7	*Bacteria:* entero- and related bacteria
		'P22-like viruses'	Enterobacteria phage P22	*Bacteria:* enterobacteria
		'ϕ29-like viruses'	Bacillus phage ϕ29	*Bacteria: Bacillus, Kurthia, Streptococcus*
	Tectiviridae	*Tectivirus*	Polyvalent phage PRD1	*Bacteria:* proteobacteria, *Bacillus, Thermus*
	Corticoviridae	*Corticovirus*	Alteromonas phage PM2	*Bacteria: Alteromonas, Vibrio*
	Plasmaviridae	*Plasmavirus*	Acholeplasma phage L2	*Bacteria: Acholeplasma* (mycoplasma)
	Lipothrixviridae	*Lipothrixvirus*	Thermoproteus virus TTV1	*Archaea: Thermoproteus, Desulfurolobus*
	Rudiviridae	*Rudivirus*	Thermoproteus virus TTV4	*Archaea: Thermoproteus, Sulfolobus*
	Fuselloviridae	*Fusellovirus*	Sulfolobus virus SSV1	*Archaea: Sulfolobus*
		Unassigned genus SNDV	Sulfolobus virus SNDV	*Archaea: Sulfolobus*
Single-stranded DNA viruses				
	Inoviridae	*Inovirus*	Coliphage Ff	*Bacteria:* entero- and related bacteria, *Thermus*
		Plectrovirus	Acholeplasma phage L51	*Bacteria: Acholeplasma, Spiroplasma* (mycoplasma)
	Microviridae	*Microvirus*	Coliphage ϕX174	*Bacteria:* enterobacteria
		Spiromicrovirus	Spiroplasma phage SpV4	*Bacteria: Spiroplasma* (mycoplasma)
		Bdellomicrovirus	Bdellovibrio phage MAC-1	*Bacteria: Bdellovibrio*
		Chlamydiamicrovirus	Chlamydia phage Chp1	*Bacteria: Chlamydia*
Double-stranded RNA viruses				
	Cystoviridae	*Cystovirus*	Pseudomonas phage ϕ6	*Bacteria: Pseudomonas*
Positive-sense, single-stranded RNA viruses				
	Leviviridae	*Levivirus*	Enterobacteriophage MS2	*Bacteria:* enterobacteria
		Allolevirus	Enterobacteriophage Qβ	*Bacteria:* enterobacteria

principle, taxa are defined from the 'bottom up', because the species is the fundamental taxonomic unit in biological systematics. Genera are then defined in terms of species, families in terms of genera and orders in terms of families.

Virus species

The ICTV definition of a virus species is: 'A virus species is a polythetic class of viruses that constitutes a replicating lineage and occupies a particular eco-

logical niche.' This means that members of a species must have a number of properties in common, but no single property need be shared by all members of the species. This definition involves subjective decisions because some virus species contain strains with greater phenotypic and/or genetic diversity than others. Species are given English vernacular names.

The criteria used to define phage species are properties such as virion morphology (e.g. fine structure, protein composition and serological relationships), DNA homology, sequence similarity and physiology (e.g. host range and burst size). These generally are adequate to identify a virus or group of viruses as distinct from other groupings. However, as for other viruses, there is an element of judgment in deciding whether a particular bacterial virus should be considered a species or a strain.

Virus genera

The ICTV definition of a virus genus is: 'A virus genus is a group of species sharing certain common characters.' Like the species definition, this involves subjective judgments, so there is significant diversity among genera in different families. Genus names end in *-virus*.

A variety of criteria have been employed to define phage genera. Properties related to DNA replication and packaging have been used for defining tailed phage genera. Other useful properties are presence of protein covalently linked to DNA 5′ termini, virus-encoded type A (Pol I) or B (Pol II) DNA polymerase and/or RNA polymerase, occurrence of unusual DNA bases, DNA replication via transposition, temperate infection with the provirus either integrated into the host chromosome or persisting as a plasmid, genome organization and host range.

A species for which considerable data are available is designated the type species of a genus, although this may not be the virus most typical of the properties of all species in a genus.

Virus families

The ICTV definition of a virus family is: 'A virus family is a group of genera sharing certain common characters.' Family names end in *-viridae*, and are defined by high-level criteria, such as virion morphology and the nature of the genome.

Virus orders

The ICTV recently introduced the taxonomic category of 'order', defined as 'a group of families sharing certain common characters'. Order names end in *-virales*. The first two orders were established for groups of animal virus families, based on similar genome organization and replication strategies. The third order, *Caudovirales*, was established in 1998 for the three families of tailed phages with linear double-stranded (ds) DNA genomes: *Myoviridae* (phages with contractile tails), *Siphoviridae* (phages with long noncontractile tails), and *Podoviridae* (phages with short tails). These families are grouped in a single order based on a set of properties involving morphology, replication, assembly and infection cycle.

Description of New Phages – The ICTV Process

The major organizational units of the ICTV are subcommittees for viruses infecting different hosts: i.e. Bacterial, Fungal, Plant, Invertebrate and Vertebrate Virus Subcommittees. At present (mid-1998), the Bacterial Virus Subcommittee consists of 19 virologists, from ten countries, with expertise in all bacterial virus families and most important bacterial host groups. *Ad hoc* study groups are formed as needed.

Over the years, the ICTV has evolved rules for virus nomenclature and procedures for establishing new taxa. New taxonomic proposals are usually initiated by subcommittee study groups, but can also be submitted by individual virologists, and require approval by the appropriate subcommittee, the ICTV Executive Committee, and finally an ICTV Plenary Session. The process is highly interactive between organizational levels within the ICTV, in an effort to assure clarity and uniformity in viral nomenclature and taxonomy.

Description of Phage Families

The most recent literature survey of phages was completed in 1995 and covered 4551 phage descriptions: 96% were tailed phages and 4% were isometric, filamentous or pleomorphic phages. The 4389 tailed phages are classified into three families, whereas the 162 other phages form ten families and one unassigned genus (**Table 1**). Hence, each tailed phage family contains many members, with significant diversity in morphology and other characteristics.

The three tailed phage families, grouped in the order *Caudovirales*, are distinguished by tail structure. Each tailed phage family contains many species, with sufficient data on a small number to enable them to be grouped into genera. There have been 1073 descriptions of phages with contractile tails (family *Myoviridae*); 2708 of phages with long, noncontractile tails (family *Siphoviridae*); and 608 of phages with short tails (family *Podoviridae*).

The taxonomy, major characteristics, and host

range of the 13 phage families and one unassigned genus are summarized below. A complete listing of the members in each taxon is in the seventh ICTV Report.

Myoviridae

Taxonomy

Family: *Myoviridae*
Genus: 'T4-like viruses'
Genus: 'P1-like viruses'
Genus: 'P2-like viruses'
Genus: 'Mu-like viruses'
Genus: 'SPO1-like viruses'
Genus: 'ΦH-like viruses'

Characteristics *Myoviridae* have contractile tails and contain linear dsDNA. Virions have a relatively large head and a long, thick, complex, contractile tail (80–455 nm × 16–20 nm), consisting of a central tube surrounded by a contractile sheath and ancillary structures (e.g. tail fibers). Heads, tails and (when present) tail fibers are assembled by separate pathways.

'T4-like viruses' (type species: coliphage T4) have elongated heads (111 × 78 nm) and tails with long, kinked fibers. The genome is about 170 kb dsDNA, circularly permuted and terminally redundant, typically containing 5-hydroxymethylcytosine and encoding a type B DNA polymerase. The genetic map is circular, and progeny DNA is packaged by a headful mechanism from concatemers formed via recombination. Infection is virulent. Host range: enterobacteria and other proteobacteria.

'P1-like viruses' (type species: coliphage P1) have icosahedral heads about 85 nm in diameter. The genome is about 100 kb dsDNA, with limited circular permutation and terminal repeats. Concatemers are formed from Θ structures by rolling circle replication and progeny DNA is packaged from concatemers by a headful mechanism from *pac* sites. Infection is temperate and prophages generally persist as plasmids. Host range: enterobacteria and other proteobacteria.

'P2-like viruses' (type species: coliphage P2) have icosahedral heads about 60 nm in diameter. The genome is about 33 kb dsDNA, with cohesive ends (*cos* sites). Infection is temperate and prophages are integrated into host chromosomes by site-specific recombination. DNA replication is via Θ structures to form covalently closed circular DNA, and progeny DNA is packaged from covalently closed circular molecules cleaved at a *cos* site. Host range: enterobacteria and other proteobacteria.

'Mu-like viruses' (type species: coliphage Mu) have icosahedral heads about 60 nm in diameter. The genome is about 43 kb dsDNA, consisting of the viral genome with variable host DNA sequences at both ends. Infection is temperate and prophages integrate into host chromosomes by site-specific recombination. DNA replication is via replicative transposition of phage genomes to random sites in the host chromosome, and progeny DNA is packaged from integrated phage DNA. Host range: enterobacteria.

'SPO1-like viruses' (type species: *Bacillus* phage SPO1) have icosahedral heads about 94 nm in diameter. The genome is 140–160 kb dsDNA, with short terminal repeats, containing hydroxymethyluracil, and encoding a type A DNA polymerase. Infection is virulent. DNA replication is via concatemer formation, and progeny DNA is assumed to be packaged by headful packaging from concatemers, with site-specific cleavage and DNA synthesis to duplicate terminal repeats. Host range: *Bacillus*.

'ΦH-like viruses' (type species: *Halobacterium* phage ΦH) infect archaebacteria. Virions have icosahedral heads about 64 nm in diameter. The genome is about 59 kb dsDNA, with limited circular permutation and terminal repeats, and containing methylcytosine. Infection is temperate and prophages persist as plasmids. Early transcription is regulated by viral antisense mRNA. DNA replication results in concatemer formation and progeny DNA is packaged from *pac* sites. Host range: *Halobacterium*.

Other members of the *Myoviridae* A large number of unassigned species have been described infecting a wide range of bacteria.

Siphoviridae

Taxonomy

Family: *Siphoviridae*
Genus: 'λ-like viruses'
Genus: 'T1-like viruses'
Genus: 'T5-like viruses'
Genus: 'L5-like viruses'
Genus: 'c2-like viruses'
Genus: 'ψM1-like viruses'

Characteristics *Siphoviridae* have long, thin, noncontractile tails (65?–825 × 7–10 nm) and contain linear dsDNA. Heads and tails are assembled separately.

'λ-like viruses' (type species: coliphage λ) are temperate, with the prophage integrated into the host chromosome by site-specific recombination. The genome is about 49 kb dsDNA, with *cos* sites. DNA replication is via Θ structures followed by rolling circle replication to produce concatemers, with progeny DNA cut and packaged from concatemers at *cos* sites. Host range: enterobacteria.

'T1-like viruses' (type species: coliphage T1) have

extremely flexible tails. The genome is about 49 kb dsDNA, with limited circular permutation and terminal repeats. DNA replication is via concatemers formed by recombination, and progeny DNA is packaged from concatemers by headful packaging from *pac* sites. Infection is virulent. Host range: enterobacteria.

'T5-like viruses' (type species: coliphage T5) have large heads and long, kinked tail fibers. The genome is about 125 kb dsDNA, with terminal repeats and single-strand gaps, and encodes a type A DNA polymerase. DNA injection is by 2-step transfer process; concatemers are formed. Infection is virulent. Progeny genomes may be packaged by a headful mechanism from concatemers, with site-specific cleavage and DNA synthesis to duplicate terminal repeats. Host range: enterobacteria, *Vibrio*.

'L5-like viruses' (type species: *Mycobacterium* phage L5) are temperate, with the prophage integrated into host chromosome by site-specific recombination. The genome is about 52 kb dsDNA with *cos* sites, encoding a type A DNA polymerase. Host range: *Mycobacterium*.

'c2-like viruses' (type species: *Lactococcus* phage c2) have prolate heads. The genome is about 22 kb dsDNA with *cos* sites, possibly encoding a type B DNA polymerase. Infection is virulent. Host range: *Lactococcus*.

'ψM1-like viruses' (type species: *Methanobacterium* phage ψM1) produce virulent infections in archaebacteria. The genome is about 30 kb dsDNA, circularly permuted and terminally redundant. Host range: *Methanobacterium*.

Other members of the *Siphoviridae* A large number of unassigned species have been described infecting a wide range of bacteria.

Podoviridae

Taxonomy
Family: *Podoviridae*
Genus: 'T7-like viruses'
Genus: 'P22-like viruses'
Genus: 'ϕ29-like viruses'

Characteristics *Podoviridae* have short, noncontractile tails, about 20 × 8 nm, and contain linear dsDNA. Heads are assembled first, DNA is packaged, and tail subunits assembled on the completed head.

'T7-like viruses' (type species: coliphage T7) produce virulent infections. The genome is about 40 kb dsDNA with short terminal repeats, encoding a type A DNA polymerase and RNA polymerase. Concatemers are formed by annealing 3′ termini of daughter molecules. DNA packaging is from concatemers via site-specific cleavage and DNA synthesis to duplicate terminal repeats. Host range: enterobacteria and other proteobacteria.

'P22-like viruses' (type species: enterobacteria phage P22) are temperate, with the prophage integrated into host chromosome by site-specific recombination. The genome is about 43 kb dsDNA, with limited circular permutation and terminal repeats, and encoding a type A DNA polymerase. DNA replication is via Θ structures followed by rolling-circle replication to produce concatemers, and packaging is from *pac* sites in concatemers. Host range: enterobacteria.

'ϕ29-like viruses' (type species: *Bacillus* phage ϕ29) produce virulent infections. The genome is about 19 kb dsDNA, with short inverted terminal repeats and encoding a type B DNA polymerase. Virus-encoded protein is covalently linked to DNA 5′ termini, to prime DNA replication from both termini to produce linear monomeric daughter molecules with protein covalently linked to 5′ termini. Host range: *Bacillus, Kurthia, Streptococcus*.

Other members of *Podoviridae* A large number of unassigned species have been described infecting a wide range of bacteria.

Tectiviridae

Taxonomy
Family: *Tectiviridae*
Genus: *Tectivirus*

Characteristics Tectiviruses (type species: polyvalent phage PRD1) are lipid-containing dsDNA phages. Virions are nonenveloped icosahedral particles, about 63 nm in diameter with spikes at the vertices. The protein capsid encloses an inner, DNA-containing, lipoprotein vesicle. Virions are normally tailless, but produce tail-like tubes, about 60 × 10 nm, upon adsorption or after chloroform treatment. The genome is about 15 kb linear dsDNA, with inverted terminal repeats and protein covalently linked to DNA 5′ termini, and encoding a type B DNA polymerase. DNA replication is protein-primed and proceeds from both termini by strand displacement. Infection is virulent. Host range: phage PRD1 and relatives infect proteobacteria with P, N, or W incompatibility plasmids (*Acinetobacter*, enterobacteria, *Pseudomonas, Vibrio* and possibly others); phage P37-14 and relatives infect *Thermus*; phages AP50, NS11 and relatives infect *Bacillus*.

Corticoviridae

Taxonomy
Famsgml_outily: *Corticoviridae*
Genus: *Corticovirus*

Characteristics Corticoviruses (type species: *Alteromonas* phage PM2) are lipid-containing dsDNA phages. Virions are nonenveloped icosahedral particles about 60 nm in diameter, consisting of two protein shells separated by a lipid bilayer and spikes at the vertices. The inner protein shell contains a transcriptase. The genome is about 9 kb covalently closed, superhelical, circular dsDNA, encoding a transcriptase. Infection is virulent. DNA replication proceeds unidirectionally. Inner protein shells of progeny virions are assembled first, filled with DNA, and completed by progressive addition of lipids, the outer shell and spikes. Host range: *Alteromonas*; an unassigned species in the family infects *Vibrio*.

Plasmaviridae

Taxonomy
Family: *Plasmaviridae*
Genus: *Plasmavirus*

Characteristics Plasmaviruses (type species: *Acholeplasma* phage L2) are dsDNA phages that infect mycoplasmas. Virions are quasi-spherical, slightly pleomorphic, enveloped particles, about 80 nm (range 50–125 nm) in diameter. No capsid is detectable. Size range is due to virion heterogeneity; at least three distinct virion forms are produced during infection. The genome is 12 kb circular, superhelical dsDNA. Infection includes both a nonlytic cytocidal productive infectious cycle and a lysogenic cycle in each infected cell. Noncytocidal infection involves progeny virus released by budding from the host cell membrane, with the host surviving as a lysogen. Lysogeny involves integration into a unique site in the host chromosome. Host range: *Acholeplasma*.

Lipothrixviridae

Taxonomy
Family: *Lipothrixviridae*
Genus: *Lipothrixvirus*

Characteristics Lipothrixviruses (type species: *Thermoproteus* virus TTV1) are dsDNA phages that infect archaebacteria. Virions are thick, enveloped, rigid rods, about 400 nm long and 40 nm in diameter. The genome is about 16 kb linear dsDNA. Infection results in virus production with lysis or establishment of a carrier state. Pieces of TTV1 DNA may be integrated into host genomes. Host range: *Thermoproteus*; an unassigned species in the family infects *Desulfurolobus*.

Rudiviridae

Taxonomy
Family: *Rudiviridae*
Genus: *Rudivirus*

Characteristics Rudiviruses (type species: *Thermoproteus* virus TTV4) are dsDNA phages that infect archaebacteria. Virions are stiff, nonenveloped, rod-shaped particles, 26–30 × 500–950 nm. The genome is 17–33 kb linear dsDNA. Host range: *Thermoproteus, Sulfolobus*.

Fuselloviridae

Taxonomy
Family: *Fuselloviridae*
Genus: *Fusellovirus*

Characteristics Fuselloviruses (type species: *Sulfolobus* virus SSV1) are dsDNA phages that infect archaebacteria. Virions are lemon-shaped, 60 × 100 nm, and slightly flexible in appearance with short tail fibers attached to one pole. The virion envelope consists of host lipids and two virus-encoded proteins; a third protein is DNA-associated. The genome is about 15 kbp circular, positively supercoiled dsDNA and, after infection, is integrated into a tRNA gene in the host chromosome. UV induction results in large numbers of particles which are released without lysis. Host range: *Sulfolobus*.

Sulfolobus Virus SNDV

Taxonomy
Unassigned genus: *Sulfolobus* virus SNDV

Characteristics *Sulfolobus* virus SNDV is a dsDNA phage that infects archaebacteria. Virions are 80 × 180 nm droplet-shaped particles. The genome is about 20 kb circular, covalently-closed dsDNA. Host range: *Sulfolobus*.

Inoviridae

Taxonomy
Family: *Inoviridae*
Genus: *Inovirus*
Genus: *Plectrovirus*

Characteristics *Inoviridae* are single-stranded (ss)DNA phages with helical symmetry. Virions are nonenveloped, rod-shaped or filamentous particles, with virion length determined by the size of the genome. The genome is circular, positive-sense ssDNA, usually in the range 5–10 kb. Productive

infection involves conversion of parental ssDNA into replicative form (RF) dsDNA, semiconservative RF replication, synthesis of progeny ssDNA by a rolling circle mechanism, and membrane-based progeny virus assembly and extrusion without cell lysis. Infected host cells survive in a carrier state. However, there are lysogenic *Vibrio* and *Xanthomonas* inovirus strains that encode integrases and viral sequences are found integrated at several host chromosome sites. Vibrio phage CTX encodes the two subunits of cholera toxin, which are expressed on conversion of the lysogen into nonlytic productive infection.

Inovirus (type species: coliphage Ff, where Ff is the collective designation for coliphages M13, f1, and fd) particles are 700–2000 × 6–8 nm, with genomes of 6–9 kb. Host range: entero- and other proteobacteria, *Thermus*.

Plectrovirus (type species: *Acholeplasma* phage L51) particles are 70–90 × 14–16 nm for *Acholeplasma* phages and 230–280 × 10–15 nm for *Spiroplasma* phages, with genomes about 4.5 kb for *Acholeplasma* phages and 8 kb for *Spiroplasma* phages. Host range: *Acholeplasma, Spiroplasma* (mycoplasmas).

Microviridae

Taxonomy
Family: *Microviridae*
Genus: *Microvirus*
Genus: *Spiromicrovirus*
Genus: *Bdellomicrovirus*
Genus: *Chlamydiamicrovirus*

Characteristics *Microviridae* are isometric ssDNA phages. Virions are nonenveloped, 22–33 nm in diameter (depending on the orientation chosen for measurement), with icosahedral symmetry. The genome is 4.4–5.4 kb circular, positive-sense ssDNA. Infection is virulent and involves adsorption to cell walls (cell membranes for *Spiromicrovirus*), parental ssDNA conversion into circular RF DNA, synthesis of progeny ssDNA from RF DNA, and progeny virus release by cell lysis (by a nonlytic cytocidal mechanism for *Spiromicrovirus*).

Microvirus (type species: coliphage ϕX174) can be divided into three major groups based on their host enzyme requirement for viral DNA replication. Host range: enterobacteria.

Spiromicrovirus (type species: *Spiroplasma* phage SpV4) infects mycoplasmas and, therefore, adsorbs to and is released from wall-less bacteria. Also, like their host cells, viruses use TGA as the tryptophan codon instead of as the 'universal' stop codon. Host range: *Spiroplasma*.

Bdellomicrovirus (type species: *Bdellovibrio* phage MAC-1) has not been extensively characterized, but is distinguished by its host. Host range: *Bdellovibrio*.

Chlamydiamicrovirus (type species: *Chlamydia* phage Chp1) replicates in *Chlamydia* reticulate bodies, and transmission is via infection of extracellular *Chlamydia* elementary bodies. Host range: *Chlamydia*.

Cystoviridae

Taxonomy
Family: *Cystoviridae*
Genus: *Cystovirus*

Characteristics Cystoviruses (type species: *Pseudomonas* phage ϕ6) are dsRNA phages. Virions are about 85 nm in diameter, consisting of an envelope enclosed 58 nm icosahedral capsid. The capsid contains RNA polymerase and three linear dsRNA molecules. The three dsRNA genome segments are 2.9, 4.1 and 6.4 kb. Infection is virulent and involves adsorption to pili, which retract bringing the virion into contact with the host outer membrane. After fusion of viral and cellular outer membranes, the capsid enters the periplasmic space. Viral RNA replication is semiconservative with viral-encoded RNA polymerase to produce progeny dsRNA. Virus release is by cell lysis. Host range: *Pseudomonas*.

Leviviridae

Taxonomy
Family: *Leviviridae*
Genus: *Levivirus*
Genus: *Allolevivirus*

Characteristics *Leviviridae* are nonenveloped, icosahedral particles about 26 nm in diameter, containing ssRNA. The genome is linear, positive-sense ssRNA, either about 3.5 kb or 4.2 kb. Infection is by adsorption to pili determined by a wide variety of different plasmids. Viral replication is via a virus-encoded replicase, which requires host translation factors as cofactors. Release is by cell lysis.

Levivirus (type species: enterobacteriophage MS2) particles contain shorter genomes (about 3.5 kb) and have a separate gene for cell lysis, which partly overlaps the replicase coding region in the +1 reading frame. Overlap with the coat protein gene is variable. Synthesis of the lysis protein is dependent on translation of the coat protein gene. Host range: enterobacteria.

Allolevivirus (type species: enterobacteriophage Qβ) particles contain longer genomes (about 4.2 kb). The extra RNA encodes a carboxy-terminal extension of the coat protein, which is expressed by occasional suppression of the coat gene termination

codon. There is no separate lysis gene. Host range: enterobacteria.

Other members of *Leviviridae* There are unassigned species infecting *Acinetobacter*, *Caulobacter* and *Pseudomonas*.

See also: **Taxonomy and classification – general.**

Further Reading

Ackermann H-W (1996) Frequency of morphological phage descriptions in 1995. *Arch. Virol.* 141: 209.

Ackermann H-W, DuBow MS (1987) *Viruses of Prokaryotes*, vols 1 and 2. Boca Raton, FL: CRC Press.

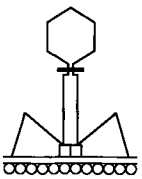
Calendar R (ed.) (1988) *The Bacteriophages*, vols 1 and 2. New York: Plenum Press.

Campbell AM (1996) Bacteriophages. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, vol. 1, p. 587. Philadelphia: Lippincott-Raven.

Fauquet C, van Regenmortel MHV, Bishop DHL *et al* (eds) (1999) *Seventh Report of the International Committee on Taxonomy of Viruses*. New York: Academic Press. (in preparation).

Maniloff J, Ackermann H-W (1998) Taxonomy of bacterial viruses: establishment of tailed virus genera and the Order *Caudovirales*. *Arch. Virol.* 143: 2051.

PHAGE TOXINS AND DISEASE



Randall K Holmes and **Michael G Jobling**, Department of Microbiology, University of Colorado Health Sciences Center, Denver, Colorado, USA

Michael P Schmitt, Laboratory of Bacterial Toxins, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland, USA

Introduction

Bacteriophages are associated with most bacteria, including pathogenic bacteria. Many bacterial pathogens produce toxic proteins that function as virulence factors and cause characteristic signs or symptoms of disease in humans or animals. The ability of some pathogenic bacteria to produce toxins is determined by specific temperate phages. When a nontoxinogenic bacterium becomes toxinogenic as a consequence of infection or lysogenization by a specific phage, the process is called phage conversion (or lysogenic conversion). Phenotypic characteristics other than toxinogenicity can also be determined by phage conversion.

Some nonconverting phages are highly homologous to converting phages, except that the determinants for toxin production are absent or nonfunctional in the nonconverting phages. The determinants for toxinogenicity in converting phages are usually not essential either for lytic growth or for lysogeny. Converting phages are similar to specialized transducing phages, except that the origin of genes that determine toxinogenicity is unknown.

Toxins that are produced as a result of phage conversion include diphtheria toxin in *Corynebacterium diphtheriae*, Shiga toxins in *Escherichia coli*, pyrogenic exotoxins in *Streptococcus pyogenes*, enterotoxins in *Staphylococcus aureus*, botulinum toxins in *Clostridium botulinum*, and cholera toxin in *Vibrio cholerae*. The converting phages in these pathogenic bacteria contain the structural genes for the corresponding toxins, but the various toxins differ greatly in biochemical structure, immunogenicity, mode of action and toxicity. This chapter emphasizes the characteristics of phage-encoded toxins that are important for pathogenesis of diseases in humans.

Diphtheria Toxin

Diphtheria is a local infection of the upper respiratory tract or skin that is caused by *C. diphtheriae* and spread from person to person by respiratory droplets or intimate contact. The characteristic lesion in the respiratory tract is a pseudomembrane that can sometimes cause obstruction of the airway. Diphtheria toxin produced at the site of local infection is distributed systemically and can cause myocarditis, polyneuritis and other complications. Respiratory diphtheria is usually caused by toxinogenic strains of *C. diphtheriae*, but cutaneous diphtheria is often caused by nontoxinogenic strains. The primary treatment for diphtheria is parenteral administration of diphtheria antitoxin to neutralize toxin that has not yet entered target cells. Antibiotics are used to eradi-

codon. There is no separate lysis gene. Host range: enterobacteria.

Other members of *Leviviridae* There are unassigned species infecting *Acinetobacter*, *Caulobacter* and *Pseudomonas*.

See also: **Taxonomy and classification – general.**

Further Reading

Ackermann H-W (1996) Frequency of morphological phage descriptions in 1995. *Arch. Virol.* 141: 209.

Ackermann H-W, DuBow MS (1987) *Viruses of Prokaryotes*, vols 1 and 2. Boca Raton, FL: CRC Press.

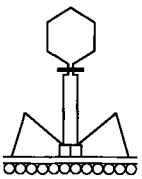
Calendar R (ed.) (1988) *The Bacteriophages*, vols 1 and 2. New York: Plenum Press.

Campbell AM (1996) Bacteriophages. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, vol. 1, p. 587. Philadelphia: Lippincott-Raven.

Fauquet C, van Regenmortel MHV, Bishop DHL *et al* (eds) (1999) *Seventh Report of the International Committee on Taxonomy of Viruses*. New York: Academic Press. (in preparation).

Maniloff J, Ackermann H-W (1998) Taxonomy of bacterial viruses: establishment of tailed virus genera and the Order *Caudovirales*. *Arch. Virol.* 143: 2051.

PHAGE TOXINS AND DISEASE



Randall K Holmes and **Michael G Jobling**, Department of Microbiology, University of Colorado Health Sciences Center, Denver, Colorado, USA

Michael P Schmitt, Laboratory of Bacterial Toxins, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland, USA

Introduction

Bacteriophages are associated with most bacteria, including pathogenic bacteria. Many bacterial pathogens produce toxic proteins that function as virulence factors and cause characteristic signs or symptoms of disease in humans or animals. The ability of some pathogenic bacteria to produce toxins is determined by specific temperate phages. When a nontoxinogenic bacterium becomes toxinogenic as a consequence of infection or lysogenization by a specific phage, the process is called phage conversion (or lysogenic conversion). Phenotypic characteristics other than toxinogenicity can also be determined by phage conversion.

Some nonconverting phages are highly homologous to converting phages, except that the determinants for toxin production are absent or nonfunctional in the nonconverting phages. The determinants for toxinogenicity in converting phages are usually not essential either for lytic growth or for lysogeny. Converting phages are similar to specialized transducing phages, except that the origin of genes that determine toxinogenicity is unknown.

Toxins that are produced as a result of phage conversion include diphtheria toxin in *Corynebacterium diphtheriae*, Shiga toxins in *Escherichia coli*, pyrogenic exotoxins in *Streptococcus pyogenes*, enterotoxins in *Staphylococcus aureus*, botulinum toxins in *Clostridium botulinum*, and cholera toxin in *Vibrio cholerae*. The converting phages in these pathogenic bacteria contain the structural genes for the corresponding toxins, but the various toxins differ greatly in biochemical structure, immunogenicity, mode of action and toxicity. This chapter emphasizes the characteristics of phage-encoded toxins that are important for pathogenesis of diseases in humans.

Diphtheria Toxin

Diphtheria is a local infection of the upper respiratory tract or skin that is caused by *C. diphtheriae* and spread from person to person by respiratory droplets or intimate contact. The characteristic lesion in the respiratory tract is a pseudomembrane that can sometimes cause obstruction of the airway. Diphtheria toxin produced at the site of local infection is distributed systemically and can cause myocarditis, polyneuritis and other complications. Respiratory diphtheria is usually caused by toxinogenic strains of *C. diphtheriae*, but cutaneous diphtheria is often caused by nontoxinogenic strains. The primary treatment for diphtheria is parenteral administration of diphtheria antitoxin to neutralize toxin that has not yet entered target cells. Antibiotics are used to eradi-

cate C. *diphtheriae*, eliminate the convalescent carrier state, and prevent spread of infection to susceptible contacts. Active immunization with diphtheria toxoid is highly effective in preventing diphtheria. Diphtheria is now rare in the United States and most developed countries, but it re-emerged as an epidemic disease in the early 1990s in the New Independent States of the former Soviet Union as a consequence of inadequate implementation of immunization programs during a period of severe socioeconomic disruption.

Toxinogenic conversion in C. *diphtheriae* was discovered in 1951, and in 1971 the structural gene for diphtheria toxin was shown to be phage-encoded. C. *ulcerans* and C. *pseudotuberculosis*, animal pathogens that occasionally cause disease in humans, can also carry tox^+ corynephages and produce diphtheria toxin. Phage β, the best characterized of the tox^+ corynephages, is an inducible, temperate phage with a polyhedral head and a long noncontractile tail. It has a linear, double-stranded, 34.7 kbp DNA genome with cohesive ends. On the vegetative map the genes for head and tail components are located at opposite ends, and the *tox* gene is located centrally between the immunity region *imm* and the host range marker *h*. The phage integrates into the host chromosome by means of site-specific recombination between the phage locus *attP* and an homologous bacterial locus *attB*. The prophage map is a circular permutation of the vegetative phage map, and the *tox* gene in the prophage is adjacent to the attachment site. This location suggests that an ancestral *tox* gene located on the bacterial chromosome may have been acquired by the phage by illegitimate recombination during prophage excision. Several tox^+ corynephages that vary with respect to virion morphology, antigenicity, and immunity specificity have homologous genomes, indicating that they constitute a family of related corynephages.

Diphtheria toxin (DT) is produced by C. *diphtheriae* as a secreted, extracellular protein of 535 amino acid residues. DT is cleaved by a protease such as trypsin or furin into an amino-terminal fragment A (21 kDa) and a carboxyl-terminal fragment B (37 kDa) that remain linked by a disulfide bond. The receptor-binding (R) domain of fragment B interacts with a receptor (heparin-binding EGF-like growth factor precursor) on susceptible cells and mediates entry of DT by endocytosis. Following acidification of the endosomes, the transmembrane (T) domain of fragment B inserts into the endosomal membrane and mediates translocation of fragment A into the cytoplasm. Fragment A catalyzes ADP-ribosylation of the unique diphthamide residue of EF-2, causing inactivation of EF-2, inhibition of protein synthesis and cell death. One molecule of fragment A in the cytoplasm can kill a susceptible eukaryotic cell. Exotoxin A from *Pseudomonas aeruginosa* ADP-ribosylates EF-2 in the same manner as DT, but it interacts with a different cell surface receptor and therefore differs from DT in its species and tissue specificity.

Production of diphtheria toxin by C. *diphtheriae* is regulated by iron, and maximal toxin production occurs during late log or stationary phase in iron-depleted medium. Transcription of the *tox* gene is repressed under high-iron growth conditions by the diphtheria toxin repressor (DtxR), an Fe^{2+}-activated global regulatory protein that also controls production of corynebacterial siderophore, utilization of ferric siderophore complexes, and probably other functions in C. *diphtheriae*. DtxR binds to promoter/operators that have a 19 bp core homologous to the palindromic consensus sequence TTAGGTTAGCC-TAACCTAA. The function of DtxR in C. *diphtheriae* is similar to that of the ferric uptake regulator (Fur) protein in *E. coli*, but DtxR and Fur bind to different consensus sequences and are therefore specific for different operators.

Shiga Toxins of *Escherichia coli*

Shigella dysenteriae type 1 causes bacillary dysentery and produces the classical Shiga toxin (Stx). Some pathogenic *E. coli* that produce disease in humans or animals produce toxins (currently designated Stx1 and Stx2) that are closely related to Stx. These *E. coli* Shiga toxins were previously called Shiga-like toxins (SLT) or Vero toxins (VT). *E. coli* that cause hemorrhagic colitis and hemolytic uremic syndrome in humans (enterohemorrhagic *E. coli* [EHEC]) typically produce moderate to large amounts of Stx1, Stx2, or both, and *E. coli* that cause edema disease in pigs produce a variant of Stx2 called Stx2e.

Classification of Shiga toxins is based on their antigenicity, amino acid sequences, and biochemical properties. Stx1 and Stx are antigenically indistinguishable and differ by a single, conservative amino acid substitution. Stx2 and Stx2e do not crossreact antigenically with Stx/Stx1, but they exhibit partial antigenic crossreactivity with each other. The amino acid sequences of Stx2 and Stx2e are more homologous with each other than with Stx/Stx1. Additional variants of Stx2 designated Stx2c and Stx2d have been characterized. The biological activity of Stx2d, but not that of Stx2, Stx2c and Stx2e, is activated by incubating the toxin in the presence of human intestinal mucus.

The operons that encode Stx1 and Stx2 are located on different phages in EHEC, and individual EHEC

strains can produce Stx1 only, one or more variants of Stx2, or both Stx1 and Stx2. The operons for Stx in *S. dysenteriae* type 1 and for Stx2e in *E. coli* are located on the bacterial chromosome. The synthesis of Stx by *S. dysenteriae* type 1 and Stx1 by *E. coli* is inhibited by high concentrations of iron in the growth medium, and this iron-dependent repression is mediated by Fur. In contrast, the synthesis of Stx2 and Stx2e by *E. coli* is not regulated by iron.

The prototype converting phages for Stx1, designated H19A and H19B, were isolated from EHEC strain H19 (O26:H11), which was obtained from an infantile diarrhea outbreak in Great Britain. Morphologically these phages have a noncontractile, flexible, long tail and a hexagonal head. DNA hybridization revealed that phage H19B is partially homologous with coliphage lambda. In phage H19B the genes that encode Stx1 are located far from the phage attachment locus. The prototype converting phage for Stx2, designated 933W, was isolated from EHEC strain 933 (O157:H7), which produces both Stx1 and Stx2. Phage 933W is morphologically distinct from the H19 phages and has a very short, noncontractile tail. A converting phage for Stx1 designated 933J, originally reported from EHEC strain 933, is indistinguishable from phage H19A, and subsequent attempts to recover phages from strain 933 in two laboratories yielded only 933W-like phages. Hybridization experiments demonstrated that EHEC strain 933 contains sequences that are homologous with DNA from phage H19A, raising the possibility that a EHEC strain 933 may contain a defective converting phage for Stx1 related to phage H19A.

Stx and the Shiga toxins of *E. coli* are all oligomeric proteins with one A polypeptide and five identical B polypeptides. Exposure of toxin to trypsin and reduction of an intrachain disulfide bond converts the A polypeptide into fragments A1 and A2. The B polypeptides bind to specific glycosphingolipid receptors in the plasma membranes of susceptible cells. The preferred receptor for Stx, Stx1 and Stx2 is globotriaosylceramide (Gb3), whereas the preferred receptor for Stx2e is globotetraosylceramide (Gb4). Binding of toxin to these specific glycolipids triggers receptor-mediated endocytosis, uptake via coated pits, and retrograde transport of toxin to the Golgi and endoplasmic reticulum. The A1 fragment is translocated to the cytosol, probably from the endoplasmic reticulum, by a mechanism that does not depend on acidification of endosomes. The final step in intoxication is inhibition of protein synthesis, which is caused by removal of a specific adenine residue from 28S rRNA in 60S ribosomal subunits by the highly specific RNA *N*-glycosidase activity of fragment A1. The intracellular action of the A1 fragment of Stx and the *E. coli* Shiga toxins is identical to that of ricin, and the active sites of these toxins have homologous structures. X-ray crystallography also demonstrated conserved three-dimensional folding between the B polypeptides of Stx/Stx1 and the heat-labile enterotoxin of *E. coli* despite lack of amino acid sequence homology.

The role of Shiga toxin in the pathogenesis of dysentery is unclear. *S. dysenteriae* type 1 and other species of *Shigella* cause bacillary dysentery, characterized by bloody, mucoid diarrhea and colonic inflammation, although dysentery caused by *S. dysenteriae* type 1 in humans or monkeys is typically more severe than that caused by *S. dysenteriae* type 1 mutants or other shigellae that produce little or no Shiga toxin. These findings suggest that Shiga toxin is not essential for the pathogenesis of dysentery but that it contributes to the severity of the disease.

Epidemiologic evidence clearly identifies EHEC as a cause of hemorrhagic colitis, but the roles of Stx1 and Stx2 in the pathogenesis of that disease are not clearly defined. Similarly, hemolytic uremic syndrome is clearly a sequela of infections caused by *E. coli* that produce high levels of Stx1, Stx2 or both, but an animal model that precisely replicates the pathology observed in humans with hemolytic uremic syndrome is not yet available. An emerging consensus from numerous studies on bacillary dysentery, hemorrhagic colitis, and hemolytic uremic syndrome, however, is that damage to endothelial cells of the vasculature in the colon and in the kidneys caused by Stx or related toxins of *E. coli* is an important aspect of the pathogenesis of these diseases. In the case of edema disease, injection of purified Stx2e into pigs mimics the naturally occurring disease. The data are compelling, therefore, that Stx2e has a direct and dominant role in the pathogenesis of edema disease.

Pyrogenic Exotoxins of *Streptococcus Pyogenes* and *Staphylococcus Aureus*

The streptococcal pyrogenic exotoxins (also known as erythrogenic toxins or scarlatinal toxins) are produced by *S. pyogenes* and cause the rash of scarlet fever. The enterotoxins of *S. aureus* cause the symptoms of staphylococcal food poisoning, and toxic shock syndrome toxin of *S. aureus* is responsible for many manifestations of toxic shock syndrome. These toxins share antigenic properties, amino acid sequences, and biological activities including pyrogenicity, enhancement of sensitivity to endotoxins of Gram-negative bacteria, and various immunomodulatory effects. They are all superantigens as described below.

Phage conversion for streptococcal pyrogenic exotoxin A (SpeA, 221 amino acids) production was demonstrated after lysogenization of *S. pyogenes* strain $T25_3$ with the temperate phage T12. Phage T12 is a double-stranded DNA phage with a 36 kbp genome that is circularly permuted and terminally redundant. The *speA* structural gene in the phage T12 genome is adjacent to *attP*. There are at least 10 different SpeA-converting phages that differ serologically and in host range. DNA sequences homologous with *speA* and with phage T12 sequences distinct from *speA* were present and physically linked in other SpeA-producing strains of *S. pyogenes* from which converting phages could not be recovered. These findings suggest that some SpeA converting phages are defective or that appropriate indicator strains for detecting them were not available.

The production of streptococcal pyrogenic exotoxin C (SpeC, 208 amino acids) by *S. pyogenes* is also controlled by phage conversion, and the genome of the SpeC converting phage CS112 is also circularly permuted with *speC* closely linked to *attP*. The genetic control of streptococcal pyrogenic exotoxin type B (SpeB, 253 amino acid mature protein) production in *S. pyogenes* is not yet clearly defined. DNA hybridization studies show that all strains of *S. pyogenes* have one copy of the *speB* gene, although only half of the strains produce detectable SpeB. Previous suggestions that SpeB production is controlled by phage conversion have not been confirmed.

In recent years there has been a resurgence of serious group A streptococcal infections and the recognition of a streptococcal toxic shock syndrome (strepTSS) that resembles staphylococcal toxic shock syndrome. Early studies in the United States showed that most isolates of *S. pyogenes* from patients with strepTSS produced SpeA, whereas isolates from most strepTSS patients from Europe and Canada produced SpeB and SpeC. Recent studies suggest that other streptococcal superantigens designated SpeF and SSA may also contribute to strepTSS. The importance of specific streptococcal superantigens in the pathogenesis of strepTSS and severe invasive streptococcal infections remains under active investigation.

The pyrogenic toxins of *S. aureus* include enterotoxins A, B, C1, C2, C3, D, E and G (228–239 amino acids) as well as toxic shock syndrome toxin 1 (TSST-1, 234 amino acids). The staphylococcal enterotoxins (designated SEA, SEB, etc.) are classified serologically, with C1, C2 and C3 representing minor serological variants. The genes that encode these toxins have been sequenced, and allelic variants have been identified for some of them. Analysis of amino acid sequences demonstrated homologies among the staphylococcal enterotoxins and SpeA, as well as homologies between SpeA and SpeC. SEA, SED, and SEE formed one closely related subgroup, whereas SEB, SEC, SEG, and SpeA formed a second closely related subgroup. SpeB and TSST-1 did not have significant amino acid sequence homology with the other toxins. X-ray crystallographic studies of these toxins are progressing rapidly and are identifying common structural motifs important for their function as superantigens.

Production of the staphylococcal enterotoxins SEA and SEE is controlled by phage conversion. Phage PS42-D has a linear 42 kbp genome with cohesive ends that encodes the *sea* gene, but *sea* in PS42-D is not immediately adjacent to *attP*. Phage PS42-D has a preferred site of integration in the *pur-ilv* region of the chromosome of *S. aureus*, and integration of PS42-D results in insertional inactivation of the gene that encodes β-hemolysin. A family of phages related to PS42-D has been identified, some of which contain the *sea* gene and some of which lack it. The *see* gene may be part of a defective phage that is related to the *sea*-containing phages. There is no evidence that the other staphylococcal pyrogenic exotoxins are phage-encoded. The *sed* gene is located on a 27.6 kbp penicillinase plasmid. The genes *seb*, *sec* and *tst* (which encodes TSST-1) are located on discrete but not well characterized genetic elements within the bacterial chromosome; they may possibly be mobile genetic elements similar to the 'hitchhiking transposons' described in other strains of *S. aureus*.

Pyrogenic exotoxins function as superantigens because they can interact with two different classes of receptors on cells of the immune system and activate large populations of T cells. They bind simultaneously to major histocompatibility complex (MHC) class II molecules on antigen-presenting cells (APC) and to T cell receptors (TCR) that express specific β chain variable regions. The resulting ternary complexes are unconventional, however, because the superantigens are not processed by the APC; they interact with the MHC class II molecules at positions that are distinct from those used for presentation of processed peptide antigens; and they interact with the TCR at positions that are distinct from those used for recognition of complexes of processed peptide antigens with MHC class II molecules. If appropriate costimulatory signals are present, the result is activation of T cells that express the appropriate Vβ chains. Superantigens can activate 5–20% of T cells, whereas the response to traditional antigens activates only 0.0001–0.01% of T cells. The activated T cells produce large amounts of several mediators, including interleukin-2, γ-interferon (IFN-γ), and tumor necrosis factor α (TNF-α), which are responsible for many of the observed biological effects. Superantigens

can induce T cell anergy if they engage TCR without delivery of appropriate costimulatory signals, and they can induce apoptosis and cause deletion of specific T cell lineages if they engage TCR on T cells that are already activated by exposure to high levels of cytokines such as IFN-γ or TNF-α. There is also evidence that the emetic action of the staphylococcal enterotoxins can be dissociated from their ability to activate T cells and might involve stimulation of mast cells to release leukotrienes.

Botulinum Toxins

Botulinum toxins are produced primarily by *C. botulinum* and cause the paralytic symptoms of botulism. Seven serologically different neurotoxins, designated A, B, C1, D, E, F and G, have been identified, and all appear to be structurally and functionally similar. Types A, B, E and F cause botulism in humans. Types C1 and D are the primary causes of animal botulism. Type G is produced by an environmental isolate of *C. botulinum*.

C. botulinum is a Gram-positive, spore-forming, anaerobe that is classified into groups I–IV based on biochemical, physiological and serological characteristics. Spores of *C. botulinum* are highly resistant to heating and boiling and can persist in the environment for years. Production of toxin requires germination of spores and anaerobic growth of the bacteria. Some strains of *C. botulinum* produce more than one type of botulinum toxin. Some isolates of *C. butyricum* and *C. baratii* have also been shown to produce botulinum toxin.

Three forms of botulism are recognized. Food-borne botulism, which involves the ingestion of preformed toxin, is the classical form in adults and is now rare. Wound botulism, which is associated with infection by *C. botulinum* at the site of an injury, is extremely rare. Infant botulism, which is usually caused by ingestion of *C. botulinum* and production of toxin within the intestinal tract, occurs more frequently, but the incidence of infant botulism in the United States is less than 100 cases per year. Infant botulism has received much attention because of a possible association with a small proportion of cases of sudden infant death syndrome.

Botulinum toxin affects the peripheral cholinergic nervous system, resulting in neuromuscular paralysis. The symptoms of botulism are quite variable and depend on the amount of toxin present. Symptoms typically begin approximately one day after ingestion of toxin, but in severe cases death may occur within 24 h. Early symptoms include weakness, double vision or inability to focus, impaired speech and difficulty swallowing, and death may result from respiratory paralysis. Treatment involves administration of antitoxin and supportive care as needed, including assisted ventilation.

Phage conversion of botulinum toxin production has only been proved for type C1 and D botulinum toxins. The C1 and D converting phages share some morphological similarities with the *E. coli* T-even phages. Electron microscopy indicates that they have hexagonal heads and long flexible tails surrounded by contractile sheaths. A study examining the DNA from five tox^+ phages that encode either the C1 or D toxins established that the phages contained double-stranded DNA genomes of either 110 kbp or 150 kbp. Restriction endonuclease profiles and DNA hybridization experiments with genomic DNA from these phages indicated that all but one of the phage DNAs were similar.

One or more isolates of the structural genes for all seven serological types of botulinum toxin, and for the related clostridial neurotoxin tetanus toxin, have been cloned and sequenced. The botulinum toxins of different serotypes vary from 1251 to 1297 amino acids in size. Amino acid sequence identity between toxin isolates of different serotypes does not usually exceed 40%. In contrast, the amino acid identity of toxin isolates within serotypes is much higher (74–100%), and the greatest differences are observed when the serologically similar toxins are produced by *C. botulinum* isolates from different groups (I–IV) or by *C. barati*.

Botulinum toxin is synthesized as a single polypeptide of approximately 150 kDa. After secretion, the toxin is typically cleaved into two components, a 100 kDa heavy chain and a 50 kDa light chain that are held together by a disulfide bond. Botulinum toxins are usually purified as high-molecular-weight complexes of the 150 kDa toxin with a 130–140 kDa protein (called the nontoxic–nonhemagglutinin component) plus a 33 kDa hemagglutinin protein; although the hemagglutinin is not present in purified botulinum toxins of types E and F. Other uncharacterized proteins are also present in some preparations of botulinum toxin. The high-molecular-weight complexes may protect the integrity of the botulinum toxins as they are exposed to gastric juices in the stomach.

Botulinum toxin blocks the release of acetylcholine from motor neurons at the myoneural junctions, resulting in flaccid paralysis. The heavy chain is believed to mediate receptor binding and translocation of toxin into the neuron, whereas the light chain acts intracellularly. The light chains of botulinum and tetanus neurotoxins were recently shown to be Zn^{2+}-dependent metalloproteases which cleave specific proteins of the neuroexocytosis apparatus at positions

that are characteristic for each toxin, thereby preventing release of acetylcholine from synaptic vesicles. The specific proteins that are targets for the action of these neurotoxins are VAMP/synaptobrevin for botulinum toxins B, D, F and G and tetanus toxin, SNAP-25 for botulinum toxins A and E, and syntaxin for botulinum toxin C1.

Cholera Toxin

Cholera is a profuse watery diarrhea produced by infection with *Vibrio cholerae* and caused mainly by the action of cholera enterotoxin (CT) on the intestinal epithelium. *V. cholerae* is a Gram-negative, motile, rod-shaped bacterium that is classified into over 140 serogroups based on O antigens. Only *V. cholerae* serogroups O1 and O139 cause epidemic cholera. *V. cholerae* O1 consists of two biotypes, classical and El Tor, that are differentiated by biochemical properties, polymyxin B resistance and bacteriophage sensitivity. The biotypes of strains that caused the first four recorded cholera pandemics (1817 through 1879) are unknown. Classical O1 strains caused the fifth and sixth cholera pandemics (1881 through 1923), and the O1 El Tor biotype has predominated in the seventh pandemic (1961 to present). *V. cholerae* O139, believed to have arisen from an El Tor strain by acquisition of novel O antigen determinants, was first identified as a cause of epidemic cholera in 1993.

V. cholerae produces toxin coregulated pili that enable it to colonize the small intestine, where it secretes CT. CT is composed of a single A polypeptide and a pentamer of five identical B polypeptides, and it is closely related to the type I and type II heat-labile enterotoxins (LT-I and LT-II) of *E. coli*. The A subunit is proteolytically nicked to generate disulfide-linked A1 and A2 peptides, and the A2 polypeptide links the A1 polypeptide to the B pentamer. Binding of B pentamer to the cell surface receptor ganglioside GM_1 triggers endocytosis of CT. The reduced A1 polypeptide is translocated to the cytoplasm, where it catalyzes the transfer of ADP-ribose from NAD to the $G_S\alpha$ subunit of a heterotrimeric regulatory G protein. This causes constitutive activation of adenylate cyclase, increases intracellular cAMP concentration, and leads to active secretion of chloride and diarrhea.

The genes for CT (*ctxA* and *ctxB*) are organized into an operon in a genetic element called CTX that is found at the same location in the chromosome of classical and El Tor biotypes of *V. cholerae*, and a second copy of the CTX element is present at an unlinked chromosomal location in the classical biotype. In both biotypes the CTX element can be present as tandemly repeated copies. Expression of the *ctx* operon and of toxin coregulated pili is positively regulated by the product of the *toxR* gene. The *ctxA* and *ctxB* genes are present in a core element, flanked by copies of 2.4–2.7 kbp repetitive sequences called RS elements. Unequal recombination between RS sequences is believed to generate the tandem duplications. The RS elements encode a site-specific recombination system capable of integrating into an 18 bp sequence in the *V. cholerae* chromosome called attRS1, which forms the junctions between RS elements and also between the CTX element and the chromosome in all *V. cholerae* strains. The core element also contains genes that were originally identified as potential virulence factors, such as *zot* (zonula occludens toxin), *ace* (accessory cholera enterotoxin), *cep* (core encoded pilin) and an unidentified gene (*orfU*).

Recent discoveries have identified the CTX genetic element as the genome of a novel, filamentous, single-stranded DNA bacteriophage (designated CTXΦ) related to coliphage M13. Among the major bacterial protein toxins, therefore, CT is the one most recently recognized to be produced as a consequence of phage conversion. The *zot* and *orfU* genes are required for phage morphogenesis, and *cep* is predicted to encode the virion capsid protein. The genes present in the RS element encode regulation, replication and integration functions. In *V. cholerae* strains possessing an attRS1 site, CTXΦ exists as an integrated prophage, but in attRS1-minus strains it can exist as an extrachromosomal, 7 kbp, double-stranded, circular prophage. This extrachromosomal prophage consists of the core element plus a copy of RS2 and attRS1, and it corresponds to the expected product of a chromosomal excision event between attRS1 sites flanking the core-RS2 element. CTXΦ is a 7 nm diameter filamentous phage with a single-stranded genome, and it uses the TCP pilus as a receptor.

CTXΦ is the first filamentous converting phage described. The *ctx* operon that encodes CT appears to be the only non-essential part in the viral genome. The coregulation of the toxin genes and the phage receptor TCP by *toxR* highlights the coevolution of phage and bacterial virulence determinants in *V. cholerae*. Only bacteria that express TCP can colonize the intestine, be infected by CTXΦ, acquire the ability to make CT, and in turn cause cholera.

Conclusions

The bacterial protein toxins described here share the characteristic that they are encoded by genes of temperate phages, but the toxins are otherwise heterogeneous in structure and function. These toxins have no essential roles in the life cycles of the

corresponding converting phages, and toxin production is regulated by mechanisms that are independent of the regulation of other phage genes. Typically, the toxin genes can be expressed from prophage, from lytically replicating phage, or from superinfecting, nonreplicating phage in bacteria with homologous lysogenic immunity.

The evolution of toxin converting phages is not well defined. Genes that encode bacterial toxins could presumably become incorporated into bacteriophage genomes by any of several mechanisms for genetic exchange. The fact that several toxin genes are located in phage genomes at sites adjacent to *attP*, however, suggests that imprecise excision of prophage and incorporation into the phage genome of segments of the bacterial genome that were originally contiguous to *attB* may be a fairly common mechanism for evolution of toxin converting phages.

In recent years the genes for many toxins and other virulence factors have been found on accessory genetic elements of bacteria such as phages, plasmids and transposons. Typically, virulence-associated genes encode nonessential functions that are advantageous for pathogenic bacteria in their animal hosts. The localization of multiple virulence-associate genes on accessory genetic elements has several potential advantages for pathogenic bacteria. The virulence genes can be maintained in the bacterial gene pool without being present in every member of the population, and appropriate groups of virulence genes can be disseminated rapidly by horizontal transfer of the accessory genetic elements within the bacterial population. The potential for successful intergeneric transfer of virulence-associated genes is also increased by their presence on accessory genetic elements that may have broad host range. Presumably, such advantages could provide the pressure for positive selection of converting phages that arose during the course of bacterial and bacteriophage evolution.

See also: **Immune response: Cell mediated immune response, General features; Lysogeny and prophage.**

Further Reading

Collier RJ (1995) Three-dimensional structure of diphtheria toxin. In: Moss J, Iglewski B, Vaughan M and Tu AT (eds) *Handbook of Natural Toxins*, vol. 8, *Bacterial toxins and virulence factors in disease*, pp. 81–93. New York: Marcel Dekker.

Domenighini M and Rappuoli R (1996) Three conserved consensus sequences identify the NAD-binding site of ADP-ribosylating enzymes, expressed by eukaryotes, bacteria and T-even bacteriophages. *Mol. Microbiol.* 21: 667.

Kotb M (1995) Bacterial pyrogenic exotoxins as superantigens. *Clin. Microbiol. Rev.* 8: 411.

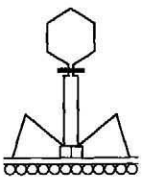
O'Brien AD and Holmes RK (1996) Protein toxins of *Escherichia coli* and *Salmonella*. In: Neidhard FC, Curtiss R, Ingraham JL *et al* (eds) *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn, vol. 2, pp. 2788–2802. Washington, DC: ASM Press.

Tonello F, Morante S, Rossetto O, Schiavo G and Montecucco C (1996) Tetanus and botulism neurotoxins: a novel group of zinc-endopeptidases. *Adv. Exp. Med. Biol.* 389: 251.

Waldor MK and Mekalanos JJ (1996) Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* 272: 1910.

PHAGE TRANSDUCTION

Werner Arber, University of Basel, Basel, Switzerland,



Definition and Discovery of Transduction

Transduction is the phage-mediated transfer of genetic information from donor to recipient bacteria followed by the phenotypic expression of transferred genetic traits in the progeny of the recipient cell.

Transduction was first described in 1952 by Norton Zinder and Joshua Lederberg. These authors investigated the possibility that pairs of genetically distinct bacterial strains of *Salmonella typhimurium* could exchange genetic traits by conjugation, a phenomenon discovered a few years before with *Escherichia coli* K-12 and requiring intercellular contact. In the

corresponding converting phages, and toxin production is regulated by mechanisms that are independent of the regulation of other phage genes. Typically, the toxin genes can be expressed from prophage, from lytically replicating phage, or from superinfecting, nonreplicating phage in bacteria with homologous lysogenic immunity.

The evolution of toxin converting phages is not well defined. Genes that encode bacterial toxins could presumably become incorporated into bacteriophage genomes by any of several mechanisms for genetic exchange. The fact that several toxin genes are located in phage genomes at sites adjacent to *attP*, however, suggests that imprecise excision of prophage and incorporation into the phage genome of segments of the bacterial genome that were originally contiguous to *attB* may be a fairly common mechanism for evolution of toxin converting phages.

In recent years the genes for many toxins and other virulence factors have been found on accessory genetic elements of bacteria such as phages, plasmids and transposons. Typically, virulence-associated genes encode nonessential functions that are advantageous for pathogenic bacteria in their animal hosts. The localization of multiple virulence-associate genes on accessory genetic elements has several potential advantages for pathogenic bacteria. The virulence genes can be maintained in the bacterial gene pool without being present in every member of the population, and appropriate groups of virulence genes can be disseminated rapidly by horizontal transfer of the accessory genetic elements within the bacterial population. The potential for successful intergeneric transfer of virulence-associated genes is also increased by their presence on accessory genetic elements that may have broad host range. Presumably, such advantages could provide the pressure for positive selection of converting phages that arose during the course of bacterial and bacteriophage evolution.

See also: **Immune response: Cell mediated immune response, General features; Lysogeny and prophage.**

Further Reading

Collier RJ (1995) Three-dimensional structure of diphtheria toxin. In: Moss J, Iglewski B, Vaughan M and Tu AT (eds) *Handbook of Natural Toxins*, vol. 8, *Bacterial toxins and virulence factors in disease*, pp. 81–93. New York: Marcel Dekker.

Domenighini M and Rappuoli R (1996) Three conserved consensus sequences identify the NAD-binding site of ADP-ribosylating enzymes, expressed by eukaryotes, bacteria and T-even bacteriophages. *Mol. Microbiol.* 21: 667.

Kotb M (1995) Bacterial pyrogenic exotoxins as superantigens. *Clin. Microbiol. Rev.* 8: 411.

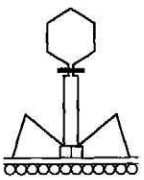
O'Brien AD and Holmes RK (1996) Protein toxins of *Escherichia coli* and *Salmonella*. In: Neidhard FC, Curtiss R, Ingraham JL *et al* (eds) *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn, vol. 2, pp. 2788–2802. Washington, DC: ASM Press.

Tonello F, Morante S, Rossetto O, Schiavo G and Montecucco C (1996) Tetanus and botulism neurotoxins: a novel group of zinc-endopeptidases. *Adv. Exp. Med. Biol.* 389: 251.

Waldor MK and Mekalanos JJ (1996) Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* 272: 1910.

PHAGE TRANSDUCTION

Werner Arber, University of Basel, Basel, Switzerland,



Definition and Discovery of Transduction

Transduction is the phage-mediated transfer of genetic information from donor to recipient bacteria followed by the phenotypic expression of transferred genetic traits in the progeny of the recipient cell.

Transduction was first described in 1952 by Norton Zinder and Joshua Lederberg. These authors investigated the possibility that pairs of genetically distinct bacterial strains of *Salmonella typhimurium* could exchange genetic traits by conjugation, a phenomenon discovered a few years before with *Escherichia coli* K-12 and requiring intercellular contact. In the

experiments with *Salmonella,* hybrid recombinants were indeed obtained. However, these also appeared under conditions in which the two bacterial strains were inhibited by a membrane filter from undergoing close intercellular contact. The filterable agent responsible for the formation of recombinants was soon identified as the endemic bacteriophage P22.

Experimental Evidence for Transduction

The basic experimental strategy to identify transduction by a phage strain is as follows. The phage is propagated in a donor bacterium prototrophic for a few identifiable traits. The vegetative propagation can be initiated by either infection or induction of lysogens for the phage in question. The resulting phage lysate is carefully sterilized in order to inactivate any remaining viable donor bacteria. Recipient bacteria chosen to be auxotrophic for the considered genetic traits are then infected with the sterile phage preparation, ideally at a multiplicity of infection of about one phage per cell. The infected bacteria are exposed to selective medium in which the input recipient cells will not grow, but in which recipients having acquired one or several genetic traits of the donor strain can propagate. Growth of a usually low number of colonies indicates that the transfer of the traits under consideration from the donor into the recipient cells has been mediated by phage particles acting as gene vectors. Obvious control experiments include the determination of mutant reversion frequencies of the recipient to prototrophy as well as tests of the sterility of the phage lysate, the sensitivity of transduction to phage-inactivating antiserum and the insensitivity of transduction to nuclease treatment of the phage lysate.

The ability of a given phage strain to transduce and the particular mechanistic features of transduction largely depend on the life style of a virus. Because of the usually low frequency of transduction and because of the often aleatoric nature of each particular transduction event, the mechanistic investigation of transduction at the molecular level largely depends on evidence collected at the level of microbial populations. Individual events can hardly be looked at directly, nor are many of their features strictly reproducible. Nevertheless, satisfactory interpretations are available to explain transduction processes displayed by a small number of thoroughly investigated model systems, including the *Salmonella* phage P22 and the coliphages P1 and lambda (λ). While some of the mechanistic features unraveled with these phages may represent general rules, others may, rather, be specifically dependent on some particular viral trait. This should serve as a caveat to the reader of this general overview on transduction and its biological significance.

Generalized versus Specialized Transduction

In early experiments it was found that some phages transduced any tested donor gene with roughly similar, although low, frequencies. This phenomenon is called *generalized transduction,* and phages P22 and P1 represent prototypes of this transduction mode. In contrast, other phages such as λ transduce only a few particular donor genes, thus the term *specialized transduction.* It soon became clear that in specialized transducing phages, the transferred donor genes are covalently linked to the phage genome, so that they also become propagated as the virus propagates either vegetatively in bacteria sensitive to the phage or as prophage in lysogenic bacteria. This is usually not so in generalized transduction, in which normally a DNA segment of the donor genome is wrapped into a viral coat without undergoing hybridization with viral DNA. Its later propagation to the progeny of the infected recipient cell will thus usually depend on recombinational integration into the recipient genome. In the absence of such integration, unilinear inheritance of the transferred genetic information can occur. This phenomenon is known as *abortive transduction.* In particular cases, in which transferred DNA carries the appropriate genetic information for its autonomous replication, the establishment can be ensured by plasmid replication and maintenance.

Uptake of Cellular Genetic Information into Transducing Viral Particles

The mode of packaging progeny viral DNA into preformed viral coats is an important characteristic of the lifecycle of each phage strain. Some phages encapsulate a so-called headful of DNA (see section below). Other phages package precisely a viral genome, the ends of which are in these cases identified by specific nucleotide sequences (see section below).

Packaging: headful of DNA Together with the virulent T-even phages of *Escherichia coli* the classical transducing phages P22 and P1 use the headful strategy for the packaging of phage particles. In the course of DNA replication, the viral genomes of these phages form long concatemeric DNA molecules composed of several genome copies arranged in tandem. DNA packaging into viral particles is then initiated by the interaction of a specific viral protein with a particular nucleotide sequence within the

genome, the *pac* site, from which packaging of the DNA then proceeds linearly until the viral coat, called the phage head, is full. DNA is then cleaved and the protein which apparently remains attached to the end of the not yet packaged part of the concatemeric DNA molecule can immediately reinitiate the packaging of another phage particle. In this way, several phage particles become sequentially packaged from the same concatemeric DNA molecule. This explains why phages encapsulating their DNA by this headful mechanism form populations of particles with circularly permuted DNA molecules. The normal phage head has a capacity to take up somewhat more DNA than that corresponding to the length of the wild-type phage genome. Therefore, the phage DNA in these viral particles is also terminally redundant, which is a requirement for successful initiation of the next cycle of reproduction of the phage. As observed with phage P1, some phages produce populations of phage coats of different size, the smaller of which encapsulate less than a genome size of DNA. Consequently, these small-headed phages are not able to reproduce upon single infection.

Occasionally, DNA packaging becomes initiated on cellular DNA rather than on the specific *pac* site on a concatemer of phage DNA. Possibly, this occurs at host DNA sequences resembling the *pac* site of the phage. Packaging of cellular DNA can then proceed in the same way as with phage DNA. Several coats chosen randomly from populations of phage coats of different size, if relevant, thus become packaged with cellular DNA segments of lengths corresponding to the capacity of the heads. This is, for example, about 40 kb for P22 coats, about 100 kb for P1 heads of normal size and about 40 kb for small-headed P1 particles; 40 kb roughly corresponds to 1% of the host chromosome.

There are alternative possibilities by which packaging of cellular DNA can also occur. Some phages such as P1 encode for site-specific recombinases and they carry in their genomes crossing-over sites at which these enzymes can act. Recombination between such a phage site and a similar DNA sequence carried on the host chromosome can bring about the fusion of a copy of the viral genome with the cellular DNA. If DNA packaging then becomes initiated at the viral *pac* site of such a cointegrate, packaging may proceed into the regions of cellular DNA. If for example a single copy of the viral genome had fused with the bacterial chromosome, subsequent packaging as proposed will produce a first viral particle with hybrid DNA between the phage and the host chromosome, and all subsequent viral particles from this packaging chain will contain only host DNA. Fusion of the phage genome with cellular DNA can also occur either by transposition-mediated cointegration or more classically by general recombination acting at portable sequence homologies, as they are, for example, provided by inserted sequence (IS) elements carried both in the host genome (see below) and in some viral genomes.

Transducing phage particles originating by one of the possibilities described usually contain only host DNA and give generalized transduction. The frequency of such transducing phage particles in phage preparations is of the order of 1% of the total phage population, both for P22 and for P1.

Packaging: discrete genomes Bacteriophage λ and its relatives also normally package their DNA from concatemeric oligomers of the phage genome. However, a specific enzyme-mediated process selects discrete phage genomes for encapsulation. A specific nucleotide sequence, called the *cos* site, identifies the genome ends at which concatemers are cleaved upon packaging. Consequently, the linear DNA molecules carried in phage particles represent a uniform population of genomes; they are neither terminally redundant nor circularly permuted.

With these phages, DNA packaging only rarely originates within cellular DNA at a DNA sequence homologous to a *cos* site. Even so, it is quite unlikely that a second *cos* site would be carried at an appropriate distance from the first, so that the DNA segment contained between the two sites would roughly correspond to the length of the phage genome, a requirement to obtain phage particles able to infect a new host cell. Indeed, phage λ does not give measurable frequencies of transduction for most of the regions of the bacterial chromosome.

In contrast, phage λ transduces genes located near the site in the chromosome at which the λ genome is carried as prophage in λ-lysogenic bacteria. λ lysogeny is the result of an infection in which the vegetative phage reproduction is repressed at an early stage and the phage genome becomes integrated into the host chromosome by phage-mediated site-specific recombination between the phage *att*P sequence and the host *att*B sequence. The normal bacterial attachment site (*att*B) for the integration of prophage λ into the *E. coli* chromosome is located between the *gal* operon and the *bio* operon. When λ-lysogenic bacteria are induced (e.g. by UV irradiation) to vegetatively produce λ phage, the λ prophage is normally excised precisely at its junctions with the cellular DNA, i.e. at the *att* sites. With a frequency of about 10^{-4} per event, however, excision is not as precise and can occur more or less randomly by so-called illegitimate recombination. The result of such recombination occurring between phage sequences

and host sequences is a hybrid genome in which part of the phage genes have been replaced by bacterial genes, which can be either the *gal* or the *bio* genes. Consequently, such specialized transducing phages may have lost some of the genes essential for vegetative phage reproduction. Such phages are therefore defective and can only reproduce by complementation with functions provided by a normal phage genome serving as 'helper'.

A model drawn by Allan Campbell explains prophage excision with the formation of a figure eight of the λ-lysogenic *E. coli* chromosome followed by a single recombination event. In independent illegitimate excision events hybrid genomes are formed which differ with regard to the extent of both the deleted phage genes and the acquired host DNA. However, hybrids always carry their substitutions adjacent to the *att* site, in accordance with the Campbell model. In fact, the *att* site of the hybrid genomes also remains hybrid; it is one of the two junctions between the prophage and the host chromosome in λ lysogens.

In theory, hybrid DNA molecules of any length could be expected to result from illegitimate prophage excision. In practice, limits are naturally set to hybrid DNA molecules to be packaged into λ phage particles. The hybrid DNA must contain the *cos* site on the phage sequences and the length of the hybrid genome must allow the formation of an infective particle, i.e. its length can at most vary between 78% and 108% of the length of the wildtype λ genome length. This has to do with the size and the stability of the phage λ head. The stability is ensured only for genome sizes varying between about 36 and 51 kb.

Specialized transducing λ phages carrying *gal* or *bio* genes are only formed upon initiation of phage reproduction in lysogenic bacteria; they do not arise upon vegetative phage growth after infection. Occasionally, the λ prophage can integrate at other chromosomal locations than *att*B. In these cases, subsequent induction of phage reproduction can lead to the formation of specialized transducing phage derivatives transferring those bacterial genes located in the immediate vicinity of the prophage location, exactly as in the normal situation with the prophage located at *att*B.

Transposition as an Alternative Source of Specialized Transducing Phage Genomes

As we have already seen, the formation of specialized λ*gal* and λ*bio* transducing phages depends on recombinational events involving the hybridization of the λ phage genome with the bacterial chromosome. The resulting hybrids are autonomous replicons which, upon vegetative reproduction of the phage, can be encapsidated into phage particles. Upon infection of new hosts, the hybrid genomes can become established and give rise to transductants. These are derivatives of the infected recipient host expressing some of the genes carried in the transducing phage.

An alternative route to obtaining specialized transducing phage under natural conditions is transposition. Transposons are mobile genetic elements which are specific DNA sequences able to insert into any selected target on DNA molecules. In addition to the genetic information required for the transposition process, transposons carry one or more genes unrelated to the transposition functions. This contrasts with the structure of prokaryotic inserted sequence (IS) elements which are defined as mobile genetic elements carrying only genetic information for their own transposition. However, an IS element can be part of a transposon. Indeed, composite transposons are defined as IS-mediated transposons which carry genetic information unrelated to transposition sandwiched between two identical IS elements, at least one of which should exert transpositional activity. Therefore, in principle any segment of a bacterial chromosome may at one time or another become part of a composite transposon, when it becomes flanked by the consecutive insertion of an IS element to its left and right sides. Other transposons may have their structural origin in other types of DNA rearrangements.

Transposons can transpose from the bacterial genome into the genome of a vegetatively reproducing phage. Alternatively, a transposon can also become inserted into a prophage carried in lysogenic bacteria. The vegetative reproduction of such prophage can later become induced. Both cases can give rise to the formation of specialized transducing phages, provided that the following conditions are fulfilled. The insertion of the transposon must not have inactivated phage functions essential for vegetative reproduction, or at least such inactivated functions must be complemented by a helper phage present in the same host cell. Furthermore the size of the hybrid genome must not be excessively large. This condition again relates to the packaging mode of the phage strain. Phages packaging a genome length of DNA cannot accommodate insertions leading to a genome size larger than that corresponding to the volume available in the phage particles. In phage λ this is about 108% of the wild-type λ genome length. Phages packaging a headful of DNA such as P1 require some terminal redundancy for recircularization of the phage genome, a prerequisite for a new cycle of

vegetative reproduction or for prophage establishment. However, insertions of large transposons into the P1 genome have been observed, so that the phage could no longer package an entire genome per phage head. Such oversized hybrids can still reproduce and lysogenize their host, but only upon infection at high multiplicity, so that entire genomes can become reconstructed by recombination occurring after infection. Upon infection at low multiplicity, such transducing phages may only give rise to generalized transduction.

Not all phage strains limit the volume available for the uptake of nucleic acids. The filamentous bacteriophages represented by phage M13 are a good example. These phages package a precise genome length in the form of a circular single-stranded DNA molecule. Therefore the length of the filamentous phage directly reflects the size of its genome. The insertion of a transposon consequently results in the production of specialized transducing phages larger than particles with wild-type genomes.

Fate of Transferred Donor DNA upon Infection of a Recipient Host

According to the definition of transduction, transfer of donor DNA must be followed by the expression of donor genes in the recipient bacteria: expression of the acquired gene functions is the basis of the monitored phenotype of the transductants. To produce this phenotype, the donor DNA must be maintained and ideally also replicated in the transductant clone.

If the donor DNA finds some homology in the recipient chromosome, the homologous segments can undergo general recombination. This type of genetic conversion is often involved in generalized transduction. It can cause stable integration also of DNA segments carried in specialized transducing phages, e.g. *gal* genes in *λgal*.

Another way of propagating acquired donor DNA is autonomous replication. This requires appropriate replicon functions. These can be provided by the vector phage. An example is specialized transducing P1 carrying a transposon. Upon lysogenization of the recipient, this phage establishes its prophage as a plasmid.

Phage P1 is also able to give generalized transduction for entire plasmids unrelated to the P1 genome. Such plasmids can become established as independent replicons after their transfer into recipient bacteria.

Rather than relying on their own replication functions, genomes of specialized transducing phages can also integrate into the recipient chromosome and then become further propagated together with the latter. This is the case when *λgal* lysogenizes its host. Thereby the intact *λgal* hybrid genome integrates into the bacterial chromosome by site-specific recombination. Alternatively, integration of hybrid transducing genomes can also be obtained by other types of recombinational events, e.g. if the transducing phage carries an IS element which finds its homologous counterpart in the recipient chromosome, so that general recombination can provide a means for the production of cointegrates. Alternatively a transposon carried in a transducing phage has a low probability of transposing, after its transfer, into the recipient chromosome and this can ensure its further inheritance.

As discussed above, transferred donor DNA is not always propagated in the progeny of the infected recipient cell. It can indeed also be maintained intact without undergoing replication, possibly being protected against nucleolytic degradation by specific proteins. This then gives rise to abortive transduction, in which the acquired DNA is unilinearly inherited and the donor gene functions become expressed and subsequently diluted in the cytoplasm of the cells of the growing clone of the transductant.

Obviously, the survival of the infected recipient cells is a prerequisite for the detection of transductants. This condition is particularly relevant for virulent phage strains such as T-even and T1 phages. By applying particular infection conditions, it could be shown that lysates of these phages also contain phage particles packaged with host DNA and therefore can give transduction.

Natural Limits Keep the Probability of Acquisition of Foreign Genes by Transduction Low

Although transduction frequencies are usually low, both general and specialized transduction can readily be observed with many host–phage systems. Ideally, mechanistic studies are made with homologous donor and recipient strains which differ by only a few mutations which serve as genetic markers. However, transduction is also observed if donor and recipient strains are only distantly related. Obviously, both donor and recipient strains must belong to the host range of the phage. In this respect it is interesting to note that populations of specialized transducing phage, e.g. P1Cm in which a gene for chloramphenicol resistance is carried in a transposon, can serve to select for host range mutants of the phage: rare Cm^r transductants of a bacterial strain that normally does not serve as a host for phage P1 may prove to contain a mutant P1Cm prophage with extended host range.

A serious barrier against the acquisition of foreign

genetic information by phage-mediated transduction as well as by other means of gene transfer such as conjugation and transformation is brought about by restriction-modification (R-M) systems. R-M systems are very widely present in most bacterial strains, and many strains harbor more than one R-M system. These systems produce enzymes with the ability to distinguish entering DNA originating in other bacterial strains from the cell's own DNA molecules. The basis for the distinction usually resides in the methylation patterns of specific nucleotide sequences. Only rarely do two different bacterial strains provide the same methylation pattern. Restriction enzymes are part of R-M systems. They not only recognize invading foreign DNA as such, but they also endonucleolytically cleave it into fragments. Normally such fragments will eventually become exonucleolytically degraded. However, before this happens, some of the DNA fragments may become integrated into the recipient chromosome by recombination and thus be stabilized. Indeed, the original fragmentation may render the acquired DNA recombinogenic. Therefore R-M systems can have the following double function. They efficiently reduce the overall chance that invading foreign DNA becomes established in the recipient cell but they might stimulate the stable acquisition of short segments of foreign DNA. In this light, acquisition of foreign genetic functions is tolerated at a low rate, but only in small steps, i.e. only relatively short DNA segments are made available for uptake into the recipient genome.

Acquisition of foreign gene functions in small portions may appear a good natural strategy if one considers the risk inherent to gene acquisition in disturbing the harmony of biological functions of the recipient strain. Such a risk is likely to be relatively small if only one new function is acquired, but much higher if many functions are acquired at once. In fact, functional compatibility between residential gene functions and the newly acquired functions represents another criterion contributing to the limits naturally set to gene acquisition.

Applications of Transduction

Shortly after the discovery of transduction, microbial geneticists realized its usefulness as a tool in fine structure mapping of bacterial genes. Measuring cotransduction frequencies of genetic markers linked on the bacterial chromosome allows one to determine the degree of genetic linkage much more precisely than do conjugation experiments.

The availability of specialized transducing phages able to lysogenize the recipient bacteria opens up the interesting possibility for complementation tests between bacterial mutants for the same biological function. Indeed, lysogens for specialized transducing phages are partially diploid if the transduced genetic information finds homologous information already on the chromosome, which is often the case. If donor and recipient are independently mutated for a given biological function, restoration of the function by specialized transduction suggests that the two mutations affect different genes and thus undergo complementation. Absence of complementation indicates that the two mutations affect the same gene.

Another application of transduction in microbial genetics is localized random mutagenesis. The transducing phage lysate is submitted to treatment by a mutagen before infection of the recipient strain. A marker linked to the gene to be mutagenized can then serve for selection of transductants. Screening easily reveals cotransductants carrying a mutation in the linked gene under study which had been the actual mutagenesis target. This method ensures that after heavy mutagenesis treatment no chromosomal genes other than the transduced ones could have been affected by the mutagenesis treatment.

Transduction can also serve to transfer plasmids from a donor to a recipient strain.

Perhaps the most important 'application' of transduction in the wide fields of fundamental and applied research is the general use of the concept of the host–vector system which has emerged from the understanding of structure and functions of specialized transducing genomes. It became obvious around 1960 from work with λ*gal* and other specialized transducing phage genomes that phage replicons can undergo the insertion of host DNA segments to form genetically stable units. Such hybrid phages can propagate in the same way as wild type phages and the passenger genes can express their functions in recipient bacteria. This knowledge has stimulated scientists to reproduce this phenomenon *in vitro*. The attempts were successful around 1970 and soon gave rise to the *in vitro* recombinant DNA techniques. By this technology, molecular genetics, which was formerly mainly limited to microbial genes, has become accessible for application to the genes of any living organism.

Transduction is an Element of Biological Evolution

Gene acquisition is an important source for microbial genomes to obtain new or altered biological functions. It depends on a single evolutionary step. Therefore, the acquisition strategy is more efficient per step of mutation than most other types of spontaneous mutagenesis which occur in the DNA

of the residential genome. The high degree of efficiency resides, among other reasons, in the universality of the genetic code and in the similarity of basic biological functions exerted by widely different living organisms. Thus, a biological function developed in the course of evolution in a particular branch of the evolutionary tree is likely to have a fair chance of also being useful in another branch of that tree after its horizontal transfer. In bacteria, phage-mediated transduction can be considered as efficient a process of gene acquisition as conjugation involving a conjugative plasmid as a gene vector and as transformation in which free DNA is taken up by recipient bacteria. As compared with these latter mechanisms, phage-mediated transduction offers the particular advantage that phage particles are quite stable over prolonged periods of time. Thus, DNA of donor bacteria packaged in transducing phage particles can some later time be re-activated upon infection of a recipient host strain.

In view of these considerations bacterial viruses thus have their particular role as promoters of microbial evolution. This function is accomplished by more or less random uptake of donor DNA and its subsequent spreading to more or less randomly encountered recipient host strains. Thus, phage-mediated transduction makes essential contributions to the steady generation of genetic variety in bacterial populations which is a prerequisite for the progressive course of biological evolution.

Virus-mediated gene transfer in eucaryotes has been documented on many occasions, but whether this phenomenon plays as important an evolutionary role in higher organisms as in procaryotes remains to be elucidated. The answer to this question is of real concern in view of the biohazard debate about *in vitro* recombinant DNA work, in particular with regard to protocols that foresee the deliberate release of organisms with recombinant DNA. Besides this practical aspect, the concept of gene acquisition as an evolutionary strategy is *per se* of interest not only from the scientific point of view, but also with regard to its philosophical dimensions.

See also: **Phage Homologous Recombination; Phages as cloning vehicles; Filamentous phages (*Inoviridae*); Host-controlled modification and restriction; Coliphage lambda (*Siphoviridae*); Lysogeny and prophage; Enterobacteria phage P1 (*Myoviridae*); Salmonella phage P22 (*Podoviridae*); T4-like phages (*Myoviridae*).**

Further Reading

Campbell A (1962) Episomes. *Adv. Genet.* 11: 110.

Margolin P (1987) Generalized transduction. In: Ingraham JL *et al.* (eds) *Escherichia coli* and *Salmonella typhimurium*, p. 1154. Washington, D.C.: American Society for Microbiology.

Nedelmann M, Sabottke A, Laufs R and Mack D (1998) Generalized transduction for genetic linkage analysis and transfer of transposon insertions in different *Staphylococcus epidermidis* strains. *Zentralbl. Bakteriol.* 287(1–2): 85.

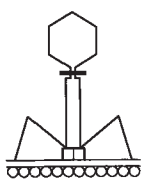
Weisberg RA (1987) Specialized transduction. In: Ingraham JL *et al.* (eds) *Escherichia coli* and *Salmonella typhimurium*, p. 1169. Washington, D.C.: American Society for Microbiology.

Yarmolinsky MB and Sternberg N (1988) Bacteriophage P1. In: Calendar R (ed.) *The Bacteriophages*, p. 291. New York: Plenum Press.

Zinder ND and Lederberg J (1952) Genetic exchange in *Salmonella*. *J. Bacteriol.* 64: 679.

PHAGES AS CLONING VEHICLES

Noreen E Murray, Division of Biological Sciences, Institute of Cell and Molecular Biology, University of Edinburgh, Kings Buildings, Mayfield Road, Edinburgh, UK



Introduction

The genomes of three coliphages have been adapted as vector systems for the purification and amplification of segments of DNA in *Escherichia coli*, the host commonly used for molecular cloning. The general principles exemplified by these well-developed vectors will be stressed rather than the specific details of particular vectors for either *E. coli* or other bacteria.

Some years before recombinant molecules could be made *in vitro*, phage lambda (λ) was the prototype cloning vehicle for those genes close to the normal

of the residential genome. The high degree of efficiency resides, among other reasons, in the universality of the genetic code and in the similarity of basic biological functions exerted by widely different living organisms. Thus, a biological function developed in the course of evolution in a particular branch of the evolutionary tree is likely to have a fair chance of also being useful in another branch of that tree after its horizontal transfer. In bacteria, phage-mediated transduction can be considered as efficient a process of gene acquisition as conjugation involving a conjugative plasmid as a gene vector and as transformation in which free DNA is taken up by recipient bacteria. As compared with these latter mechanisms, phage-mediated transduction offers the particular advantage that phage particles are quite stable over prolonged periods of time. Thus, DNA of donor bacteria packaged in transducing phage particles can some later time be re-activated upon infection of a recipient host strain.

In view of these considerations bacterial viruses thus have their particular role as promoters of microbial evolution. This function is accomplished by more or less random uptake of donor DNA and its subsequent spreading to more or less randomly encountered recipient host strains. Thus, phage-mediated transduction makes essential contributions to the steady generation of genetic variety in bacterial populations which is a prerequisite for the progressive course of biological evolution.

Virus-mediated gene transfer in eucaryotes has been documented on many occasions, but whether this phenomenon plays as important an evolutionary role in higher organisms as in procaryotes remains to be elucidated. The answer to this question is of real concern in view of the biohazard debate about *in vitro* recombinant DNA work, in particular with regard to protocols that foresee the deliberate release of organisms with recombinant DNA. Besides this practical aspect, the concept of gene acquisition as an evolutionary strategy is *per se* of interest not only from the scientific point of view, but also with regard to its philosophical dimensions.

See also: **Phage Homologous Recombination; Phages as cloning vehicles; Filamentous phages (*Inoviridae*); Host-controlled modification and restriction; Coliphage lambda (*Siphoviridae*); Lysogeny and prophage; Enterobacteria phage P1 (*Myoviridae*); Salmonella phage P22 (*Podoviridae*); T4-like phages (*Myoviridae*).**

Further Reading

Campbell A (1962) Episomes. *Adv. Genet.* 11: 110.

Margolin P (1987) Generalized transduction. In: Ingraham JL *et al.* (eds) *Escherichia coli* and *Salmonella typhimurium*, p. 1154. Washington, D.C.: American Society for Microbiology.

Nedelmann M, Sabottke A, Laufs R and Mack D (1998) Generalized transduction for genetic linkage analysis and transfer of transposon insertions in different *Staphylococcus epidermidis* strains. *Zentralbl. Bakteriol.* 287(1–2): 85.

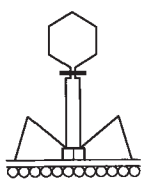
Weisberg RA (1987) Specialized transduction. In: Ingraham JL *et al.* (eds) *Escherichia coli* and *Salmonella typhimurium*, p. 1169. Washington, D.C.: American Society for Microbiology.

Yarmolinsky MB and Sternberg N (1988) Bacteriophage P1. In: Calendar R (ed.) *The Bacteriophages*, p. 291. New York: Plenum Press.

Zinder ND and Lederberg J (1952) Genetic exchange in *Salmonella*. *J. Bacteriol.* 64: 679.

PHAGES AS CLONING VEHICLES

Noreen E Murray, Division of Biological Sciences, Institute of Cell and Molecular Biology, University of Edinburgh, Kings Buildings, Mayfield Road, Edinburgh, UK



Introduction

The genomes of three coliphages have been adapted as vector systems for the purification and amplification of segments of DNA in *Escherichia coli*, the host commonly used for molecular cloning. The general principles exemplified by these well-developed vectors will be stressed rather than the specific details of particular vectors for either *E. coli* or other bacteria.

Some years before recombinant molecules could be made *in vitro*, phage lambda (λ) was the prototype cloning vehicle for those genes close to the normal

prophage attachment site in the bacterial chromosome, but subsequently more generally for genes flanking a large number of secondary attachment sites distributed around the bacterial chromosome. The λ genome was therefore an obvious substrate for manipulation to produce receptor molecules, or vectors, to which DNA fragments from any source could be covalently linked *in vitro* by DNA ligase, recovered in bacterial cells and consequently amplified *in vivo*.

Any genome that is to serve as a vector must be isolated in a double-stranded form and have a target for the specific endonuclease that will be used to open the genome as a prerequisite to the insertion of foreign DNA. Insertion must not interfere with any essential vector functions; the recombinant molecule must replicate and yield either a plaque or a lysogenic colony. On the other hand, insertional inactivation of a nonessential gene associated with a recognizable phenotypic change can provide a screen or even a selection for recombinant phages.

λ Vectors

Three factors have greatly influenced the role of λ vectors. First, the efficient recovery of recombinant genomes following *in vitro* packaging, i.e. the encapsidation of recombinant genomes within phage heads and the addition of tails thereby permitting adsorption to bacterial receptors and the consequent injection of DNA into the recipient cell. Second, the large amounts of readily accessible DNA and proteins exposed in plaques permit very sensitive detection of either specific nucleotide sequences by their hybridization to appropriate probe sequences, or the protein domains by their binding to antibodies or other ligands including oligonucleotides. Third, the amenability of phage λ to genetic analyses, including the maintenance of its genome as a prophage, one copy per bacterial chromosome and the ready transfer of phage DNA from one *E. coli* strain to another. The first two factors are most relevant to the effective recovery and screening of genomic and cDNA libraries, the third to genetic analysis of cloned genes, particularly those of *E. coli*.

The cutting and packaging of λ DNA *in vivo* is directed by specific sequences (*cos*) in concatemeric (multimeric) λ genomes; cutting regenerates the cohesive ends of the linear λ genome. Although this process is sequence specific, it is influenced significantly by the distance between the specific (*cos*) sequences. These must be separated by a minimum distance, well below the optimum, and may be separated by some few kb of DNA above the optimum. Some vectors, therefore, offer efficient recovery of small fragments (up to 10 kb), others efficient recovery of large (~20 kb) DNA fragments. In general, the former are insertion vectors and the latter replacement vectors (see below), but in either case the size selectivity of *in vitro* packaging can be used to preferentially recover recombinant rather than vector molecules.

Since λ has a big genome (48.6 kb), making vectors usually requires not only the use of deletions to make space, but the removal of superfluous restriction targets so that cutting is confined to specific cloning sites. Targets can be removed by using known deletions or substitutions, and by specific mutations, sometimes selected *in vivo* but also *in vitro* using restriction enzymes to cut, and hence select against, genomes with restriction targets.

Insertion Vectors

These simple vectors are deleted for nonessential DNA (up to approximately 9 kb) and they retain single targets (cloning sites) for one or more restriction enzymes. Insertion of DNA fragments of appropriate length yields a recombinant molecule that is packaged in preference to a vector genome. Ideally, insertion of the DNA is associated with inactivation of a nonessential coding sequence and a recognizable phenotypic change. The most commonly used screen relies on insertional inactivation of a *lacZ* gene within the vector and the combined use of $lacZ^-$ bacteria and a substrate (X-gal) that when hydrolysed by the product of the *lacZ* gene (β-galactosidase) yields a nondiffusible blue pigment. Insertional inactivation of the *lacZ* gene correlates with a failure to hydrolyse X-gal and a white rather than a blue plaque. An alternative screen is provided by insertional inactivation of the gene encoding the phage repressor (*c*I) and the consequent 'clear' rather than 'turbid' plaque morphology. The use of a specific host (hfl^-) in which cI^+ phages establish repression and consequently fail to reproduce vegetatively provides a genetic selection for recombinant phages dependent on the insertional inactivation of the *c*I gene.

Insertion vectors are used for cloning cDNA or relatively short DNA fragments (<11 kb), vectors of larger genome length (~5 kb less than λ^+) being chosen for smaller inserts, those of shorter length (8–9 kb less than λ^+) for larger inserts.

Replacement Vectors

A λ genome deleted for all nonessential genes (40% of its genome) is too small to be packaged. It follows that full advantage of the cloning capacity of a λ vector requires a derivative with a dispensable

'stuffer' fragment. When the stuffer fragment is excised it must be replaced with a donor DNA fragment, if the genome is to be packaged. In some vectors the retention of a stuffer fragment can be detected by virtue of a genetic tag; a *lacZ* gene or phage genes (*red* and *gam*). Special selective hosts can discriminate against the vector genomes, although such genetic selections are not essential since a variety of enzymatic tricks can reduce or eliminate the background of vector genomes. Replacement vectors are used for cloning larger fragments of DNA, often in the range of 20 kb. The stuffer fragment is frequently flanked by synthetic nucleotide sequences including targets for a variety of restriction enzymes, and commonly by promoter sequences recognized only by their cognate RNA polymerases (e.g. phage T3 or T7). Efficient transcription of one strand of DNA can be driven *in vitro* by the addition of T3 RNA polymerase, that of the other by T7 RNA polymerase. Labeled transcripts of peripheral fragments of the insert provide probes to facilitate chromosome walking.

Screening of genomic libraries is usually achieved by hybridization of probe sequences to the DNA released in plaques following denaturation and transfer to nitrocellulose filters. An alternative screen uses genetically tagged plasmid probes, and homology-dependent *in vivo* recombination is recognized by acquisition of the genetic tag, commonly a short sequence specifying the *supF* tRNA.

Expression Vectors

The DNA fragments are often inserted within a coding sequence that has a good promoter and Shine–Dalgarno sequence so that efficient controlled expression of a fusion polypeptide can be achieved for in-frame insertions. Frequently expression vectors take advantage of the *lacZ* gene and its control sequences. The fusion polypeptides may not be enzymatically active, but nevertheless they are frequently stable, can be purified and provide antigenic substrates. Screening by radioimmunoassay is very effective. Phage λ can be adapted to amplify gene products, particularly prokaryotic gene products, using either a λ promoter or the promoter of the relevant gene. The recombinant genome can be maintained in the prophage state and amplification achieved following prophage induction.

M13 Vectors

M13 plaques, like those of λ, offer efficient screens by DNA hybridization, but M13 has the special virtue of yielding large quantities of single-stranded DNA. From very small volumes of M13 suspensions, sufficient DNA is quickly prepared to prime a variety of sequencing reactions. M13 is the preferred vector wherever single-stranded DNA is required, including not only DNA sequencing, but site-directed mutagenesis and the assay and analysis of mRNA. M13 genomes with large inserts are readily packaged but are at a replicative disadvantage, and therefore it is preferable to use M13 for cloning small DNA fragments.

Since the phage genome is single-stranded the replicative form of the virus is used as vector DNA. M13 has little surplus DNA but multiple cloning sites built into the 5′part of a *lacZ* coding sequence have been incorporated within an intergenic interval. The N-terminal, but incomplete, *lacZ* polypeptide is functional in interallelic complementation with a defective host-encoded β-galactosidase; the latter lacks some amino acids within the N-terminal portion of the polypeptide. The complementation between polypeptides defective in either the α or ω parts of their sequences is detected by hydrolysis of the usual chromogenic substrate (X-gal). Insertional inactivation of the α component encoded by M13 provides a ready screen for recombinant derivatives.

Phage P1

Most recently the molecular understanding of phage P1 has permitted the harnessing of the large cloning capacity of this phage. DNA fragments up to approximately 100 kb can be recovered. The long recombinant molecule, though linear on entering a bacterium, is converted into a circular plasmid by a site-specific recombinase, Cre (Cyclization *re*combination protein), acting to break and join the DNA identified by two target sites, *lox* (locus for crossing-over), one in each arm of the vector DNA.

The P1 Cre-*lox* system is now used to promote site-specific recombination *in vitro* and *in vivo* even in eukaryotic cells. *In vitro,* Cre-mediated recombination between one *lox* site in a terminally labeled, linear, double-stranded oligonucleotide and a second *lox* site in a circular plasmid molecule, converts the plasmid into a terminally labeled linear molecule. Alternatively, Cre can catalyze the reverse reaction and circularize the DNA flanked by two *lox* sequences. Similarly, *in vivo,* Cre can promote site-specific recombination to integrate a circular DNA substrate into another DNA molecule or it can excise, as a circular molecule, the DNA between two *lox* sites. In this way, for example, a plasmid sequence flanked by *lox* sequences can be excised from a phage vector or some other genome.

Hybrid Systems

Plasmid/phage

Cosmids are plasmids that include the specific sequence of λ (*cos*) which is essential for packaging DNA into λ phage particles. Since plasmids are generally small, a large insert (35–45 kb) sandwiched between two cosmid genomes can provide the necessary separation between two *cos* sequences to promote packaging of the plasmid and donor DNA. The concept is an extension of that used in λ replacement vectors, but cosmids do not require a stuffer fragment since the vector DNA is prepared as a plasmid. In theory the phage particle will only package recombinant genomes, and these are efficiently transmitted to *E. coli*. Within the bacterium the linear genome circularizes via the cohesive ends of the *cos* sequence, and is then maintained as a large plasmid. Cosmids, like plasmids, have small genomes, but they take advantage of *in vitro* packaging to facilitate the efficient cloning of large DNA fragments. In a similar way the most significant feature of M13, and its relatives fd and fl, can be bestowed on plasmid vectors to produce a 'phagemid'. The ability to produce and package single-stranded plasmid DNA following infection with the appropriate phage (M13 or a relative) is conferred by the insertion of the appropriate *cis*-acting elements.

Plasmid expression vectors frequently use a λ promoter as their means of achieving efficient transcription of a cloned gene. Control of transcription is provided by temperature-sensitive repressor encoded by the repressor gene of λ (*c*I). This gene may be within the expression plasmid, within a compatible plasmid or within the host chromosome. As previously mentioned, many λ vectors include other phage promoters, particularly those recognized by only their cognate RNA polymerase. Some plasmids also incorporate these promoters to elicit efficient transcription *in vitro* or as a component of an *in vivo* expression system.

Multicomponent phage vectors

Some vectors aim to take advantage of the best features of λ, M13 and plasmids. The first of these (λZAP) is an insertion vector commonly used to express fusion polypeptides. A combination of M13 and plasmid (phagemid) are carried in a λ vector. The phagemid can be excised *in vivo* from its carrier phage following superinfection with M13 (or f 1) because the plasmid sequence is suitably flanked by signals from phage f 1 for the initiation and termination of DNA synthesis. The single-stranded DNA is made and forms a circular molecule that replicates, and can be maintained in the plasmid form. Its presence is selected by addition of the appropriate drug. Because the excised phagemid incorporates an f 1 (M13-like) origin of replication, it can be recovered in single-stranded form on infection with an f 1 or M13 helper phage. λZAP, like any λ vector, offers the high efficiency of packaging *in vitro* and sensitive screening of plaques. It then allows the experimenter to separate the plasmid *in vivo* from its superfluous λ carrier DNA, thereby providing the virtues of both plasmids and M13. The particular multicomponent vector described has a variety of cloning sites within the α coding sequence of β-galactosidase and hence has a screen for recombinant phages. The cloning sites are flanked by phage T3 and T7 promoters and appropriate terminators to facilitate efficient *in vitro* transcription of either strand of cloned DNA.

Phage vectors for other bacteria

Phage vectors have been developed for only a minority of bacterial genera, particularly Streptomycetes, Bacilli and Mycobacteria. Most cloning of microbial genes has been done in *E. coli*. Both shuttle and promiscuous plasmids permit transfer of cloned sequences between *E. coli* and other bacteria. Cosmids based on promiscuous plasmids have been particularly valuable.

See also: **Coliphage lambda (*Siphoviridae*); Enterobacteria phage P1 (*Myoviridae*); Phage ecology, evolution and speciation; Vectors: Animal viruses, Plant viruses.**

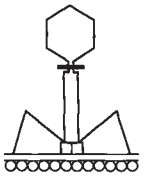
Further Reading

Relevant Chapters in Bacterial Genetic Systems (1991) *Methods Enzymol.* 204.

Chapters on vectors in *Molecular Cloning* (1989) by J Sambrook *et al.* (Nolan, C. ed.), 2nd edn, vol 1. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sternberg NL (1992) Cloning high molecular weight DNA Fragments by the bacteriophage p1 system. *Trends Genet.* 8: 11.

PHAGES IN INDUSTRIAL FERMENTATIONS



Mary Ellen Sanders, Dairy and Food Culture Technologies, Littleton, Colorado, USA

Introduction

Industrial fermentations rely on the growth and end-product formation of microbes. These processes include the production of many types of food, amino acids, organic acids and diverse, non-native proteins from recombinant microbes. Any interference with these processes is an economic liability.

Bacteriophage (or phage) can kill bacteria with astounding efficiency. A single phage may take only 60 min to complete its replication cycle, which could result in 200 new phages. Released into the environment, these new phages are free to find susceptible hosts and repeat the cycle. These kinetics suggest that, theoretically, one phage could yield over 1 billion phages within 4 h. (In contrast, a bacterium with a 30 min generation time, replicating via binary fission, would yield only 256 progeny in the same 4 h period.) Although not all phages replicate this dramatically it is apparent that phage can constitute a major threat to industries which rely on bacterial growth. Due to their biological nature, bacteriophage can be difficult to predict and to control, and as such can be the source of significant concern to industrial microbiologists.

Factors that Predispose Industries to Phage Problems

Certain industrial processes are more susceptible to bacteriophage problems than others. A combination of factors is responsible for this. Some production practices increase bacteriophage problems. However, it also seems that there may be poorly understood inherent biological factors that lead some bacteria to be more susceptible to destruction by phage than others. The following is a description of some factors that put a fermentation process at high risk for phage problems.

The process must rely on bacterial growth or metabolism. This may be the central process, such as lactic acid production in milk fermentations, but may also include secondary processes such as the malolactic fermentation in wine, the lactic acid production in sour dough bread, or diacetyl production in buttermilk.

There must be a means of contamination of the fermentation. Phage may enter a process either from external sources from air, equipment, or raw material or through the culture itself. The lysogenic or phage carrier states account for this avenue of phage entry, although modern starter culture production practices minimize the likelihood of this occurrence. Food fermentations are conducted with nonsterile raw materials and sometimes in exposed, open air fermentation vessels. Opportunities for phage contamination and dispersal abound in this environment. Other industrial fermentations including amino acid production or fermentations for recombinant DNA products can be more easily controlled by using sterile growth media, self-contained fermentation vessels, careful inoculation procedures, select production strains and contamination control. However, bacteriophage are difficult to keep out of even these systems due to their small size, especially if the fermentation requires input of oxygen. Therefore, even supposedly aseptic fermentations have reports of bacteriophage-induced failure.

The biological nature of the strains and phage present in a fermentation process dictate the speed and extent of phage destructiveness. Some phages develop to high levels very quickly, resulting in a rapid, thorough destruction of host populations. Others develop at a slow rate, detectable but insignificant to the overall bacterial metabolism and growth. This replication rate seems to be a result of both host and phage functions. Some industrial strains are known for developing devastating phages, and some phage types are known for rapid replication rates. Regardless, if phages are limited to slow development, little interruption of the fermentation process results.

The potential impact of destructive phage is augmented with some equipment and processes. In some processes, the scale-up of starter inoculum requires a commitment to the exact strains that will be used for fermentation several days in advance. A switch to a different production strain due to emerging levels of destructive phage is very difficult. If starter scale-up is not necessary (for example through the use of highly concentrated starter cultures) or can be done in less time, much more flexibility with strain substitution can be achieved, allowing effective response to detection of phage.

The necessity of adhering to an inflexible production schedule also amplifies the effects of phage on a

fermentation. Some fermentations are lengthy, and extending the time of fermentation can, under some circumstances, serve to overcome phage-induced 'slow downs' without significantly harming fermentation efficiency.

Production facilities designed with attention to personnel, material and product flow through the plant, air quality, separation of seed culture, and easily cleaned and sanitized equipment help reduce phage problems. Processes that do not produce aerosols, do not require excessive mixing, and lend themselves to adequate cleaning and sanitation can also effectively decrease the destructive effects of phage.

An industry that has a broad assortment of defined bacterial production strains has a greater chance of coping with phage problems than an industry with one, unique production strain. Strain substitution is an effective means of dealing with phage infections. The success of this approach, however, depends on the availability of suitable numbers of substitute strains that both resist current phage and carry out the desired metabolic transformation. It is not always a trivial matter to isolate these strains, and the genetic pool of available strains may not be extensive.

Processes that rely on a large number of bacterial strains seem to have a complementary large diversity of phage strains. The approach of the dairy industry to limit production strains to blends of well-characterized strains instead of mixtures of many undefined strains has worked to limit the phage populations and diversity in cheese plants.

Detection of Phage Problems

Not all fermentation delays are due to phage. The presence of chemical inhibitors, antibiotics, variability of physical parameters such as temperature, pH, dissolved oxygen and nutrient feed, and improper preparation of starter inoculum can all contribute to fermentation inhibition. The challenge is to differentiate these factors from true bacteriophage-induced fermentation failure.

The occurrence of starter failure is usually easy to detect. A drop in metabolic activity or growth is detected by whatever parameters are used to monitor the fermentation. Unfortunately, the root cause of starter failure is harder to determine, and the lack of technical resources of some industries impedes investigation into exact causes. The presence of a destructive bacteriophage can sometimes be especially difficult to determine due to inadequately defined detection methods. Results must then be differentiated from inhibition due to chemical inhibitors.

Since bacteriophage cannot be seen with the naked eye, alternative assays must be developed. Most commonly, phage are cultivated and their presence inferred from the results. Spot or plaque assays on agar plates and broth lysis are frequently used. However, laboratory assays for phage are dependent on the presence and concentration of cations, pH, temperature and media nutrients. Perhaps most importantly, purified, single-strain indicator bacteria must be available on which to assay the phage and these strains must be in the proper physiological state during assay. In some cases, mixed undefined, or multiple, defined strain combinations are used for fermentation. Production facilities may not have access to the component strains of these blends. In the case of commercially available defined strain blends, the strain manufacturer may be able to perform a meaningful assay for phage presence. However, in the case of undefined, mixed culture blends, even the culture producers may not know the strain composition of the blend. Assay for phage on a mixture of strains will often result in false-negative results, as phage against a single strain in the blend would likely be masked by growth of the other phage-insensitive strains. Furthermore, assay conditions may interfere with a definitive lytic response in the laboratory that may have been prevalent under production conditions. Therefore, it can be complicated to develop a consistent and reliable phage assay, especially for a phage population that may be continually changing. The ability of phage to replicate on hosts can also depend on a variety of environmental factors, which, when changed, may result in shifts in host range or efficiency of plating.

Even in the face of these difficulties, phage detection procedures have been developed for many phage and are regularly used during production.

Industries Affected by Phage Infection

Reliable data on the severity of phage infection of industrial fermentations are difficult to obtain. This is primarily because release of this information raises confidentiality concerns of private industry. Therefore, inference must be made from publications citing incidences (although not degrees) of phage infection or reporting developments to cope with phage problems. Such information suggests that phage problems are pervasive, although assignment of an accurate monetary value on lost or downgraded product is impossible. Phages have been responsible for disturbing industrial production of glutamic acid, butanol-acetone, natto (fermented soy beans), antibiotics, enzymes, casein, sausage, wine, sourdough bread, yogurt and cheese, as well as sewage treatment. Phage problems with industrial strains of *Clostri-*

dium, Bacillus, Lactobacillus, Lactococcus, Streptomyces, Escherichia, Streptococcus, Leuconostoc, Pseudomonas, Brevibacterium and *Corynebacterium* have been published.

The segment of the food industry that seems most seriously affected by phage is the fermented dairy foods industry. Scientific papers abound on the incidence and control of phage infecting dairy fermentations. Virtually all different types of fermented dairy products are involved, including hard cheeses, cottage cheese, yogurt and fermented milks. Phages infect the bacteria responsible for production of acid, diacetyl, extracellular polysaccharides and carbon dioxide. These factors are important to the final flavor, body and texture of different dairy products, and their absence decreases the value and, in the case of lactic acid, the safety of the food.

A relatively new aspect of the fermentation industry is the use of bacteria to produce proteins encoded by genes derived from other sources. Examples of eucaryotic proteins now produced by bacteria include chymosin, interferon, bovine somatotropin, insulin, human growth hormone, blood clotting factors and tissue plasminogen activator. This list is not complete and is expanding rapidly. Much effort has been placed on the development of efficient expression systems for these foreign genes, and inadvertent product loss through phage infection is troublesome. Although this industry is much better suited to control phage through using sterile production media, sterile air and aseptic fermentation technique, its reliance on single unique production strains increases susceptibility. Scientists involved with the scale-up of processes to production size should be aware of the threat of phage infection and design equipment, processes and production facility layout to provide a measure of defense against such problems.

Although not the process of choice today, acetone and butanol were once produced by the fermentation of molasses by some *Clostridium* species. Phage infection of this fermentation resulted in a significant decrease in process efficiency. The introduction of continuous fermentation compounded the problem. Phage-resistant mutants provided some relief, but host-range phage mutants evolved to attack newly released production strains. Finally, the rotation of phage unrelated strains brought the process into control.

Control of Phage in Industrial Fermentations

The control of phage in industrial processes is usually multifaceted. It frequently combines one or more of the following:

- *Sanitation* In considering equipment and processes, it is most important to accept that the single best intervention for preventing phage attacks is a properly sanitized production facility. This includes the valves, pipes, connectors, joints and other equipment areas that can harbor microbes of any type, including phage. Furthermore, any opportunity for crosscontamination of product containing replicating bacteria with product further upstream is an opportunity for phage to gain a foothold early in the process, in time to cause a fermentation problem.
- *Phage-resistant strain isolation or development* (see below).
- *Judicial starter use* Several approaches to starter use have been recommended, and the success of each seems to depend on the particular application. In some fermentations (especially foods), mixed, undefined blends of starter strains are used. A measure of phage control can be achieved with this approach as the bacterial populations are sufficiently diverse to allow bacterial components of the mixture which are resistant to infecting phage to carry out the fermentation. Some fermentation delay may result as these, perhaps minor, components of the strain mixture grow and become dominant. However, a totally dead fermentation is rarely experienced. In some cases during starter culture scale-up, starter cultures are deliberately contaminated with phage from the current production runs so that the phage-resistant populations can emerge. The problems with this approach are that fermentation times can be unpredictable and product characteristics dependent on bacterial metabolism can vary due to variation in the dominant strains executing the fermentations. Modern production facilities are frequently not willing to live with this lack of consistency.

An opposite approach is the isolation of production strains resistant to phages currently in the fermentation environment. Ideally, strains with broad-based resistance to phage are found or developed. These strains can then be used as single strains or as defined blends. They may be used repeatedly or may be rotated with other defined, phage-unrelated strains. Diligent analysis of phage in the fermentation environment helps predict which defined strains may suffer phage attack during production. The use of defined strains allows more predictability in product quality and process efficiency. Also, pure strains are available to assay for contaminating phage. The difficulty

with this approach is obtaining a suitable number and quality of strains to formulate the blends and to use as strain substitutes as phage appear. Also, since fewer strains are involved in executing the fermentation, the chances of complete starter failure increase.

Clearly, the starter system chosen for the fermentation must be managed carefully to control fermentation problems.

- *Use of phage-inhibitory media or other antiphage chemicals* Divalent cations such as Ca^{2+} and Mg^{2+} are required by many phage for adsorption to cell surfaces. These cations can be chelated by phosphates, citrates or oxalate. Other antiphage agents include nonionic detergents, antibiotics, spermine, ascorbic acid, phytic acid, glutathione and Fe^{2+}. Since these agents can also be inhibitory to production strains, an antiphage agent must have a more limited effect on host metabolism than on phage survival to be useful. Some industrial fermentation broths can be formulated to contain these agents. In the case of food fermentations, presence of these or other agents can be considered adulteration. Therefore, these agents may be added to starter propagation media to provide some control over phages developing in the starter scale-up process.
- *Phage detection* An ongoing program designed to detect phages against production strains can be very effective at identifying a phage problem before it builds to a truly destructive level.

Development of Phage-resistant Strains

The development of phage-resistant derivatives of production strains offers an approach to contend with phage infection of industrial strains. Diverse strategies have been taken throughout the fermentation industry, and efforts have met with different degrees of success. In a comprehensive approach, strain improvement efforts should be coupled with excellent sanitation and culture handling practices. In particularly phage-contaminated environments, the release of new, improved strains is not a permanent solution, but an intervention that will avert phage problems in the immediate future. It is a testament to the ability of phage to evolve and emerge that they can frequently, and with what seems to be creative ability, overcome many efforts to limit them.

In the laboratory, the isolation of mutants resistant to any particular phage is usually an easy task. Spontaneous mutation frequencies are sometimes as high as 1 in 10^4. Unfortunately, the resulting mutants are likely to have only shifted their phage sensitivity spectrum, and not broadened it. In addition, even though no genetic linkage may exist between phage resistance and fermentative characteristics, strains isolated as resistant to particular phages may suffer fermentative deficiencies. Therefore, isolation of mutants that possess resistance to a broad range of potentially lethal phage and still retain essential fermentative activities, is a challenge.

To develop a phage-resistant strain, the cell must be able to interfere with phage infection or proliferation at some point along the phage's lytic life cycle. Mutations in genes responsible for phage adsorption, passage of phage DNA into the host cell, transcription and translation of phage genes and expression of phage lysin could all interfere with phage development. Much larger changes in a cell's ability to resist phage could come from recombinant techniques, which would allow the introduction of entire gene systems, rather than relying on simple mutations, which would likely have a narrower effect. Bacterial systems that interfere with phage include abortive infection, a mechanism which greatly reduces the replication rates of phages and often destroys the bacterial cell prior to phage release, and restriction and modification, in which unmodified invading phage DNA containing recognized DNA sequences is cleaved and thereby destroyed.

A directed genetic approach has been successful for developing improved lactococci for dairy fermentations. In some lactococci, naturally occurring, self-transmissible plasmids were found to encode phage resistance genes. The phage resistance mechanisms included restriction and modification and abortive infection. When these plasmids were transferred into appropriately selected recipient strains, strains with improved, broad-spectrum phage resistance resulted. Although not resistant to all phages, these strains were significantly improved. Their phage resistance had a broader spectrum and fermentative activities were not diminished. This approach required characterized phage resistance genes, a method to introduce the genes into a host strain, a means to select the improved strain, and plasmid stability and gene expression in the new strain.

A different approach was taken to avert phage attack of a *Lactobacillus casei* strain used for fermentation of a dairy-based product. A troublesome lytic phage was found to be a lytic variant of a lysogenic phage carried by the production strain. The shift from lysogeny to virulence in the phage was caused by transposition of an insertion sequence from the *L. casei* strain to the phage. Prophage curing of the starter strain removed the threat of that phage from the fermentation.

In another case, phage problems were seen with a

strain of *Escherichia coli* used to produce insulin. Scientists averted problems by cloning genes for the restriction/modification system *Hha*II into the production strain. Expression of these genes decreased phage survival and permitted fermentations to proceed.

Strains used in the production of acetone and butanol, glutamic acid and fermented dairy products have been mutated to phage resistance in an effort to derive phage-resistant strains. In each case, mutants permitted only short-term freedom from phage attack.

A method of interfering with phage development has been tried with lactococci used in dairy fermentations. Expression of antisense mRNA targeted against a conserved phage gene has afforded some protection against phage in the laboratory. High copy numbers of the antisense mRNA strand presumably interfere with phage gene transcription. Although resistance levels were not very high, improvements in this strategy may possibly provide another method of developing cell-directed phage resistance to phage.

Summary

Phages abound where bacterial replication occurs. Phages have a dynamic ability to increase in numbers very rapidly at the expense of the host strain. This can lead to the rapid cessation of a bacterial viability and metabolic activity. Phages are ubiquitous and difficult to control. The isolation of phage-resistant mutants can provide temporary relief from phage problems, but the appearance of host range phage mutants usually undermines this approach. The introduction of whole gene systems and multiple levels of phage defense will more likely result in strains with a sustained ability to resist phage. Use of carefully developed defined strains, along with diligent sanitation efforts and careful process control, will usually result in control of phage.

See also: **History of virology: Bacteriophages; Lysogeny and prophage; Host-controlled modification and restriction; Phages as cloning vehicles.**

Further Reading

Dinsmore PK and Klaenhammer TR (1995) Bacteriophage resistance in *Lactococcus*. *Mol. Biotechnol.* 4: 297.

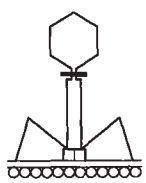
Garvey P, van Sinderen D, Twomey DP, Hill C and Fitzgerald GF (1995) Molecular genetics of bacteriophage and natural phage defence systems in the genus *Lactococcus*. *Int. Dairy J.* 5: 905.

International Dairy Federation (1991) *Practical Phage Control*. Arlington, VA: United States National Committee of the International Dairy Federation.

Ogata S (1980) Bacteriophage contamination in industrial processes. *Biotechnol. Bioeng.* 22 (suppl. 1): 177.

Sanders ME (1987) Bacteriophages of industrial importance. In: Goyal SM, Gerba CP and Bitton G (eds) *Phage Ecology*, p. 211. Chichester: Wiley.

PHAGES IN SOIL



Stanley T Williams and **A Martin Mortimer**, School of Biological Sciences, University of Liverpool, Liverpool, UK

Jackie D Parry, Division of Biological Sciences, I.E.N.S., Lancaster University, Lancaster, UK

Introduction

The soil presents a highly complex and variable environment for its microbial community. At the macroenvironmental level the activity and survival of bacteria is influenced by many factors, such as the amount and type of energy supplies and the gross abiotic factors, for example aeration, temperature and pH. Superimposed on these spatial and temporal variations are the even more subtle fluctuations at the microenvironmental level in the microbial growth sites within the soil mass. Thus, for example, it is well known that microsites for anaerobic bacterial metabolism (e.g. inside clay aggregates) occur even in grossly aerobic soils. The complexities of the soil environment have two major consequences for the microbial ecologist. Firstly, this habitat provides the greatest number and diversity of isolates. One gram of a fertile soil can provide viable counts of 10^9 bacteria and more than 100 different taxa. Even this is an

strain of *Escherichia coli* used to produce insulin. Scientists averted problems by cloning genes for the restriction/modification system *Hha*II into the production strain. Expression of these genes decreased phage survival and permitted fermentations to proceed.

Strains used in the production of acetone and butanol, glutamic acid and fermented dairy products have been mutated to phage resistance in an effort to derive phage-resistant strains. In each case, mutants permitted only short-term freedom from phage attack.

A method of interfering with phage development has been tried with lactococci used in dairy fermentations. Expression of antisense mRNA targeted against a conserved phage gene has afforded some protection against phage in the laboratory. High copy numbers of the antisense mRNA strand presumably interfere with phage gene transcription. Although resistance levels were not very high, improvements in this strategy may possibly provide another method of developing cell-directed phage resistance to phage.

Summary

Phages abound where bacterial replication occurs. Phages have a dynamic ability to increase in numbers very rapidly at the expense of the host strain. This can lead to the rapid cessation of a bacterial viability and metabolic activity. Phages are ubiquitous and difficult to control. The isolation of phage-resistant mutants can provide temporary relief from phage problems, but the appearance of host range phage mutants usually undermines this approach. The introduction of whole gene systems and multiple levels of phage defense will more likely result in strains with a sustained ability to resist phage. Use of carefully developed defined strains, along with diligent sanitation efforts and careful process control, will usually result in control of phage.

See also: **History of virology: Bacteriophages; Lysogeny and prophage; Host-controlled modification and restriction; Phages as cloning vehicles.**

Further Reading

Dinsmore PK and Klaenhammer TR (1995) Bacteriophage resistance in *Lactococcus*. *Mol. Biotechnol.* 4: 297.

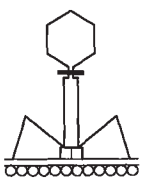
Garvey P, van Sinderen D, Twomey DP, Hill C and Fitzgerald GF (1995) Molecular genetics of bacteriophage and natural phage defence systems in the genus *Lactococcus*. *Int. Dairy J.* 5: 905.

International Dairy Federation (1991) *Practical Phage Control*. Arlington, VA: United States National Committee of the International Dairy Federation.

Ogata S (1980) Bacteriophage contamination in industrial processes. *Biotechnol. Bioeng.* 22 (suppl. 1): 177.

Sanders ME (1987) Bacteriophages of industrial importance. In: Goyal SM, Gerba CP and Bitton G (eds) *Phage Ecology*, p. 211. Chichester: Wiley.

PHAGES IN SOIL



Stanley T Williams and **A Martin Mortimer**, School of Biological Sciences, University of Liverpool, Liverpool, UK

Jackie D Parry, Division of Biological Sciences, I.E.N.S., Lancaster University, Lancaster, UK

Introduction

The soil presents a highly complex and variable environment for its microbial community. At the macroenvironmental level the activity and survival of bacteria is influenced by many factors, such as the amount and type of energy supplies and the gross abiotic factors, for example aeration, temperature and pH. Superimposed on these spatial and temporal variations are the even more subtle fluctuations at the microenvironmental level in the microbial growth sites within the soil mass. Thus, for example, it is well known that microsites for anaerobic bacterial metabolism (e.g. inside clay aggregates) occur even in grossly aerobic soils. The complexities of the soil environment have two major consequences for the microbial ecologist. Firstly, this habitat provides the greatest number and diversity of isolates. One gram of a fertile soil can provide viable counts of 10^9 bacteria and more than 100 different taxa. Even this is an

understimate, as the number detected depends on the isolation procedure used and whether or not the bacteria are inherently culturable. Secondly, it is still difficult to differentiate between active and inactive propagules within the sample studied. Therefore, there are still many problems in determining the extent and sites of bacterial activity in soil.

Any bacterium that is, at least occasionally, active in the soil is a potential host for phages. Commonly used approaches for detecting phages in soil have indicated that soil contains numerous phages capable of infecting many members of the diverse bacterial community. Although there has been no attempt to estimate the total concentration of phage particles in a soil sample, the limited data currently available indicate that a gram of fertile soil would contain numbers of phages in excess of the 10^9 viable counts for their bacterial hosts. Indeed, it has been suggested that phages may be the most numerous genetic object in the soil habitat.

Therefore, the ecology of phages in soil clearly merits more attention. Phages may exert some control on the many biochemical transformations of their hosts, either by direct lysis of key bacteria or indirectly as agents of genetic change. They also provide a novel opportunity to apply and test the concepts of host–parasite interactions. At a more practical level, the presence of phage in a sample that is active against bacterial taxa of interest can be a rapid means of screening large numbers of soils for the presence of selected bacteria. Also, the introduction of phages could potentially be used as a control method to limit the population size of engineered bacteria that have been introduced into soil.

This entry aims to address the current objectives, methods and problems of studying the roles of phages in soil. There will be some emphasis on phage infecting *Streptomyces*, a genus that is widespread in soil and to which most of our research has been directed.

Detection of Phages in Soil

Two basic approaches have been used to detect and isolate phages from soils. These are (1) enrichment procedures, which demonstrate the existence of one or more phage types in the sample; and (2) direct counts, which provide an estimate of the number of phages *in vivo* which are active against selected host(s).

Enrichment procedures

The most widely used enrichment procedure involves incubating a soil sample with a high concentration of the potential host cells. Within the soil, those phages that have the ability to infect this host will increase in number during this time. The period of incubation depends on the growth cycle of the host, as does the growth medium. Ideally both of these variables should be determined before attempting an enrichment procedure. Depending on the aims of the exercise, it may be appropriate to use a mixture of soil samples or a combination of putative hosts.

After incubation, the larger particles of soil and microbial material are removed by centrifugation and the supernatant is passed through a filter that excludes bacteria (e.g. 0.45 μm). The filtrate is then spotted on to a lawn of the putative host(s), preferably using the double-layer method. Any plaques or confluent lysis appearing after incubation indicates the presence of phage active against the host(s). Phages can then be purified by sequential isolation, dilution and testing against the host. High-titer stocks of specific phages can be prepared by plating them against their host, removing areas of high lysis, soaking them in a suitable nutrient medium at 4°C, filtering the supernatant and storing the suspension at 4°C. The viability of such stocks should be checked at 6-monthly intervals.

This method has proved to be both simple and effective, resulting in the isolation of phages specific to a variety of soil bacteria. A positive result also indicates that the host, or a close relative, is present in the soil sample(s) tested.

An alternative method dispenses with the addition of a high concentration of the potential host cells. Samples of soil are added to broth and incubated to stimulate growth of indigenous bacterial propagules and, hence, multiplication of their phages. Cell-free filtrates can then be spotted on to lawns of any potential hosts of interest. The rates of detection of actinophages (phage infecting actinomycetes) compare favorably with those obtained by the host enrichment method. Examples of actinophages detected in a range of soils by this method are given in **Table 1.**

Direct counts

Unlike enrichment procedures, direct counts aim to determine the concentration of phages present in a sample *in vivo*. Therefore, soil samples of known weight are suspended, diluted and filtered to remove bacteria. Dilutions of the filtrates are then plated on to lawns of putative host(s). The number of phage plaques developing indicates the number of phage particles per dry weight of soil, which are active against the host used.

Not all attempts to obtain direct counts have been successful and there are several possible reasons for

Table 1 Examples of actinophages in soils detected by nonhost enrichment

Propagation taxa	*No. of soils in which detected*
Streptomyces craterifer	37
Amycolatopsis spp.	37
Saccharopolyspora rectivirgula	17
Microtetraspora africana	11
Nocardioides albus	10
Actinomadura kijaniata	6
Saccharomonospora viridis	6
Amycolata autotrophica	4

this. Firstly, it is essential that the host strains used are known to be indigenous members of the soil or soil microhabitat used. Secondly, it is important that these strains are, or have been recently, active in the soil. It is well known that the soil mass is often relatively poor in energy sources for organotrophic bacteria and that these supplies are often concentrated in sites such as plant root regions. Also, there is much evidence to suggest that many common bacterial isolates (e.g. *Bacillus*, *Streptomyces*) are often obtained from resting spores within the sample. Consequently, the highest direct counts have been obtained from energy-rich habitats, such as garden compost and mushroom compost (10^3–10^4 PFU g^{-1} for *Streptomyces* phage (similar counts for thermophilic *Bacillus* phage)). Finally, the efficiency of the procedures used to extract phage particles from soil must be considered. Whatever the extraction method, its efficiency will be influenced by the physical nature of the soil. If a known titer of phage is added to sandy, loam and clay soils under controlled laboratory conditions, the percentages recovered usually decrease sequentially. This is probably due to the adsorption of phage particles to soil colloids. In addition, there are several variables in the extraction procedure. In our studies of the recovery of streptomycete phages added to three sterile soils, the best results were obtained by reciprocal shaking of the soil suspension in a nutrient broth with 0.1% (w/v) egg albumin at pH 8.0, followed by centrifugation of extracts at 1200*g* for 15 min. However, filter sterilization of the extracts resulted in lower recoveries of phage (34–87%) than those obtained using chloroform sterilization (93–100%). The latter method provided direct counts of phages active against selected *Streptomyces* spp. from arable and garden soils ranging from 0 to 10^3 PFU g^{-1} soil. Nevertheless there is much scope for the improvement of procedures to obtain accurate direct counts of phages in soil.

Stability of Free Phages in Soil

The methods used to detect or enumerate phages in soil are based on the assumption that phages exist as free particles in the sample. Therefore, it is pertinent to consider the factors that determine their stability in soil. Most data have been obtained by adding known titers of phages to natural and sterilized soil samples under laboratory conditions. The latter approach has the advantage of distinguishing between the survival of phages and the effect of factors on the activity of potential host bacteria (unless natural samples are incubated at low temperatures which preclude host growth). Additional information has been provided by studies on phage stability in laboratory broth suspensions. Relatively few studies of this topic have been made and many of the results have been equivocal. For example, when streptomycete phages were added to a range of soils, loss of viability was generally greater in natural soil. However, there was no significant difference in the stability of streptomycete phages added to natural and sterile sand dune soils; total inactivity of added phages occurred after 5 weeks. The half-life values of streptomycete phages added to sterilized sand dune soil stored at 4°C ranged from 1.19 to 18.9 days. Thus, it seems likely that free phage particles have a variable, but ecologically significant, capacity for stability in soil.

Various soil abiotic factors influence the stability of phage particles in soil. The presence and type of colloidal particles may have an effect on stability, but also influences the efficiency of extraction methods due to adsorption by phages. It is not always easy to differentiate between these two effects. In our experiments, four phages were added to a sand dune soil supplemented with 8% (w/w) kaolin or montmorillonite. Three phages showed increased half-lives in the presence of kaolin and one in the presence of montmorillonite. Previous data suggested that streptomycete phages were more readily adsorbed to kaolin than to montmorillonite.

Soil pH is another important factor influencing phage stability. Most studies have been conducted using typical neutrophilic hosts, active in a range from about pH 5.0 to pH 8.0. It has proved difficult to isolate phages from acidic soils (< pH 5.0) and laboratory studies on phage survival in soil tend to mirror this picture. The recovery of titers of streptomycete phages added to soils of different pH ranged from 0 (pH 3.7–4.2) to 10^3–10^4 PFU g^{-1} (pH 6.1–8.0). When four streptomycete phages were added to a sand dune soil (pH 6.8) and a pine forest soil (pH 5.9), half-lives in the former were larger in each case.

Soil temperatures vary considerably with locality, season and depth; most bacterial isolates react as

mesophiles with optimum growth at 25–30°C but phages are generally more stable at lower temperatures. The ideal storage temperature for most phages in broth suspension is 4°C, and when streptomycete phages were stored in sterile soil, half-lives were larger at 4°C than 20°C; however, the phages infecting thermophilic hosts, such as *Bacillus* spp. and *Thermoactinomyces* spp., which are active in self-heating composts, have an equivalent stability and activity at temperatures from 40 to 55°C.

Soil moisture is a continually fluctuating factor and has a major influence on bacterial growth, primarily through its effect on aeration; therefore, it clearly affects the activity of potential hosts. However, there is little evidence that moisture levels have a significant influence on free phage particles *per se*.

Phage–Host Interactions in Soil

Most studies on phage–host interactions in soil have been based on the assumption that the phages are virulent and undergo a typical lytic cycle, hence this article is mainly concerned with virulent phages. The possibility of phages forming lysogenic relationships cannot, however, be ruled out. It has been suggested that lysogeny is a mutually beneficial interaction for both host and phage. The phage can persist for long periods in the prophage state, protected from adverse environmental conditions, while the host is provided with homoimmunity to infecting phages. However, recent work has shown that the carriage of phages may be detrimental to the survival of the host as lysogens of a *Streptomyces* sp. were shown to die off in nonsterile soil, whereas the nonlysogen survived.

With regards to virulent phages, whatever the effects of environmental factors on free phages in soil their continued presence in soil depends on their chance adsorption to a suitable metabolically active host bacterium. Free phage particles presumably behave as passive entities in the soil suspension. Two factors may mitigate the randomness of phage–host encounters. Firstly, most phages are probably concentrated in microsites where their previously lysed hosts were active. Secondly, there is some evidence that both host cells and phage particles can adsorb to colloidal materials, thus increasing their proximity. If a potentially successful encounter occurs, its subsequent development will depend on the influence of environmental factors on adsorption, penetration and the level of host activity after infection.

The latter is clearly influenced by the quantity and nature of the energy supplies, temperature, aeration and pH. Information on the effect of environmental factors on reversible and irreversible adsorption is limited and confined mainly to reactions of phages added to sterile soil or broth cultures under laboratory conditions. Phage–streptomycete interactions usually have low infection efficiencies (10–50%), probably due to incomplete adsorption. The proportion of phages that is reversibly adsorbed is not dependent on host concentration (as streptomycetes are mycelial organisms, germlings are used as infectable units). Irreversible adsorption has been shown to depend on temperature, pH, time and host concentration. Increases in the latter lead to more adsorptions than those obtained by increasing phage concentrations, indicating that availability of adsorption sites may be a limiting factor. Subsequent to infection, it has been shown that the age and physiological state of the host influences the burst size, with a tenfold increase occurring in well-germinated spores (18 h) compared with newly germinated ones (4 h).

The temporal abundance of phages in soil is a reflection of the dynamics of host–phage interactions occurring within the microbial community. Such interactions reflect considerable complexity and have only recently begun to be examined in soil microcosms. Intrinsically, phage abundance is coupled to host abundance, and in turn host abundance will be a function of available resources within the soil environment. This coupling may, however, be an indirect one because hosts exhibit susceptibility to phages at specific stages in their life cycle. In single-cell bacteria, only actively growing cells are potentially infectable. In filamentous bacteria, the number of successful encounters between phage and hosts will be relative to the number of infectable host units, i.e. mycelial tips and germinating spores. In turn the abundance of infectable units will be proportional to resources for available host population growth. At the extremes, resource renewal may result in host populations being continuously available, as in bacteria at steady-state equilibrium or, when resource renewal is punctuated, periods where (1) absolute host numbers are low, and (2) where the number of infectable units are low. In actinomycetes, the abundance of infectable units as tips will be related to the resources available for filamentous vegetative growth. This abundance is increased when sporulation is triggered by resource exhaustion, with subsequent germination of spores occurring on resource renewal. Phasic fluctuation of infectable units in time, driven by resource utilization and renewal at the microsite level, is therefore a backdrop which may part regulate phage numbers over time. The extent to which a stable distribution of infectable units occurs over time will depend on the extent to which resources for growth are repetitively/continuously

renewed, which in turn may reflect the relative homogeneity of the soil environment as a whole.

Single species dynamics – phage components

Simplistically, absolute numbers of phages at a point in time will depend upon the gains arising from replication within hosts and the losses incurred through processes leading to inviability. In the absence of hosts, phages exhibit a 'type-specific' death rate that is constant within a given environment, enabling persistence of the virion bank within the soil to be quantified as a half-life. For actinophages, half-lives have been reported in the range of 1.19–18.9 days.

Burst size, the number of phages produced by a successful infection, may vary over three orders of magnitude, typically from *c.* 10 to 1000 PFU per infection. Temperature is an important determinant in phage reproduction, influencing key parameters of yield. Latent periods are shortened with elevated temperatures, whereas burst sizes are increased.

The probability of successful infection of a host by phages is a relatively rare event (10^{-5}–10^{-10}), and comparative studies suggest that infection efficiencies per unit volume are elevated in soil as opposed to broth culture. This may be a direct reflection of differences in surface area available for phage–host interaction in the two systems. The colloidal properties of soils are significant determinants of infection efficiency, as phages and bacteria may adsorb to clay colloids. The clay particles may act to concentrate phages and bacteria in the same microsite in the soil and, on the other hand, may protect bacteria from phage attack by differential adsorption.

Single species dynamics – host components

Rates of change in host numbers over time, in the absence of phages, will depend on the gains made through reproduction and the loss through death. In single-species bacterial populations, intrinsic rates of increase and carrying capacities of particular environments are common parameters in the descriptions of population dynamics. The steady-state conditions and the return times to steady state are governed by the form of the relationship between growth rate, the concentration of resource(s) limiting growth and the magnitude of the perturbations from equilibrium. Characteristically the generation times of unicellular hosts are short and population responses to resource renewal may be extremely rapid. In filamentous forms, developmental times are of longer duration and hence response times may be slower than in unicells. Filamentous hosts may also retain a bank of dormant spores at the same time as filamentous growth and sporulation is occurring. The finite rate of increase, often most conveniently measured immediately after sporulation, is therefore the sum of the contributions from the active fraction of the population (sporulating individuals) and the proportion of viable spores persisting over generations. In streptomycetes in natural soil, multiplication rates may vary up to a thousandfold. Significant variation in the proportion of spores persisting over a cycle of sporulation have been reported to range from 30 to 55%.

Experiments in which spore production has been measured from differing initial concentrations of inoculum into a constant volume of medium or soil have clearly pointed to density-dependent regulation of finite rates of increase in filamentous hosts. Such regulation is more likely to occur during spore production than in mortality of germinated spores. Although few actinomycetes have been considered in detail, in streptomycetes (1) both intra- and interspecific competition has been demonstrated, and (2) simple mixtures of streptomyces species may coexist at lowered equilibrium population densities than exhibited by individual components in monoculture. In long-term soil microcosms, streptomycete populations may persist in mixture, fluctuating in size between 10^5 and 10^6 CFU g^{-1} soil.

Host–Phage Interactions

The few empirical studies using soils have shown that prolonged coexistence between an individual host (single-cell or actinomycete) and a phage may occur, in which both components exhibit stability around an equilibrium mean density. In a bacteriophage–host association, the equilibrium density of both components is characterized by considerable constancy. In actinophage–host interactions, the host displays oscillations around a mean equilibrium with host numbers increasing during a sporulation phase in a periodic manner. Phage abundance shows a tendency to track host abundance.

In sterile soil cultures of *Bacillus subtilis* populations, the presence of a virulent phage lowered host equilibrium population size by a factor of ten, and phage densities were considerably lower than host densities. In contrast, the introduction of a temperate phage did not alter host equilibrium density from that observed when the host was cultured alone.

Streptomycete-virulent phage interactions in sterile soil also result in long-term persistence of phage, with a concomitant lowering of mean host abundance, although fluctuations in both components of at least one order of magnitude occurred around mean equilibrium levels. Also, the interaction with a

temperate phage resulted in the population density of the lysogen being lower than that of the nonlysogen counterpart.

Theoretical studies of predator–prey relations in other biological systems have pointed to the importance of the nature of the predator and prey responses in determining the outcome of a one-sided antagonistic interaction. In particular, the precise responses by phages to host density and host aggregation can have important effects (either stabilizing or destabilizing) on the interaction. The limited experimental evidence suggests that (1) the rate of phage 'predation' may increase as streptomycete density increases, and hence larger host populations suffer more mortality than smaller ones; and (2) the spatial heterogeneity of the soil environment may provide refuges for the host in which protection from phage infection may occur. In the former case there is a tendency to destabilize the interaction, leading to phage extinction. In the latter, the scale of spatial heterogeneity is important in determining stabilizing effects to provide a constant number or constant proportion of refuges. Besides these purely ecological considerations, temperature and lysogeny have been argued to be coevolutionary responses by phage to stabilize the interaction.

Experimental studies have shown that, on first introduction of phages to a host culture in soil, the phages may undergo an initial epidemic followed by a stable equilibrium. Analytical studies have indicated that phage–host interactions may be sensitive to initial starting conditions (relative abundance of the two components) but the form of dynamic behaviour (stable equilibrium, dampened oscillations, unstable fluctuations) is crucially dependent upon infection efficiency, burst size and intrinsic rate of increase of the host.

Community Dynamics

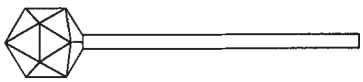
Both single-cell bacterial and streptomycete communities in soil may display considerable species richness and the role of phage and host in the maintenance of each other's diversity is yet to be unequivocally demonstrated. Laboratory studies of interspecific competition in simple (two species) mixtures of both bacteria and streptomycetes have indicated that competitive exclusion may occur, but the extent to which phage may mediate competitive interactions and ensure coexistence of competing hosts is unknown. Such a stabilizing influence will necessarily lead to phage persistence. Differential host susceptibility is another factor that may lead to long-term stability in host–phage interaction, and phages typically show wide variation in host range. The functional significance of lysogeny in the ecology of phages is still obscure but it has been suggested that prophages are much more common than free virions. If long-term phage persistence is related to lysogeny, phage diversity will be a function of host range and community interactions amongst hosts and other taxa in soil. The importance of these interactions during successional processes in soils remains to be unraveled.

See also: ***Bacillus subtilis* phages.**

Further Reading

Williams ST, Mortimer AM and Manchester L (1987) Ecology of soil bacteriophage. In: Goyal SM, Gerba CP and Bitton G (eds) *Phage Ecology*, p. 157. New York: Wiley.

PHAGES OF *STREPTOCOCCUS THERMOPHILUS*



Harald Brüssow, Nestlé Research Center Lausanne, Lausanne, Switzerland

Industrial Background

Lactic acid bacteria are used as so-called starters to transform milk into a variety of products. *Streptococcus thermophilus* is used in the production of mozzarella cheese and in symbiosis with *Lactobacillus delbrueckii* in the production of yoghurt and Swiss-type cheese. Streptococci, lactococci, lactobacilli and the well-studied model bacterium *Bacillus subtilis* belong to an evolutionary related group of the low

temperate phage resulted in the population density of the lysogen being lower than that of the nonlysogen counterpart.

Theoretical studies of predator–prey relations in other biological systems have pointed to the importance of the nature of the predator and prey responses in determining the outcome of a one-sided antagonistic interaction. In particular, the precise responses by phages to host density and host aggregation can have important effects (either stabilizing or destabilizing) on the interaction. The limited experimental evidence suggests that (1) the rate of phage 'predation' may increase as streptomycete density increases, and hence larger host populations suffer more mortality than smaller ones; and (2) the spatial heterogeneity of the soil environment may provide refuges for the host in which protection from phage infection may occur. In the former case there is a tendency to destabilize the interaction, leading to phage extinction. In the latter, the scale of spatial heterogeneity is important in determining stabilizing effects to provide a constant number or constant proportion of refuges. Besides these purely ecological considerations, temperature and lysogeny have been argued to be coevolutionary responses by phage to stabilize the interaction.

Experimental studies have shown that, on first introduction of phages to a host culture in soil, the phages may undergo an initial epidemic followed by a stable equilibrium. Analytical studies have indicated that phage–host interactions may be sensitive to initial starting conditions (relative abundance of the two components) but the form of dynamic behaviour (stable equilibrium, dampened oscillations, unstable fluctuations) is crucially dependent upon infection efficiency, burst size and intrinsic rate of increase of the host.

Community Dynamics

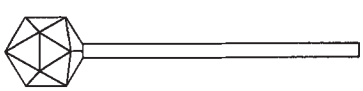
Both single-cell bacterial and streptomycete communities in soil may display considerable species richness and the role of phage and host in the maintenance of each other's diversity is yet to be unequivocally demonstrated. Laboratory studies of interspecific competition in simple (two species) mixtures of both bacteria and streptomycetes have indicated that competitive exclusion may occur, but the extent to which phage may mediate competitive interactions and ensure coexistence of competing hosts is unknown. Such a stabilizing influence will necessarily lead to phage persistence. Differential host susceptibility is another factor that may lead to long-term stability in host–phage interaction, and phages typically show wide variation in host range. The functional significance of lysogeny in the ecology of phages is still obscure but it has been suggested that prophages are much more common than free virions. If long-term phage persistence is related to lysogeny, phage diversity will be a function of host range and community interactions amongst hosts and other taxa in soil. The importance of these interactions during successional processes in soils remains to be unraveled.

See also: ***Bacillus subtilis* phages.**

Further Reading

Williams ST, Mortimer AM and Manchester L (1987) Ecology of soil bacteriophage. In: Goyal SM, Gerba CP and Bitton G (eds) *Phage Ecology*, p. 157. New York: Wiley.

PHAGES OF *STREPTOCOCCUS THERMOPHILUS*



Harald Brüssow, Nestlé Research Center Lausanne, Lausanne, Switzerland

Industrial Background

Lactic acid bacteria are used as so-called starters to transform milk into a variety of products. *Streptococcus thermophilus* is used in the production of mozzarella cheese and in symbiosis with *Lactobacillus delbrueckii* in the production of yoghurt and Swiss-type cheese. Streptococci, lactococci, lactobacilli and the well-studied model bacterium *Bacillus subtilis* belong to an evolutionary related group of the low

GC branch of Gram-positive Eubacteria. During growth the lactic streptococci ferment milk sugar (lactose) into lactic acid. The concomitant drop in pH leads to a coagulation of the milk which creates the food matrix. The low pH also protects the product against spoilage organisms.

Phage attack has always been a major problem in the dairy industry. Bacteriophages kill the starter and thus prevent proper fermentation. There are endogenous and exogenous sources of phages in the dairy industry. The endogeneous source is intimately linked with the starter. Many valuable starter bacteria, including lactic streptococci, are lysogenic, i.e. they contain an integrated copy of a temperate bacteriophage in their genome. Between 1 to 10% of starter strains release infectious phage after induction with mitomycin C. The prophage can also be activated under conditions of physiological stress of the bacterial cell. Starters frequently experience temperature, water, sugar, salt and concentration stress under conditions of industrial production. The majority of phage problems in the dairy industry are, however, linked to virulent phages which enter the factory through airborne and waterborne transmission routes from exogenous environmental sources, most likely raw milk. Production problems in a yoghurt factory start with a phage contamination level of 10^3 PFU ml^{-1}. First signs are a slowed acidification or texture problems of the yoghurt. Loss of entire starter batches are not rare. Occasionally, the phage problem is transmitted into the production line resulting in the loss of the product. Once introduced into the production line, phages can hide out for weeks, if not months in hard-to-disinfect infection pockets. Since a larger yoghurt plant processes 50 000 liters of milk daily, the stakes are high and phage attacks can be very costly. The economical implications are even more serious in cheese production where many dairies process half a million liters of milk daily. The conditions of cheese manufacturing favor the growth of phages. Not only are the milk volumes greater, but the raw milk is heated to lower temperatures and for shorter times than in yoghurt production. In addition, cheese production is an open process; during separation of the cheese whey large quantities of phage-loaden aerosols and liquids are created which contaminate the whole factory. In contrast, during yoghurt production the fermented milk is contained in a closed tubing system with minimal exposure to the environment. To minimize the negative effects of phage infections, the mozzarella industry uses complex starter mixes or even undefined starter blends. The yoghurt industry relies on few starter strains used in rotation to prevent the amplification of phages beyond a critical threshold.

Ecology

A longitudinal survey of *S. thermophilus* phages was conducted over several years in a mozzarella factory. The dairy used a complex, undefined mixture of starter cells and open, continuous fermentation tanks. Twelve distinct lytic groups of phages showing 11 distinct restriction patterns were observed over a two-year observation period. Multiple phage isolates from the same lytic group showed no nucleotide sequence changes over time. Dairy factories are thus not evolution machines creating new phage types. The diversity of the factory phages reflects the diversity of the phages attained in their natural environment. A direct proof of a link between factory phages and phages from the environment is, however, difficult to provide, as the phage concentration in the environment is very low compared to the infestation level of the factory (10^4–10^7 PFU ml^{-1}). To document these invasion events, an intervention study was conducted. The complex, undefined starter system of the factory was replaced by a defined starter system composed of cells which were unable to propagate the resident phage population. The intervention led to a marked decrease of the factory-specific phage population. Within a week three new phage types had colonized the factory (**Fig. 1**). Restriction analysis demonstrated that the new phages were not derivatives of the resident phage population. They were identical to phages found at low titers (10–130 PFU ml^{-1}) in the raw milk delivered to the factory during the intervention trial.

Classification of Virulent Phages

Virulent *S. thermophilus* phages from several collections have been characterized. All phages possess the same basic morphology consisting of an isometric head and long, noncontractile tail. In some phages, a thin fiber is attached to a terminal plate (**Fig. 2**). Morphologically they are classified as group B1 *Siphoviridae* (**Table 1**).

The largest collection covered 30 years of factory surveys from Italy and France. It contained about 100 distinct phage isolates. The phages differed in restriction pattern (more than 60 distinct restriction patterns were observed) or host range. Extensive DNA crosshybridization was observed between these phages. Host range analysis allowed the separation of yoghurt phages into two lytic groups, I and II. The taxonomic differentiation of these two lytic groups was biologically meaningful as it was corroborated by independent techniques (serology, protein and DNA analysis). Lytic group I phages were *cos*-site phages, whereas lytic group II phages were *pac*-site phages.

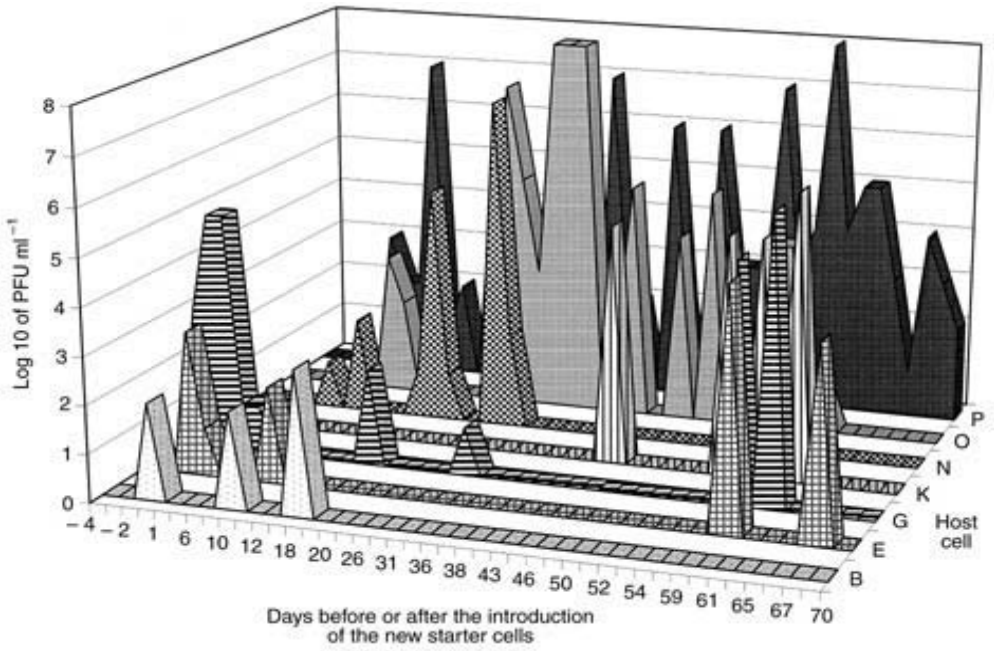


Figure 1 Cheese whey *S. thermophilus* phage titers expressed as log 10 PFU ml^{-1} observed on the indicated host cell on a given day before (negative numbers) or after the introduction of new starter strains (see text). Note the appearance of raw milk phages on host cells N, O and P. (Reproduced with permission from Bruttin A, Desiere F, d'Amico N *et al* (1997) Molecular ecology of *Streptococcus thermophilus* bacteriophage infections in a cheese factory. *Appl. Environ. Microbiol.* 63: 3144.)

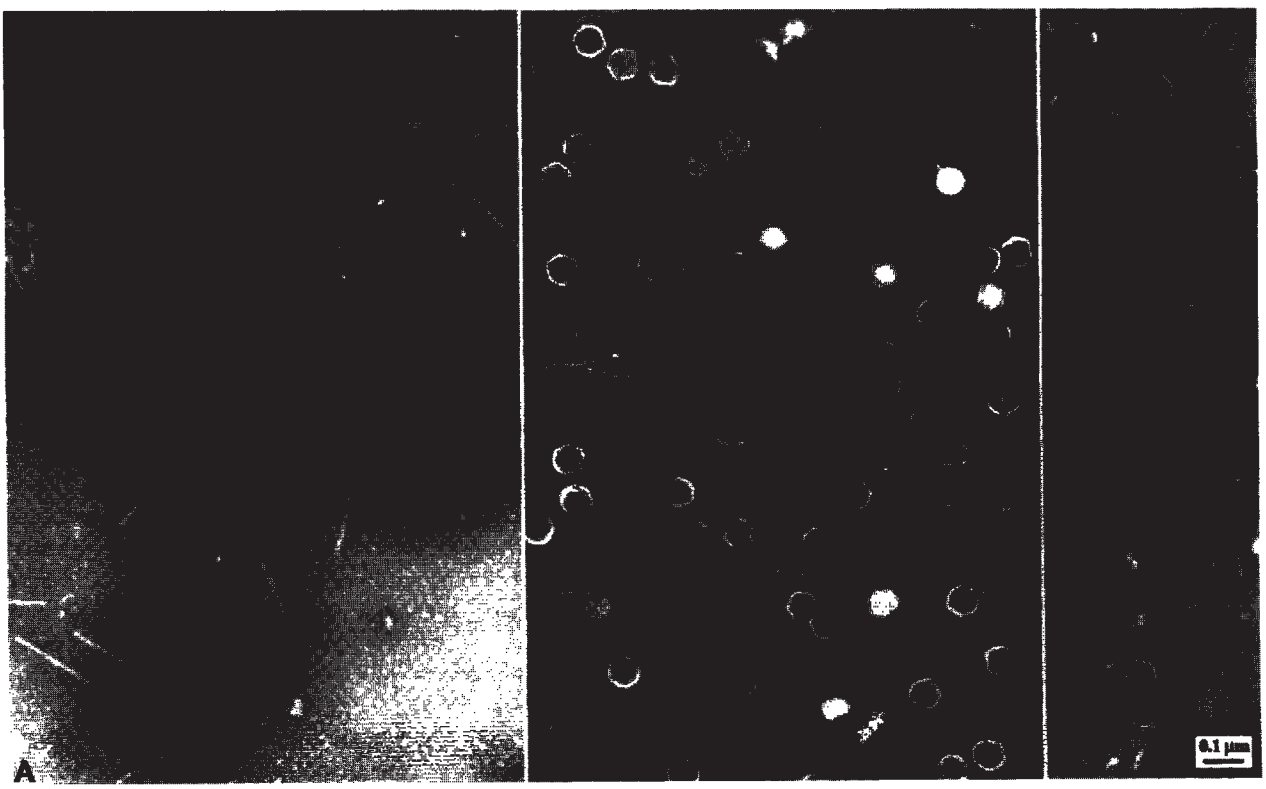


Figure 2 Electron micrographs of virulent *S. thermophilus* bacteriophages Sfi19 (**A**: Lytic group I *cos*-site phage), Sfi11 (**B**: lytic group II *pac*-site phage) and tailless Sfi11 (**C**). Bar, 0.1 µm. (Reproduced with permission from Lucchini S, Desiere F and Brüssow H (1998) The structural gene module in *Streptococcus thermophilus* bacteriophage ϕSfi11 shows a hierarchy of relatedness to Siphoviridae from a wide range of bacterial hosts. *Virology* 246: 63.)

Table 1 *Streptococcus thermophilus* phages in numbers

Growth	Growth medium	Milk, M17 broth
	Growth temperature	40°C
	Eclipse period	30 min
	Rise period	20 min
	Burst size	140–200
Morphology	Head	65 nm
	Tail	230–260 nm
	Tail fiber	50–400 nm
Taxonomy	Order	*Caudavirales*
	Family	*Siphoviridae* Group B
Genome		ds DNA, 31–45 kb
Classification:	*cos*-site	
Yoghurt	Virulent	Prototype Sfi19 (lytic group I, serotype 1) DNA: 37 392 bp; 44 ORFs Proteins: minor: 155, 105, 82, 64, 55, 44 kDa major: 33, 28 kDa
	Temperate	Prototype Sfi21 (lytic group I, serotype 1) DNA: 40 739; 52 ORFs
	pac-site	
	Virulent	Prototype Sfi11 (lytic group II, serotype 2) DNA: 39 807 bp; 51 ORFs Proteins: minor: 160, 120, 82, 74, 70, 60 kDa major: 44, 27, 18 kDa
	Temperate	Prototype 01205 DNA: 43 075 bp; 57 ORFs
Cheese	Many lytic groups	

Antisera against lytic group I phages did not neutralize lytic group II phages and vice versa. In addition, DNA probes were defined that were able to differentiate phages belonging to either lytic group I or II. The *cos*-site phages showed two major structural proteins, whereas *pac*-site phages demonstrated three major proteins.

In contrast, cheese phage isolates possessed a narrow host range and consisted of many lytic groups; within 41 isolates 34 different lytic groups were observed. The limited number of lytic groups in yoghurt-derived phages may reflect the restricted number of specialized starter strains used in yoghurt production.

Genomics of Virulent Phages

Phage Sfi19 (ϕSfi19) was chosen as the type phage for virulent lytic group I *cos*-site phages. It has a genome size of 37 kb. Interestingly, all 44 predicted genes are encoded on the same DNA strand (**Fig. 3, Table 2**). In accordance with the modular theory of phage evolution, genes of related functions are found in clusters. A DNA packaging module is found around the *cos*-site at the arbitrarily defined left genome end. It is followed by a cluster of putative head genes and then a tail DNA module. Downstream of the structural gene modules, which filled up the left half of the phage genome, there is a lysis cassette, then a remnant of a lysogeny module, a DNA replication module and finally a region of unknown attribution. With one exception the closest database matches of the predicted proteins are to predicted proteins from other phages attacking Gram-positive bacteria. The exception is the first viral ClpP protease. This gene differs substantially from that encoded by the streptococcal host genome.

A remarkable feature of the ϕSfi19 genome structure is the similarity of gene clusters from ϕSfi19 to clusters of adjacent genes in other phages. The compared phage genome segments show a similar topological organization of genes and significant similarity of their predicted protein sequences. Specifically, the DNA packaging region in ϕSfi19 resembles that of *Lactobacillus casei* phage a2; the major head-to-tail region resembles that of *Leuconostoc oenos* phage L10; the lysis cassette resembles that of *Streptococcus pneumoniae* phage Dp-1 and finally the DNA replication region shows similarity to two *Lactococcus lactis* phages, BK5-T and 7-2.

ϕSfi11 was chosen as the type phage for virulent lytic group II *pac*-site phages. It has a genome size of 40 kb. Despite being longer than ϕSfi19, its overall structure is strikingly similar (**Fig. 3**). All 51 predicted ϕSfi11 genes are encoded on the same DNA strand, two similarly situated open reading frames (ORFs)

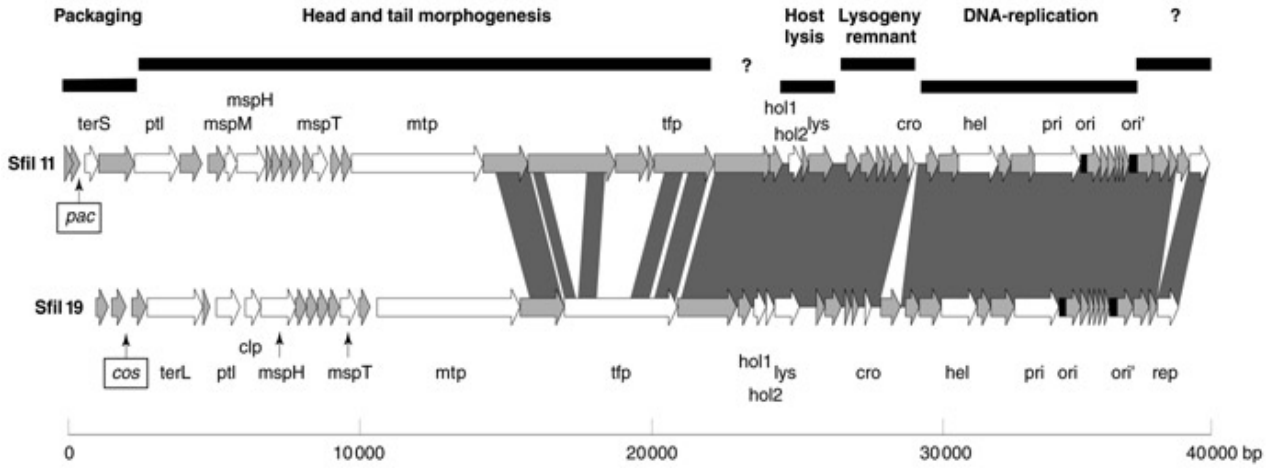


Figure 3 Alignment of the genome maps from phages Sfi11 and Sfi19. ORFs are represented by arrows and are annotated (see **Table 2** for abbreviations). Regions of sequence similarity are connected by shading.

are of unusual length for prokaryotic genes and the modular structure is exactly identical. Over the DNA replication module both phages show such a high degree of sequence similarity that one has to postulate a recent DNA exchange process between both phage types. The only exception is a gene from ϕSfi19 with a highly repetitive sequence structure (18 repeats of 18 bp), which has no complement in Sfi11. Over the DNA packaging and morphogenesis modules no sequence similarity was detected, despite a similar genetic organization. In this region, adjacent genes from phage Sfi11 demonstrated sequence similarities with adjacent genes from *Bacillus subtilis* phage SPP1 (DNA packaging) and *Lactococcus lactis* phage TP901-1 (morphogenesis module). At the end of the tail module, a transition zone was identified where regions with sequence similarity are interspersed with regions lacking any sequence similarity. When the *tfp* region was compared between phages Sfi11 and ϕ01205 a similar intermingling of short conserved and non-conserved protein domains was observed. Since both phages are otherwise so similar (see

Table 2 The genes of *Streptococcus thermophilus* phage Sfi21

Gene	*Amino acids*	*Function*	*Comments*
cos		cohesive end	15 nt 3′ overhang; dyad symmetry
terL	623	terminase, large subunit	NTP-binding motif (WalkerA)
ptl	384	portal protein	
clp	221	clpP-protease	First viral clp protease
mspH	397	major structural protein Head	Proteolytic cleavage
mspT	203	major structural protein Tail	
mtp	1560	minor tail protein	
tfp	1276	tail fiber protein	Collagen-like repeats; anti-receptor?
hol1	141	holin	Host viability loss
hol2	87	holin	Dual start site, host viability loss
lys	288	lysin	*N*-acetylmuramoyl-L-alanine amidase
attP		attachment site phage	Site of integrase action
int	359	integrase	Recombinase
imm	203	immunity	Strong antiphage element against heterologous virulent phages
mpm	122	metallo proteinase motif	
cI	127	cI repressor	Helix-turn-helix motif, antiphage element against homologous phage
cro	75	cro-repressor	Helix-turn-helix motif
ant	287	antirepressor	Helix-turn-helix motif
hel	443	helicase	DEAH-box, Walker A motif
pri	508	primase	Walker A motif
ori		origin of replication	Drives plasmid replication, very strong antiphage element

below), but differed in host range, the protein is a candidate for the streptococcal antireceptor. The antireceptor interacts with the phage receptor structure on the bacterial cell.

Temperate Phages

The prototype *cos*-site temperate phage ϕSfi21 was isolated from a French yoghurt starter. The starter spontaneously released infectious phages at a low level (10^3 PFU ml^{-1}). A number of cells could be lytically infected with ϕSfi21. The one-step growth characteristics of ϕSfi21 are similar to that of virulent *S. thermophilus* phages. A small percentage of the infected cells (<0.01%) survived the infection to yield lysogens. Phage DNA was site-specifically integrated into a tRNA gene of the host genome. The prophage DNA coexisted with unintegrated phage DNA of monomer genome size.

Extensive crosshybridization between individual restriction fragments of ϕSfi21 and a large panel of virulent *S. thermophilus* phages suggested a close relationship between temperate and virulent *S. thermophilus* phages. Crosshybridization with ϕSfi21 DNA ranged from a single restriction fragment (differing from phage to phage) to all fragments. Lytic group I phages showed the closest relationship to ϕSfi21.

The prototype *pac*-site temperate ϕO1205 was isolated from a Greek yoghurt. In contrast to ϕSfi21, ϕO1205 could only infect the cured lysogen and did not protect the lysogen against superinfection with virulent phages. A further *pac*-site temperate phage, ϕTP-J34, was isolated from a German yoghurt. The lysogenic host released mainly defective phages lacking the tail structure. The few infectious particles released could infect the cured lysogen. The presence of the prophage induced a phenotypic change in the aggregation behavior of the host cell (lysogenic conversion).

Genomics of Temperate Phages

ϕSfi21 has a genome size of 41 kb with cohesive ends. In contrast to virulent phages, not all ORFs are encoded on the same DNA strand. The exception are four adjacent genes covering the core genetic information for the lysogenic life style (**Fig. 4**). Otherwise, the genome of ϕSfi21 closely resembles the genome of the lytic group I *cos*-site ϕSfi19. In fact, over major parts of the genome the two phages show an identical gene order. The genes differ from each other only by point mutations (mainly at the third codon position) and small deletions/insertions. Overall the two phages differ by 10% bp changes. The changes are unevenly distributed; over the packaging and morphogenesis modules the change rate is high (20%), whereas over the lysis and replication modules only few (<1%) base pair changes are observed. Interestingly, both phage genomes differ only over the lysogeny module and the DNA replication modules. The virulent ϕSfi19 shows a duplication of the phage origin of replication, whereas the temperate phages ϕSfi21 and ϕO1205 possess only the origin downstream of the primase gene (see below).

ϕO1205 has a genome of 43 kb and contains a *pac*-site. It resembles ϕSfi21 in several respects: all but four genes of the core lysogeny module are coded on the same DNA strand; the lysogeny module is located at an identical genome position; the overall modular structure is identical. ϕO1205 is closely related to virulent *S. thermophilus* phages, but in contrast to ϕSfi21 the closest similarity is with *pac*-site phages of lytic group II. Major differences between ϕO1205 and lytic group II ϕSfi11 are located in three regions: the lysogeny module, the DNA replication region and the putative tail fiber gene. Over the remaining regions the two phages differed more or less uniformly by 10% base pair changes except for the highly conserved DNA replication module.

Lysogeny Module

In the lysogeny module of ϕSfi21 there is the following gene order: *int* (integrase), *imm* (superinfection immunity), *mpm* (metalloproteinase motif protein), *cI* (phage repressor) and in the opposite orientation *cro* (phage repressor), *ant* (antirepressor) (**Fig. 4**). This geometry creates a potential genetic switch structure between the two phage repressor genes. The lysogeny module is flanked on one side by the lysis cassette and on the other side by the DNA replication module. This gene constellation is found in many *Siphoviridae* infecting an evolutionarily related group of Gram-positive bacteria (*Streptococcus, Lactococcus, Lactobacillus, Bacillus*). Numerous sequence similarities link the predicted proteins of *Siphoviridae* from these bacteria suggesting a common evolutionary origin also for their phages. Recombination events have, however, reshuffled the genes of this module; sequence alignments of the lysogeny modules from different streptococcal phages reveal areas of high sequence conservation interspersed with regions of low sequence similarity. Several transition zones are found within genes. Contrary to the initial formulation of the modular theory of phage evolution the unit of the evolutionary exchange in streptococcal phages is not a group of functionally related genes, but can be as small as a single gene. Exchange reactions can even occur within

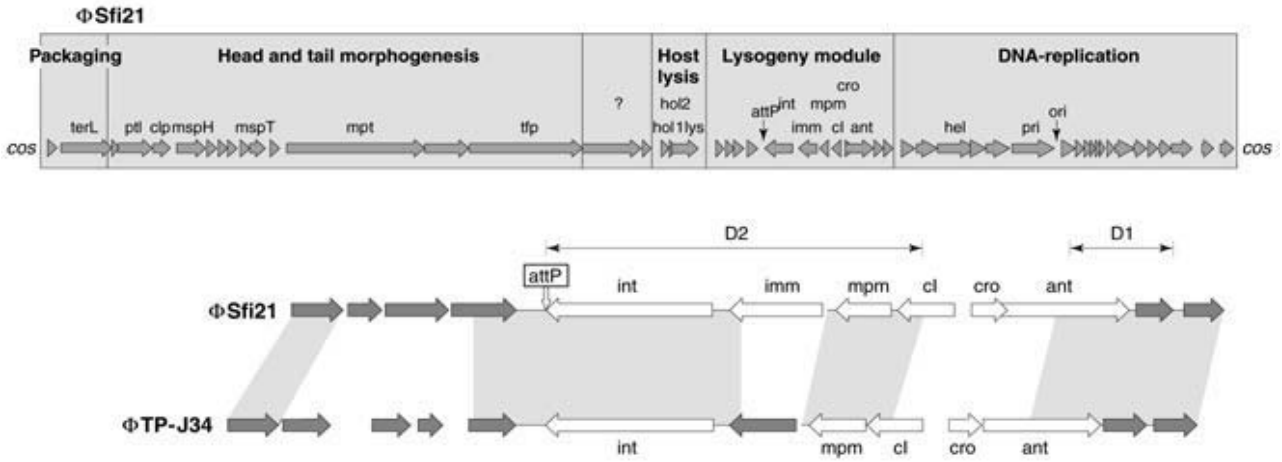


Figure 4 Genome map of the temperate *cos*-site phage Sfi21 (above). An amplified view of its lysogeny module is shown below in comparison with the *pac*-site phage TP-J34. Regions of sequence similarity are connected by shading. D1 and D2, deletions.

genes, possibly between gene segments encoding distinct protein domains. Several genes from the lysogeny module of ϕSfi21 were biologically characterized. The integrase mediates the integration of phage DNA into the chromosome of several *S. thermophilus* cells via site-specific recombination. Sequencing of the phage and bacterial attachment sites (*attP, attB, attR, attL*) identified a 40 bp identity region which, unusually, overlapped both the phage integrase gene for 18 bp and the terminal 11 bp of a host tRNA gene. A phage DNA segment covering *attP* and the phage integrase contained all the genetic information for faithful integration of a nonreplicative plasmid into the bacterial *attB* site. ϕO1205 and ϕTPJ-34 use a molecularly identical integration system.

When the indicator cell Sfi1 was lysogenized with ϕSfi21, the cell was protected against superinfection with the temperate phage and with 15 out of 19 virulent *S. thermophilus* phages (notably not ϕSfi19). The immunity functions could be attributed by cloning experiments to two phage genes. Interestingly, the two genes showed complementary activities. Transformation of an indicator cell with the cI repressor protected the cell against superinfection with the homologous temperate phage and its deletion mutants, but not against virulent phages. The CI repressor showed a helix-turn-helix DNA binding motif. Gel-shift experiments demonstrated binding to the putative genetic switch region between the *cI* and *cro* genes. Protection of the lysogenic cell against superinfection with virulent phages is achieved by the phage gene located upstream of the integrase. Transformation of the indicator cell with this gene protected the cell against 12 virulent phages, but not the homologous phage.

The Cro and Ant proteins showed both helix-turn-helix DNA binding motifs and sequence similarity to a repressor from a lambdoid coliphage 434 and an antirepressor from coliphage P1. A gene downstream of *ant* encodes a protein with a weak excisionase motif. However, a spontaneous ϕSfi21 deletion mutant (D1 in **Fig. 4**) showed that neither *ant* nor *xis*? are necessary for establishment of lysogeny or induction of the prophage.

The Lysis Cassette

Three genes constitute the lysis cassette of *S. thermophilus* phages. In contrast to other phage systems, two holins (pore-forming proteins) precede the lysin gene (**Fig. 3.**). One holin shows a dual start site suggesting a holin–antiholin control circuit. The lysin gene suggests a modular design. The N-terminal domain encoding the amidase shows similarity to other phage lysins. The C-terminal domain encoding the substrate-binding part resembles host proteins. In an infectious variant phage the lysin gene was actually split into two parts separated by 1 kb. The intervening DNA showed links to endonucleases found in self-splicing group I introns. At late, but not early times after infection both parts of the lysin messenger were rejoined. Both holin genes reduced the viability of the *Escherichia* coli host, when their expression was induced. Overexpression of the lysin gene in *E. coli* yielded a 31 kDa protein. Its specific lytic activity on streptococcal cell walls was demonstrated by an *in situ* detection assay.

Origin of Replication and Phage Resistance

Seven adjacent genes from the DNA replication module are the most conserved part of the *S. thermophilus* phage genomes. More than 70% of the phages from different collections contained this DNA segment. The very low sequence variability observed in this module makes it an attractive target for the design of engineered phage resistance mechanisms. This genome segment codes for four genes with nucleoside triphosphate binding motifs (Walker A motif), one of which is a distant member of the helicase superfamily, whereas another shows sequence similarity to DNA primases. Downstream of the putative primase gene noncoding DNA has been identified which possesses conspicious DNA repeats. This AT-rich DNA segment shows 80% sequence identity to a region following the *repA* gene of the cryptic *S. thermophilus* plasmid pST1. In fact, when cloned into a low copy number plasmid it acts as an origin of replication driven by phage infection. The inhibitory activity is plasmid copy number dependent and operates by blocking the accumulation of phage DNA during infection. Protein(s) in φSfi21-, but not in φSfi19-infected cells interact with the putative phage origin. In fact, φSfi19 is insensitive to the inhibition mediated by the φSfi21 origin. The φSfi19 origin differs in 14 nucleotide positions from the φSfi21 origin. When cloned on a plasmid the φSfi19 origin protects the indicator cell against infection with φSfi19 and five further phages insensitive to the φSfi21 origin. Together the two origins protect the indicator cell against infection with 23 out of 25 phages tested. None of the phages was able to form plaques on the starter containing the cloned phage origin. This is one of the broadest and strongest phage inhibitory activities described in the lactic acid bacteria field. In combination with the superinfection immunity gene, it might provide the basis for the development of phage resistant starters for yoghurt and mozzarella factories.

Structural Gene Cluster

The genetic organization of the DNA packaging and the head and tail morphogenesis region from φSfi21 resembles closely that of phages infecting evolutionary very unrelated hosts, the Gram-negative Eubacterium *Escherichia* and even the Archaeon *Methanobacterium* (**Fig. 5**). From the *cos*-site to the gene coding for the major tail protein, there is a surprising similarity in gene order between φSfi21 and lambdoid coliphages. Comparable genes have been found at identical positions; φSfi21 genes which could not be attributed showed a very similar size to the corresponding genes from these coliphages.

Sequence similarity of streptococcal structural phage proteins is limited to phages infecting Gram-positive bacteria. A hierarchy of sequence relatedness has been observed that correlated approximately with the evolutionary distance between the bacterial hosts (*S. thermophilus* ≫ *Lactococcus* > *Leuconostoc* ≈ *Bacillus* ≈ *Lactobacillus* ≫ *Mycobacterium* ≈ *Streptomyces*). Sequence similarity is closest to phage BK5-T infecting *Lactococcus lactis*, which is the closest evolutionary relative of *S. thermophilus* in this bacterial branch. The major head genes of these two phages could be aligned resulting in a 50% base pair sequence identity. The similarity to all other phages was only detectable at the amino acid sequence level of the predicted proteins.

No sequence similarity has been seen between φSfi21 and lambdoid phages, including φHK97. Nevertheless, striking structural similarities have been observed. Both major head proteins show a comparable length, a comparable secondary structure prediction and proteolytic cleavage at a comparable position. Both head genes are preceded by a protease gene. A possible interpretation is that *Siphoviridae* from Eubacteria (and Archaea?) share a common, although very distant ancestor for this genome region.

Phage Diversity

A number of mechanisms could theoretically create phage diversity. The classical mechanism is modular DNA exchange. Streptococcal phages have certainly exchanged their DNA replication modules. No other known mechanism can explain this high level of sequence conservation (99.8% at the bp level) over long DNA segments between otherwise clearly distinct phages. However, modular exchanges with non-*S. thermophilus* phage DNA have not been observed. The highest similarity in this category was a 50% base pair identity with a lactococcal phage. This similarity probably represents evolutionary relationships.

Genes from related *S. thermophilus* phages differ by numerous point mutations. The investigated phages demonstrated an average 10% base pair difference for phages belonging to the same subgroup. It is likely that this figure will rise when sequence data from *S. thermophilus* phages of other ecological or geographical origin become available. Accumulation of point mutations therefore seems to drive streptococcal phage evolution as it drives animal and plant virus evolution. In fact, phylogenetic trees could be constructed for individual phage proteins (e.g. integrase). These trees do not necessarily reflect the

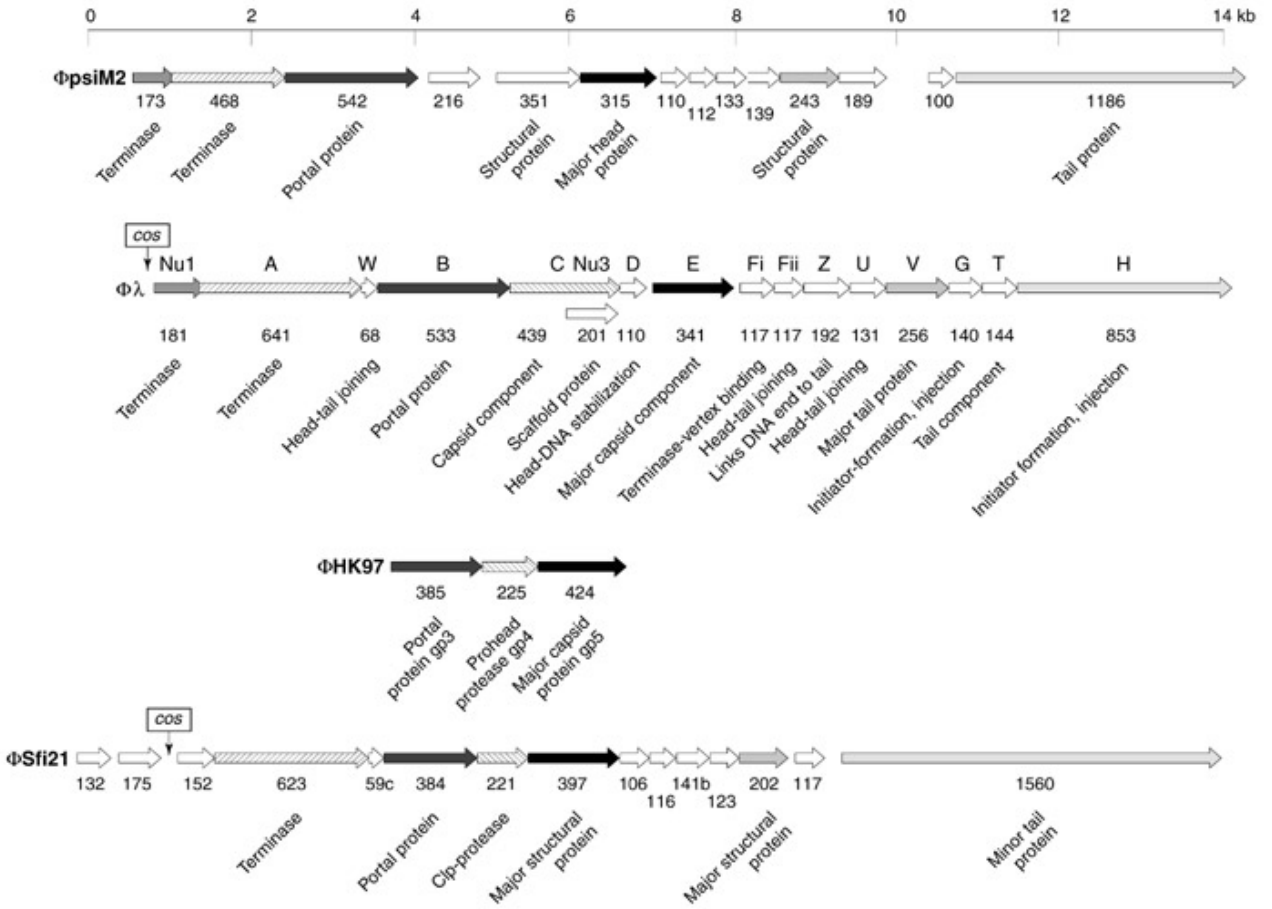


Figure 5 Comparison of the DNA packaging and morphogenesis modules from phage Sfi21, *E. coli* phages λ and HK97 and archaeavirus psiM2. Corresponding genes are marked by the same shading. Numbers give codon length.

evolutionary relationships of the corresponding phages, but only that of individual phage genes.

Genes from related *S. thermophilus* phages frequently differ by insertion/deletion events. These processes might create variability and thus the raw material for selection. Several spontaneous deletions have been observed repeatedly. One occurred in the putative tail fiber gene of φSfi21 which contains four collagen-like tripeptide GXY repeats. A 0.8 kb long DNA segment flanked by a 53 bp repeat was lost. Slippage of the DNA polymerase or unequal crossover events might have created this event. In other spontaneous deletions the phage integrase seems to be involved. During serial passage of φSfi21 a DNA segment spanning from the *attP* site to a site within the *cI* repressor gene was reproducibly lost (D2 deletion in **Fig. 4**). The latter site possesses a 4 bp repeat from one border of the attachment site. This deletion transforms the temperate φSfi21 into a virulent phage. Interestingly, wild-type virulent phages contain most of the genes surrounding this deleted region, although in a rearranged fashion, but none of the genes from the integrase to the cI repressor. It is thus tempting to speculate that the virulent *S. thermophilus* phages were created from temperate phages through recombination processes. Interestingly, the putative intron in the lysin gene of a variant streptococcal phage contained an endonuclease gene with bioinformatic links to self-mobilizing introns. This intron was near the rest of the lysogeny module which experienced extensive recombination. Recombinant phages might be produced during double infections, but such recombinants remain to be characterized molecularly. *S. thermophilus* phages are a young research field. Molecular data have only become available in the last four years. Due to its industrial importance the field will rapidly develop. *S. thermophilus* phages will also be an interesting model system to address those questions of phage evolution which can not be investigated with lambdoid coliphages.

See also: **Coliphage lambda (*Siphoviridae*); Phages in industrial fermentations; T1-like phages (*Siphoviridae*); T5-like phages (*Siphoviridae*).**

Further Reading

Brüssow H, Bruttin A, Desiere F, Lucchini S and Foley S (1997) Molecular ecology and evolution of *Streptococcus thermophilus* bacteriophages – a review. *Virus Genes* 16: 95.

Bruttin A, Foley S and Brüssow H (1997) The site-specific integration system of the temperate *Streptococcus thermophilus* bacteriophage φSFi21. *Virology* 237: 148.

Desiere F, Lucchini S and Brüssow H (1998) Evolution of *Streptococcus thermophilus* bacteriophage genomes by modular exchanges followed by point mutations and small deletions and insertions. *Virology* 241: 345.

Foley S, Lucchini S, Zwahlen M-C and Brüssow H (1998) A short non-coding viral DNA element showing characteristics of a replication origin confers bacteriophage resistance to *Streptococcus thermophilus*. *Virology* 250: 377.

Lucchini S, Desiere F and Brüssow H (1998) The structural gene module in *Streptococcus thermophilus* bacteriophage Sfi11 shows a hierarchy of relateness to *Siphoviridae* from a wide range of bacterial hosts. *Virology* 246: 63.

Stanley E, Fitzgerald GF, Le Marrec C, Fayard B and van Sinderen D (1997) Sequence analysis and characterization of φO1205, a temperate bacteriophage infecting *Streptococcus thermophilus* CNRZ1205. *Microbiology* 143: 3417.

Phocid Distemper Virus *see* **Rinderpest and Distemper Viruses**

PHYTOREOVIRUSES (*REOVIRIDAE*)

Bradley I. Hillman, Department of Plant Pathology, Foran Hall, Cook College, Rutgers University, 59 Dudley Rd, New Brunswick, New Jersey, USA

Donald L. Nuss, Center for Agricultural Biotechnology, Rm. 5115c – Plant Sciences Bldg., University of Maryland, College Park, Maryland, USA

History

Plant reoviruses are members of the family *Reoviridae*. They are distinguished by a segmented double-stranded (ds) RNA genome, a transmission mechanism that involves replication in the insect vector, and a propensity to induce tumors in infected plant hosts. Consequently, viruses within this group have attracted attention as potential model systems for studying a range of biological and molecular interactions and processes. From a historical perspective, plant reoviruses have played important roles in several significant scientific developments. Studies with rice dwarf virus (RDV) and wound tumor virus (WTV) were crucial in confirming the once controversial hypothesis that some insect-borne plant viruses actually replicate in the vector. The technique of density gradient centrifugation was developed during attempts to purify WTV. Efforts to understand the details of plant reovirus transmission led to the establishment of a continuous cell culture line from a plant virus insect vector. Several members of this group can cause considerable damage to crop plants and therefore are also of interest from an agronomic perspective.

Complete sequence data are now available for two plant-associated reoviruses and partial sequence data for several others, allowing for informed comparisons within this part of the *Reoviridae* family, and with animal-infecting reoviruses.

Taxonomy and Classification

Plant-associated reoviruses are subdivided into three genera based on particle structure, number and size of dsRNA segments, and vector. Members of the genus *Phytoreovirus* are leafhopper transmitted and have particles that contain 12 genomic segments. Members of the genus *Fijivirus* contain 10 genomic segments and are planthopper transmitted. Members of the genus *Oryzavirus* are also planthopper transmitted and have particles that contain 10 segments of dsRNA, but these differ in size and structure from those of the genus *Fijivirus*, as noted below.

The genus *Phytoreovirus* currently consists of three members: WTV, RDV and rice gall dwarf virus (RGDV). A fourth possible member that was recently identified, tobacco leaf enation virus (TLEV), would represent only the second reovirus capable of infecting

Further Reading

Brüssow H, Bruttin A, Desiere F, Lucchini S and Foley S (1997) Molecular ecology and evolution of *Streptococcus thermophilus* bacteriophages – a review. *Virus Genes* 16: 95.

Bruttin A, Foley S and Brüssow H (1997) The site-specific integration system of the temperate *Streptococcus thermophilus* bacteriophage φSFi21. *Virology* 237: 148.

Desiere F, Lucchini S and Brüssow H (1998) Evolution of *Streptococcus thermophilus* bacteriophage genomes by modular exchanges followed by point mutations and small deletions and insertions. *Virology* 241: 345.

Foley S, Lucchini S, Zwahlen M-C and Brüssow H (1998) A short non-coding viral DNA element showing characteristics of a replication origin confers bacteriophage resistance to *Streptococcus thermophilus*. *Virology* 250: 377.

Lucchini S, Desiere F and Brüssow H (1998) The structural gene module in *Streptococcus thermophilus* bacteriophage Sfi11 shows a hierarchy of relateness to *Siphoviridae* from a wide range of bacterial hosts. *Virology* 246: 63.

Stanley E, Fitzgerald GF, Le Marrec C, Fayard B and van Sinderen D (1997) Sequence analysis and characterization of φO1205, a temperate bacteriophage infecting *Streptococcus thermophilus* CNRZ1205. *Microbiology* 143: 3417.

Phocid Distemper Virus *see* **Rinderpest and Distemper Viruses**

PHYTOREOVIRUSES (*REOVIRIDAE*)

Bradley I. Hillman, Department of Plant Pathology, Foran Hall, Cook College, Rutgers University, 59 Dudley Rd, New Brunswick, New Jersey, USA

Donald L. Nuss, Center for Agricultural Biotechnology, Rm. 5115c – Plant Sciences Bldg., University of Maryland, College Park, Maryland, USA

History

Plant reoviruses are members of the family *Reoviridae*. They are distinguished by a segmented double-stranded (ds) RNA genome, a transmission mechanism that involves replication in the insect vector, and a propensity to induce tumors in infected plant hosts. Consequently, viruses within this group have attracted attention as potential model systems for studying a range of biological and molecular interactions and processes. From a historical perspective, plant reoviruses have played important roles in several significant scientific developments. Studies with rice dwarf virus (RDV) and wound tumor virus (WTV) were crucial in confirming the once controversial hypothesis that some insect-borne plant viruses actually replicate in the vector. The technique of density gradient centrifugation was developed during attempts to purify WTV. Efforts to understand the details of plant reovirus transmission led to the establishment of a continuous cell culture line from a plant virus insect vector. Several members of this group can cause considerable damage to crop plants and therefore are also of interest from an agronomic perspective.

Complete sequence data are now available for two plant-associated reoviruses and partial sequence data for several others, allowing for informed comparisons within this part of the *Reoviridae* family, and with animal-infecting reoviruses.

Taxonomy and Classification

Plant-associated reoviruses are subdivided into three genera based on particle structure, number and size of dsRNA segments, and vector. Members of the genus *Phytoreovirus* are leafhopper transmitted and have particles that contain 12 genomic segments. Members of the genus *Fijivirus* contain 10 genomic segments and are planthopper transmitted. Members of the genus *Oryzavirus* are also planthopper transmitted and have particles that contain 10 segments of dsRNA, but these differ in size and structure from those of the genus *Fijivirus*, as noted below.

The genus *Phytoreovirus* currently consists of three members: WTV, RDV and rice gall dwarf virus (RGDV). A fourth possible member that was recently identified, tobacco leaf enation virus (TLEV), would represent only the second reovirus capable of infecting

dicotyledonous plants. All members of this genus are morphologically similar, e.g. they lack capsid spikes prominent on particles of other plant reoviruses, contain 12 genomic RNA segments that exhibit similar electrophoretic profiles, and are transmitted exclusively by cicadellid leafhoppers. The genus *Fijivirus*, whose name is derived from its type member Fiji disease virus (FDV), consists of three principal members: FDV, maize rough dwarf virus (MRDV) and oat sterile dwarf virus (OSDV). Several other plant-infecting reoviruses described in the literature are now considered geographical races of members of this genus. Viruses related to MRDV include rice black streaked dwarf virus (RBSDV), pangola stunt virus (PSV) and cereal tillering disease virus (CTDV). *Arrhenatherum* blue dwarf virus (ABDV) and Lolium enation viruses (LEV) are related to OSDV. Members of the genus *Fijivirus* have genomes consisting of 10 segments that exhibit a characteristic electrophoretic profile quite distinct from that exhibited by genomes of the members of the genus *Phytoreovirus* and are transmitted exclusively by delphacid planthoppers. A possible member of the genus *Fijivirus*, *Nilaparvata lugens* reovirus (NLRV), was tentatively assigned to the genus based on sequence, structure and a planthopper association. However, although NLRV may transit through rice plants as a means of transmission from planthopper to planthopper, it does not replicate in these plants. The third plant-associated genus, *Oryzavirus*, consists of the type member rice ragged stunt virus (RRSV) and the *Echinochola* ragged stunt virus (ERSV). Members of this genus resemble members of the genus *Fijivirus* in terms of number of genomic segments and transmission by delphacid planthoppers, but differ in virus particle morphology and the electrophoretic profiles of the genomic RNA segments.

Additional support for the subdivision of plant-associated reoviruses into these three genera comes from comparative sequence analysis of the terminal domains of respective genomic segments. All genomic segments of each member of the genus *Phytoreovirus* contain the conserved terminal oligonucleotide sequences (+) 5′-GGU/CA----U/CGAU-3′ (only terminal sequences of the coding strand are shown). In contrast, two confirmed members of the genus *Fijivirus*, MRDV and RBSDV, have the conserved oligonucleotide sequences (+) 5′-AAGUUUUU----GUC-3′. Terminal sequences of the possible member NLRV are (+) 5′-AGU----GUUGUC-3′. The consensus conserved terminal oligonucleotide sequence for members of the genus *Oryzavirus* is (+) 5′-GUAAA----GUGC-3′. Thus, the nature of the genomic conserved terminal oligonucleotide sequences for the plant-infecting members of the family *Reoviridae* appear to be genus specific, providing an additional taxonomic criterion.

Virion Structure

Plant reovirus particles consist of two protein shells surrounding a core composed of dsRNA genomic segments and associated proteins. For members of the genus *Phytoreovirus*, the core consists of 12 dsRNA genomic segments tightly associated with three structural proteins surrounded by a shell of well-defined capsomers. This structure is further surrounded by a less well-defined outer shell consisting of two more loosely associated proteins. The complete particle is approximately 70–80 nm in diameter. RDV was recently crystallized, and its structure is now better known. Crystallized RDV particles are 693 Å (69.3 nm) in diameter. The outer capsid contains 780 protein subunits, thought to be the 46 kDa P8 protein, that are linked by disulfide bonds to form 260 trimeric capsomers. Members of the other plant reovirus genera have a slightly different morphology, characterized by the presence of prominent spikes protruding from the inner capsid. Purified RRSV particles exhibit additional differences in that they appear to contain only one protein shell.

Several enzymatic activities are associated with the purified core structures. These include an RNA-dependent RNA polymerase responsible for mRNA synthesis, and two enzymatic activities involved in the RNA-capping reactions: an mRNA-guanyltransferase and a guanine 7-methyltransferase. An additional RNA-modifying activity, an mRNA 2′-O-methyltransferase, has also been demonstrated in purified WTV preparations. From an evolutionary perspective, this observation is interesting, since this enzymatic activity is not present in plant cells.

Genome Organization

Complete sequences are now available for one member of the genus *Phytoreovirus* (RDV) and one proposed member of the genus *Fijivirus* (NLRV). Partial sequences of several other plant reoviruses are also available. Although each of the 10 or 12 segments that constitute the viral genome is unique in terms of nucleotide sequence, most dsRNA segments of the genera *Phytoreovirus* and *Fijivirus* conform to a similar basic organizational strategy. For example, all dsRNA segments of the RDV genome and the nine sequenced segments of the WTV genome contain conserved terminal sequences. It has not yet been determined whether the 5′ terminus of the coding strand is capped with an m^7G^5 ppp structure as occurs for other members of the family *Reoviridae*. The

other terminus, however, appears to be a perfect base-paired duplex and lacks any homopolymeric stretches. Segment-specific regions of inverted complementarity (inverted repeats) from 3 to 17 residues in length are found adjacent to the conserved sequences. Most segments contain one long open reading frame (ORF). For dsRNA segments of the genus *Phytoreovirus*, the ORF is on the strand containing the conserved 5′ terminal sequence GGU/CA∼, and is flanked by a relatively short (18–63 nucleotides) 5′ noncoding region and a slightly longer (93–492 nucleotides) 3′ noncoding region. Segment 12 of both WTV and RDV contains a short ORF located downstream from the large ORF that encodes the nonstructural protein Pns12, and RDV segment S12 contains other short ORFs that may be partially duplicated and rearranged. It is unclear whether any of the smaller ORFs of WTV or RDV S12 is expressed *in vivo*. However, the expression of two small out-of-phase ORFs embedded within the main RDV S12 ORF has been reported for infected rice tissue.

In vitro expression of cloned WTV and RDV genomic dsRNAs has allowed the assignment of virally-encoded polypeptides to their cognate genomic segments. More detailed structure–function studies have recently been performed on several RDV-encoded proteins. In addition, alignment analysis of the predicted amino acid sequences of the major inner capsid protein of the three members of genus *Phytoreovirus* have provided convincing evidence that these three viruses evolved from a common ancestor.

Interestingly, both MRDV segment S6 and RBSDV segment S7 revealed coding strands that contained two large nonoverlapping ORFs consisting of 363 and 310 codons and separated by 52 nucleotide intercistronic regions. This configuration is unique among the family *Reoviridae*. Only the 5′ proximal ORF was expressed in cell-free translation systems, and it is unknown whether the 3′proximal ORF is expressed *in vivo*. The finding that the second ORF is absent in the corresponding segment of NLRV, the putative member of the genus *Fijivirus* that does not replicate in its plant host, suggests that the second ORF may be important to infection of plants. As indicated above, the genomic segments of MRDV and RBSDV, both members of the genus *Fijivirus*, contain the same conserved terminal oligonucleotide sequences (+AAGUUUUU----GUC-3′), indicating the likelihood of a common ancestor for these two viruses.

Sequence information for members of genus *Oryzavirus* is currently somewhat limited. However, it is clear from RRSV and ERSV sequence data gathered to date that the structural motif consisting of conserved terminal oligonucleotides and adjacent segment-specific inverted repeats is also a feature for this plant reovirus genus. Coding features for members of the genus *Oryzavirus* also seem to be consistent with reovirus genome organization.

Several pieces of evidence suggest that plant reovirus particles may contain one copy of each of the 10 or 12 segments that comprise the viral genome. This evidence is based primarily on studies with WTV, and includes the following observations. Genomic RNA isolated from purified virus particles contains equimolar amounts of each segment and, based on extrapolations of dilution–infectivity curves, it is predicted that an infection can be initiated by a single virus particle. The mechanisms responsible for the apparent selective packaging of one copy of each viral RNA segment into a single particle are not well understood. Molecular characterization of internally deleted defective-interfering (DI) RNAs present in some transmission-defective WTV populations clearly established that the sequence information necessary for replication and packaging of genomic segments resides within the terminal domains of the segment. It was shown further that DI RNAs displaced only the genomic segment from which they were derived and did not interfere with the replication or packaging of unrelated genomic segments. Combined, these observations suggested that each genomic segment must contain at least two operational recognition domains: one that specifies that the RNA is viral and not cellular, and a second that distinguishes one genomic RNA segment from another. It has been postulated that the initial events leading to the replication of WTV genomic segments involve the specific recognition and packaging of viral transcripts, rather than dsRNA segments, followed by subsequent synthesis of the genomic noncoding strand. It has been postulated further that the conserved terminal oligonucleotides and the segment-specific inverted repeats may serve as recognition domains, with the terminal inverted repeats serving to specify the conformational properties of the viral transcripts as a result of intramolecular base-pairing.

Gene Expression

There are numerous technical difficulties associated with studying virus replication and gene expression *in planta*. In contrast, the cultured cell lines derived from leafhopper vectors provide very convenient systems for examining these processes for the plant reoviruses. All three members of the genus *Phytoreovirus* have been shown to multiply readily in their leafhopper vector and cultured cells derived from these vectors. Infection of both the leafhopper vector and cultured

vector cells results in a productive, usually asymptomatic, persistent infection. Infection of cultured vector cells by WTV has been examined in detail and was found to have the following characteristics. Infected cultures fail to exhibit any apparent cytopathology either during the initial (acute) stages of infection or after the onset of persistence. Persistently infected cells exhibit the same morphology, pattern of cellular protein synthesis and growth rate as uninfected cells and do not suffer from periodic crises. Essentially all cells in the persistently infected culture remain infected for hundreds of cell passages. Infectious virus is recoverable from persistently infected cells and can initiate a new round of acute and persistent infection. The kinetics of viral protein and RNA synthesis has also been measured in WTV-infected vector cells. Based on distinct changes in the level of viral gene expression, the infection was divided into an acute phase (the first 5 days after inoculation) and a persistent phase (beginning with the first cell passage). Viral specific polypeptide and viral genomic RNA accumulation was observed to increase to a maximum level during the first 5 days following inoculation and then to decrease rapidly to approximately 5% of the maximum level beginning with the first cell passage. In contrast, viral specific mRNAs were present at approximately the same level in the acute phase and in the early stage of the persistent phase of infection. Moreover, viral transcripts isolated from persistently infected cells were found to be translated inefficiently *in vitro*, reflecting the situation observed in infected cells. These results were interpreted as suggesting that a form of post-translational regulation of viral gene expression operates in vector cells persistently infected with WTV. The recent successful culture of planthopper cell lines will allow similar studies with members of the other plant reovirus genera.

Plant reovirus gene structure is generally simple, with apparent monocistronic messages in most dsRNA segments. In cases of possibly more complex gene structures, expression *in vivo* of coding regions in addition to the major segment-associated polypeptide (e.g. WTV or RDV S12; MRDV S6 or RBSDV S7, all discussed elsewhere) has not been confirmed. Post-translational processing has been identified for one plant reovirus protein. Genomic segment S8 of RRSV, type member of the genus *Oryzavirus*, encodes a structural protein that has been identified serologically in virus particles. The complete 67 kDa S8 product was recently shown to be processed autocatalytically to a 46 kDa product. The 43 kDa protein identified as the major capsid protein of RRSV, P8, was thought to be the result of further proteolytic processing of the 46 kDa protein.

Epidemiology

The epidemiology of the monocot-infecting reoviruses is tied to the presence of the crop and the hopper vector. Many of the monocot-infecting reoviruses are found in tropical regions, so the cycle from the agronomically important plant host to the vector and back to the plant host is often difficult to break. Complicating this, wild grasses may serve as alternate hosts for these viruses. In tropical and subtropical regions, overwintering of the virus is often unnecessary because of the constant presence of plant and vector. However, overwintering of virus in eggs may be an important survival mechanism in temperate regions.

Prevention and Control

Several members of this genus were once responsible for serious crop losses and were expensive to control, but advances in the development of effective control measures have resulted in reduced impact of these viral diseases. The two main approaches for control of reoviruses that infect graminaceous hosts are breeding for resistance and chemical control of insect vectors. Although intensive breeding for resistance was only recently initiated, several rice varieties resistant to RDV and other reoviruses are now available. Chemical control of insect vectors has been a reasonably effective preventative measure when vector populations were otherwise low, but has not been effective under high vector pressure. Pathogen-derived resistance involving genetic engineering approaches, for example transforming plants with coat protein or other viral genes as has been successfully deployed against numerous other RNA plant viruses, have been tried to a limited extent with RDV and RRSV. None of the three structural genes transformed into rice plants to date have effectively reduced virus infection.

Geographic and Seasonal Distribution

Plant-infecting reoviruses have been isolated on every continent. Members of the genus *Phytoreovirus* have been identified only in North America (WTV), Asia (RDV and RGDV), and Africa (TLEV). WTV is of no economic significance and has been isolated only twice, in both cases serendipitously, but RDV and RGDV are commonly found infecting rice crops and have been reported to cause severe damage under certain growing conditions and when viruliferous insect vector population levels are high. Members of the genus *Fijivirus* are more widely distributed, found in the South Pacific, South America, Europe and Asia. Both confirmed members of the genus *Oryzavirus*

have been found only in Asia and are so far of limited but potential economic importance. Seasonal distribution of plant reoviruses is associated with presence of the crop and vector.

Plant Host Range and Symptom Expression

The recorded host range of plant reoviruses, with the exception of WTV and TLEV, is limited to the Gramineae. Severe stunting or dwarfing is the most common symptom of reovirus infection of graminaceous plants. Additional symptoms include distortion of the leaves, including marginal serration, flecking or streaking, or the development of abnormally dark-green coloration. These symptoms are generally accompanied by vein swelling, enations or hyperplastic growth on the abaxial leaf surface. WTV is the only confirmed reovirus known to infect dicotyledonous plants, and has a wide experimental range extending to more than 40 species in 20 plant families. The experimental host range of the other possible member found in dicotyledonous plants, TLEV, has not yet been determined. Symptoms of WTV infection include stunting and leaf distortion, but the most notable symptom is neoplastic growth of phloem tissue, the degree and duration of which are dependent on the host. For example, WTV infection of *Trifolium* spp. results in vein enlargement, whereas infection of *Melilotus* spp. results in the formation of massive stem and root tumors. Wounding of infected plants tends to promote tumor formation, thus the virus name. The molecular basis for tumor induction by plant reoviruses is unknown. Among the plant-infecting reoviruses, only RDV fails to induce neoplasia. RDV is also atypical in that its replication is not limited to the phloem tissue of infected plants.

Transmission and Tissue Tropism

Infection of plant hosts by the plant reoviruses requires the aid of an insect vector; members of this virus group are not spread by mechanical transmission. The mode of transmission is referred to as propagative because virus multiplication occurs in the insect vector as a necessary part of the transmission process. The virus is acquired during feeding on infected plants. It sequentially infects different tissues of the vector, eventually reaching the salivary gland, and is then transmitted to uninfected plants via the salivary fluid during subsequent feeding. This process can take from 13 to 30 days to complete, depending on environmental conditions.

As indicated above, members of genus *Phytoreovirus* are transmitted by leafhoppers, whereas members of the other genera are transmitted by planthoppers. Transmission of a particular plant reovirus is limited to a single or several vector species, e.g. WTV is transmitted only by *Agallia constricta* Van Duzee, *Agallia quadripunctata* Provancher and *Agalliopsis novella* (Say). In contrast to the situation in infected plants, multiplication of virus in the insect vector results in no apparent pathological consequences, although reductions in fecundity of MRDV- and RDV-infected vectors have been reported. Transovarial transmission to progeny has also been reported for several of the plant reoviruses. Continuous cell culture lines have been generated from several leafhopper species that serve as plant reovirus vectors, and recently a continuous culture was developed from embryonic cells of a planthopper. These cultured vector cells support virus multiplication, thus providing a valuable system for studying viral gene expression and replication as well as details of virus–vector interaction. Although the plant reoviruses do not form plaques on the vector cell monolayers, virus titers can be readily determined with the aid of a fluorescence focus assay.

Maintenance of plant reoviruses exclusively in a plant host can result in the generation of virus populations that are deficient or defective in their ability to be transmitted by the insect vector. This phenomenon has been studied in some detail for WTV. The loss of transmissibility was accompanied by a loss in the ability to infect cultured vector cells and the generation of internally deleted forms of specific genomic segments that acted as DI RNAs. In selected isolates, certain segments, e.g. segments S2 or S5, appeared to be missing. Although these isolates retained the ability to induce tumors in systemically infected vegetatively propagated plants, they were completely defective in insect transmissibility. It has been suggested that one or both of these segments are required for replication in the insect vector but are dispensable for replication in the plant host. This hypothesis was partially tested for RDV by selective removal of the outer capsid protein P2 (the S2 product) with carbon tetrachloride. Progressive removal of the P2 protein resulted in a progressive decrease in infectivity to insect cells. Subsequent studies demonstrated that an RDV mutant lacking P2 was transmission-defective but able to replicate in plants, confirming the importance of the S2 gene product for infection of insect cells. The S5 gene product of RDV, P5, was recently identified as a minor core protein with NTP binding properties, and not an outer coat protein as previously believed. The specific roles of P2 or P5 in infection of insect cells remains unknown.

With the exception of RDV, plant reoviruses are

confined to the phloem of infected plants. The galls or enations often associated with plant infection represent modified phloem that serves as a site of virus replication and accumulation.

Future

Many of the fascinating biological properties exhibited by the plant reoviruses remain poorly understood. Unfortunately, the lack of infectious cDNA clones of plant reoviruses prevents mechanistic studies from proceeding as fast as they might. Recent completion of plant-associated reovirus sequences, including NLRV, is providing important information for comparison of plant reoviruses with some of the more thoroughly studied animal reoviruses. Studies using expressed proteins are allowing for examination of the roles of individual dsRNA segments and their gene products in the infection process. Questions about the molecular basis of such processes as virus-induced neoplasia, viral persistence, vector range and the recognition, sorting and packaging of a segmented RNA genome will be addressed in the coming years.

See also: **Defective interfering viruses; Persistent viral infection; Reoviruses (*Reoviridae*): General features; Molecular biology; Plant reoviruses.**

Further Reading

Boccardo G and Milne RG (1984) Plant reovirus group (no. 294). In: *CMI/AAB Descriptions of Plant Viruses*. Wellesbourne, Warwick, UK: Association of Applied Biologists.

Francki RIB and Boccardo G (1983) The plant *Reoviridae*. In: Joklik WK (ed.) *The Reoviridae*, p. 571. New York: Plenum.

Holmes IH, Boccardo G, Estes MK *et al* (1995) *Reoviridae*. In: Murphy FA *et al.* (eds) *Sixth Report of the International Committee on Taxonomy of Viruses*, p. 208. New York: Springer-Verlag.

Kimura I and Omura T (1988) In: Harris KF (ed.) *Advances in Disease Vector Research*, vol 5, p. 300. New York: Springer Verlag.

Nobuhiko N, Koizumi M, Watanabe H and Noda H (1996) Complete nucleotide sequence of the *Nilaparvata lugens* reovirus: a putative member of the genus *Fijivirus*. *J. Gen. Virol.* 77: 139.

Nuss DL and Dall DJ (1990) Structural and functional properties of plant reovirus genomes. *Adv. Virus Res.* 38: 249.

Uyeda I, Kimura I and Shikata E (1995) Characterization of genome structure and establishment of cell lines for plant reoviruses. *Adv. Virus Res.* 45: 249.

Zhu Y, Hemmings AM, Iwasaki K *et al* (1997) Details of the arrangement of the outer capsid of rice dwarf phytoreovirus, as visualized by two-dimensional crystallography. *J. Virol.* 71: 8899.

PICORNAVIRUSES – INSECT (*PICORNAVIRIDAE*)

Paul D Scotti, The Horticulture and Food Research Institute of New Zealand Ltd, Mt Albert Research Centre, Auckland, New Zealand

Peter D Christian, CSIRO Division of Entomology, Canberra, Australia

Introduction

'Insect picornavirus' is often misused as a generic term for any small, spherical virus isolated from insects. This term is regularly, and often incorrectly, applied to all unenveloped particles less than 40 nm in diameter without considering the physicochemical properties of the virions. With one exception none of these have been shown to have properties that would indicate they are true picornaviruses. They will therefore be referred to as 'picorna-like' meaning they have *at least* single-stranded, positive-sense RNA genomes of about 2.5–3.0 million daltons (MDa) molecular weight and 25–30 nm diameter, nonenveloped isometric particles containing (usually) three capsid proteins present in equimolar amounts. Excluding nodaviruses and tetraviruses, there are about 60 of these small viruses. Most of these are not sufficiently well characterized to assign to any existing taxonomic group. However 20 or so have physicochemical properties that allow them to be termed picorna-like (**Table 1**). They are unassigned viruses, for genus, in the family *Picornaviridae*.

Properties of the Genome

Four of the insect picorna-like viruses have had their genome structures elucidated through determination of their complete sequence. Infectious flacherie virus

confined to the phloem of infected plants. The galls or enations often associated with plant infection represent modified phloem that serves as a site of virus replication and accumulation.

Future

Many of the fascinating biological properties exhibited by the plant reoviruses remain poorly understood. Unfortunately, the lack of infectious cDNA clones of plant reoviruses prevents mechanistic studies from proceeding as fast as they might. Recent completion of plant-associated reovirus sequences, including NLRV, is providing important information for comparison of plant reoviruses with some of the more thoroughly studied animal reoviruses. Studies using expressed proteins are allowing for examination of the roles of individual dsRNA segments and their gene products in the infection process. Questions about the molecular basis of such processes as virus-induced neoplasia, viral persistence, vector range and the recognition, sorting and packaging of a segmented RNA genome will be addressed in the coming years.

See also: **Defective interfering viruses; Persistent viral infection; Reoviruses (*Reoviridae*): General features; Molecular biology; Plant reoviruses.**

Further Reading

Boccardo G and Milne RG (1984) Plant reovirus group (no. 294). In: *CMI/AAB Descriptions of Plant Viruses*. Wellesbourne, Warwick, UK: Association of Applied Biologists.

Francki RIB and Boccardo G (1983) The plant *Reoviridae*. In: Joklik WK (ed.) *The Reoviridae*, p. 571. New York: Plenum.

Holmes IH, Boccardo G, Estes MK *et al* (1995) *Reoviridae*. In: Murphy FA *et al.* (eds) *Sixth Report of the International Committee on Taxonomy of Viruses*, p. 208. New York: Springer-Verlag.

Kimura I and Omura T (1988) In: Harris KF (ed.) *Advances in Disease Vector Research*, vol 5, p. 300. New York: Springer Verlag.

Nobuhiko N, Koizumi M, Watanabe H and Noda H (1996) Complete nucleotide sequence of the *Nilaparvata lugens* reovirus: a putative member of the genus *Fijivirus*. *J. Gen. Virol.* 77: 139.

Nuss DL and Dall DJ (1990) Structural and functional properties of plant reovirus genomes. *Adv. Virus Res.* 38: 249.

Uyeda I, Kimura I and Shikata E (1995) Characterization of genome structure and establishment of cell lines for plant reoviruses. *Adv. Virus Res.* 45: 249.

Zhu Y, Hemmings AM, Iwasaki K *et al* (1997) Details of the arrangement of the outer capsid of rice dwarf phytoreovirus, as visualized by two-dimensional crystallography. *J. Virol.* 71: 8899.

PICORNAVIRUSES – INSECT (*PICORNAVIRIDAE*)

Paul D Scotti, The Horticulture and Food Research Institute of New Zealand Ltd, Mt Albert Research Centre, Auckland, New Zealand

Peter D Christian, CSIRO Division of Entomology, Canberra, Australia

Introduction

'Insect picornavirus' is often misused as a generic term for any small, spherical virus isolated from insects. This term is regularly, and often incorrectly, applied to all unenveloped particles less than 40 nm in diameter without considering the physicochemical properties of the virions. With one exception none of these have been shown to have properties that would indicate they are true picornaviruses. They will therefore be referred to as 'picorna-like' meaning they have *at least* single-stranded, positive-sense RNA genomes of about 2.5–3.0 million daltons (MDa) molecular weight and 25–30 nm diameter, nonenveloped isometric particles containing (usually) three capsid proteins present in equimolar amounts. Excluding nodaviruses and tetraviruses, there are about 60 of these small viruses. Most of these are not sufficiently well characterized to assign to any existing taxonomic group. However 20 or so have physicochemical properties that allow them to be termed picorna-like (**Table 1**). They are unassigned viruses, for genus, in the family *Picornaviridae*.

Properties of the Genome

Four of the insect picorna-like viruses have had their genome structures elucidated through determination of their complete sequence. Infectious flacherie virus

Table 1 Some properties of insect 'picorna-like' viruses

Virus	*RNA (MDa)*	*Capsid (kDa)*	*Density (CsCl)*	*Diameter (nm)*
		CrPV–DCV[a]		
Cricket paralysis virus	2.5–2.8	35, 34, 30, 7	1.34	27
Drosophila C virus (dipteran)	2.5–3.0	31, 30, 28, 9	1.34	27
		Hymenopteran viruses (bees/wasps)		
Acute bee paralysis virus	3.1	35, 33, 24, 9	1.34	30
Berkeley bee picornavirus	2.8	37, 35, 33		30
Black queen cell virus	2.8	34, 32, 29, 6	1.34	30
Egypt bee virus		41, 30, 25	1.37	30
Kashmir bee virus (all strains)		41, 37, 25, 6	1.37	30
Sacbrood virus	2.6	32, 28, 25	1.33	30
Slow bee paralysis virus		46, 29, 27	1.37	30
		Dipteran viruses (flies/mosquitoes)		
Ceratitis V virus		39, 34, 27	1.35	24
Drosophila A virus		73, 42, 32	1.37	30
Drosophila P virus		48, 29, 26	1.36	25–30
Kawino virus	2.6	33, 30, 27, 7	1.33	28
Queensland fruit fly virus	2.9	42, 37, 31	1.34	30
		Hemipteran viruses (true bugs)		
Aphid lethal paralysis virus	3.3	34, 32, 31	1.34	26–28
Himetobi P virus	2.8	37, 33, 28	1.35	29
Nezara viridula virus-1	3.1	32, 32, 31	1.34	29
Plautia stali intestine virus[a,b]	9.1 kb	33, 30, 26, 4.5		30
Rhopalosiphum padi virus	2.9 (9 kb)	31, 30, 28	1.37	27
Triatoma virus	3.0	39, 37, 33	1.39	30
		Lepidopteran viruses (moths/butterflies)		
Gonometa virus		37, 32, 29, 12	1.35	32
Infectious flacherie virus[c]	2.4 (9.7 kb)	35, 33, 31, 12	1.38	26–27
Latoia viridissima virus	2.9	31, 30	1.34	30
Lymantria ninayi virus	2.8	38, 33, 32	1.32	29

[a]Genome organization is 'calici-like' with capsid protein coding region at the 3′ end.
[b]Could also be grouped with CrPV and DCV.
[c]Genome organization suggests IFV is a 'true' picornavirus with single ORF and polymerase region located at the 3′ end.
RNA weights are in megadaltons (MDa) and/or in kilobases (kb); a blank for RNA means no value has been reported. Only major capsid proteins are listed (kDa). The densities of virions in CsCl are reported densities and have been estimated using a number of different methods and in a variety of conditions so they should not be taken as either the 'best estimates' nor are they directly comparable.

(IFV) of the silkworm has a genome structure similar to that of the mammalian picornaviruses. *Drosophila* C virus (DCV), *Plautia stali* intestinal virus (PsIV) and *Rhopalosiphum padi* virus (RhpV) have a quite different genomic organization. In contrast to the picornaviruses these viruses have the RNA-dependent RNA polymerase (RDRP) located at the 5′ end of the genome and the coat proteins at the 3′ end. Rather than producing a single polyprotein these viruses have two distinct open reading frames (ORFs) separated by an untranslated region of around 200 bases and available evidence suggests that the two ORFs are translated independently. We will refer to these viruses as 'di-cistronic picorna-like viruses' (**Fig. 1**). There are, therefore, at least two distinct groups of viruses among the insect picorna-like viruses: true picornaviruses and di-cistronic picorna-like viruses.

The CrPV/DCV Complex

Until 1997, the best characterized insect picorna-like viruses were cricket paralysis virus (CrPV) (**Fig. 2**), originally isolated from the orthopterans *Teleogryllus commodus* and *T. oceanicus* in Australia, and isolated from the dipteran *Drosophila melanogaster*. CrPV was discovered during the course of a mass-rearing program of the Australian field cricket, *T. commodus*, in the late 1960s. Young crickets became paralyzed and died and the disease, which spread rapidly and killed about 95% of the colony, was shown to be caused by the virus now called 'cricket paralysis virus'. Similar outbreaks in laboratory and commercial colonies of crickets have been observed elsewhere. DCV was isolated from laboratory populations of *D. melanogaster* in the late 1960s, during a

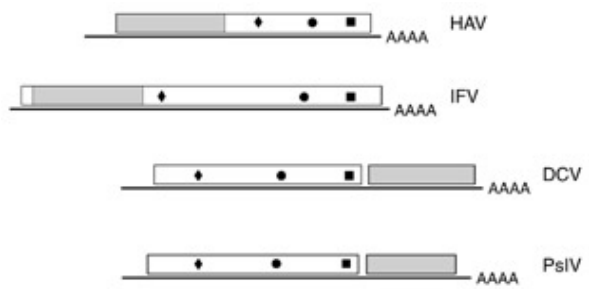


Figure 1 Genome organization of *Drosophila* C virus (DCV) and *Plautia stali* intestinal virus (PsIV) compared with that of infectious flecherie virus (IFV) and hepatitis A virus (HAV) (*Picornaviridae*: *Hepatovirus*). The capsid protein coding regions are shown by the shaded boxes and the RNA-dependent RNA polymerase (RDRP) regions by the unshaded boxes. Within the RDRP region the helicase-like domains (◆), cysteine protease domains (●) and RNA polymerase domains (■) are indicated.

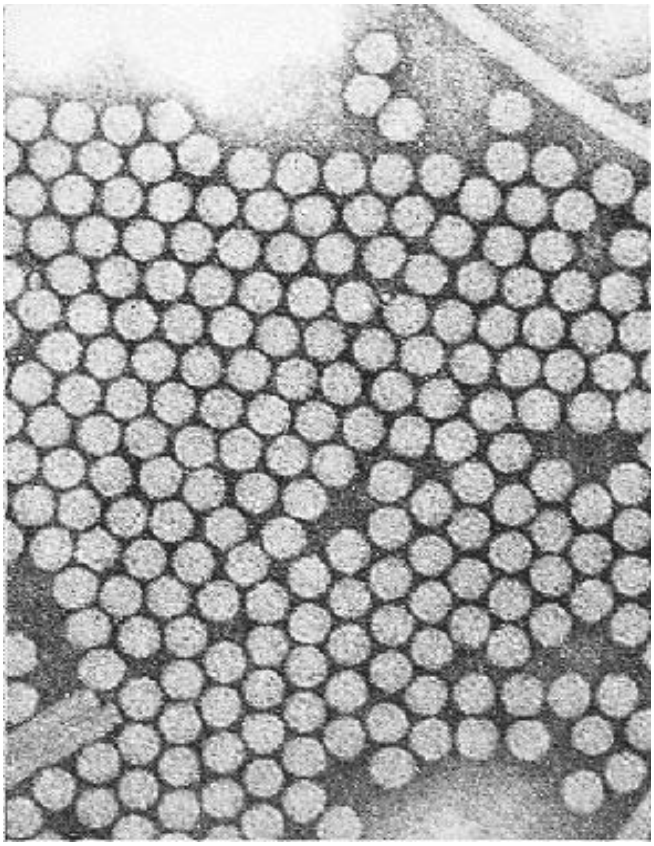


Figure 2 Electronmicrograph of cricket paralysis virus. The icosahedral virus particles are 25–27 nm in diameter; tobacco mosaic virus rods, 18 nm in diameter, were included as a reference standard.

general study of the incidence of small viruses in flies. When extracts of dead flies were injected into healthy adult *Drosophila*, the flies died within three days and the virus multiplied to higher titers than the other viruses being studied at that time. DCV has subsequently been identified in a number of species of *Drosophila*.

CrPV is a rather ubiquitous virus with a wide host range (**Table 2**) so the name is historical rather than categorical. In fact the host range of CrPV is extensive and covers over 40 species that have been tested from five orders of insects. CrPV did not multiply in the silkworm, *Bombyx mori*, or a species of cockroach nor in a locust species. CrPV has also not been shown to replicate in coleopterans, although only a few species have been tested. In insect hosts, both CrPV and DCV have frequently been found as inapparent infections, detected only by bioassay or serological tests. These infections may go completely undetected until a total population collapse occurs. Two DCV isolates that were tested multiplied in the Mediterranean fruit fly, *Ceratitis capitata*, but not in the cricket *Gryllus bimaculatus*. CrPV, however, multiplies in this cricket species. One strain of CrPV was shown to be highly infectious for *C. capitata* and killed the majority of adult flies fed with a virus suspension.

CrPV replicates in most, but not all, *D. melanogaster* cell lines tested and in several lepidopteran and mosquito cell lines. In some cultured *D. melanogaster* cell lines, CrPV causes a distinct cytopathic effect (cpe) which normally leads to cell lysis. DCV has only been observed to produce a cpe in *D. melanogaster* cells. In these cells, DCV causes cell clumping and cell detachment and in some instances results in cell lysis. DCV has also been detected as a persistent infection in several *D. melanogaster* cell lines. The fact that CrPV and DCV replicate in cultured cell lines has allowed their replicative strategies to be quite extensively studied.

CrPV and DCV show serological crossreactivity although they are not identical, a fact which is confirmed by other data. There has also been a report of crossreactivity between CrPV and the vertebrate cardiovirus (*Picornaviridae*), encephalomyocarditis virus (EMCV). CrPV antigen reacted with EMCV antibody, shown to be IgG. Since CrPV and EMCV are clearly unrelated this probably reflects a structural convergence rather than shared ancestry.

There are a number of isolates of CrPV and these can be separated into two distinct serological groups; one group consists of all the isolates from Australasia and the other group contains the two North American isolates. This correlates with differences in polyacrylamide gel electrophoresis profile of capsid polypeptides and that seen from polymerase chain reaction–restriction endonuclease digest (PCR-REN) analysis of the genomes. CrPV isolates can be distinguished serologically from DCV isolates. No serological

Table 2 Unclassified small RNA viruses (SRVs) (< 40 nm in diameter) and picorna-like viruses isolated from insects

Unclassified SRVs	*Picorna-like viruses*
	Lepidopteran (moths/butterflies) viruses
Epicerura pergrisea virus	Cricket paralysis virus
Galleria cell line virus	*Gonometa* virus
Heliothis armigera virus	Infectious flacherie virus
Hippotion eson nonoccluded VLP	*Latoia viridissima* virus
Parasa lepida VLP	*Lymantria ninanyi* virus
Pectinophora gossypiella virus	
Pteroteinon laufella virus-1	
Pteroteinon laufella virus-2	
Sesamia calamastis PVI	
Sesamia cretica virus	
Teinorhyncha umbra virus	
Turnaca rufisquamata virus	
	Dipteran (flies/mosquitoes) viruses
Blowfly VLP	*Ceratitis* V virus
Lucilia VLP3	Cricket paralysis virus
Aedes taeniorhynchus virus	*Drosophila* A virus
Culex cavaticus virus	*Drosophila* C virus
Culex pipiens virus	*Drosophila* P virus/iota virus
Culex tarsalis virus-2	Queensland fruit fly virus
Culicoides variipennis virus	
Dacus oleae virus	
Drosophila G virus[a]	
Drosophila RS virus[a]	
Kelp fly virus[a]	
	Hymenopteran (bees/wasps) viruses
Microplitis croceipes VLP	Acute bee paralysis virus
Deformed wing virus	Berkeley bee picornavirus
Arkansas bee virus[a]	Black queen cell virus
Chronic bee paralysis virus[a]	Cricket paralysis virus
Bee virus X and Y[a]	Egypt bee virus
Cloudy wing virus	Kashmir bee virus
	Sacbrood virus
	Slow bee paralysis virus
	Hemipteran (true bugs) viruses
Bemisia tabaci VLP	Aphid lethal paralysis virus
Pink sugarcane mealybug VLP	Himetobi P virus
Myzus persicae VLP	*Nezara viridula* virus-1
Rhopalosiphum maidis VLP	*Plautia stali* intestine virus
Sitobion avenae virus[a]	*Rhopalosiphum padi* virus
	Triatoma virus
	Coleopteran (beetle) viruses
Diabrotica virgifera VLP	
Graphoderus occidentalis VLP	
Ceratoma trifurcata VLP	
Scolytus cytoplasmic VLP	
	Orthoptera (grasshopper/locust) viruses
Grasshopper crystalline array virus[a]	Cricket paralysis virus
	Isoptera (termite) viruses
Termite paralysis virus[a]	

Table 2 continued

Unclassified SRVs	*Picorna-like viruses*
	Acarine (mite) viruses
Panonychus citri VLP1	
Panonychus citri VLP2	
Panonychus citri VLP3	
Panonychus ulmi VLP	

[a]Many of the unclassified SRVs have only been identified from histopathological studies and only those observed to have a cytoplasmic location are included. Some of the unclassified SRVs have been reasonably well characterized and are clearly not picorna-like; these are included for completeness.

subgrouping of DCV isolates has been observed. The general serological picture for CrPV/DCV is that of a complex of related, but not identical, virus strains. Since these isolates are often from totally different geographical regions and, in the case of CrPV, from a variety of hosts, it is not surprising that such variability exists.

Despite the serological crossreactivity, molecular data indicate that CrPV and DCV are quite distinct. For example, cDNA hybridization studies revealed essentially no homology between CrPV and DCV genomes. Sequence analysis confirmed this but showed that in the region encoding the capsid proteins CrPV and DCV have around 78% similarity (66% identity) at the amino acid level. cDNA hybridization results showed that the different isolates of DCV were 60–85% homologous at the nucleotide level whereas PCR-restriction nucleotide digest analysis indicated a higher degree of nucleotide sequence homology, around 90–95%. Similar results have been obtained among CrPV strains using the latter methodology.

Sequence analysis of the DCV genome demonstrates that it is 9264 nucleotides long and contains two large ORFs. These are separated by 191 nucleotides of untranslated sequence containing several stop codons in all three reading frames. The 5′ ORF encodes a protein with similarities to the RNA-dependent RNA polymerase, helicase and protease domains of several other small RNA viruses from plants and animals (**Fig. 1**). The ORF at the 3′ end encodes the capsid proteins. This latter ORF is unusual in that it lacks a methionine initiation codon and no subgenomic RNA is produced; this suggests that the capsid polyprotein is translated through an internal ribosome entry site. The organization of the 3′ end of CrPV appears to be the same as DCV which also has no apparent methionine initiation codon for the coat protein-containing ORF.

In *D. melanogaster* cells, CrPV shuts down virtually all host cell protein synthesis within several hours after infection. This contrasts sharply with DCV which shows less ability to shut down host cell functions. The fact that CrPV eventually results in extensive cpe whereas DCV does not may be a reflection of this ability. CrPV structural protein synthesis is similar to DCV. Processing of precursors into structural proteins is more rapid than for poliovirus, mengovirus or EMCV. The processing of precursor polypeptides is primarily by virus-induced protease(s) but *in vitro* data suggest that *Drosophila* proteases may also be involved. Coat proteins are produced in supramolar excess compared with the nonstructural proteins, supporting the model for independent initiation of the two ORFs.

A major difficulty arises in identifying reported picorna-like viruses as CrPV strains since a number of isolates of 'picorna-like viruses' or 'small RNA viruses' from insects have never been tested serologically to see if they are CrPV-related. Even if this is done, some viruses could easily be missed or misclassified. For example, $CrPV_{BRK}$, is serologically distinct from most other isolates – a feature borne out by its capsid polypeptide pattern and by PCR-REN analysis of the 3′ end of its genome. Far higher concentrations of heterologous antisera had to be used to get a detectable reaction. An isolate of CrPV from *Pseudoplusia includens*, now called $CrPV_{ARK}$ and known to be closely related to $CrPV_{BRK}$, would not have been recognized as a CrPV strain if the conditions for serological testing had not been radically adjusted.

Picorna-like Viruses of Hymenopterans (Wasps and Bees)

With its wide host range it is not surprising that CrPV has been isolated from the hymenopterans, the European honeybee, *Apis mellifera*, the common wasp, *Vespa vulgaris*, and the German wasp, *V. germanica*. However, there have been a number of other picorna-like viruses isolated from the honeybee. A 'filterable virus', identified years later as sacbrood virus (SBV), was isolated in 1913 from *A. mellifera*

and later from the Asiatic hive bee *A. cerana*. Despite their economic importance as pollinators, which far outweighs their value as honey producers, only a few laboratories worldwide have studied the bee viruses. Most of the isolations and characterization studies were carried out at the Rothamsted Experimental Station in England by Leslie 'Bill' Bailey in a 20-year period beginning in the mid-1960s. **Table 2** lists the eight viruses from a total of 15 different viruses, that have properties that merit them being designated picorna-like (see also **Table 1**).

The causative agent of sacbrood was first described in 1913 but it was not until 1964 that it was found to have picorna-like virus properties (SBV). Prior to this, in 1963, acute bee paralysis virus (ABPV) had been described as having picorna-like properties. Although capable of causing paralysis when injected into adult honeybees, ABPV was not initially reported to be associated with any naturally occurring disease symptoms in the UK. However, it has since been found to kill larvae, pupae and adult bees in colonies in other parts of the world when found in association with the parasitic mite *Varroa jacobsoni*. In the UK ABPV is most frequently detected during the late summer months when colony numbers are at their greatest but drops quickly to a low level over the autumn and winter months. ABPV particles are icosahedral, composed of four capsid proteins and contain single-stranded RNA.

Kashmir bee virus (KBV) was initially isolated from *A. cerana* in Kashmir and India. Several strains of KBV were subsequently isolated from the European honeybee in Australia and New Zealand as well as from the wasp *V. germanica* and has since been shown to be pandemic in *A. mellifera* populations. Initially it was thought to cause extensive mortality in hives. Later studies showed that KBV normally exists in a latent state but that infections with other bee pathogens such as the microsporidian *Nosema apis* and the foul brood bacterium *Melisococcus pluton* can trigger virus replication leading to death. Some data suggest that KBV has a serological relationship to ABPV. However, this may be a nonspecific reaction similar to the serological crossreactivity detected between CrPV and the mammalian picornavirus EMCV or an example of convergent evolution since they can both be transmitted through the salivary glands. Molecular studies on the genome of KBV and ABPV will likely resolve these alternatives.

Other picorna-like viruses have been reported for the European honeybee: Berkeley bee paralysis virus (BBPV), black queen cell virus (BQCV), Egypt bee virus (EBV), and slow paralysis virus (SPV). A 30 nm virus, called deformed wing virus, was isolated from *A. mellifera* in Japan but no detailed characterization has been done so this isolate is not included in **Table 1**.

Slow paralysis virus was isolated from *A. mellifera* bees in Britain during field surveys for bee virus X in the early 1970s. It has been found occasionally in honeybees in the UK and causes paralysis of adult *A. mellifera* bees approximately 12 days after injection. As the name implies this aspect of its pathology distinguishes it from the faster-acting acute and chronic paralysis viruses. BQCV is commonly found in association with *N. apis*. As the name implies it was first isolated from dead pupae found in queen cells that exhibited a dark brown to black discoloration. The virus is not particularly infectious when fed to larvae or adult bees or injected into the latter. Nevertheless it is a common infection in beehives in the UK, particularly during the early summer months. Apart from the original isolation and description, little is known of either BBPV or EBV.

Experimental manipulations such as the injection of buffer solutions or sera into pupae, or raising the incubation temperature, can induce replication of inapparent viral infections. Conversely, injections of antisera specific to a particular virus into pupae can neutralize or suppress the induction of that virus if it is present. Some experiments using such techniques have suggested a replicative hierarchy among some honeybee viruses. For instance in one series of experiments carried out in Australia, in the absence of suppression, injection with buffer induced primarily KBV. If KBV was neutralized then SBV and BQCV were induced with SBV out-replicating BQCV. If both KBV and SBV were suppressed then BQCV replicated to high levels.

Except for the $CrPV_{BEE}$ isolate, none of the bee viruses have been found to replicate in any insect cell line and no bee cell line exists. This means that detailed studies on the replication of this important class of viruses is lacking although there are substantial data on their biology and pathology.

Picorna-like Viruses of Dipterans (True Flies)

Although dipterans, such as mosquitoes, have been well studied because of their ability to transmit pathogenic viruses of humans or animals, surprisingly few viruses have been discovered which are pathogenic to the insects themselves. In addition to *Drosophila* C virus, only five other viruses merit inclusion in **Table 1**. These include Kawino virus (KV), isolated from the mosquito *Mansonia uniformis* in Kenya, and the picorna-like viruses isolated from the Mediterranean fruit fly, *Ceratitis capitata* (*Ceratitis* V virus), the Queensland fruit fly, *Dacus*

tryoni (Queensland fruit fly virus QFFV) and *D. melanogaster* (*Drosophila* P and A viruses, DPV and DAV, respectively).

KV has been relatively well characterized and shows the typical capsid protein profile of a picornavirus. However, the genome does not appear to contain a poly(A) region. It replicates in several mosquito cell lines but only produced a cpe in one *Aedes* line. Unfortunately, no studies have been made on its replication and the virus appears to be 'lost'. QFFV is relatively well characterized and the RNA shown to have a poly(A) tract. Unfortunately the virus is not infectious to the *Drosophila* cell lines 1 and 2 (in which DCV and CrPV replicate) so detailed studies on replication have not been done.

DPV was originally discovered in apparently healthy laboratory populations of *D. melanogaster* when studies showed that infected flies had a lower life expectancy and reduced fecundity and fertility. Like DPV, DAV is only mildly pathogenic to flies and frequently occurs as inapparent infections in laboratory populations. Both DPV and DAV replicate in *Drosophila* cells but neither produce the lytic cpe commonly seen with DCV. Both viruses have been commonly found in natural populations of *D. melanogaster*, and in the case of DAV it has also been found in natural populations of other *Drosophila* species.

A fourth picorna-like virus is also known from *Drosophila*, namely the virus 'iota' (DiV), originally isolated from *D. immigrans* populations collected in France in the early 1970s. DiV is serologically related to DPV but has not been well characterized physically and so has not been included in **Table 1**. DiV has been shown to confer CO_2 sensitivity to infected flies, similar to the totally unrelated insect rhabdovirus, sigma virus. Although common in *D. immigrans* populations in France in the early 1970s, within five years it seemed to have completely disappeared and could no longer be isolated from natural populations of flies.

Picorna-like Viruses of Hemipterans (True Bugs)

Hemipterans, such as aphids and leafhoppers are important as vectors of plant viruses and human or veterinary diseases. As with the dipteran viruses there have not been extensive recordings of pathogenic viruses found in them. Only five of the ten isolates of small RNA viruses or virus-like particles found in Hemipterans have been characterized sufficiently to be considered picorna-like (**Table 1**).

The first virus, RhpV, pathogenic to a hemipteran, was isolated from the aphid *Rhopalosiphum padi* in Illinois and later reported from laboratory colonies in South Africa. Another aphid virus, aphid lethal paralysis virus (ALPV) was also isolated from *R. padi* colonies but is quite distinct from RhpV in its physicochemical properties as well as being much more pathogenic, causing paralysis and eventual death of the infected aphids. RhpV has also been shown to occur in laboratory and field populations of other *Rhopalosiphum* species as well as aphid species of other genera. ALPV host range is also not limited to *R. padi*.

No cultured cell lines have been found to support the replication of Rhpv and ALPV and therefore detailed studies of their replication strategies have not been carried out. Therefore, for both viruses, little is known of their replicative strategies or affinities. However, cell-free translations of the genomic RNA of RhpV and ALPV showed that large proteins were synthesized and that for ALPV the putative polyprotein was cleaved into smaller proteins which reacted with antisera to purified virions. Recent sequence data indicate that RhpV has a genomic organization similar to DCV/CrPV, i.e. dicistronic with the coat proteins encoded at the 3′ end of the genome, although full details of the sequence are not currently available.

Recently, a virus (PsIV) was isolated from the intestines of the brown winged green bug *Plautia stali*. This virus appears to have a dicistronic genomic organization like that of DCV and CrPV. However, the two ORFs are in the same frame and separated by only one stop codon, allowing for the possibility of read-through expression of ORF 2. The putative ORF 1 contains identifiable core motifs for the RNA helicase, cysteine protease and the RNA polymerase (**Fig. 1**) but overall shows very low amino acid similarity to the RDRP region of DCV (52% similarity, 27% identity). ORF 2 contained sequences corresponding to regions of the capsid proteins and like the RDRP region has low amino acid identity with the DCV capsid proteins (51% similarity, 29% identity).

Himetobi P virus (HiPV) was originally isolated from the leafhopper *Laodelphax striatellus* (called 'himetobi' in Japan) but subsequently detected in laboratory and field populations of other species. The virus NVV-1 has been isolated from another leafhopper, *Nezara viridula*. A VP4 has not been recorded for either of these viruses nor have they apparently been tested in the several leafhopper cells lines described so, again, these viruses cannot be classified at this time.

The only recorded insect virus isolate from a hemipteran affecting vertebrates is *Triatoma* virus, which was isolated from a species in Argentina which

transmits the human protozoan parasite which causes Chagas' disease. Although some physical characters have been described there are no reports of attempts to infect cell lines.

Picorna-like Viruses of Lepidopterans (Moths and Butterflies)

Although many insect viruses affecting lepidopterans have been described, stimulated mainly by the pest status of many moth species in agriculture, horticulture and forestry, these viruses are mainly the large occluded viruses (baculoviruses, entomopoxviruses and cytoplasmic polyhedrosis viruses). Relatively few picorna-like viruses have been described although there have been numerous reports of 'virus-like particles' and electron micrographs of 'crystalline arrays' in the literature of the past 20–30 years. Some of these could well be 'CrPVs' as in the case of the virus reported from *Pseudoplusia includens* but which turned out to be $CrPV_{ARK}$. Other isolates have only been partially characterized.

The best characterized isolate is infectious flacherie virus (IFV) obtained from the silkworm *B. mori* in Japan. Besides the characteristics of IFV illustrated in **Table 1**, the ssRNA genome has been translated *in vitro* and shown to synthesize a large 130–200 kDa protein reminiscent of the precursor polypeptides seen for the true picornaviruses. The 3′ terminus is polyadenylated although the 5′ terminus appears to lack a VPg cap. Like the vertebrate picornaviruses, the genome consists of a single long ORF of 9255 nucleotides with the replicase-related proteins located at the 3′ end and the structural proteins located at the 5′ end of the genomic RNA (**Fig. 1**). Although it has a somewhat larger genome than most mammalian picornaviruses, IFV appears to be the best (and presently only) insect virus candidate for inclusion in the *Picornaviridae*.

Three other lepidopteran viruses, *Gonometa* virus (GV), *Latoia viridissima* virus (LvV) and *Lymantria ninayi* virus (LnV) are sufficiently characterized to regard them as picorna-like. GV was isolated from field populations of insects which had been devastated by an epizootic in Uganda in the 1960s and may possibly be the same as or similar to a virus found in *Pachymetana* during the same period. Although GV has long been regarded as a possible insect picornavirus because of its physical characteristics it has essentially been 'lost to science'. The few existing samples do not replicate in any of the insect cell lines tested and since the insect host is not readily available, it is not known whether the few laboratory stocks of the virus are even viable. LvV and LnV, like GV, were both isolated from insects collected during natural disease epizootics. Apart from their initial characterization little else is known about these latter three lepidopteran picorna-like viruses.

Potential as Biological Control Agents

Insect picorna-like viruses have been recorded in a number of different insect families including some, such as the hemipterans and the orthopterans (e.g. grasshoppers, locusts, crickets) where the occluded viruses do not occur frequently. The picorna-like viruses might be useful for these groups as well as others. There are several recorded examples of the picorna-like viruses being used as control agents. Infestations of the moth *Gonometa podocarpi* in pine plantations in Uganda were controlled using extracts of dead insects killed by GV and applying these to pest populations where the virus was absent. Like GV, LvV was shown to be an effective biocontrol agent when extracts of diseased cadavers were applied to apparently healthy populations of the pest insects.

Although not tested in the field, CrPV has shown itself quite capable of devastating insect populations and was originally discovered when it triggered a population collapse of crickets. CrPV has also been shown to kill adult Mediterranean fruit flies in laboratory tests where several days after feeding the flies 80% died. CrPV was also shown to be the most effective agent for killing the olive fruit fly, being superior to the other viruses tested, including two baculoviruses and an iridovirus.

Although CrPV can be grown in cultured cell lines and in large scale, most of the picorna-like viruses do not grow, or have not been tested for growth, in established insect cell lines. Although this indicates that commercial production could be difficult, recent developments in the production and assembly of viruses in nonpermissive systems may be particularly applicable to the insect picorna-like viruses. Detailed environmental impact evaluations are also essential for any bioinsecticide, not only for potential interactions between the viruses and vertebrates but also with regard to nontarget insect species, particularly with viruses like CrPV which have a demonstrated broad host range. However a wide host range could also be considered an advantage since a particular virus could be used against a range of insect pests and considering the local ecology and environment, as well as the method of application, could make the picorna-like viruses useful agents.

See also: **Cardioviruses (*Picornaviridae*); Polioviruses (*Picornaviridae*): General features, Molecular biology.**

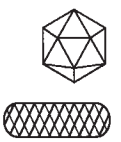
Further Reading

Christian PD and Scotti PD (1998) The picorna-like viruses of insects. In: Miller L and Ball A (eds) *The Insect Viruses*. New York: Plenum Press.

Scotti PD, Longworth JF, Plus N, Croizier G and Reinganum C (1981) The biology and ecology of strains of an insect small RNA virus complex. *Adv. Virus Res.* 26: 117.

Piry Virus *see* **Chandipura, Piry and Isfahan Viruses**

PLANT PARARETROVIRUSES (*CAULIMOVIRIDAE*)



Contents

Caulimoviruses: General Features

James E Schoelz and **June E Bourque**, Plant Pathology, University of Missouri, Columbia, Missouri, USA

History

Caulimoviruses are a group of plant viruses composed of double-stranded (ds) DNA encapsidated into an icosahedral virion. Caulimoviruses have been a subject of study because they cause economic losses in their respective hosts; in addition, they have made significant contributions to our knowledge of the molecular biology of plant viruses. The genome of cauliflower mosaic virus (CaMV), the type member of the genus, was the first of the plant viruses shown to contain DNA rather than RNA as genetic material. Furthermore, it was the first viral genome to be completely sequenced and cloned into bacterial plasmids in an infectious form. The caulimoviruses have also been extensively studied as a model for host–pathogen interactions and because their replication and expression strategies are unusual for plant viruses. For example, CaMV was the first plant virus to be shown to replicate by reverse transcription of an RNA intermediate into dsDNA.

In the early 1980s CaMV was thought to have some promise as a vector for foreign genes in plants. The effort to convert CaMV into a virus-based vector was scaled back when it was shown that the virus genome could tolerate only small insertions. A few small genes, such as dihydrofolate reductase and interferon, were eventually expressed in plants via a CaMV vector. However, other plant viruses, in particular tobacco mosaic virus, potato virus X and tomato bushy stunt virus, have been shown to be much more versatile as vectors for foreign genes.

Although CaMV has had only limited utility as a plant virus vector, the virus continues to have a great impact on plant biotechnology. The CaMV genome has served as a source for the most widely used promoter in plant genetic engineering. The 35S promoter of CaMV is capable of directing a high level of transcription in most types of plant tissues. This promoter was used to drive expression of the first transgene introduced into transgenic plants, and it is still the primary choice for expression of transgenes for both research and commercial applications.

Taxonomy and Classification

Members of the *Caulimovirus* genus, which has 11 definitive species (**Table 1**), have recently been assigned to the *Caulimoviridae* family. The virus genome is composed of circular, dsDNA approximately 8.0 kb in length that contains one or more single-stranded discontinuities. The viral DNA is encapsidated into isometric virions, 45–50 nm in diameter. In infected plants most virions are usually

Further Reading

Anjaneyulu A, Satapathy MK and Shukla VD (1994) Rice tungro. New Delhi: Oxford and IBH Publishing.

Chancellor TVB and Thresh JM (eds) (1996) Epidemiology and management of rice tungro disease. Chatham, UK: Natural Resources Institute.

Hull R (1996) Molecular biology of rice tungro viruses. *Ann. Rev. Phytopathol.* 34: 275.

Badnaviruses

Benham E. Lockhart and **Neil E. Olszewski**, Department of Plant Pathology and Department of Plant Biology, University of Minnesota, St Paul, Minnesota, USA

Taxonomy and Classification

The genus *Badnavirus* consists of plant viruses with nonenveloped bacilliform virions measuring 120–150 × 30 nm containing a circular double-stranded DNA (dsDNA) genome 7.5–8 kb in size (**Fig. 1**). The majority of badnaviruses are transmitted by mealybugs, and some are also seed-transmitted. The genus currently contains 13 definitive members: *Commelina* yellow mottle virus (CoYMV), the type member; *Aglaonema* bacilliform virus (AgBV); cacao swollen shoot virus (CSSV); rice tungro bacilliform (RTBV); banana streak virus (BSV); canna yellow mottle virus (CaYMV); citrus mosaic badnavirus (CMBV); *Kalanchoë* top-spotting virus (KTSV); *Dioscorea* bacilliform virus (DBV); pineapple bacilliform virus (PBV); *Piper* yellow mottle virus (PYMV); sugarcane bacilliform virus (ScBV); and *Schefflera* ringspot virus (SRV). Viruses with similar particle morphology occurring in *Aucuba, Colocasia, Mimosa, Rubus, Spiraea* and *Yucca* are possible additional members of the genus.

Badnaviruses differ from other bacilliform plant viruses in particle size and composition, and in genome type. The plant rhabdoviruses have significantly larger bacilliform particles surrounded by a lipid-containing envelope, and have a single-stranded RNA (ssRNA) genome. The bacilliform virions of alfalfa mosaic virus (AMV) are less than one-third the size of badnaviruses, and also contain ssRNA. Badnaviruses differ from caulimoviruses, the only other known group of dsDNA plant viruses, in particle morphology, genome size and organization, vector relationships and histopathology.

It is noteworthy that a number of clonally propagated plant hosts of badnaviruses (sugarcane, citrus, banana, black pepper, betel, taro, schefflera) have their centers of origin or diversity in southeast Asia or Australasia. This suggests, but does not provide direct evidence, that badnaviruses may have originated in this part of the world.

Virus Structure and Composition

Badnavirus particles average 120–150 × 30 nm in size, but particles ranging in size from 30 nm spheres to bacilliform particles up to 1000 nm long may occur. Some isolates of BSV, CSSV and PYMV produce significant numbers of abnormally long particles. The particles have a sedimentation coefficient of approximately 200S, and a buoyant density in CsCl of 1.31 g cm^{-3}. The virions are stable in Cs_2SO_4 but not in CsCl. Purified virus preparations sediment as a single component in both rate zonal and isopycnic density gradient centrifugation, indicating that particles of different lengths contain approximately equal ratios of nucleic acid and protein. Purified virus preparations have uncorrected A_{260}/A_{280} ratios of 1.3–1.4. There is no information on extinction coefficient values.

Badnavirus virions contain a major capsid protein of 39 kDa. Proteolysis by proteases generates a second major polypeptide of 37 kDa. Larger virus-related polypeptides are also detected by immunoblotting, and these most probably represent various fragments of the polyprotein from which the capsid protein is released by post-translational processing. The CoYMV capsid protein is not glycosylated, and there is no evidence for the presence of any other non-protein components in the capsid. Analysis of the particle structure of RTBV using optical diffraction

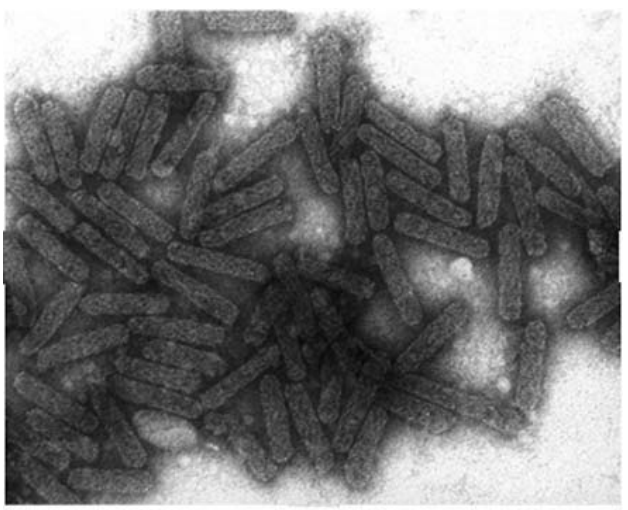


Figure 1 Particles of sugarcane bacilliform virus (ScBV) negatively stained with sodium phosphotungstate. Scale bar = 100 nm.

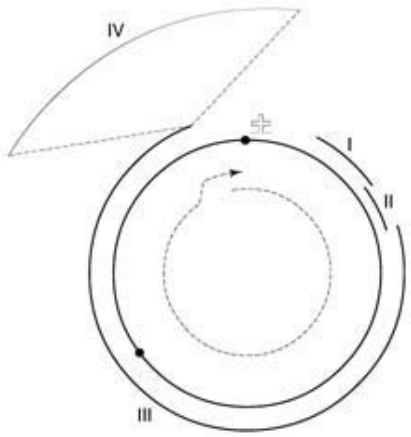


Figure 2 The generalized organization of the viral transcript, genome and open reading frames (ORFs). The dotted arrow indicates the badnavirus transcript. The solid circle indicates the DNA genome and the dots mark the locations of the site-specific discontinuities. The cloverleaf indicates the $tRNA^{Met}$ binding site. The solid arcs indicate the locations of ORFs I–III, which are present in all badnaviruses, and the thinner outermost arc indicates the relative location of ORF IV of RTBV.

of electron micrographs indicates that the tubular portion of the particle has a structure based on an icosahedron cut across its threefold axis, with a structural repeat of 10 nm and nine rings of hexamer subunits.

Badnavirus virions contain a single species of dsDNA, which has a buoyant density of 1.57 g cm^{-3} in CsCl–ethidium bromide. Under nondenaturing conditions, the DNA migrates in agarose gels as three or more bands corresponding to linear, circular and knotted configurations. Electron microscopic examination of purified virus and ultrathin sections of infected tissue reveal that the virions have an electron-translucent central core. However, there is no information on the nature of protein–DNA interaction within the nucleocapsid.

Genome Structure

The various badnaviruses encapsidate a circular dsDNA which, depending on the virus, ranges in size from 7.5 to 8.0 kb. This circular DNA molecule is relaxed, rather than supercoiled, because each strand is interrupted by a site-specific discontinuity (**Fig. 2**). The plus and minus strand discontinuities are located adjacent to the sequences that are believed to prime the synthesis of these strands, suggesting that the discontinuities are generated during replication of the genome.

Virus Replication and Multiplication

The badnaviruses are pararetroviruses that replicate by reverse transcription of a terminally redundant transcript that is longer than the genome (**Fig. 2**). This process is carried out by a virus-encoded reverse transcriptase (RT) and RNase H. Minus-strand synthesis is believed to be primed when $tRNA^{Met}$ anneals near the 5′ end of the viral transcript. In the case of CoYMV and similar to other retroelements, plus-strand synthesis appears to initiate at a polypurine-rich region. In contrast, RTBV plus-strand synthesis does not appear to initiate at a polypurine-rich region.

Molecular Biology

Only the minus strand of the genome is transcribed. Although it has not yet been demonstrated, transcription is probably similar to that of the caulimoviruses, in that it involves host RNA polymerase II and occurs on a nuclear-localized, covalently closed minichromosome. All badnaviruses examined produce a transcript that is terminally redundant and between 120 and 270 nt longer than genome length. The terminally redundant transcript is believed to serve both as a polycistronic mRNA and as the template for genome replication. In addition, a small portion of the RTBV transcript is spliced to produce an mRNA-encoding open reading frame (ORF) IV, the terminal ORF of the polycistronic mRNA. This smaller mRNA appears to be the only one capable of expressing ORF IV.

In general, the polycistronic mRNA contains three ORFs (I–III) arranged in a tight tandem array (**Fig. 2**). However, the RTBV RNA contains an additional ORF, ORF IV. In addition to the larger protein-encoding ORFs, the leader of the polycistronic RNA also contains several very small and apparently noncoding ORFs. Translation of the polycistronic mRNA employs both a translational shunt to avoid the untranslated ORFs of the leader and leaky scanning by ribosomes allowing the expression of ORFs II and III.

ORF III, which is the largest and with the exception of RTBV the last ORF of the mRNA, encodes a large polyprotein that is proteolytically processed to yield a protein involved in the cell-to-cell movement of the virus, the capsid protein, an aspartic protease, a RT and an RNase H. The polyprotein is probably processed by the aspartic protease. The cell-to-cell movement protein is a structural component of tubules extending between adjacent cells that serve as a conduit for virion movement between cells. Although the functions of the proteins encoded by the

other ORFs are unknown, the ORF I and ORF II proteins of CoYMV and RTBV are known to be associated with virions and the ORF II proteins of CSSV and RTBV bind nucleic acids *in vitro*.

Geographic Distribution and Economic Importance

Badnaviruses occur primarily in the tropics and subtropics in vegetatively propagated or perennial crops. The notable exception is RTBV, whose host, rice, is a seed-propagated annual. RTBV occurs in Asia and southeast Asia, where it may be a major yield-limiting factor in some localities. Cacao swollen shoot virus occurs in several countries in West Africa, and is also a disease of major economic importance. Banana streak virus is the most commonly occurring virus in *Musa*, and probably occurs in all banana- and plantain-growing areas of the world. Some BSV isolates cause severe damage and crop loss, and the occurrence of this virus in improved varieties has been a hindrance to movement of this germplasm. PYMV, which occurs on both black pepper (*Piper nigrum*) and betel vine (*P. betle*) in southeast Asia, has a pronounced effect on plant growth, and may have been one of the factors contributing to the decline of black pepper cultivation in the former Indochina. ScBV, some isolates of which are serologically closely related to BSV, occurs worldwide in nearly all clones of noble sugarcane (*Saccharum officinarum*), but is less prevalent in newer commercial *Saccharum* hybrids. Badnaviruses occur frequently in mixed infections with other viruses, and there is some evidence of mutually synergistic effects on virus replication and symptom development in such mixed infections.

Host Range and Symptomatology

Badnaviruses occur in both monocotyledonous (e.g. RTBV, CoYMV, ScBV) and dicotyledonous (e.g. CSSV, CMBV, PYMV, SRV) hosts. The individual viruses typically have very restricted natural and experimental host ranges, frequently limited to a few species within a single plant genus. The exceptions are CSSV and ScBV, which can infect several different genera and families. The most common symptom of badnavirus infection is a yellow foliar mottle.

A characteristic feature of badnavirus infection is seasonal variation in virus titer and symptoms expression. This periodicity of virus replication and symptom development is correlated with seasonal temperature changes.

Serology

Badnaviruses are only moderately immunogenic. Repeated immunization of rabbits yields antisera with homologous enzyme immunoassay (EIA) titers of 1:250 000. Both EIA and immunoelectron microscopy (IEM) can be used for virus detection and for studying serological relationships within and between badnavirus species.

There is a general lack of serological crossreactivity between members of the badnavirus group, and individual badnaviruses may have one or more distinct serotypes (e.g. CSSV, ScBV, BSV, KTSV, SRV). EIA and IEM tests indicate serological relationships between BSV and ScBV, and between CSSV, SRV and DBV. ScBV has the greatest degree of serological diversity among its isolates, and polyclonal antisera raised against a mixed pool of ScBV antigens crossreact with the majority of other badnaviruses.

Virus–Host Relationships

Except in the early stages of infection, badnaviruses typically occur in low concentration in host tissue. RTBV has been reported to be phloem-limited in rice, but several other badnaviruses (e.g. CoYMV, PYMV, SRV, CaYMV) are not tissue-limited, and their virions have been observed in epidermal, mesophyll and vascular cells. Virions only occur in the cytoplasm, and may appear in palisade-like arrays or in random aggregates. The particles do not occur in the proteinaceous inclusion bodies (viroplasms) typical of caulimoviruses. The absence of a proteinaceous inclusion body from badnaviruses is corroborated by the absence from the badnavirus genome of a coding region analogous to the caulimovirus ORF VI. Although badnavirus virions have not been observed in nuclei of infected cells, filamentous structures of unknown function have been observed in nuclei of ScBV-infected sugarcane. Badnavirus infection also appears to induce internal structural modifications in mitochondria. Tubular structures containing virions traverse the wall between adjacent cells in CoYMV-infected *Commelina diffusa*. These structures presumably occupy modified plasmodesmatal openings, and appear to play a role in cell-to-cell trafficking of virions.

Many *Musa* genotypes contain integrated BSV genomic sequences, and there is evidence for similar integration of badnavirus genome sequences in other plant species such as sugarcane and canna. The mechanism of integration of viral sequences has not been determined, and the number, nature and possible

biological significance of these viral sequences are not known.

Transmission

Eight of the 13 definitive members of the badnavirus group (CMBV, CSSV, CoYMV, BSV, KTSV, PYMV, ScBV, SRV) are transmitted by mealybugs (*Pseudococcidae*). The mode of transmission is semipersistent and nonpropagative. Vectors can transmit virus after brief acquisition periods (15–30 min), but the rate and duration of transmissibility increase with acquisition feeding up to 24 h. After a 24 h acquisition feeding period, mealybugs retain the ability to transmit virus for up to 72 h. Mealybug vectors of badnaviruses include *Planococcus citri, Planococcus kenyae, Planococcoides njalensis, Pseudococcus longispinus, Ferrisia virgata, Saccharicoccus saccharoides* and *Dysmicoccus boninsis*. The mealybug vectors are able to acquire and transmit badnaviruses at all stages of the life cycle.

In contrast with the majority of badnaviruses, RTBV is transmitted in a semipersistent manner by leafhopper vectors, principally *Nephotettix* spp. Leafhopper transmission of RTBV occurs only in association with a helper virus, rice tungro spherical virus (RTSV), which has an ssRNA genome. Transmission of RTBV does not require the presence of RTSV, but only the presence of a helper component coded for by the RTSV genome. Although the mealybug-transmitted badnaviruses commonly occur in nature in mixed infections with other insect-transmitted viruses, it has been clearly demonstrated that they are not dependent on these viruses for transmission by their mealybug vectors.

Three definitive or proposed members of the badnavirus group (KTSV, CoYMV and Mimosa bacilliform virus) are seed-transmitted at rates of 10–80% and KTSV is also pollen-transmitted. BSV is also seed-transmitted in banana. Except for RTSV and CaYMV, the other definitive members of the badnavirus group are transmitted by mechanical inoculation.

Epidemiology, Detection and Control

The epidemiology of plant diseases caused by badnaviruses is determined primarily by the fact that the majority of these viruses occur in vegetatively propagated crops, have restricted host ranges and are transmitted by mealybug vectors which are relatively sedentary and do not disperse rapidly or over great distances.

Clonal propagation from virus-infected stock plants therefore represents the principal means of spread of badnavirus diseases. Short-distance spread by mealybug vectors occurs when foliage contact within the crop canopy permits the vectors to crawl from infected to healthy plants. Because of their restricted host ranges, spread of badnaviruses occurs primarily within a single cultivated species, and apart from the case of CSSV in West Africa, there is little evidence that noncrop plants play an important role in the epidemiology of these diseases. There is also no evidence that badnaviruses can be spread by mechanical contact, by cutting tools, or during cultural operations.

RTBV differs fundamentally from the other badnaviruses in its epidemiology. RTBV infects a seed-propagated annual crop, and is spread by an aerial vector capable of rapid, as well as long-distance, dispersal. The practice of multiple cropping of rice in parts of Asia and southeast Asia provides a source of both virus and vector for infection of new fields.

Control of the majority of mealybug-transmitted badnaviruses occurring in clonally propagated crops can be accomplished effectively by use of virus-free propagating stock. The principal difficulty lies in developing sensitive and reliable virus-indexing methods. Badnaviruses usually occur in low concentration in their host plants, many of which contain high levels of tannins, mucilage and other compounds which may interfere with assay procedures. In addition, badnavirus-infected plants may produce symptomless, virus-free foliage for several months at a time before symptoms reappear, thereby compromising the reliability of disease detection based on visual inspection. Enzyme immunoassay EIA offers a sensitive and reliable method for detection of badnaviruses when viral antigen occurs in plant tissue. The applicability of this technique is restricted, however, by the occurrence of serologically distinct badnavirus populations within a given crop (e.g. cacao, banana, sugarcane, *Kalanchoë*). Detection and identification methods based on polymerase chain reaction (PCR) amplification of common badnavirus genomic sequences appear to offer a more reliable and sensitive indexing method for propagating stock. However, the reliability of this method may be compromised by the existence of badnavirus genomic sequences integrated into host plant DNA. Virus detection using immunocapture PCR (IC-PCR) can be used to avoid potential problems caused by the presence of integrated viral sequences. The production of virus-free plants by apical meristem culture has been used successfully with a variety of clonally propagated crop plants infected by RNA viruses. Initial results with ScBV in sugarcane suggests that this technique may not be successful in the case of badnavirus infection.

On the basis of characteristics of the host plant and

disease epidemiology, RTBV-induced disease in rice is amenable to control strategies not relevant to the other badnavirus diseases. Breeding and selection of virus- or vector-resistant varieties is a prime option in the case of rice, but is less readily accomplished in clonally propagated crop plant hosts of other badnaviruses. Control of vector populations by cultural or chemical methods is also far more relevant to RTBV than to other badnaviruses.

See also: **Plant pararetroviruses (*Caulimoviridae*): Caulimoviruses: general features, Caulimoviruses: molecular biology.**

Further Reading

Cheng C-P, Lockhart, BEL and Olszewski NE (1996) The ORF I and II proteins of *Commelina* yellow mottle virus are virion-associated. *Virology* 223: 263.

Hay J, Grieco F, Druka A, Pinner M, Lee S-C and Hull R (1994) Detection of rice tungro bacilliform virus gene products *in vivo*. *Virology* 205: 430.

Hibino H (1983) Relation of rice tungro bacilliform and rice tungro spherical virus with their vector *Nephotettix virescens*. *Ann. Phytopathol. Soc. Jpn* 49: 545.

Hohn T and Futterer J (1997) The proteins and functions of plant pararetroviruses: knowns and unknowns. *Crit. Rev. Plant Sci.* 16: 133.

Jacquot E, Keller M and Yot P (1997) A short basic region supports nucleic acid binding activity in the rice tungro bacilliform virus open reading frame 2 product. *Virology* 239: 352.

Tzafrir I, Ayala-Navarrette L, Lockhart BEL and Olszewski NE (1997) The N-terminal portion of the 216-kDa polyprotein of *Commelina* yellow mottle badnavirus is required for virus movement but not for replication. *Virology* 232: 359.

PLANT RESISTANCE TO VIRUSES

Contents

Natural Resistance

RSS Fraser, Society for General Microbiology, Reading, UK

Importance of Resistance in Disease Control

Plant viruses cause serious economic losses in many crops, in three main ways. Infection normally reduces yield biomass; an important example is the effect of the complex of related viruses known as barley yellow dwarf on all major cereal crops. In crops which are sold on the basis of appearance, such as ornamentals and many fruits and vegetables, blemishing and distortion as a result of virus infection can lead to complete loss of marketability, even in cases where yield biomass is only slightly reduced. Finally, costs are incurred if eradication or avoidance programs are enforced, for example in production and certification of virus-tested seed potatoes.

In contrast to the control of fungal diseases of plants, no chemical pesticides are available for routine use as direct antiviral agents. It has, therefore, been necessary to develop a number of alternative control strategies. These include control of the biotic vectors of particular plant viruses, use of virus-free seed or planting material of vegetatively propagated crops, good cultural practices to minimize transmission, and deployment of a number of different mechanisms of plant resistance. Naturally occurring resistance mechanisms, the topic of this article, have been of most use in crop protection until now, but several types of genetically engineered resistance mechanisms, using viral and nonviral genes, are now becoming available. In both types of resistance, it is important to study the mechanisms involved, to increase the effectiveness with which resistance can be used in crop protection, and to understand more fundamental questions about the molecular interactions between plants and viruses.

Types of Natural Resistance Mechanism

Plant viruses are very diverse in their structure, mode of replication and transmission, and in their pathogenic effects on their hosts. Perhaps not surprisingly,

functions alone were not responsible for the complementation.

Control

PC1SV-CVB isolate was found to be economically important in some of the peanut growing areas in the state of Andhra Pradesh. Reports on the economic importance of SoyCMV are not available. No efforts have so far been made to devise any strategies for controlling PC1SV or SoyCMV.

See also: **Plant pararetroviruses: Badnaviruses; Plant pararetroviruses (*Caulimoviridae*): Cassava vein mosaic virus, Caulimoviruses: general features, Caulimoviruses: molecular biology; Plant pararetroviruses: Rice tungro bacilliform virus.**

Further Reading

Ducasse DA, Mushegian AR and Shepherd RJ (1995) Systemic infection of solanaceous hosts by peanut chlorotic streak caulimovirus is temperature dependent and can be complemented by coinfection with figwort mosaic caulimovirus. *Phytopathology* 85: 286.

Hasegawa A, Verver J, Shimada A *et al* (1989) The complete sequence of soybean chlorotic mottle virus DNA and the identification of a novel promoter. *Nucleic Acids Res.* 17: 9993.

Iwaki M, Isogawa Y, Tsuzuki H and Honda Y (1984) Soybean chlorotic mottle, a new caulimovirus on soybean. *Plant Dis.* 68: 1009.

Mushegian AR, Wolff JA, Richins RD and Shepherd RJ (1995) Molecular analysis of the essential and non-essential genetic elements in the genome of peanut chlorotic streak caulimovirus. *Virology* 206: 823.

Reddy DVR, Richins RD, Rajeshwari R *et al* (1993) Peanut chlorotic streak virus, a new caulimovirus infecting peanuts (*Arachis hypogaea*) in India. *Phytopathology* 83: 129.

Richins RD, Broos T, Ducasse A *et al* (1998) Organization and transcription of the peanut chlorotic streak virus genome. *Arch. Virol.* (in press).

Satyanarayana T, Broglio EP, Sreenivasulu P *et al* (1995). Genome characterization of a new strain of peanut chlorotic streak virus causing chlorotic vein banding disease of groundnut (*Arachis hypogaea* L.) in India. *Plant Pathol.* 44: 966.

PLANT PARARETROVIRUSES

Contents

Rice Tungro Bacilliform Virus

Roger Hull, John Innes Centre, Norwich Research Park, Colney, Norwich, UK

General Features

Rice tungro disease was first recognized in the experimental fields at the International Rice Research Institute, The Philippines in 1963 and identified as a distinct virus disease in 1965. However, previous descriptions of disorders in rice (*Oryza sativa*) would indicate that it has been present in the Philippines, and probably elsewhere in southeast Asia from at least the 1930s. Since its recognition as a virus disease, tungro has been reported in most of the rice-growing countries of south and southeast Asia (**Fig. 1**).

Tungro is unusual in that it has a multitude of local names, there being at least 40 names including 'tungro' and 'accep na pula' in The Philippines and names such as 'penyakit merah' in Malaysia and 'mentek' in Indonesia; this plurality of names reflects the importance with which farmers regarded the disease. The disease is generally endemic but has caused periodic major outbreaks in various countries in this region. Much of the increase in impact of tungro has been attributed to the 'green revolution', which resulted in large areas of rice with high fertilizer inputs and overlapping cropping of new susceptible cultivars, conditions highly suitable to rapid spread of the viruses. Overall this disease is now considered to be one of the major constraints to rice production in this region, with annual losses in excess of \$US 1.5×10^9; this does not include the costs of insecticides to control the virus vector.

It was not until 1978 that it was realized that tungro disease is caused by a complex of two viruses, rice

Figure 1 Distribution (shaded area) of rice tungro disease in south and southeast Asia.

tungro spherical virus (RTSV), which causes few symptoms and is leafhopper transmitted, and rice tungro bacilliform virus (RTBV), which is the major contributor to symptom production. RTBV on its own is not leafhopper transmitted but is so in the presence of RTSV. Thus, RTSV confers the leafhopper transmissibility to the complex and RTBV confers most of the symptom production.

Symptoms and Host Range

The symptoms of tungro vary with rice cultivar but in many cultivars the disease causes stunting of the plant, red or orange colouring of the leaves and a reduction of tiller numbers. Early infection can cause death of some very susceptible cultivars and, in many other cultivars, results in considerable loss of yield. RTBV (and RTSV) is restricted to the vascular tissues of infected plants being found in both the phloem and xylem.

Rice is the principal host of the tungro viruses but either or both of them can infect wild rice species (e.g. *O. australiensis, O. barthii, O. brachyantha, O. eichengeri, O. fatua, O. glaberrima, O. nivara, O. officinalis, O. perennis, O. punctata, O. redleyi, O. rufipogon*) and some other graminaceous species. However, some of these reports need treating with caution as reliable confirmation of infection by either or both viruses was not available at the time the tests were conducted.

Virus Transmission

As noted above, RTBV is transmitted by leafhoppers only in the presence of RTSV. The major vector is the rice green leafhopper, *Nephotettix virescens*, and it can also be transmitted by some other leafhopper species such as *N. nigropictus, N. malayensis, N. parvus* and *Recilia dorsalis*. The relationship of both viruses with the leafhopper vector is semipersistent, with acquisition in a matter of minutes and ability to be able to transmit immediately. The retention of transmissibility increases with length of acquisition feeding. Neither of the two viruses is transmissible mechanically but cloned RTBV has been transmitted by agroinoculation (see below).

Virus Control

The major approaches to controlling tungro are cultural, use of insecticides against the vector and the deployment of resistance. Cultural techniques mainly aim at avoiding the main peaks of vector occurrence and having a crop-free period which would reduce sources of infection. There is widespread use of insecticides but, as the disease is transmitted in a semipersistent manner, and as rice is usually grown on small plots, this is not particularly effective. There are two major types of resistance, that against the insect vector and tolerance against RTBV. The initial breeding programs used the vector resistance, which did not prove to be particularly durable. Current breeding programmes are utilizing various forms of natural resistance to the two viruses. That against RTBV is tolerance, which is found in the cultivar Utri Merah. Various transgenic approaches to conferring resistance to RTBV are currently being tested.

Virus Classification

RTBV belongs to the *Badnavirus* genus of the plant pararetrovirus family, the *Caulimoviridae*. It has bacilliform particles which contain a double-stranded DNA genome of about 8.0 kb. Because of some differences in genome organization RTBV is placed in a separate group to the other badnaviruses.

Virus Particles

RTBV has bacilliform particles of 30 nm diameter and usually about 130 nm length (**Fig. 2**). However, in some isolates longer particles in excess of 300 nm are found. The structure of the particles is based on a $T = 3$ icosahedron cut across its threefold axis with the tubular portion being made up of rings of hexamer subunits and a repeat distance of about 10 nm. The particles have an s_{20w} of approximately 200 and a buoyant density in cesium chloride of approximately 1.36 g ml^{-1}.

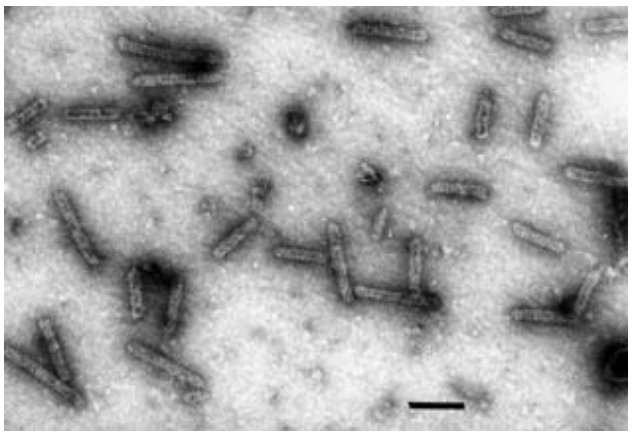


Figure 2 Electron micrograph of particles of RTBV negatively stained with uranyl acetate. Bar = 100 nm.

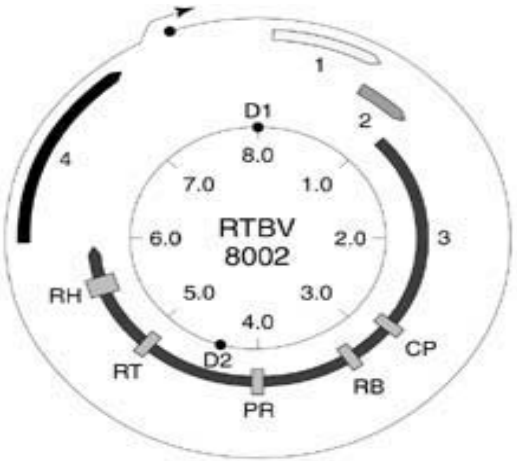


Figure 3 Genome organization of RTBV. The inner circle represents the double-stranded DNA genome with the positions of the two discontinuities, D1 and D2, indicated. The arcs show the positions of the four open reading frames (ORF 1–4). ORF 1 is shaded lighter as it does not have a conventional AUG start codon. The positions of the sequence motifs for coat protein (CP), RNA binding 'cys' sequence (RB), aspartate protease (PR), reverse transcriptase (RT) and RNase H (RH) on ORF 3 polyprotein are indicated. The outer circle represents the 35S RNA with terminal redundancy, the spot being the 5′ end and the arrow head the 3′ end.

Viral Genome

The virus particles contain a double-stranded DNA genome of 7934–8002 bp, depending on strain. As with all plant pararetroviruses, the genome has discontinuities at specific sites and, as with other badnaviruses, RTBV has one discontinuity in each strand. These discontinuities are related to the replication of the genome and are discussed further below.

Genome Organization

RTBV is similar to other retro- and pararetroviruses in that the genomic DNA is transcribed asymmetrically, with all the coding capacity being on one strand. RTBV has four open reading frames (ORFs) which potentially encode proteins of more than 10 kDa (**Fig. 3**). The first three ORFs are closely packed and interface with the sequence ATGA, the ATG being the start codon of the downstream ORF and the TGA the stop codon for the upstream ORF. ORF 4 is separated from ORF 3 by a short noncoding region and ORF 1 is separated from ORF 4 by a longer noncoding region.

ORF 1

This ORF, which follows a 666 nt leader sequence of the genome length transcript, potentially encodes a protein of 24 kDa. It is unusual in not having an AUG start codon in any of the three reading frames and using AUU as a start codon. Although no function is known for the product of this ORF, it is found within virus particles.

ORF 2

This ORF encodes a protein of 12 kDa which is located on purified virus particles. The C-terminus of this protein has a nucleic acid-binding activity but no real function has yet been ascribed to it.

ORF 3

The protein of 194 kDa encoded by ORF 3 is a polyprotein which is processed to give at least the viral coat protein, an aspartate protease, reverse transcriptase (RT) and ribonuclease H (RH). Thus, it contains the analogues of *gag* and *pol* of reverse transcribing elements. The N-terminal portion of this polyprotein is processed to give a 40 kDa protein with some sequence similarities with cell-to-cell movement proteins found in other plant viruses. However, its function is uncertain as RTBV is limited to vascular tissue and is not found in mesophyll tissue, where viral movement proteins are thought to operate. The next section of the polyprotein is processed to give two 37 kDa species which make up the viral coat protein. The C-terminal of these species contains the 'cys' motif, $CXCX_2CX_4HX_4C$, characteristic of the RNA-binding site of retroviral *gag* proteins. It also contains the additional 'cys' sequence ($CX_2CX_{11}CX_2CX_4CX_2C$) which is apparently unique to all badnaviruses. The aspartate protease is identified by its core motif, DSG, and is a protein of about 20 kDa. Similarly, the RT and RH regions are identified by their characteristic amino acid motifs. This region is processed into products of 62 and

55 kDa, both of which have RT activity but only the 55 kDa protein having RH activity.

ORF 4

This is an ORF unique to RTBV, not being found in other badnaviruses. It encodes a product of 46 kDa with a sequence motif characteristic of a leucine zipper. No function has yet been ascribed to it.

Expression of the Gene Products

The genome is transcribed to give a transcript (35S RNA) with a terminal redundancy of 215 or 216 nt which, as with most retro- and pararetroviruses, is the template for both replication (see below) and expression of the gene products. In the agroinoculation procedure mentioned above, which was used to show that the sequenced genome was biologically active, a construct was made which would express this 35S RNA. This construct was inserted into the T DNA of *Agrobacterium,* the bacteria transformed with the plasmid and the bacterial paste suspension inoculated into rice plants.

The core promoter for the 35S RNA is a characteristic TATA sequence 32 nucleotides upstream of the transcript start and has enhancing elements both upstream and downstream in the transcribed region. The transcript is terminated at a poly(A) signal sequence.

The expression of RTBV gene products from the 35S RNA has several unique features. There is strong evidence for a 'ribosome shunt' mechanism by which ribosomes bypass most of the leader sequence. The 'shunt donor' is close to the 5′ end of the RNA and the 'shunt acceptor' is between the first and second ATT sequences in ORF I. This ATT is an inefficient start codon, having only about 10% of the efficiency of an AUG codon in a good context. Thus, many of the ribosomes will bypass it in the uninitiated state. Similarly, the AUG codon at the start of ORF 2 is in a weak context and so a significant proportion of the ribosomes will leak through uninitiated. The next AUG codon, which is at the start of ORF 3, is in a good context and should recruit the rest of the ribosomes. Thus, the first three ORFs are expressed by a cascade of 'leaky' ribosomes from the same mRNA. ORF 4 is expressed from an mRNA spliced from the 35S RNA, the splice donor being close to the 5′ end of the RNA and the splice acceptor being just in ORF 4, leaving an intron of 6.3 kb. The AUG start codon for this ORF comes from a short ORF supplied by the splice donor.

One of the differences that RTBV has from most reverse transcribing elements is that the analogues of *gag* and *pol* are on the ORF 3 polyprotein. The *gag* protein is required in much higher amounts than the *pol* and in almost all reverse transcribing elements their production is regulated either by frameshift or by readthrough of a weak stop codon. The *copia* retroelement has a polyprotein containing both *gag* and *pol* but these are differentially expressed by a splicing mechanism. No such mechanism has been found for RTBV and it is possible that the second 'cys' sequence may control the expression of these two gene products.

Viral Replication

The replication mechanism of RTBV closely resembles that of other plant pararetroviruses, such as cauliflower mosaic virus (CaMV), with transcription of a supercoiled form of the viral genome by host DNA-dependent RNA polymerase II in the nucleus, to give the 35S RNA and reverse transcription of the 35S RNA by virus-encoded reverse transcriptase in the cytoplasm. Replication intermediates have been found which have features very similar to those of CaMV. As with nearly all plant reverse transcribing elements the (−)-strand DNA synthesis on the RTBV 35S RNA transcript is primed by $tRNA^{met}_{init}$ but the priming of the (+)-strand DNA synthesis is not at a polypurine-rich region. The discontinuities in the viral genome are at these priming sites.

Virus Variation

Many of the early descriptions of variants of tungro were before it was recognized that the disease was caused by two viruses and did not distinguish between RTBV and RTSV. There are few characterized biological variants of RTBV but molecular studies have shown both local microvariation and regional variants. The microvariation is of the order of 1–2% at the nucleotide level and is that expected from a replication system that does not have proofreading. At the regional level there are two distinct strains of the virus, one from southeast Asia and the other from the Indian subcontinent. The nucleotide sequences of these two strains are about 75% similar and at the amino acid level the similarity varies from 88% (ORF 2) to 56% (ORF 4). The Indian strain is characterized by a deletion of 68 nucleotides in the non-coding region when compared with the southeast Asian strain. No serological differences can be detected between these strains.

See also: **Plant pararetroviruses (*Caulimoviridae*): Caulimoviruses: general features, Caulimoviruses: molecular biology, Badnaviruses, Cassava vein mosaic virus, Legume caulimoviruses.**

Further Reading

Anjaneyulu A, Satapathy MK and Shukla VD (1994) Rice tungro. New Delhi: Oxford and IBH Publishing.

Chancellor TVB and Thresh JM (eds) (1996) Epidemiology and management of rice tungro disease. Chatham, UK: Natural Resources Institute.

Hull R (1996) Molecular biology of rice tungro viruses. *Ann. Rev. Phytopathol.* 34: 275.

Badnaviruses

Benham E. Lockhart and **Neil E. Olszewski**, Department of Plant Pathology and Department of Plant Biology, University of Minnesota, St Paul, Minnesota, USA

Taxonomy and Classification

The genus *Badnavirus* consists of plant viruses with nonenveloped bacilliform virions measuring 120–150 × 30 nm containing a circular double-stranded DNA (dsDNA) genome 7.5–8 kb in size (**Fig. 1**). The majority of badnaviruses are transmitted by mealybugs, and some are also seed-transmitted. The genus currently contains 13 definitive members: *Commelina* yellow mottle virus (CoYMV), the type member; *Aglaonema* bacilliform virus (AgBV); cacao swollen shoot virus (CSSV); rice tungro bacilliform (RTBV); banana streak virus (BSV); canna yellow mottle virus (CaYMV); citrus mosaic badnavirus (CMBV); *Kalanchoë* top-spotting virus (KTSV); *Dioscorea* bacilliform virus (DBV); pineapple bacilliform virus (PBV); *Piper* yellow mottle virus (PYMV); sugarcane bacilliform virus (ScBV); and *Schefflera* ringspot virus (SRV). Viruses with similar particle morphology occurring in *Aucuba, Colocasia, Mimosa, Rubus, Spiraea* and *Yucca* are possible additional members of the genus.

Badnaviruses differ from other bacilliform plant viruses in particle size and composition, and in genome type. The plant rhabdoviruses have significantly larger bacilliform particles surrounded by a lipid-containing envelope, and have a single-stranded RNA (ssRNA) genome. The bacilliform virions of alfalfa mosaic virus (AMV) are less than one-third the size of badnaviruses, and also contain ssRNA. Badnaviruses differ from caulimoviruses, the only other known group of dsDNA plant viruses, in particle morphology, genome size and organization, vector relationships and histopathology.

It is noteworthy that a number of clonally propagated plant hosts of badnaviruses (sugarcane, citrus, banana, black pepper, betel, taro, schefflera) have their centers of origin or diversity in southeast Asia or Australasia. This suggests, but does not provide direct evidence, that badnaviruses may have originated in this part of the world.

Virus Structure and Composition

Badnavirus particles average 120–150 × 30 nm in size, but particles ranging in size from 30 nm spheres to bacilliform particles up to 1000 nm long may occur. Some isolates of BSV, CSSV and PYMV produce significant numbers of abnormally long particles. The particles have a sedimentation coefficient of approximately 200S, and a buoyant density in CsCl of 1.31 g cm^{-3}. The virions are stable in Cs_2SO_4 but not in CsCl. Purified virus preparations sediment as a single component in both rate zonal and isopycnic density gradient centrifugation, indicating that particles of different lengths contain approximately equal ratios of nucleic acid and protein. Purified virus preparations have uncorrected A_{260}/A_{280} ratios of 1.3–1.4. There is no information on extinction coefficient values.

Badnavirus virions contain a major capsid protein of 39 kDa. Proteolysis by proteases generates a second major polypeptide of 37 kDa. Larger virus-related polypeptides are also detected by immunoblotting, and these most probably represent various fragments of the polyprotein from which the capsid protein is released by post-translational processing. The CoYMV capsid protein is not glycosylated, and there is no evidence for the presence of any other non-protein components in the capsid. Analysis of the particle structure of RTBV using optical diffraction

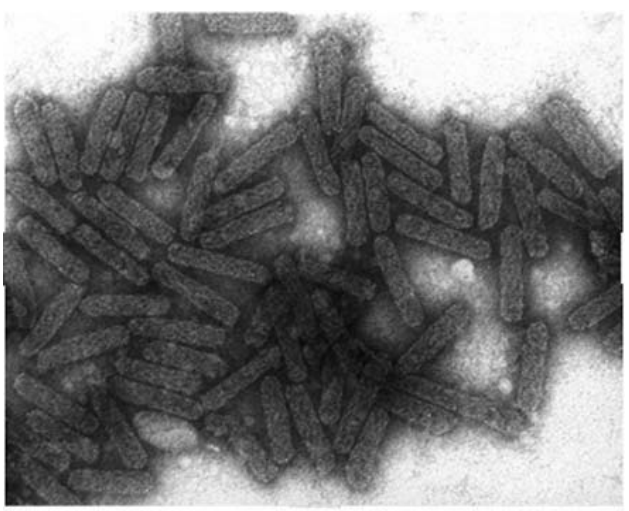


Figure 1 Particles of sugarcane bacilliform virus (ScBV) negatively stained with sodium phosphotungstate. Scale bar = 100 nm.

caulimoviruses the precapsid protein is cleaved at both ends removing its very acidic termini. This process might be coupled to virus assembly and maturation. The capsid protein is both methylated and glycosylated. The Pol protein is cleaved to yield the aspartic proteinase and reverse transcriptase/RNase H. At least some of these cleavages occur by the action of the viral proteinase. In contrast, the ORF III product is cleaved by a host cysteine proteinase. The genome arrangement and the presence of protease domains suggest that protein processing also occurs in CVMV and PVCV. It can be assumed that this is even more extensive, since the primary translation products are fewer and larger.

See also: **Plant pararetroviruses: Badnaviruses; Plant pararetroviruses (*Caulimoviridae*): Cassava vein mosaic virus, Caulimoviruses: general features, Legume caulimoviruses; Plant pararetroviruses: Rice tungro bacilliform virus; Vectors: Plant viruses.**

Further Reading

Bonneville J and Hohn T (1993) A reverse transcriptase for cauliflower mosaic virus. The state of the art, 1991. In: Skalka N and Goff S (eds) *Reverse Transcriptase,* Cold Spring Harbor: Cold Spring Harbor Laboratory Press. p. 357.

Covey SN (1991) Pathogenesis of a plant pararetrovirus: CaMV. *Semin. Virol.* 2: 151.

Fütterer J and Hohn T (1996) Translation in plants – rules and exceptions. *Plant Mol. Biol.* 32: 159.

Goldbach R and Hohn T (1996) Plant viruses as gene vectors. In: Bryant JA (ed.) *Methods in Plant Biochemistry,* vol. 10b, *Molecular Biology,* p. 103. San Diego: Academic Press.

Hohn T and Fütterer J (1997) The proteins and functions of plant pararetroviruses: knowns and unknowns. *Crit. Rev. Plant Sci.* 16: 133.

Hull R (1992) Genome organization of retroviruses and retroelements: evolutionary considerations and implications. *Semin. Virol.* 3: 373.

Kiss-László Z and Hohn T (1996) Pararetro- and retrovirus RNA: splicing, non-splicing and export. *Trends Microbiol.* 4: 480.

Lam E (1994) Analysis of tissue specific elements in the CaMV 35S promoter. In: Nover L (ed.) *Results and Problems in Cell Differentiation,* vol. 20, p. 181. Berlin: Springer.

Rothnie HM, Chapdelaine Y and Hohn T (1994) Pararetroviruses and retroviruses: a comparative review of viral structure and gene expression strategies. *Adv. Virus Res.* 44: 1.

Sanfaçon H (1992) Regulation of mRNA formation in plants: lessons from the cauliflower mosaic virus transcription signals. *Can. J. Bot.* 70: 885.

Cassava Vein Mosaic Virus

Alexandre de Kochko, International Laboratory for Tropical Agricultural Biotechnology, La Jolla, California, USA

History, Taxonomy and Classification

The symptoms induced on cassava plants by cassava vein mosaic virus (CsVMV) were recognized as early as 1940 but the virus itself was first described by Kitajima and Costa in 1966. Based on preliminary observations, which showed that it has isometric particles and that its genome consisted of double-stranded DNA (dsDNA), it was tentatively classified in the *Caulimovirus* genus. More recent work led to the characterization of its genomic organization and showed important features which differ from caulimoviruses as well as from badnaviruses, the two genera of plant dsDNA formerly recognized. In response to these results and additional observations made on other plant dsDNA viruses, executive committee of the International Committee on Taxonomy of Viruses (ICTV) created a new genus (not yet named) with CsVMV designated as the type species. It was also decided to create and name *Caulimoviridae,* the family which contains all the plant dsDNA viruses including the genera *Caulimovirus, Badnavirus,* and three newly created genera. One includes CsVMV, another the former caulimoviruses infecting legumes (Soybean chlorotic mottle virus, SbCMV, and peanut chlorotic streak virus, PCSV) and the third has rice tungro bacilliform virus (RTBV) as its type species.

Geographic Distribution, Host Range and Virus Propagation

CsVMV was described in Brazil, with detailed accounts of its presence in the states of São Paulo and Ceará, but this limited description is certainly a consequence of the very few studies carried out on this virus. It infects plants from the cultivated cassava species, *Manihot esculenta* Crantz, which originated from the Amazon basin. Its complete host spectrum is not known, in particular no surveys have been made on wild cassava relatives. The impact on cassava production seems to be limited. Caulimoviruses are aphid-transmitted but in the case of CsVMV the natural vector is unknown. As cassava is mainly reproduced vegetatively, the virus is propagated very easily through cuttings. Experimentally it is possible to infect cassava plants by introducing an infectious clone using microbombardment. It has also been

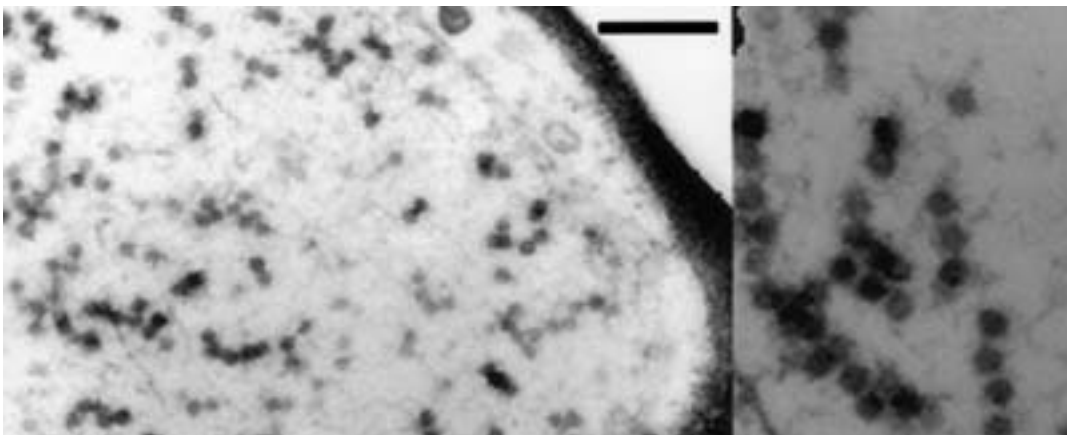


Figure 1 Electron microscopy views of CsVMV particles: (**A**) in inclusion bodies, ×40 000, bar = 250 nm; (**B**) as a cluster in the cytoplasm, ×80 000, bar = 125 nm.

shown that several dsDNA viruses are mechanically transmissible, namely cauliflower mosaic virus (CaMV) and cacao swollen shoot virus (CSSV).

The symptoms developed by the plants while in tissue culture, include discoloration of the veins, mottling, reduced leaf lamina and necrosis, starting from the tip and extending to the whole leaf. When the plants are grown under growth chamber conditions, the appearance of symptoms is very slow; only old leaves, over one month old, present symptoms similar to those observed in tissue culture except that the necrosis does not extend to the entire leaf.

Virus Structure and Genome Organization

Particles of CsVMV were observed by electron microscopy (**Fig. 1**). They appear to be isometric and about 50 nm in diameter. The particles accumulate in inclusion bodies in the cytoplasm of infected cells. The shape and the size of the virions of CsVMV are similar to those of CaMV which contain 420 subunits of a single coat protein (CP) arranged with $T = 7$ icosahedral symmetry. No studies have been made on CsVMV ultrastructure. CsVMV encodes its CP as a portion of a polyprotein which undergoes a post-translational processing. The exact size of the CP has not yet been determined.

The genome of CsVMV is a single circular molecule of a dsDNA 8159 nucleotides long. **Figure 2** depicts the organization of the genome which contains four open reading frames (ORFs), with a possible fifth one, and a large intergenic region in which is located a promoter suspected to govern the transcription of a genome length, terminally redundant RNA. No biological studies were made on CsVMV, all the following information results from predictions deduced from sequence comparison and similarities with the genomes of plant dsDNA viruses. The large ORF 1 of CsVMV contains domains that are equivalent to the CP and the movement protein (MP) of caulimoviruses. ORF 3 shares a high degree of similarity with ORF 5 of the caulimoviruses, and the second half of ORF 3 of the badnaviruses and RTBV, which encode an aspartic proteinase (PR) and a reverse transcriptase (RT) including a RNase H domain. The aspartic PR is responsible for the post-translational processing of the polyproteins resulting from the translation of ORF 4 and ORF 5 of caulimoviruses and ORF 3 of badnaviruses.

Several unique features were identified in the genome organization of CsVMV that led to the creation of a new genus with CsVMV as the type species. The low proportion of GC, 25% vs. 34–39% for caulimoviruses, the presence of a large ORF 1, and the relative order of encoded functions. All the retroelements, including plant and animal pararetroviruses, retroviruses and retrotransposons, have in their genome a continuum between the so-called *gag* (CP) and *pol* (PR+RT) genes. The genome of CsVMV in contrast, displays first the CP (*gag*) followed by the MP and an additional ORF (ORF 2) instead of having the PR-RT (*pol*) immediately downstream of the CP (**Fig. 3**). ORF 4 of CsVMV shares a low but significant level of similarity with ORF 6 of caulimoviruses. This ORF encodes a protein involved in various functions. It plays a major role in the regulation of the translation of the polycistronic RNA (it is called transactivator or TAV in that case) and it is the main component of the viral inclusion body matrix (in that case it is called inclusion body protein or IBP).

Replication

The genome of CsVMV contains all the necessary

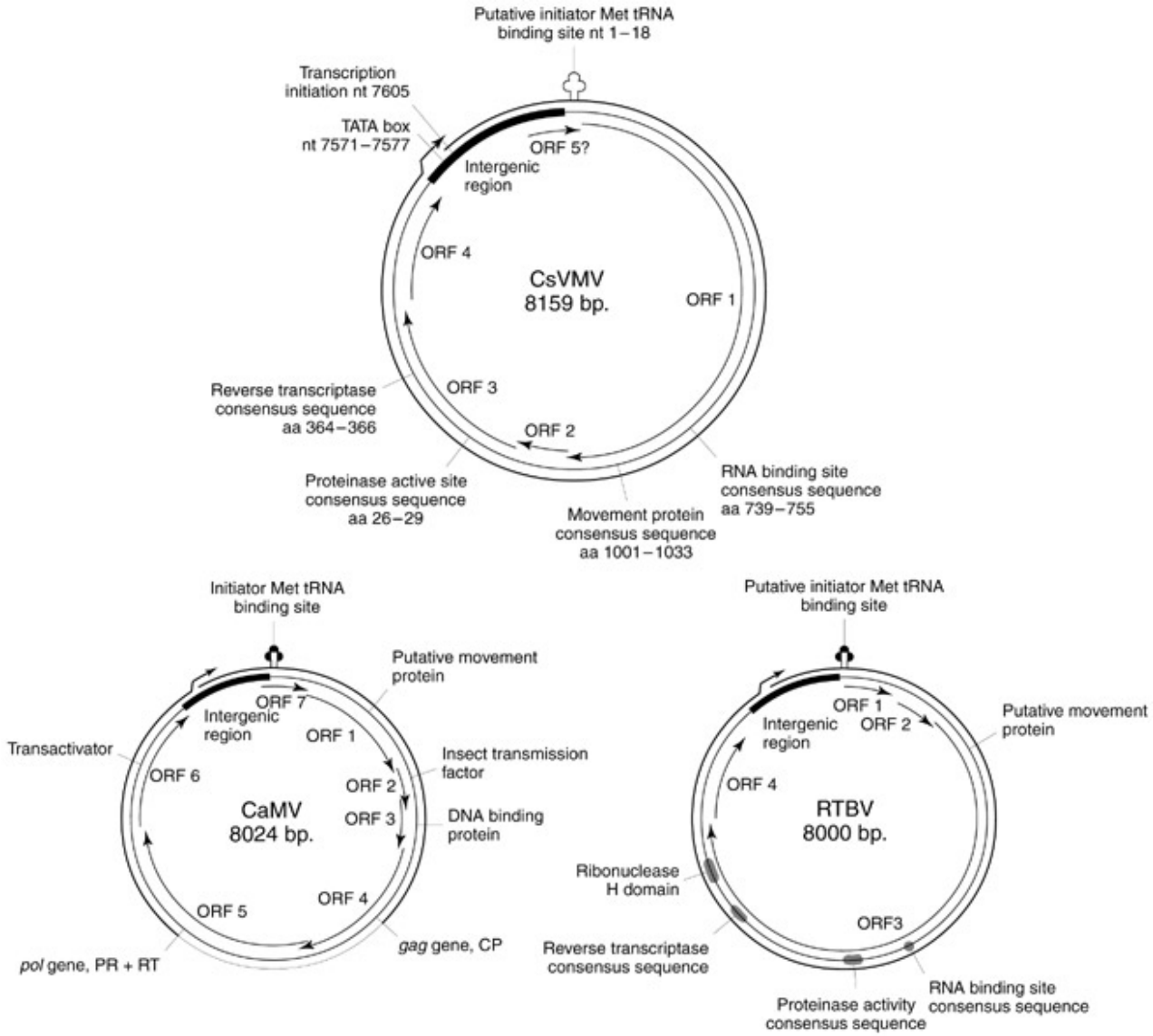


Figure 2 Genomic maps of CsVMV, CaMV and RTBV. Inner circles represent the genomic DNA on which the identified functions have been indicated. Outer circle represents the terminally redundant genome-length RNA transcript. The arrows inside the circle represent the ORFs. CsVMV has 4 ORFs (maybe 5) with a long polygenic ORF 1. CaMV, the type species of the *Caulimovirus* genus, has 7 ORFs, and RTBV, type species of a new genus of plant dsDNA viruses, has 4 ORFs; badnaviruses have three ORFs (see **Fig. 3**).

elements to make it compatible with the model of replication for the plant pararetroviruses. After infection of the host cell, the viral DNA migrates to the nucleus where the replication cycle begins with the synthesis of a terminally redundant genome-length RNA under the control of the genomic viral promoter. This synthesis is performed by a host RNA polymerase. The resulting transcript is used as a template by the viral-encoded RT when primed by a host methionine tRNA which recognizes a complementary sequence on the transcript. The RT synthesizes a minus-strand DNA and the RNase H degrades the RNA strand in the heteroduplex RNA–DNA leaving one or more specific short RNA fragments. The RT, acting now as a DNA-dependent DNA polymerase, uses these RNA fragments to prime the synthesis of the plus strand of viral DNA resulting in the circular double-stranded viral genome containing as many gaps as short RNA fragments were left. Except for the viral promoter which has been shown to be active, no experimental data have been obtained to demonstrate that CsVMV replication follows this cycle. Nevertheless, predictions deduced from sequence similarities indicate that this model should

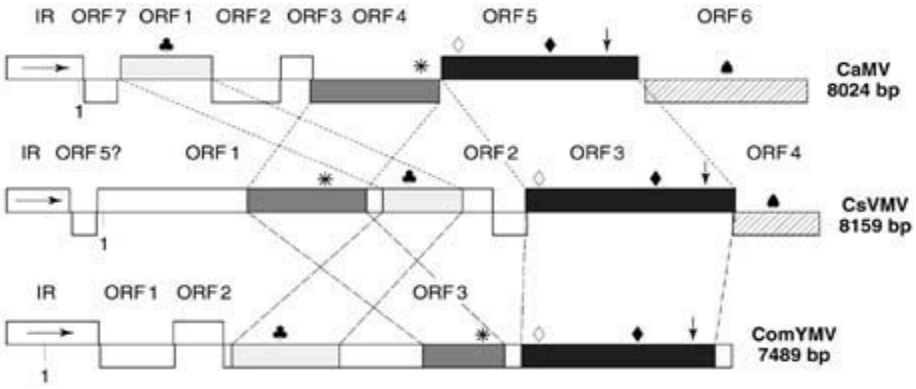


Figure 3 Comparison between genomic organization of CsVMV, CaMV, the type species of the genus *Caulimovirus*, and commelina yellow mottle virus (ComYMV) the type species of the genus *Badnavirus*. The three genomes start at the beginning of the large intergenic region for clarity purposes. ORFs or ORF segments encoding similar putative function are linked by vertical lines; the number 1 indicates the origin of DNA replication. ♣, MP active site; ✳, RNA binding site; ◇, PR active site; ◆, RT active site; ♠, TAV active center; ⸸, RNAse H consensus sequence.

be applicable to CsVMV. After assembly and accumulation in the infected cell, CaMV moves as a virion to adjacent cells through plasmodesmata where the viral MP contributes to the elaboration of tubular structures. As CsVMV encodes a predicted MP similar to the one of CaMV, it is probable that the cell to cell spread occurs by a mechanism comparable to that of CaMV.

Promoter Function and Organization

Verdaguer *et al* have shown that similarly to the 35S promoter of CaMV, the expression of CsVMV promoter is strong and constitutive. In unpublished results the same authors described that this expression requires a synergistic or combinatorial interaction between different *cis*-elements which individually confer tissue specificity and/or variation in the strength of expression. **Figure 4** represents a functional map of the CsVMV promoter. This promoter is a valuable tool for genetic engineering and a good alternative to 35S. It can be used for expressing transgenes in dicotyledonous as well as monocotyledonous plants. Its organization permits the expression of the introduced gene(s) to be modulated by removing some of the *cis*-elements.

See also: **Plant pararetroviruses: Badnaviruses; Plant pararetroviruses (*Caulimoviridae*): Caulimoviruses: general features, Caulimoviruses: molecular biology, Legume caulimoviruses; Plant pararetroviruses: Rice tungro bacilliform virus.**

Further Reading

Calvert LA, Ospina MD and Shepherd RJ (1995) Char-

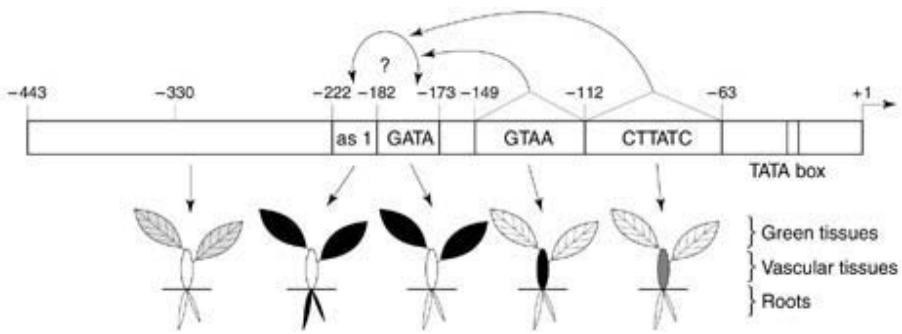


Figure 4 Functional design of the CsVMV promoter. Effect of the different regions of the promoter on gene expression in transgenic plant are represented. Three types of tissue are considered: green tissue, vascular tissue and roots. Dark area represents strong expression, gray area indicates lower expression and white area symbolizes undetectable expression. Arrows indicate the synergistic interactions between different domains of the promoter. Sequence motifs that might play an important role for CsVMV promoter expression are also mentioned. The numbers indicate the position relative to the transcription start site (+1). Adapted from Verdaguer *et al*, unpublished results.

acterization of cassava vein mosaic virus: a distinct plant pararetrovirus. *J. Gen. Virol.* 76: 1271.

Hohn T and Fütterer J (1991) Pararetroviruses and retroviruses: a comparison of expression strategies. *Semin. Virol.* 2: 55.

Hull R (1992) Genome organization of retroviruses and retroelements: evolutionary considerations and implications. *Semin. Virol.* 3: 373.

Kiss-Laslo Z, Blanc S and Hohn T (1995) Splicing of cauliflower mosaic virus 35S RNA is essential for viral infectivity. *The EMBO Journal* 14: 3552–3562.

Kochko de A, Verdaguer B, Taylor N, Carcamo R, Beachy RN and Fauquet C (1998) Cassava vein mosaic virus (CsVMV), the type species for a new genus of double stranded DNA plant virus? *Archives of Virology* 143, 945–962.

Laco G, Kent SBH and Beachy RN (1995) Analysis of the proteolytic processing and activation of the rice tungro bacilliform virus reverse transcriptase. *Virology* 208: 207.

Verdaguer B, Kochko de A, Fux CI, Beachy RN and Fauquet C (1998) Functional organization of the cassava vein mosaic virus (CsVMV) promoter. *Plant Molecular Biology* 37: 1055–1067.

Legume Caulimoviruses

D. V. R. Reddy, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, India

R. D. Richins, Department of Plant Pathology, University of California, Riverside, California, USA

History

The occurrence of a legume caulimovirus was first reported in 1980. The virus, subsequently identified as Peanut chlorotic streak virus (PC1SV), was found to occur naturally on peanut (groundnut, *Arachis hypogaea*) in the state of Andhra Pradesh in India. The first comprehensive report on a legume caulimovirus, Soybean chlorotic mottle (SoyCMV), was published in 1984. The virus was observed during surveys in Aichi Prefecture in 1981. To date PC1SV and SoyCMV appear to be the only caulimoviruses which were reported to occur under natural conditions on legumes.

Taxonomy and classification

DNA-containing plant viruses are included under the family *Caulimoviridae*. PC1SV and SoyCMV are in the genus Caulimovirus.

Virion Structure and Proteins

Virus particles are icosahedrical, c. 50 nm in diameter. Similar to other caulimoviruses they produce inclusions in cytoplasm which are circular or ovoid and consist of an amorphous, vacuolated, electron-dense matrix. The virus particles are embedded in the matrix.

Strains

No strains have so far been reported for SoyCMV. However a strain of PC1SV, designated as 'Chlorotic Vein-banding' strain (PC1SV-CVB) has been reported, which produces distinct symptoms on peanut. It differs from PC1SV in host range, symptoms and also in the physical map.

Geographic Distribution

SoyCMV occurs in Japan although its distribution in the various Prefectures in Japan is currently not known. PC1SV occurs in the states of Andhra Pradesh, Karnataka, Maharasthra and Tamil Nadu in India.

Symptoms and Host Range

Symptoms are here only given for the principal hosts. PC1SV in peanut causes reduced leaflets, chlorotic streaks and stunting. Symptoms produced by SoyCMV on soybean are mosaic or mottle on leaves and stunting of plants. Some soybean cultivars have been reported to produce different symptoms. A typical feature with both the viruses is the appearance of symptoms after three weeks of inoculation. The host range is given in **Table 1.**

Serological Relationships

PC1SV and SoyCMV do not react serologically with each or with a number of other caulimoviruses which include cauliflower mosaic (CaMV), figwort virus (FWV) or carnation etched ring virus (CERV). The serological tests employed were ELISA and agar gel double diffusion.

Transmission

PC1SV and SoyCMV are readily transmitted by mechanical sap inoculations. Although the majority of caulimoviruses are transmitted by aphids, attempts to transmit PC1SV or SoyCMV, both nonpersistently and persistently, by a range of aphids have so far been unsuccessful. PC1SV could not be transmitted by *Aphis craccivora* or *Myzus persicae* and SoyCMV by *Acyrthosiphon pisum, A. craccivora, Aulacorthum*

natural variants that respond in an altered manner to pathogenesis by CaMV, and these plants may be used to explain how viruses cause disease.

In addition to contributing to molecular virology, it is likely that new strategies for controlling diseases caused by caulimoviruses will emerge. Although plants have been genetically engineered to resist infection by many types of plant viruses, genetic engineering has not been used yet to create transgenic plants with resistance to caulimoviruses. As transgenic plants have already been made that express antisense or sense versions of CaMV genes, it is possible that genetic engineering for control of caulimoviruses will be an option in the near future.

See also: **Plant pararetroviruses: Badnaviruses; Plant pararetroviruses (*Caulimoviridae*): Caulimoviruses: molecular biology; Pathogenesis: Plant viruses; Plant resistance to viruses: Natural resistance; Vectors: Plant viruses.**

Further Reading

Covey SN (1991) Pathogenesis of a plant pararetrovirus: CaMV. *Semin. Virol.* 2: 151.

Hull R (1984) Caulimovirus group. *CMI/AAB Descriptions of plant viruses*, no. 295. Kew: Commonwealth Mycological Institute/Association of Applied Biologists.

Matthews REF (1991) *Plant Virology* 3rd edn. New York: Academic Press.

Rothnie HM, Chapdelaine Y and Hohn T (1994) Pararetroviruses and retroviruses: a comparative review of viral structure and gene expression strategies. *Adv. Virus Res.* 44: 1.

Schoelz JE, Qiu SG and Wintermantel WM (1995) Genetic and environmental factors that determine the ability of cauliflower mosaic virus to systemically infect solanaceous hosts. In: Bills DD and Kung S (eds) *Biotechnology and Plant Protection: Viral Pathogenesis and Disease Resistance*, p. 313. Singapore: World Scientific Publishing.

Caulimoviruses – Molecular Biology

Thomas Hohn, Frederick Miescher Inst., Basel, Switzerland

Properties of the Virion and Inclusion Bodies

Caulimoviruses are icosahedral ($T = 7$), 45–50 nm in diameter and sediment between 200 and 250S. The main capsid components of the type member cauliflower mosaic virus (CaMV) are proteins with mobilities of 37 and 44 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis derived from open reading frame (ORF) IV. In addition, CaMV particles contain minor amounts of other polypeptides, a 15 kDa nucleic acid binding protein derived from ORF III, protease and reverse-transcriptase derived from ORF V, and host casein kinase II. In cells infected with CaMV or a number of other caulimoviruses large numbers of virus particles accumulate in typical stable inclusion bodies, the matrix of which is the virus-encoded multifunctional inclusion body protein of 62 kDa, derived from ORF VI. Inclusion bodies are visible in the light microscope and can be stained with phloxin.

Properties of the Genome

The caulimoviral genome is built from 7200–8200 bp. It exists in infected plant nuclei as supercoiled DNA bound to nucleosomes (minichromosome), in infected cytoplasm as RNA with a 180 nucleotides (nt) terminal redundancy, and in virus particles as open circular ds DNA. The open form is due to nicks at specific sites of the (+)- and (−)-strand with short 5′ overlaps (**Fig. 1**), remnants of the reverse transcription process, which are apparently removed in the infected nucleus.

The sequence of many of the caulimoviruses is known. According to sequence and ORF arrangement four groups of caulimoviruses can so far be distinguished: CaMV-like, PCSV-like, CVMV and PVCV (**Table 1** and **Fig. 2**). CaMV-like caulimoviruses contain seven ORFs in the order VII (dispensable, unknown function), I (cell-to-cell movement), II (aphid transmission), III (nucleic acid binding protein), IV (capsid protein precursor), V (protease, reverse transcriptase) and VI (multifunctional protein [inclusion body matrix protein, translation transactivator]). PCSV-like caulimoviruses differ by containing three (A, B, C), instead of two ORFs between ORFs I and III (**Fig. 2**). The ORF arrangements in

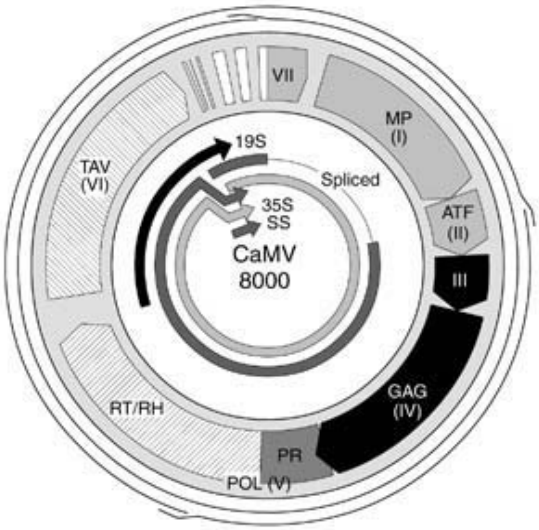


Figure 1 Map of cauliflower mosaic virus. On the outside the open circular double-stranded viral DNA with its overhangs is symbolized. The main circle shows the arrangement of open reading frames and on the inside the primary transcript and the essential spliced RNA are shown.

CVMV and PVCV are considerably different. In both cases some of the ORFs are combined to form super-ORFs (**Fig. 2**). Furthermore, in CVMV the domain corresponding to the CaMV movement protein is shifted to the middle of the genome, and, most interestingly, in PVCV an integrase motif was detected, which is absent from all other caulimoviruses and also from the related badnaviruses.

Properties of the Proteins

Movement protein

CaMV (and probably all the other caulimoviruses) move from cell to cell as particles through tubular structures spanning the cell walls between adjacent cells. In CaMV the 37 kDa movement protein, coded for by ORF I, is responsible for the formation of these tubules and also provides their main component.

Aphid transmission factor

In general, aphids transmit caulimoviruses. In the case of CaMV, ORF II codes for the 18 kDa aphid transmission factor (ATF). Some of the viral inclusion bodies in CaMV-infected cells consist mainly of ATF. ATF interacts with virus particles and with tubulin and it can be assumed that it also interacts with the cuticulum lining the aphid's stylet. ATF mutants normally infect host plants on mechanical inoculation. Viruses lacking ATF can still be transmitted by aphids if the insects have previously been in contact with wild-type ATF either by feeding on plants infected with a related virus or on nutrient solutions supplied with artificially produced ATF.

Small nucleic acid binding protein

The product of CaMV ORF III, a nucleic acid binding protein of 15 kDa, is found in the viral inclusion bodies and in small amounts also in virus particles. A processed form of 11 kDa lacking the C-terminus has lost the nucleic acid binding activity. An intact ORF III is essential for virus infectivity; however, so far, it is not known whether p15 or its processed form is an obligate component of infectious virus particles or whether it functions only transiently, e.g. in assembly.

Capsid proteins and their precursor

The product of CaMV ORF IV has a molecular weight of 56 kDa and an electrophoretic mobility of 80 kDa. It is flanked by very acidic regions, which are removed from the mature capsid proteins which have a mobility corresponding to proteins of 44 and

Table 1 Caulimoviruses

Type	*Abbreviation*	*Name*	*Main host family*	*Accession*
CaMV-like	CaMV	Cauliflower mosaic	*Cruciferae*	V00141 and others
	CERV	Carnation etched ring	*Caryophyllaceae*	X04658
	FMV	Figwort mosaic	*Scrophulariaceae*	X06166
	SVBV	Strawberry vein banding	*Rosaceae*	X97304
PCSV-like	PCSV	Peanut chlorotic streak	*Leguminosae*	U13988
	SCMV	Soybean chlorotic mottle	*Leguminosae*	X15828
CVMV	CVMV	*Cassava* vein mosaic	*Euphorbiaceae*	U20341
PVCV	PVCV	*Petunia* vein clearing	*Solanaceae*	U95208
Unassigned	CV	*Cestrum*	*Solanaceae*	
	DMV	*Dahlia* mosaic	*Compositae*	
	HRLV	Horseradish latent	*Cruciferae*	
	MMV	*Mirabilis* mosaic	*Nyctaginaceae*	

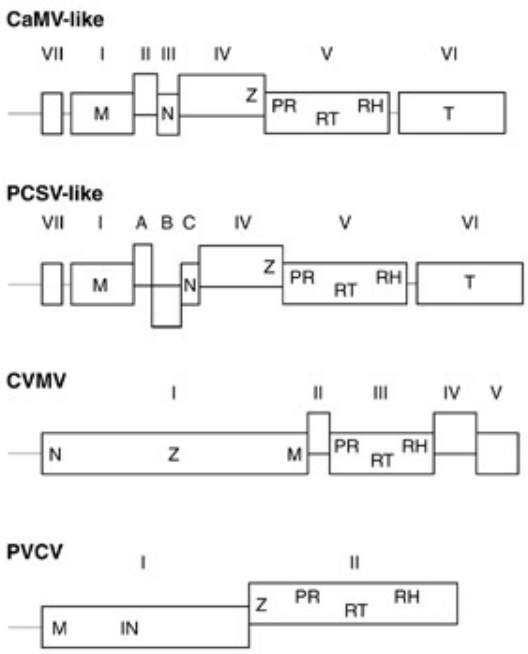


Figure 2 Open reading frame arrangement of caulimoviruses. Examples for each of the known classes are shown. For CaMV the ORFs code for dispensable unknown function (VII), movement protein (I), aphid transmission factor (II), small nucleic acid binding protein (III), capsid protein precursor (IV), Pol polyprotein (V) and inclusion body protein/translation transactivator (VI). The major known motifs indicative for protein functions are shown: M, movement protein motif; N, histone-like nucleic acid-binding motif; Z, Zn-finger; PR, protease motif; RT, reverse transcriptase motif; RH, RNase H motif; T, translation transactivator homology; IN, integrase motif.

37 kDa, although their actual molecular weight is probably smaller. The sequence of p37 is included within p44 and thus antibodies against p37 react also with p44. p44 but not p37 is phosphorylated. It is not known whether both types of proteins are required for full infection, or whether one of these types is the true functional capsid protein. It might be that p44 is the mature form of the capsid protein and p37 a degradation product or p37 might be the mature form and p44 still a precursor. Both p37 and p44 include a zinc-finger motif, which is implicated in RNA binding. Furthermore, large stretches of basic amino acids at the C-termini of p37 and p44 constitute DNA- and RNA-binding motives.

Pol-polyprotein, protease, reverse transcriptase/RNAseH

CaMV ORF V corresponds to the Pol ORF of retroviruses. It produces a polyprotein which is cleaved during virus production into a 15 kDa aspartic proteinase and a 60 kDa reverse transcriptase/RNAse H. It is not known whether the RNAse H functions as part of the 60 kDa protein or whether it is released by another cleavage reaction. One has to assume that the proteinase also functions as part of the polyprotein, i.e. when it releases itself. Caulimoviruses do not have an integrase and consequently their DNA is not integrated into the host genome. A notable exception is PVCV (**Fig. 2**) which exists in an integrated form and has integrase consensus sequences in its genome.

Inclusion body protein/translation transactivator

The 62 kDa inclusion body protein of CaMV- and PCSV-like caulimoviruses is encoded by ORF VI. It has no significant homologies to any other known viral or host gene. The inclusion body protein has been implicated in virus assembly, reverse transcription, host range determination, symptom severity, control of polycistronic translation and seclusion of virus functions. Perhaps as a consequence of the role of the ORF VI protein in translation, all CaMV proteins including foreign proteins carried by a CaMV vector can be found within the inclusion bodies. A subdomain in its center has been assigned to the translational transactivation and other domains have been found to bind RNA unspecifically. Furthermore the protein interacts with capsid protein.

Virus Stability

Both the viral inclusion bodies and the virus particles are very stable. Dissolution of the inclusion bodies to obtain virus particles requires treatment with 1 M urea over a long period. Virus particles aggregate at acidic pH and disintegrate at very high pH (0.1 M NaOH). Virus particles can also be disintegrated by boiling in sodium dodecyl sulfate/dithiothreitol or by treatment with proteinase K and phenol extraction.

Genome Replication

As in retroviruses, replication occurs by production of a terminally redundant RNA by transcription in the nucleus and its reverse transcription in the cytoplasm. Also as in retroviruses, and unlike hepadnaviruses, a tRNA primer (met-ini-tRNA) is used as the primer for (−)-strand DNA synthesis from the RNA template. The RNA template is digested by RNase H following the reverse transcriptase. However, oligo(G) stretches are resistant to this digestion and remain bound to the (−)-strand DNA where they act as primers for (+)-strand synthesis. The number of (+)-strand synthesis initiation events varies between one and three major events in different caulimoviruses and even within strains from a particular virus. Minor

initiation events also occur. The synthesis of both the strands overshoots, creating short overhangs that cannot be repaired in the cytoplasm due to lack of repair exonuclease and ligase in this compartment. Accordingly, the packaged DNA contains open circular DNA. On introduction of viral DNA into the nucleus of an infected cell, the overhangs are removed and supercoiled circular DNA is formed. This interacts with histones to form minichromosomes. Caulimovirus DNA, probably with the exception of PVCV DNA, does not integrate into the host chromosome obligatorily. The template for transcription is the supercoiled circular DNA.

Transcription

Transcription in caulimoviruses is unidirectional. All caulimoviruses produce a transcript covering the total genome plus about 180 nt, such that the RNA is terminally redundant. This RNA is called 35S RNA due to its sedimentation behavior. The terminal redundancy is caused by the polyadenylation signal located on the circular DNA 180 bp downstream of the transcription start site being ignored by the polymerase at its first passage (see below). CaMV-like caulimoviruses produce a second transcript, the 19S RNA, covering ORF VI and encoding for the inclusion body protein/translation transactivator.

The 35S promoter of CaMV, FMV and probably other caulimoviruses is very strong and quasi-constitutive, i.e. it is expressed in nearly all types of cells and at all developmental stages. This constitutivity is caused by a number of different enhancer elements, each with some specificity for certain cell types. Some of the corresponding transcription factors have been identified, e.g. ASF-1, ASF-2 and CAF recognizing a TGACG motif, a GATA motif and CA-rich region, respectively.

RNA Processing

Full-length RNA made from the circular DNA template includes a polyadenylation signal. If this is used on the first encounter, a short stop RNA of 180 nt is formed; if used on the second encounter full-length terminally redundant RNA is formed. The role of the short-stop RNA, if any, is not known.

The CaMV polyadenylation signal consists of an AAUAAA sequence, which determines the cleavage site 13 nt downstream of it. Occasionally cryptic signals are used in addition. In contrast to the animal system, single point mutations in the AAUAAA signal are partially tolerated by the plant system. Polyadenylation enhancers are located upstream of the AAUAAA signal and not downstream as in most animal cases. In CaMV and FMV these elements are tandem repeats of UAUUUGUA.

In addition to the primary CaMV 35S transcript, alternatively spliced versions of it have been detected. By removing an intron extending from close to the end of the leader to a position within ORF II, a mRNA is created in which ORF III is the first ORF and ORF IV the second. Additional splicing events were found using the same splice acceptor but using donors within ORF I. These led to ORF I–ORF II in-frame fusions, the function of which is not known. Whether the ratio of spliced to unspliced RNA is controlled, for example by nuclear export of unspliced RNA as in human immunodeficiency virus is not known.

Translation Mechanism

The 35S RNA of caulimoviruses and also its spliced derivatives serve as polycistronic mRNAs for the viral proteins. The ORFs of these viruses closely follow each other, are opened by efficiently recognized start codons and usually also contain internal start codons. In plant protoplasts and in transgenic plants most of the downstream ORFs are poorly expressed in the absence of the inclusion body protein/translation *trans*-activator encoded by ORF VI, interestingly the only ORF that is translated from a monocistronic mRNA. *Trans*-activation activity has been demonstrated for CaMV and FMV, and probably resides also in the corresponding ORFs of the other CaMV-like and PCSV-like caulimoviruses. *Trans*-activation is thought to be based on a reinitiation mechanism.

Another special feature of the caulimovirus (and also the badnavirus) 35S RNAs is their 600–700 nt-long leader, which is rather large for eukaryotic RNAs. These leaders contain several small ORFs and include a large hairpin structure. These are features that usually make an RNA a poor messenger, since initiation factors and/or 40S ribosomes initiate scanning at the cap of an RNA moving in the 3′ direction until an AUG codon is encountered and translation proper begins, whereas translation from AUGs further downstream is precluded. To overcome this problem caulimoviruses employ a shunting mechanism for translation initiation, whereby scanning is initiated at the cap as usual, but the scanning complex bypasses (shunts) most of the leader to reach the first longer ORF. This mechanism is unlike the internal initiation mechanism used by enteroviruses.

Protein Processing

Many of the caulimovirus proteins are processed, i.e. in CaMV-like (and by analogy probably the other)

caulimoviruses the precapsid protein is cleaved at both ends removing its very acidic termini. This process might be coupled to virus assembly and maturation. The capsid protein is both methylated and glycosylated. The Pol protein is cleaved to yield the aspartic proteinase and reverse transcriptase/RNase H. At least some of these cleavages occur by the action of the viral proteinase. In contrast, the ORF III product is cleaved by a host cysteine proteinase. The genome arrangement and the presence of protease domains suggest that protein processing also occurs in CVMV and PVCV. It can be assumed that this is even more extensive, since the primary translation products are fewer and larger.

See also: **Plant pararetroviruses: Badnaviruses; Plant pararetroviruses (*Caulimoviridae*): Cassava vein mosaic virus, Caulimoviruses: general features, Legume caulimoviruses; Plant pararetroviruses: Rice tungro bacilliform virus; Vectors: Plant viruses.**

Further Reading

Bonneville J and Hohn T (1993) A reverse transcriptase for cauliflower mosaic virus. The state of the art, 1991. In: Skalka N and Goff S (eds) *Reverse Transcriptase,* Cold Spring Harbor: Cold Spring Harbor Laboratory Press. p. 357.

Covey SN (1991) Pathogenesis of a plant pararetrovirus: CaMV. *Semin. Virol.* 2: 151.

Fütterer J and Hohn T (1996) Translation in plants – rules and exceptions. *Plant Mol. Biol.* 32: 159.

Goldbach R and Hohn T (1996) Plant viruses as gene vectors. In: Bryant JA (ed.) *Methods in Plant Biochemistry,* vol. 10b, *Molecular Biology,* p. 103. San Diego: Academic Press.

Hohn T and Fütterer J (1997) The proteins and functions of plant pararetroviruses: knowns and unknowns. *Crit. Rev. Plant Sci.* 16: 133.

Hull R (1992) Genome organization of retroviruses and retroelements: evolutionary considerations and implications. *Semin. Virol.* 3: 373.

Kiss-László Z and Hohn T (1996) Pararetro- and retrovirus RNA: splicing, non-splicing and export. *Trends Microbiol.* 4: 480.

Lam E (1994) Analysis of tissue specific elements in the CaMV 35S promoter. In: Nover L (ed.) *Results and Problems in Cell Differentiation,* vol. 20, p. 181. Berlin: Springer.

Rothnie HM, Chapdelaine Y and Hohn T (1994) Pararetroviruses and retroviruses: a comparative review of viral structure and gene expression strategies. *Adv. Virus Res.* 44: 1.

Sanfaçon H (1992) Regulation of mRNA formation in plants: lessons from the cauliflower mosaic virus transcription signals. *Can. J. Bot.* 70: 885.

Cassava Vein Mosaic Virus

Alexandre de Kochko, International Laboratory for Tropical Agricultural Biotechnology, La Jolla, California, USA

History, Taxonomy and Classification

The symptoms induced on cassava plants by cassava vein mosaic virus (CsVMV) were recognized as early as 1940 but the virus itself was first described by Kitajima and Costa in 1966. Based on preliminary observations, which showed that it has isometric particles and that its genome consisted of double-stranded DNA (dsDNA), it was tentatively classified in the *Caulimovirus* genus. More recent work led to the characterization of its genomic organization and showed important features which differ from caulimoviruses as well as from badnaviruses, the two genera of plant dsDNA formerly recognized. In response to these results and additional observations made on other plant dsDNA viruses, executive committee of the International Committee on Taxonomy of Viruses (ICTV) created a new genus (not yet named) with CsVMV designated as the type species. It was also decided to create and name *Caulimoviridae,* the family which contains all the plant dsDNA viruses including the genera *Caulimovirus, Badnavirus,* and three newly created genera. One includes CsVMV, another the former caulimoviruses infecting legumes (Soybean chlorotic mottle virus, SbCMV, and peanut chlorotic streak virus, PCSV) and the third has rice tungro bacilliform virus (RTBV) as its type species.

Geographic Distribution, Host Range and Virus Propagation

CsVMV was described in Brazil, with detailed accounts of its presence in the states of São Paulo and Ceará, but this limited description is certainly a consequence of the very few studies carried out on this virus. It infects plants from the cultivated cassava species, *Manihot esculenta* Crantz, which originated from the Amazon basin. Its complete host spectrum is not known, in particular no surveys have been made on wild cassava relatives. The impact on cassava production seems to be limited. Caulimoviruses are aphid-transmitted but in the case of CsVMV the natural vector is unknown. As cassava is mainly reproduced vegetatively, the virus is propagated very easily through cuttings. Experimentally it is possible to infect cassava plants by introducing an infectious clone using microbombardment. It has also been

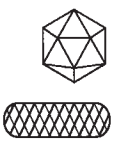
Further Reading

Christian PD and Scotti PD (1998) The picorna-like viruses of insects. In: Miller L and Ball A (eds) *The Insect Viruses*. New York: Plenum Press.

Scotti PD, Longworth JF, Plus N, Croizier G and Reinganum C (1981) The biology and ecology of strains of an insect small RNA virus complex. *Adv. Virus Res.* 26: 117.

Piry Virus *see* **Chandipura, Piry and Isfahan Viruses**

PLANT PARARETROVIRUSES (*CAULIMOVIRIDAE*)



Contents

Caulimoviruses: General Features

James E Schoelz and **June E Bourque**, Plant Pathology, University of Missouri, Columbia, Missouri, USA

History

Caulimoviruses are a group of plant viruses composed of double-stranded (ds) DNA encapsidated into an icosahedral virion. Caulimoviruses have been a subject of study because they cause economic losses in their respective hosts; in addition, they have made significant contributions to our knowledge of the molecular biology of plant viruses. The genome of cauliflower mosaic virus (CaMV), the type member of the genus, was the first of the plant viruses shown to contain DNA rather than RNA as genetic material. Furthermore, it was the first viral genome to be completely sequenced and cloned into bacterial plasmids in an infectious form. The caulimoviruses have also been extensively studied as a model for host–pathogen interactions and because their replication and expression strategies are unusual for plant viruses. For example, CaMV was the first plant virus to be shown to replicate by reverse transcription of an RNA intermediate into dsDNA.

In the early 1980s CaMV was thought to have some promise as a vector for foreign genes in plants. The effort to convert CaMV into a virus-based vector was scaled back when it was shown that the virus genome could tolerate only small insertions. A few small genes, such as dihydrofolate reductase and interferon, were eventually expressed in plants via a CaMV vector. However, other plant viruses, in particular tobacco mosaic virus, potato virus X and tomato bushy stunt virus, have been shown to be much more versatile as vectors for foreign genes.

Although CaMV has had only limited utility as a plant virus vector, the virus continues to have a great impact on plant biotechnology. The CaMV genome has served as a source for the most widely used promoter in plant genetic engineering. The 35S promoter of CaMV is capable of directing a high level of transcription in most types of plant tissues. This promoter was used to drive expression of the first transgene introduced into transgenic plants, and it is still the primary choice for expression of transgenes for both research and commercial applications.

Taxonomy and Classification

Members of the *Caulimovirus* genus, which has 11 definitive species (**Table 1**), have recently been assigned to the *Caulimoviridae* family. The virus genome is composed of circular, dsDNA approximately 8.0 kb in length that contains one or more single-stranded discontinuities. The viral DNA is encapsidated into isometric virions, 45–50 nm in diameter. In infected plants most virions are usually

Table 1 Definitive members of the genus *Caulimovirus*

Virus	*Transmission by aphids*	*Host families*[a]	*Distribution*
Blueberry red ringspot (BRRV)	No	*Ericaceae*	North America
Cauliflower mosaic (CaMV)	Yes	*Cruciferae* (*Solanaceae*)	Worldwide
Carnation etched ring (CERV)	Yes	*Caryophyllaceae*	Worldwide
Dahlia mosaic (DaMV)	Yes	*Compositae* (*Amaranthaceae*) (*Chenopodiaceae*) (*Solanaceae*)	Worldwide
Figwort mosaic (FMV)	Yes	*Scrophulariaceae* (*Solanaceae*)	North America
Horseradish latent (HRLV)	Yes	*Cruciferae*	Europe
Mirabilis mosaic (MMV)	Yes	*Nyctaginaceae*	North America
Peanut chlorotic streak (PClSV)	No	*Leguminosae* (*Solanaceae*)	India
Soybean chlorotic mottle (SoyCMV)	No	*Leguminosae*	Japan
Strawberry vein banding (SVBV)	Yes	*Rosaceae*	Nearly worldwide
Thistle mottle (ThMV)	ND	*Compositae*	Europe

ND, not determined.
[a] The plant family that has been found to be infected in nature by a caulimovirus is listed first. The experimental host range is indicated in parentheses.

embedded within amorphous inclusions. The most closely related virus genera are the badnaviruses, which also infect plants, and the hepadnaviruses, which infect birds and mammals. All three genera replicate by reverse transcription of an RNA intermediate and their viral genome does not integrate into host chromosomes as a part of the replication cycle. Two features can be used to distinguish the badnaviruses from the caulimoviruses. Badnaviruses form bacilliform particles rather than icosahedral particles, and the genome organization of the badnaviruses differs considerably from the caulimoviruses. Collectively, the caulimoviruses, badnaviruses, and hepadnaviruses have been called pararetroviruses to emphasize their differences in replication strategy from the true retroviruses.

Geographic Distribution

CaMV, carnation etched ring (CERV) and dahlia mosaic viruses (DaMV) are found worldwide, wherever their hosts are grown (**Table 1**). Strawberry vein banding virus (SVBV) has been reported in North and South America, Australia and Europe. The distribution of most of the other caulimoviruses is limited to a single continent, although this fact may simply reflect an incomplete search for these viruses. Figwort mosaic (FMV) and mirabilis mosaic viruses (MMV) have only been found in limited areas in the USA. In contrast, blueberry red ringspot (BRRV) has been found throughout the USA, where it causes economically significant losses in highbush blueberries. Horseradish latent (HRLV) and thistle mottle viruses (ThMV) have only been found in Europe. Peanut chlorotic streak virus (PClSV) is widely distributed in India, while soybean chlorotic mottle virus (SoyCMV) has only been recovered from a few samples in Japan. The symptoms of SoyCMV, however, are similar to the potyvirus, soybean mosaic virus, and consequently, infections caused by SoyCMV might have been mistaken for other viruses that infect soybean.

Host Range and Virus Propagation

The host range of each of the caulimoviruses is fairly narrow. In nature, caulimoviruses generally infect plants within a single family, although their experimental host range may extend to the members of one or two other families (**Table 1**). The most information about the host range of caulimoviruses has been gained from CaMV, which has been studied more extensively than other caulimoviruses. CaMV can infect a broad range of plants in the cruciferae family, but crucifers are not uniformly susceptible to infection. In fact, the degree of susceptibility of *Brassica* species to CaMV infections is regulated by the host at the level of CaMV RNA that is available for reverse transcription. In *Brassica* species such as turnip (*B. rapa* subspecies *rapifera*), CaMV 35S RNA can

accumulate to a high level. CaMV is primarily propagated in turnips because it infects this host under a broad range of environmental conditions and virions accumulate in infected leaves to higher levels than in other hosts. In contrast to turnips, *Brassica* species such as kohlrabi (*B. oleracea* subspecies *gongylodes*) remain nearly symptomless and only a very low level of viral RNA accumulates in infected cells. Interestingly, the reaction of cauliflower (*B. oleracea* subspecies *botrytis*) to CaMV infection is intermediate between that of turnip and kohlrabi, although CaMV can cause severe losses in cauliflower crops.

Strains of CaMV can also be distinguished by their ability to infect solanaceous species in the genera *Datura* and *Nicotiana*. Some CaMV strains are able to systemically infect several solanaceous species, including *N. bigelovii, N. edwardsonii* and *D. stramonium*, while other CaMV strains elicit a plant defense response that limits the infection to the inoculated leaf. By making exchanges of DNA between the two types of CaMV strains, it has been shown that solanaceous host range is conditioned by a complex interaction between the host, viral strain, and the lighting conditions under which the infected plants are grown. Three separate genes, genes II, IV and VI (**Fig. 1A**), have been shown to determine the ability of CaMV strains to infect *Nicotiana* and *Datura* species systemically. Genes II and IV may affect host specificity through an interaction with the CaMV gene VI rather than through a direct interaction with host factors. In contrast, the gene VI product likely interacts directly with host factors. The gene VI protein of some CaMV strains elicits a hypersensitive defense response (HR) in solanaceous hosts, while other variants of gene VI elicit only chlorotic primary lesions and actually facilitate systemic infection. It is not clearly understood at this time how the CaMV gene VI product mediates the host response.

Taken together, the host range studies in the cruciferae and the solanaceae indicate that at least two mechanisms may operate to limit the host range of CaMV. First the host can regulate the capacity for replication by controlling the level of transcription of viral RNA, which would effectively limit the level of reverse transcription. Second, the host can recognize and target its defense response against individual strains of CaMV to limit their spread to the first infected leaf, while other strains are able to evade the plant defense response.

Genetics

Because the genomes of caulimoviruses are composed of circular dsDNA, it has been relatively easy to clone an infectious form of the entire viral genome into bacterial plasmids using molecular techniques. Consequently, infectious plasmid clones exist for many of the caulimoviruses. In the case of CaMV, infections can be initiated with cloned DNA simply by excising the viral DNA from the vector and gently rubbing this DNA mixture on to turnip leaves that have been dusted with an abrasive. It has been possible to identify symptom and host range determinants of CaMV by exchanging viral DNA segments *in vitro* between different cloned strains. The nucleotide sequences of CaMV strains are fairly homologous, differing from each other by only about 5% on average, and most exchanges between strains will result in an infectious, hybrid virus.

Exchange of genetic material between CaMV strains can also occur quite effectively *in vivo*. Wild-type virus can be readily recovered from plants co-inoculated with viruses that contain lethal mutations in different genes. Several mechanisms have been proposed to describe recombination between CaMV strains, but the most common method is probably a copy choice mechanism in which the reverse transcriptase jumps from one viral template to another in the plant cell. CaMV strains are also adept at acquiring CaMV sequences from transgenic plants. As with recombination between wild-type CaMV strains, the CaMV sequences present in the transgene mRNA can effectively replace the analogous viral sequences and become incorporated into the viral genome during the replication process.

Evolution

All viruses that replicate by reverse transcription have one genomic feature in common. The genes encoding the structural proteins (*gag*) are always arranged upstream from the polymerase (*pol*) genes. Although the position of the *gag* gene is conserved, there is essentially no nucleotide sequence homology between capsid proteins of the caulimoviruses and the true retroviruses. In contrast to the *gag* gene, the *pol* genes of the caulimoviruses have two domains, which specify reverse transcriptase and RNase H functions, that have a significant level of homology with the *pol* genes of the retroviruses. The *pol* gene of the caulimoviruses lacks an intregrase domain, which distinguishes it from the true retroviruses. It has been suggested that the functions required for reverse transcription have been conserved amongst all retroelements, while different virus groups have acquired new genes for infection of their respective hosts. Consequently, the caulimoviruses, have a gene for

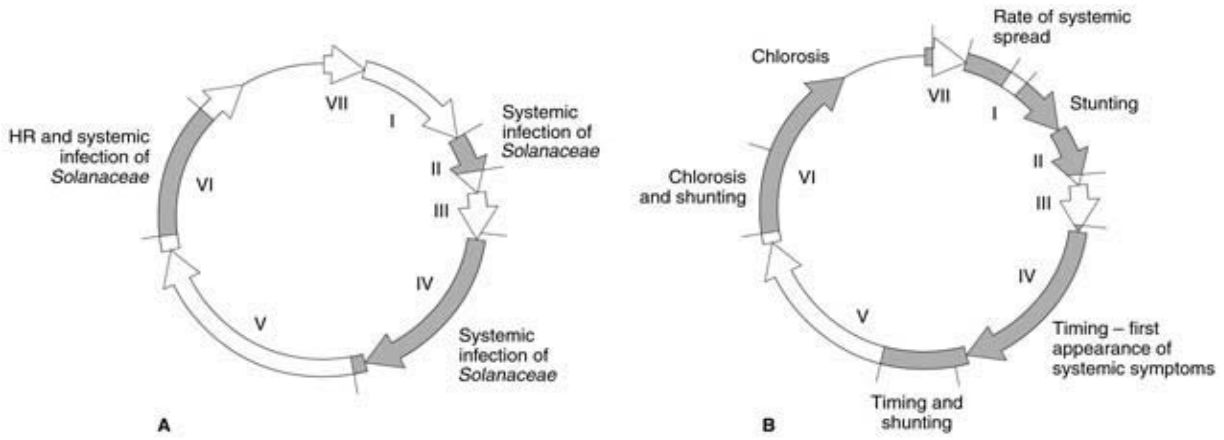


Figure 1 CaMV regions that influence solanaceous host range and pathogenicity. The positions of the genes of CaMV that comprise the circular genome are indicated by the arrows. Gene VII is nonessential and its function is unknown. Gene I encodes a protein required for cell-to-cell movement protein. Gene II encodes a protein required for aphid transmission. Gene III encodes a protein that binds nucleic acids and may be a minor component of the virions. Gene IV encodes the major capsid protein. Gene V encodes a polymerase that has reverse transcriptase, RNase H, and protease activities. Gene VI encodes a protein described as a translational transactivator that is necessary for efficient translation of the polycistronic 35S RNA. (**A**) The CaMV genes that determine the hypersensitive plant response (HR) or influence systemic infection of solanaceous species are delimited by stippling. (**B**) The CaMV regions that influence the development of specific symptoms in turnips are delimited by stippling.

cell-to-cell movement, a gene that is required for the infection of plants by plant viruses.

Caulimoviruses have evolved through the accumulation of point mutations and by recombination between strains. The mutation rate that would occur in the infection cycle in a single plant has been estimated to be $4–6 \times 10^{-4}$ changes per nucleotide. A comparison of the nucleotide sequences of nine isolates of CaMV has indicated that recombination has occurred frequently between strains in nature. A phylogenetic analysis of the sequences, which represent CaMV strains collected worldwide, also indicated that the viruses could be divided into two distinct lineages: North American and non-North American. It was concluded that the major factor that determines relatedness is the host geographic distribution.

Serologic Relationships and Variability

CaMV is serologically related to DaMV, HRLV, CERV and SVBV. Although CaMV and HRLV both infect crucifers, HRLV virions react only weakly to CaMV antiserum, evidence that HRLV is distinctly different from CaMV and not simply a CaMV strain. Interestingly, although DaMV and CaMV virions are closely related serologically, nucleic acid hybridization experiments indicate only a low level of homology. No antigenic relationships have been found between the other caulimoviruses. Virions of BRRV do not crossreact with CaMV antiserum, and MMV virions are unrelated serologically to CaMV, CERV or DaMV. SoyCMV is not serologically related to CaMV, CERV or even to PClSV, the other caulimovirus that infects legumes.

Transmission and Tissue Tropism

Most caulimoviruses are transmitted in a relatively nonspecific manner by as many as 27 species of aphids. The mode of transmission has been characterized as 'semipersistent'. The virions are acquired rapidly by the aphid, and can be transmitted immediately upon acquisition. Virions can be maintained by the aphid for as little as 5 h up to 3 days, but are not retained after the aphid molts and are thus not passed on to aphid progeny. The 18 kDa aphid transmission factor (ATF), encoded by gene II (**Fig. 1**), is required for transmission by aphids.

Although it is not known exactly how P18 mediates aphid transmission, it has been demonstrated for CaMV that P18 can bind to virions. It has been suggested that P18 may protect the virion from proteolytic enzymes in the aphid, or may function to bind the virion to the aphid stylet. Neither SoyCMV, BRRV, nor PClSV appear to be transmitted by aphids. It is not known if alternate vectors exist for these viruses. Interestingly, an analysis of the PClSV genomic sequence has shown that it does not have a gene homologous to the aphid transmission factor of CaMV.

Caulimoviruses are not transmitted by seed or

pollen, but most, with the exception of SVBV and blueberry red ringspot virus, can initiate infections after mechanical inoculation. DaMV has been shown to be transmitted by vegetative propagation of dahlia tubers, while BRRV has been transmitted through grafting and may be spread primarily through the use of infected plant material.

CaMV does exhibit some degree of tissue tropism, as virions accumulate to much higher levels in leaves than in stems and roots. An analysis of the viral nucleic acids present in root tissues indicates that roots contain relatively high levels of supercoiled viral DNA, but very low levels of viral RNA transcripts. Low transcript levels would in turn lead to a reduction in reverse transcription into viral DNA and reduced accumulation of virions. In contrast to roots, leaves contain low amounts of supercoiled DNA, high levels of viral transcripts and high levels of virions. Therefore, it has been suggested that transcription of the supercoiled DNA, a key point in the replication cycle of CaMV, is differentially regulated by leaves and roots.

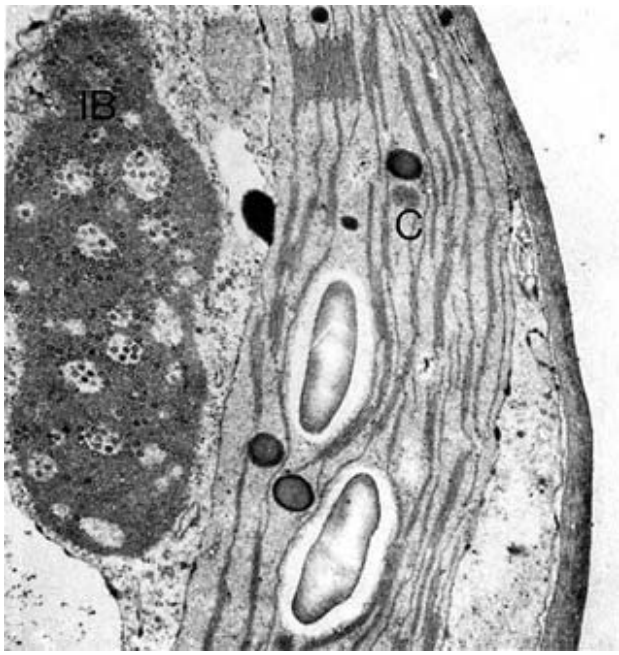


Figure 2 Amorphous inclusion body of cauliflower mosaic virus. IB, an amorphous, vacuolated inclusion body with CaMV virions embedded within it; C, a chloroplast. (Courtesy of Robert Shepherd.)

Pathogenicity

Caulimoviruses induce a variety of systemic symptoms in their hosts, from ringspots to necrosis and mosaics. With the exception of CaMV, very little is known about which viral genes are responsible for symptoms or how these viruses cause disease. In the case of CaMV, several studies have focused on mapping the CaMV genes responsible for specific symptoms. It is important to note, however, that the assignment of symptom determinants reflects the differences between the two CaMV strains whose genomes are exchanged. Therefore, although symptom characters have been associated with specific regions of the CaMV genome, other CaMV regions, which might be revealed in exchanges with additional CaMV strains, could also be involved. Furthermore, the environment can have a significant impact on the development of symptoms. It has been shown that some cauliflower varieties that exhibit severe symptoms in the greenhouse may have only mild symptoms in the field, and the opposite situation has also been found. Nevertheless, the mapping studies do provide a starting point for understanding how CaMV induces symptoms in its hosts.

Several studies are in agreement that gene VI of CaMV influences the intensity of chlorotic symptoms that typify an infection in turnips (**Fig. 1B**). In addition to gene VI, chlorosis was influenced by sequences within the large intergenic region. Gene VI can also affect the degree of stunting in turnips, perhaps by regulating the expression of other viral genes. Interestingly, gene VI is one of the few plant virus genes that elicits virus-like symptoms when expressed in transgenic plants, and these symptoms largely match those associated with gene VI expressed from the viral genome. Transgenic plants that express the gene VI product exhibit chlorosis and mottling, stunting and necrosis.

Other regions of CaMV have been found to influence the timing and severity of symptoms. The severity of stunting in turnips is affected by sequences within portions of genes I and II and gene V, in addition to gene VI (**Fig. 1B**). The initial appearance of systemic symptoms, characterized as either a rapid or delayed systemic infection, was determined by sequences within genes III, IV or V. The rate of spread of the virus to other leaves, after the first appearance of systemic symptoms, was determined by sequences within genes VII and I. Further research will now be directed towards determining whether the viral gene products interact directly with the host to elicit a specific symptom or whether the viral gene may alter some aspect of viral replication that subsequently alters the symptoms in the host.

Pathology

The most prominent characteristic noted within caulimovirus-infected cells is the presence of amorphous inclusions (**Fig. 2**). The inclusion bodies are

not bound by a membrane, can range in diameter from 5–20 μm, and occur in virtually all types of plant cells. The inclusions can be visualized with a light microscope in strips of epidermal tissue after staining with phloxine B, and are a diagnostic feature of CaMV infections. Usually infected plant cells contain just one or two inclusion bodies. Close examination of these amorphous inclusion bodies using electron microscopy reveals two types which can be easily differentiated. One type contains many vacuoles and consists of an electron-dense, granular matrix that is composed primarily of the CaMV gene VI protein. A second, electron-lucent, type is made up of the CaMV 18 kDa ATF protein. In addition to the electron-lucent inclusions, the ATF protein of CaMV is capable of forming paracrystalline structures that colocalize with plant microtubules. Most virus particles are associated with either one type of amorphous inclusion or the other, but a few virions can be found free in the cytoplasm, within enlarged plasmodesmata, or in the nucleus. In one case, virions of CaMV strain CM4-184 have been localized within chloroplasts.

A second unusual characteristic of caulimovirus infections is that the plasmodesmata between infected cells can become enlarged, and virions of DaMV and CaMV have been found within these enlarged plasmodesmata. The presence of CaMV virions in the plasmodesmata has led to the suggestion that CaMV virions may move from cell to cell in the form of virions, although an alternative model postulates that CaMV moves into adjacent cells in the form of ribonucleoprotein complexes. The alteration in plasmodesmata size is dependent upon the CaMV gene I product, a cell-to-cell movement protein. In addition, the gene I product induces the formation of long tubular structures that extend away from the cell surface in studies with infected plant protoplasts. The tubular extensions are not unique to CaMV, as they have also been observed in protoplasts infected with cowpea mosaic virus and red clover mottle comovirus.

Epidemiology and Control

Caulimovirus infections of economically important hosts are initiated by viruliferous aphids or through grafting or planting of infected plant material. CaMV is most likely to cause a problem in areas where successive crops of crucifers are grown. Virus surveys of brassica crops in Britain indicated that the two viruses encountered most frequently were CaMV and the potyvirus, turnip mosaic virus. CaMV has been considered to be the most important virus disease of winter cauliflower in Britain. CaMV symptoms are most severe under low temperature conditions. In addition, high levels of sunshine inhibit virus infections. SVBV infections of strawberry are considered to be a minor problem, but the virus can cause a greater degree of loss when present in plants with other viruses, such as strawberry crinkle virus.

Diseases caused by caulimoviruses can be controlled using the same strategies that are effective for most plant viruses. Sources of resistance to CaMV in cabbages were identified as early as 1951, while resistance to CaMV in Brussels sprout has recently been found. Although no cauliflower varieties are completely resistant, the symptoms of some varieties are milder than others. In addition to resistance, cross protection, the deliberate inoculation of a mild strain to protect against more severe strains, has been demonstrated in Brussels sprout. Some effort has been directed towards creating transgenic cauliflower plants resistant to CaMV infection. Transgenic cauliflower plants that express the capsid gene and an antisense construct of gene VI have been created and are now being evaluated for their ability to resist CaMV infection. In the case of BRRV, DaMV and SVBV, it is important to start with virus-free planting material. Insecticide sprays to control the spread of the aphid vector are recommended for DaMV and SVBV, but are not considered economically feasible for CaMV.

Future Perspectives

Research on the caulimoviruses will continue to contribute to a general understanding of how viruses replicate and express their genomes, and also how plant viruses cause disease. The 35S RNA is considered to be one of the most complex eucaryotic, polycistronic messages. Although much has been learned about its expression, there are still gaps in our understanding. For example, the gene VI product is required for translation of the 35S RNA, but it is not known how it influences the host translational apparatus. The caulimoviruses will also add to our knowledge of how viruses cause disease. The host range and symptom determinants of CaMV have been identified. The challenge now will be to determine how these viral genes interact with the host. Perhaps the best approach to understanding how plant virus genes influence host range and symptomatology would be to identify their host counterparts through traditional plant breeding or through mutagenesis and then to isolate the gene(s) by map-based cloning techniques. One of the hosts of CaMV is *Arabidopsis*, a favorite plant of molecular biologists because of its ease of manipulation and small genome size. Thus, it may be possible to select *Arabidopsis* mutants or

natural variants that respond in an altered manner to pathogenesis by CaMV, and these plants may be used to explain how viruses cause disease.

In addition to contributing to molecular virology, it is likely that new strategies for controlling diseases caused by caulimoviruses will emerge. Although plants have been genetically engineered to resist infection by many types of plant viruses, genetic engineering has not been used yet to create transgenic plants with resistance to caulimoviruses. As transgenic plants have already been made that express antisense or sense versions of CaMV genes, it is possible that genetic engineering for control of caulimoviruses will be an option in the near future.

See also: **Plant pararetroviruses: Badnaviruses; Plant pararetroviruses (*Caulimoviridae*): Caulimoviruses: molecular biology; Pathogenesis: Plant viruses; Plant resistance to viruses: Natural resistance; Vectors: Plant viruses.**

Further Reading

Covey SN (1991) Pathogenesis of a plant pararetrovirus: CaMV. *Semin. Virol.* 2: 151.

Hull R (1984) Caulimovirus group. *CMI/AAB Descriptions of plant viruses*, no. 295. Kew: Commonwealth Mycological Institute/Association of Applied Biologists.

Matthews REF (1991) *Plant Virology* 3rd edn. New York: Academic Press.

Rothnie HM, Chapdelaine Y and Hohn T (1994) Pararetroviruses and retroviruses: a comparative review of viral structure and gene expression strategies. *Adv. Virus Res.* 44: 1.

Schoelz JE, Qiu SG and Wintermantel WM (1995) Genetic and environmental factors that determine the ability of cauliflower mosaic virus to systemically infect solanaceous hosts. In: Bills DD and Kung S (eds) *Biotechnology and Plant Protection: Viral Pathogenesis and Disease Resistance*, p. 313. Singapore: World Scientific Publishing.

Caulimoviruses – Molecular Biology

Thomas Hohn, Frederick Miescher Inst., Basel, Switzerland

Properties of the Virion and Inclusion Bodies

Caulimoviruses are icosahedral ($T = 7$), 45–50 nm in diameter and sediment between 200 and 250S. The main capsid components of the type member cauliflower mosaic virus (CaMV) are proteins with mobilities of 37 and 44 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis derived from open reading frame (ORF) IV. In addition, CaMV particles contain minor amounts of other polypeptides, a 15 kDa nucleic acid binding protein derived from ORF III, protease and reverse-transcriptase derived from ORF V, and host casein kinase II. In cells infected with CaMV or a number of other caulimoviruses large numbers of virus particles accumulate in typical stable inclusion bodies, the matrix of which is the virus-encoded multifunctional inclusion body protein of 62 kDa, derived from ORF VI. Inclusion bodies are visible in the light microscope and can be stained with phloxin.

Properties of the Genome

The caulimoviral genome is built from 7200–8200 bp. It exists in infected plant nuclei as supercoiled DNA bound to nucleosomes (minichromosome), in infected cytoplasm as RNA with a 180 nucleotides (nt) terminal redundancy, and in virus particles as open circular ds DNA. The open form is due to nicks at specific sites of the (+)- and (−)-strand with short 5′ overlaps (**Fig. 1**), remnants of the reverse transcription process, which are apparently removed in the infected nucleus.

The sequence of many of the caulimoviruses is known. According to sequence and ORF arrangement four groups of caulimoviruses can so far be distinguished: CaMV-like, PCSV-like, CVMV and PVCV (**Table 1** and **Fig. 2**). CaMV-like caulimoviruses contain seven ORFs in the order VII (dispensable, unknown function), I (cell-to-cell movement), II (aphid transmission), III (nucleic acid binding protein), IV (capsid protein precursor), V (protease, reverse transcriptase) and VI (multifunctional protein [inclusion body matrix protein, translation transactivator]). PCSV-like caulimoviruses differ by containing three (A, B, C), instead of two ORFs between ORFs I and III (**Fig. 2**). The ORF arrangements in

acterization of cassava vein mosaic virus: a distinct plant pararetrovirus. *J. Gen. Virol.* 76: 1271.

Hohn T and Fütterer J (1991) Pararetroviruses and retroviruses: a comparison of expression strategies. *Semin. Virol.* 2: 55.

Hull R (1992) Genome organization of retroviruses and retroelements: evolutionary considerations and implications. *Semin. Virol.* 3: 373.

Kiss-Laslo Z, Blanc S and Hohn T (1995) Splicing of cauliflower mosaic virus 35S RNA is essential for viral infectivity. *The EMBO Journal* 14: 3552–3562.

Kochko de A, Verdaguer B, Taylor N, Carcamo R, Beachy RN and Fauquet C (1998) Cassava vein mosaic virus (CsVMV), the type species for a new genus of double stranded DNA plant virus? *Archives of Virology* 143, 945–962.

Laco G, Kent SBH and Beachy RN (1995) Analysis of the proteolytic processing and activation of the rice tungro bacilliform virus reverse transcriptase. *Virology* 208: 207.

Verdaguer B, Kochko de A, Fux CI, Beachy RN and Fauquet C (1998) Functional organization of the cassava vein mosaic virus (CsVMV) promoter. *Plant Molecular Biology* 37: 1055–1067.

Legume Caulimoviruses

D. V. R. Reddy, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, India

R. D. Richins, Department of Plant Pathology, University of California, Riverside, California, USA

History

The occurrence of a legume caulimovirus was first reported in 1980. The virus, subsequently identified as Peanut chlorotic streak virus (PC1SV), was found to occur naturally on peanut (groundnut, *Arachis hypogaea*) in the state of Andhra Pradesh in India. The first comprehensive report on a legume caulimovirus, Soybean chlorotic mottle (SoyCMV), was published in 1984. The virus was observed during surveys in Aichi Prefecture in 1981. To date PC1SV and SoyCMV appear to be the only caulimoviruses which were reported to occur under natural conditions on legumes.

Taxonomy and classification

DNA-containing plant viruses are included under the family *Caulimoviridae*. PC1SV and SoyCMV are in the genus Caulimovirus.

Virion Structure and Proteins

Virus particles are icosahedrical, c. 50 nm in diameter. Similar to other caulimoviruses they produce inclusions in cytoplasm which are circular or ovoid and consist of an amorphous, vacuolated, electron-dense matrix. The virus particles are embedded in the matrix.

Strains

No strains have so far been reported for SoyCMV. However a strain of PC1SV, designated as 'Chlorotic Vein-banding' strain (PC1SV-CVB) has been reported, which produces distinct symptoms on peanut. It differs from PC1SV in host range, symptoms and also in the physical map.

Geographic Distribution

SoyCMV occurs in Japan although its distribution in the various Prefectures in Japan is currently not known. PC1SV occurs in the states of Andhra Pradesh, Karnataka, Maharasthra and Tamil Nadu in India.

Symptoms and Host Range

Symptoms are here only given for the principal hosts. PC1SV in peanut causes reduced leaflets, chlorotic streaks and stunting. Symptoms produced by SoyCMV on soybean are mosaic or mottle on leaves and stunting of plants. Some soybean cultivars have been reported to produce different symptoms. A typical feature with both the viruses is the appearance of symptoms after three weeks of inoculation. The host range is given in **Table 1.**

Serological Relationships

PC1SV and SoyCMV do not react serologically with each or with a number of other caulimoviruses which include cauliflower mosaic (CaMV), figwort virus (FWV) or carnation etched ring virus (CERV). The serological tests employed were ELISA and agar gel double diffusion.

Transmission

PC1SV and SoyCMV are readily transmitted by mechanical sap inoculations. Although the majority of caulimoviruses are transmitted by aphids, attempts to transmit PC1SV or SoyCMV, both nonpersistently and persistently, by a range of aphids have so far been unsuccessful. PC1SV could not be transmitted by *Aphis craccivora* or *Myzus persicae* and SoyCMV by *Acyrthosiphon pisum, A. craccivora, Aulacorthum*

Table 1 Host range of PC1SV and SoyCMV

Host	*PC1SV*	*SoyCMV*
Arachis hypogaea	+	–
Canavalia ensiformis	+	NA[a]
Cyamopsis tetragonoloba	+	NA
Datura stramonium	+	–
Glycine max	+[b]	+[c]
Dolichos lablab	NA	+
Nicandra physaloides	+	NA
Nicotiana benthamiana	+	NA
N. clevelandii	+	–
N. edwardsonii	+	NA
N. glutinosa	+	–
N. rustica	+	NA
N. tabacum	+	–
Petunia hybrida	+	–
Phaseolus vulgaris	+	+
Spinacia oleracea	+	–
Vigna radiata	+	–
V. unguiculata	+	+

[a] Data not available.
[b] Veinal necrosis followed by systemic symptoms of chlorotic spots and veinal necrosis.
[c] Mosaic or mottle and leaf curling on some cultivars.

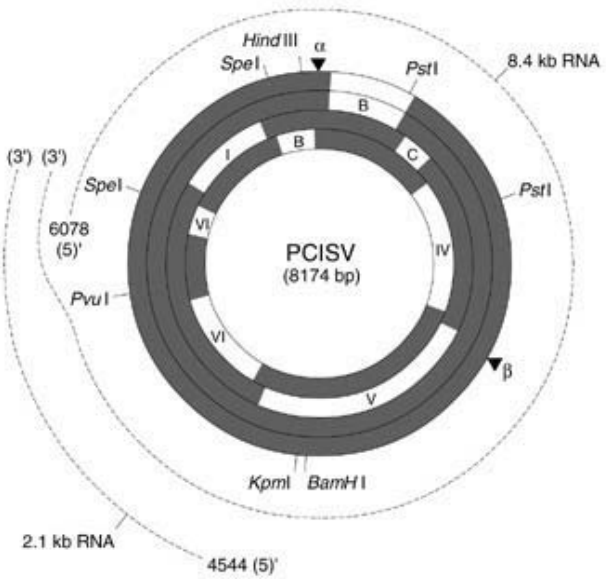


Figure 1 Gene and transcript map of PC1SV DNA. The position of the major open reading frames as they occur around the circular, PC1SV DNA are indicated. The ORFs are identified based on observed relationships with spatially homologous ORFs occurring in other caulimovirus genomes. The location of the single-stranded discontinuities (▼ and ▼) are also shown. The two major mRNA transcripts are illustrated by the dashed lines. Selected restriction endonuclease sites are also indicated.

solani or *M. persicae*. Neither of the viruses are seed transmitted.

Molecular Biology

With respect to genomic organization, gene expression and gene products, there are a variety of features which distinguish the legume caulimoviruses from other *Caulimoviridae*. First, the circular, double-stranded DNA genomes of SoyCMV and PC1SV contain 8175 and 8174 bp, respectively, making them 140–430 bp larger than the genomes of other caulimoviruses. The CVB strain of PC1SV appears also to contain about 8.2 kb of DNA, although a number of restriction site polymorphisms have been observed. SoyCMV and PC1SV appear to encode for eight major open reading frames (ORFs) compared to seven observed in CaMV, CERV and FMV (**Fig. 1**). The additional ORF occurs between ORF I (the movement protein) and ORF IV (the major capsid protein). For both SoyCMV and PC1SV, the three ORFs which occur between ORFs I and IV show little or no apparent similarity to ORFs observed in other caulimoviruses. Therefore they have been designated Ib, II and III for SoyCMV and A, B and C for PC1SV.

The location of the tRNA primer binding site (PBS), utilized during genomic replication, is also distinctive among the legume caulimoviruses. The PBS occurs 3′ to the movement protein (ORF I) in the genomes of SoyCMV and PC1SV. In contrast, the PBS occurs 5′ to the movement protein gene in the genomes of CaMV, CERV and FMV.

Transcription

Another distinguishing feature of the legume caulimoviruses is that their genomes contain only one intergenic region (IGR) which occurs between ORFs VI and VII. The IGR contains several putative regulatory domains which are also observed in the larger IGR of other caulimoviruses. The small IGR which occurs upstream of gene VI in the genomes of CaMV, CERV and FMV is not observed in the SoyCMV and PC1SV genomes. Nevertheless, two major transcripts, of 2.1 and 8.4 kb, respectively, have been observed in PC1SV-infected hosts (see **Fig. 1**). The larger transcript is likely a terminally redundant, replicative intermediate RNA. As with the other caulimoviruses, most of the polypeptides encoded by the PC1SV (and probably SoyCMV) genome are probably translated from this '35S-like' RNA. The smaller transcript hybridizes specifically to ORF VI of PC1SV, and thus appears to be an ORF VI subgenomic mRNA. A 5.7 kb minor transcript is also observed which may specify ORF V. A strong promotor within ORF III (about 200 bp 3′ to the start of ORF IV) has been

identified in the SoyCMV genome. However, transcript data are currently unavailable for SoyCMV, and consequently the role of this promotor during virus infection is yet to be established.

Gene products

Based on similarities in the deduced amino acid sequence and on mutational analyses, ORFs I, IV, V and VI of SoyCMV and PC1SV appear to specify the viral movement protein, major capsid protein, reverse transcriptase and transactivating proteins, respectively. Immunosorbent polymerase chain reaction (PCR) technique has been used to confirm the involvement of the ORF I protein in virus movement. Mutations to ORF I were shown to abolish virus movement, but not replication.

Limited amino acid sequence similarity is also observed between the PC1SV ORF I polypeptide, and the 30 kDa movement protein of tobacco mosaic virus (TMV). Interestingly, the cell-to-cell movement functions in PC1SV have been successfuly used to complement the movement of a 30 kDa-mutant of TMV.

A variety of mutation analyses have demonstrated that ORFs A and B are not essential for PC1SV replication or movement. Mutations to ORFs IV, V, VI and C are usually lethal. Similar studies have not yet been performed on SoyCMV. Nevertheless, based on the characteristically high proline content of their translation product, it seems likely that ORF C of PC1SV and ORF III of SoyCMV may encode the minor capsid protein. A low degree of similarity was observed between the putative ORF B protein of PC1SV and the ORF II protein of SoyCMV. Nevertheless, the role of these gene products, as well as the gene products of ORF 1b of SoyCMV and ORF A of PC1SV are yet to be determined.

The salient features of ORFs IV and V of SoyCMV and PC1SV are essentially the same as that for the other caulimoviruses. However, the degree of relatedness differs markedly with respect to ORF VI. The predicted amino acid sequence of the SoyCMV and PC1SV ORF VI (transactivating) products are significantly smaller than that of the other caulimovirus ORF VI proteins. The putative ORF VI gene products for SoyCMV and PC1SV are 53 and 49 kDa, respectively, compared with 56–58 kDa for CaMV, CERV and FMV ORF VI. In addition, the PC1SV gene VI protein shows little sequence to the CaMV, CERV and FMV homologues; however, a notable degree of similarity is evident between the gene VI proteins of PC1SV and SoyCMV. Phylogenetic analyses have been performed to determine the evolutionary relationships between the various plant pararetroviruses. One such analysis based on the relationships of the reverse transcriptase domains is shown in **Fig. 2**. The cladogram clearly shows that SoyCMV and PC1SV form a distinct clade with 100% of the trees containing the branch in 100 bootstrap replications.

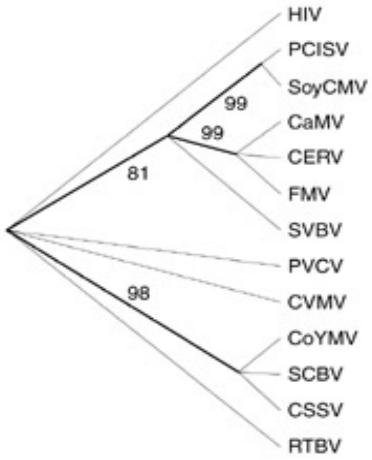


Figure 2 Cladogram depicting the phylogenetic relationships of conserved domains within the reverse transcriptase genes of members of the *Caulimoviridae* (rooted to a similar domain within the *pol* gene of human immunodeficiency virus (HIV). Parsimony was employed to generate a 70% consensus tree using the PAUP 3.1 program. Numbers above selected branches indicate the percentage of trees containing the branch for the 100 bootstrap replications.

Transactivation

The caulimoviruses have evolved a rather unique mechanism to express their genomes. Most viral gene products are translated from a single, polycistronic mRNA in caulimovirus-infected hosts in apparent violation of the 5′ proximal rule. The mechanism of this so-called translational *trans*-activation is not completely understood, but substantial evidence has been accumulated to support the involvement of the gene VI polypeptide for CaMV and FMV. For the study of transactivation by the PC1SV ORF VI protein, *Nicotiana edwardsonii* suspension cells and a 'nested' GUS reporter gene have been used. Transactivation was much stronger at 35°C than at 24°C (relative to a PC1SV 35S-GUS control). This observation may begin to provide a molecular explanation for the high temperature required by PC1SV to systemically infect many of its hosts. Support for the temperature-dependent transactivation of PC1SV came from studies which utilized FMV to complement the low temperature (25°C) systemic spread of PC1SV in doubly infected *Datura inoxia* plants. Systemic spread was not observed when PC1SV gene I mutants were co-inoculated with FMV suggesting that the FMV cell-to-cell transport

functions alone were not responsible for the complementation.

Control

PC1SV-CVB isolate was found to be economically important in some of the peanut growing areas in the state of Andhra Pradesh. Reports on the economic importance of SoyCMV are not available. No efforts have so far been made to devise any strategies for controlling PC1SV or SoyCMV.

See also: **Plant pararetroviruses: Badnaviruses; Plant pararetroviruses (*Caulimoviridae*): Cassava vein mosaic virus, Caulimoviruses: general features, Caulimoviruses: molecular biology; Plant pararetroviruses: Rice tungro bacilliform virus.**

Further Reading

Ducasse DA, Mushegian AR and Shepherd RJ (1995) Systemic infection of solanaceous hosts by peanut chlorotic streak caulimovirus is temperature dependent and can be complemented by coinfection with figwort mosaic caulimovirus. *Phytopathology* 85: 286.

Hasegawa A, Verver J, Shimada A *et al* (1989) The complete sequence of soybean chlorotic mottle virus DNA and the identification of a novel promoter. *Nucleic Acids Res.* 17: 9993.

Iwaki M, Isogawa Y, Tsuzuki H and Honda Y (1984) Soybean chlorotic mottle, a new caulimovirus on soybean. *Plant Dis.* 68: 1009.

Mushegian AR, Wolff JA, Richins RD and Shepherd RJ (1995) Molecular analysis of the essential and non-essential genetic elements in the genome of peanut chlorotic streak caulimovirus. *Virology* 206: 823.

Reddy DVR, Richins RD, Rajeshwari R *et al* (1993) Peanut chlorotic streak virus, a new caulimovirus infecting peanuts (*Arachis hypogaea*) in India. *Phytopathology* 83: 129.

Richins RD, Broos T, Ducasse A *et al* (1998) Organization and transcription of the peanut chlorotic streak virus genome. *Arch. Virol.* (in press).

Satyanarayana T, Broglio EP, Sreenivasulu P *et al* (1995). Genome characterization of a new strain of peanut chlorotic streak virus causing chlorotic vein banding disease of groundnut (*Arachis hypogaea* L.) in India. *Plant Pathol.* 44: 966.

PLANT PARARETROVIRUSES

Contents

Rice Tungro Bacilliform Virus

Roger Hull, John Innes Centre, Norwich Research Park, Colney, Norwich, UK

General Features

Rice tungro disease was first recognized in the experimental fields at the International Rice Research Institute, The Philippines in 1963 and identified as a distinct virus disease in 1965. However, previous descriptions of disorders in rice (*Oryza sativa*) would indicate that it has been present in the Philippines, and probably elsewhere in southeast Asia from at least the 1930s. Since its recognition as a virus disease, tungro has been reported in most of the rice-growing countries of south and southeast Asia (**Fig. 1**).

Tungro is unusual in that it has a multitude of local names, there being at least 40 names including 'tungro' and 'accep na pula' in The Philippines and names such as 'penyakit merah' in Malaysia and 'mentek' in Indonesia; this plurality of names reflects the importance with which farmers regarded the disease. The disease is generally endemic but has caused periodic major outbreaks in various countries in this region. Much of the increase in impact of tungro has been attributed to the 'green revolution', which resulted in large areas of rice with high fertilizer inputs and overlapping cropping of new susceptible cultivars, conditions highly suitable to rapid spread of the viruses. Overall this disease is now considered to be one of the major constraints to rice production in this region, with annual losses in excess of \$US 1.5×10^9; this does not include the costs of insecticides to control the virus vector.

It was not until 1978 that it was realized that tungro disease is caused by a complex of two viruses, rice

breeder should attempt to predict the likely durability of a resistance gene, by testing it against as many isolates of the virus as possible, at an early stage of the breeding program.

However, another problem is that the genetic base of resistance to plant viruses is rather limited, and in many crops no resistance to particular diseases is known. Screening of wild relatives and gene bank collections will undoubtedly reveal further natural resistance genes, although at a cost. The various techniques for developing resistance by introduction of transgenes offers an excellent prospect of broadening the basis of control.

See also: **Vector transmission of plant viruses; Pathogenesis: Plant viruses; Plant resistance to viruses: Engineered resistance; Plant virus disease – economic aspects; Transformation: Plant viruses; Vectors: Plant viruses.**

Further Reading

Brunt AA, Crabtree K, Dallwitz MJ, Gibbs AJ and Watson L (1996) *Viruses of Plants: Descriptions and Lists from the VIDE Database*. Wallingford: CAB International.

Fraser RSS (1990) Genetics of resistance to viruses. *Annu. Rev. Phytopathol.* 28: 179.

Hadidi A, Khetarpal RK and Koganezawa H (eds) (1998) *Plant Virus Disease Control*. St Paul: American Phytopathological Society Press.

Lartey R, Ghoshroy S, Sheng J and Citovsky V (1997) Transport through plasmodesmata and nuclear pores: cell-to-cell movement of plant viruses and nuclear import of *Agrobacterium* T-DNA. In: McCrae MA, Saunders JR, Smyth CJ and Stow ND (eds) *Molecular Aspects of Host–Pathogen Interactions*. Society for General Microbiology Symposium Series, Vol. 55, p. 253. Cambridge: Cambridge University Press.

Matthews REF (1991) *Plant Virology*, 3rd edn. San Diego and London: Academic Press.

Slusarenko A, Fraser RSS and Van Loon LC (eds) (1999) *Mechanisms of Resistance to Plant Diseases*, 2nd edn. Dordrecht: Kluwer.

Engineered Resistance

C Michael Deom, University of Georgia, Athens, Georgia, USA

Introduction

Engineered resistance (or genetically engineered resistance) to viral pathogens refers to the introduction and expression of foreign genes, termed transgenes, in plants such that expression of a transgene interferes with the virus life cycle and thereby confers resistance to the virus. Advances in plant transformation technologies for a variety of plant species have resulted in the introduction of transgenes from a variety of sources that confer resistance to viral pathogens. The transgenes are stably integrated into the plant genomes, are heritable, and resistance segregates with the transgenes. In many cases, transgenic plants have been generated that have resistance to viruses by mechanisms that do not occur naturally and could not have been introduced into the plants by traditional breeding techniques. Genetically engineered virus resistance in crop plants shows great promise in efficiently reducing losses due to virus disease and complements traditional plant breeding.

Transgenes are designed as chimeric genes under the control of a promoter and contain nonviral sequences at the 3′ end to provide transcription termination and polyadenylation signaling. With few exceptions the preferred promoter has been the 35S promoter from the plant DNA virus cauliflower mosaic virus, which is constitutively expressed and drives high levels of expression of transgenes in most plant tissues. In some cases, the transgene is placed under the control of a tissue-specific promoter to localize expression of the transgene.

Pathogen-derived Resistance

The transformation of plants to confer virus resistance was an initial focus of plant biotechnology. The most widely applied and effective strategy presently used for this purpose is pathogen-derived resistance (PDR): resistance obtained by expressing viral-derived transgenes (genes or genomic sequences) in transgenic plants (**Table 1**). This strategy has provided some degree of virus resistance in almost all hosts to which it has been applied. In all cases, the level of PDR is variable, depending on both the type of virus from which the transgene sequence is derived and on the individual transgenic plant line. In some instances, transgenic plants have been developed that are essentially immune to virus infection. Therefore, biologically screening a number of transgenic plant lines is required to identify those having the highest levels of genetically engineered virus resistance and acceptable agronomic traits. Unfortunately, the resistance mechanisms are poorly understood at the cellular and molecular levels. PDR can be divided into two broad categories, depending on whether the resistance mechanism is mediated by protein or nucleic acid. In some instances it is not clear whether the resistance is conferred by expression of the protein or the nucleic acid encoded by the transgene and in

Table 1 Examples of pathogen-derived, genetically engineered virus resistance

Virus genus	*Virus*[a]	*Transgene*[b] *sequence*	*Host*
Alfamovirus	Alfalfa mosaic virus	CP	Alfalfa, tobacco, tomato
Begomovirus	African cassava mosaic	Rep	*Nicotiana benthamiana*
	Tomato mottle virus	MP	Tobacco
	Tomato yellow leaf curl virus	CP	Tomato
Bromovirus	Brome mosaic virus	MP	Tobacco
Carlavirus	Poplar mosaic virus	CP	*N. benthamiana*
	Potato virus S	CP	Potato, *N. debneyii*
Comovirus	Bean pod mottle virus	CP	Soybean
	Cowpea mosaic virus	MP	*N. benthamiana*
		Rep	*N. benthamiana*
Cucumovirus	Cucumber mosaic virus	CP	Cantalope, cucumber, petunia, squash, tobacco, tomato
		Rep	Tobacco
		satellite	Tobacco, tomato, petunia
Luteovirus	Potato leafroll virus	CP	Potato
		MP	Potato
		Rep	Potato
Nepovirus	Arabis mosaic virus	CP	Tobacco
	Grapevine chrome mosaic virus	CP	Tobacco
	Grapevine fanleaf virus	CP	Tobacco
	Tobacco ringspot virus	CP	Tobacco
		satellite	Tobacco
Potexvirus	Cymbidium mosaic virus	CP	*N. benthamiana*
	Potato virus X	CP	Potato
		Rep	Tobacco
	Potato acuba mosaic virus	CP	Tobacco
	White clover mosaic virus	MP	*N. benthamiana*
Potyvirus	Bean yellow mosaic virus	CP-3′	*N. benthamiana*
	Lettuce mosaic virus	CP	Lettuce, tobacco
	Maize dwarf mosaic virus	CP	Corn
	Papaya ringspot virus	CP	Papaya, plum, tobacco
	Passionfruit woodiness virus	CP	Tobacco
	Peanut stripe virus	CP	*N. benthamiana*
	Plum pox virus	CP	*N. clevelandi, N. benthamiana,* plum, tobacco
	Potato virus Y	CP	Potato, tobacco
		Rep	Tobacco
		NIa	Tobacco
	Soybean mosaic virus	CP	Tobacco
	Tobacco etch virus	CP	Tobacco
		6-kDa/VPg	Tobacco
	Tobacco vein mottling virus	CP	Tobacco
	Vanilla necrosis virus	CP	*N. benthamiana*
	Watermelon mosaic virus 2	CP	Cantalope, *N. benthamiana,* squash
	Zucchini yellow mosaic virus	CP	Squash, cantalope, *N. benthamiana,* tobacco
Tenuivirus	Rice stripe virus	CP	Rice
Tobamovirus	Tobacco mosaic virus	CP	Tobacco, tomato
		MP	Tobacco
		Rep	Tobacco
Tobravirus	Tobacco rattle virus	CP	Tobacco
	Pea early-browning virus	Rep	*N. benthamiana*
Tombusvirus	Cymbidium ringspot virus	Rep	*N. benthamiana*

Table 1 Continued

Virus genus	*Virus*[a]	*Transgene*[b] *sequence*	*Host*
Tospovirus	Groundnut ringspot virus	N	Tobacco
	Tomato chlorotic spot virus	N	Tobacco
	Tomato spotted wilt virus	N	Chrysanthemum, *N. benthamiana*, lettuce, tobacco, tomato
		NSm	Tobacco
	Impatiens necrotic spot virus	N	*N. benthamiana*
Tymovirus	Turnip yellow mosaic virus	3′	Rapeseed

CP, coat protein; CP-3′ coat protein plus adjacent 3′ untranslated region; MP, movement protein; N, nucleoprotein; NSm, nonstructural protein encoded on the middle RNA; Rep, replicase; VPg, virus protein genome linked; 3′, 3′ untranslated region.
[a] Virus from which the transgene sequence was derived.
[b] Virus sequence or portion of virus sequence introduced into transgenic plants.

some cases both mechanisms may function. The discussion below is based on interpretations and on conclusions drawn from supportive or suggestive evidence characteristic of either protein- or nucleic acid-mediated PDR. Much is yet to be determined on the specific mechanism(s) that confer PDR.

Protein-mediated resistance

Protein-mediated resistance is directed either by transgenes that express the wild-type proteins or modified transgenes that express defective or truncated proteins. With protein-mediated resistance the degree of protection is positively correlated with the level of transgene mRNA and protein accumulation. The first demonstration of PDR for virus resistance was using coat protein-mediated resistance (CPMR). Viral coat proteins (CP) are abundant structural proteins that are well studied and CP genes are easily obtained for production of transgenic plants. CPMR results from the expression of a wild-type viral CP gene in transgenic plants and the resistance conferred ranges from moderate to high. Typical of CPMR, the number of infection sites is decreased and viral systemic movement is inhibited, reduced, or delayed. Although more than one mechanism is likely to be responsible for protection conferred by CPMR, at least part of the protection is believed to result from transgenic CPs blocking uncoating of virions immediately following entry of virus into healthy cells. Upon entry into a healthy cell of a host, virions normally disassemble and initiate an infection, but in newly infected cells expressing transgenic CP the equilibrium between uncoating and coating is believed to be shifted towards coating and stabilization of incoming virus particles. CPMR, apparently free of nucleic acid-mediated resistance, has been demonstrated in tobacco with CP genes derived from alfalfa mosaic alfamovirus (ALMV), potexvirus X (PVX), and tobacco mosaic tobamovirus (TMV). CPMR is narrow, limited to closely related strains of the same type species or closely related members of the same viral genus.

A number of genes encoding nonstructural proteins have also been used for protein-derived PDR. The expression in plants of full-length viral replicase genes or truncated or mutated versions of the replicase genes conferred effective resistance to virus infection. Replicase-mediated resistance has been demonstrated in at least four virus genera: groups: alfamovirus, luteovirus, tobamovirus and potyvirus. Expression of functional, full-length replicase genes may interfere with the timing of replication and/or transcription of an infecting virus, altering the order of events that are critical during the virus' life cycle and resulting in resistance. Alternatively, constitutively expressed replicase proteins may induce defense responses that lead to resistance. On the other hand, resistance mechanisms to virus infection in transgenic plants expressing truncated or altered replicase proteins likely result from dominant-negative mutations. Genes with dominant-negative mutations encode proteins that, when overexpressed, disrupt the activity of the wild-type protein. Mutant replicases interact with host and/or viral components and block equivalent interactions with wild-type replicase proteins that are required for replication and infection. The resistance spectrum induced by altered replicase proteins is narrow and is only active against closely related strains of the virus from which the replicase was derived. The narrow resistance spectrum mirrors the specificity of viral replicase proteins, indicating that replicase-mediated resistance may be directed towards competition for viral components rather than towards cellular components that are more likely conserved between plants.

Viral movement proteins (MP) also confer resistance to virus infection in transgenic plants when

expressed as dominant-negative mutants. A number of plant viruses encode an MP that potentiates cell-to-cell and systemic movement of virus progeny. Movement proteins dilate plasmodesmata, intercellular channels through which virus progeny move, and chaperone virus progeny RNA from cell to cell. Expression of defective MPs or a wild-type MP from a virus that does not naturally move from cell to cell in the transgenic plants species confers resistance to some plant viruses. The modified MPs interfere with normal functions of wild-type MPs and block cell-to-cell and/or systemic movement of progeny virus. The expression of modified MPs in transgenic plants confers a broad spectrum of resistance to virus infection. A defective TMV MP expressed in transgenic tobacco inhibited disease development by other tobamoviruses, as well as viruses representing five additional virus genera: alfamo-, caulimo-, cucumo-, tobra- and nepovirus. The resistance was characterized by a delay in the appearance of systemic disease symptoms, which correlated with a decrease in virus accumulation in upper uninoculated leaves. Likewise, plants expressing modified potato leafroll luteovirus (PLRV) MPs conferred resistance against infection by members of the potyvirus and potexvirus groups in addition to PLRV. In one instance, the expression in tobacco of the MP of brome mosaic bromovirus (BMV), a virus that does not move in tobacco, interferes with intercellular movement of TMV. In the latter case, the expression of a wild-type MP in a nonhost plant appears to be mimicking a dominant-negative mutant. The success of both strategies implies that MPs from very different viruses have some functions that are similar and can be exploited for a broad-based resistance to virus infection.

Nucleic acid-mediated resistance (homologous-dependent resistance)

The more common mechanism conferring PDR is mediated by RNA. With RNA-mediated resistance the level of mRNA accumulation from virus-derived transgenes does not correlate with the degree of resistance, although the mechanism that confers resistance is highly sequence specific. Moreover, RNA-mediated resistance is conferred by modified transgenes that express untranslatable RNAs. Analysis of RNA-mediated resistance in transgenic plants expressing viral CP-gene sequences established an association between resistance and post-transcriptional suppression of transgene expression, indicating that the two phenomena are related. Finding that gene silencing of nonviral transgenes results from a post-transcriptional cosuppression mechanism supports this interpretation. In gene silencing, the post-transcriptional mechanism results in the cosuppression of the transgene and the homologous cellular gene. Similarly, in transgenic plants expressing viral transgenes, post-transcriptional transgene silencing suppresses expression of the transgene and accumulating viral RNA, which shares sequence homology with the transgene. Post-transcriptional cosuppression of the viral transgene and viral RNA explains why resistance does not show a positive correlation with the level of transgene mRNA. Virus resistance resulting from this type of mechanism is referred to as homology-dependent resistance. Because a high degree of sequence homology is required for homology-dependent resistance, it is not unexpected that the resistance in the systems that have been investigated are highly strain specific. When transgene sequences as small as approximately 400 nucleotides are expressed alone, homology-dependent resistance can be conferred. When fused to nonviral (nontarget) DNA sequences, transgene segments as short as approximately 100 nucleotides can confer homology-dependent resistance, although when expressed alone the small virus-derived transgene segments are ineffective in conferring resistance.

Homology-dependent resistance is thought to be mediated by small antisense RNAs that are produced by cellular encoded RNA-dependent RNA polymerases, using transgene mRNA as a template. The duplex formed between viral-transgene antisense RNAs and viral RNA and transgene mRNA could be degraded by RNAases specific for double-stranded RNA. Alternatively, the RNA duplex formed could arrest translation and affect RNA stability indirectly. Both possibilities could reduce the accumulation of transgene mRNA and viral RNA. In addition, viral RNA levels could be affected directly if the RNA duplex inhibited translation of a viral product required for replication or inhibited replication by blocking access of viral replication machinery to viral RNA.

Homology-dependent resistance has been described extensively in transgenic plants expressing replicase, coat protein and protease gene sequences from PVX, tobacco etch potyvirus (TEV), potato potyvirus Y (PVY), PLRV and tomato spotted wilt tospovirus (TSWV). Indeed, it is likely that homology-dependent resistance is the resistance mechanism functioning in a number of examples of PDR where there is no correlation between transgene mRNA levels and resistance.

Other nucleic acid-mediated resistance strategies

Expression in transgenic plants of viral antisense

RNAs to block translation and/or replication has been used with limited success. Antisense-mediated resistance developed against RNA viruses (e.g. cucumo-, potex-, poty- and tobamoviruses) has been weak to moderate and is only effective at low virus inoculum. In contrast, expression of antisense RNA to a replicase protein of a DNA virus (geminivirus) was effective, showing reduced symptoms that correlated with the level of antisense RNA. Because DNA viruses replicate in the nucleus where antisense RNA levels are more abundant, this strategy may be more effective against DNA viruses.

Several groups of viruses can acquire satellite RNAs: small RNAs (approximately 300 nucleotides) that require a helper virus for replication, but are usually not related by sequence homology to the helper virus. Satellite RNAs, which are not essential to the helper virus, can enhance or ameliorate symptoms, depending on the host. Because of their ability to suppress symptoms, satellite RNAs have been candidates for genetically engineered resistance. Inoculation of transgenic tobacco plants expressing satellite RNAs of tobacco ringspot tobravirus (TRSV) or cucumber mosaic cucumovirus (CMV) with the respective helper viruses resulted in the amplification of the transgene-derived satellite RNAs to a high level, presumably because the helper virus replicated the satellite RNAs. In each case, helper virus accumulation was greatly reduced and symptom development was largely suppressed, especially in noninoculated leaves. While this strategy appears promising, the utility of using satellite-mediated resistance is in question because only a few nucleotide changes are required to change a satellite that suppresses symptoms to one that enhances disease symptoms and subsequent yield losses.

Defective interfering nucleic acids (DIs) represent another type of nucleic acid that has been exploited for engineered resistance. DI-RNAs or DI-DNAs are deletion mutants of infectious viral genomes that are associated with RNA or DNA viruses, respectively, and generally suppress symptom development. While DIs are incapable of autonomous replication, they do retain the necessary sequences to be replicated. Presumably, because the DI is smaller than the virus genome, it competes efficiently against the infectious viral genome during replication and is subsequently amplified at the expense of the virus genome. DI-DNAs of two geminiviruses expressed in transgenic plants conferred virus resistance. The resistance was characterized by amplification of the DI-DNAs, symptom amelioration, and a significant decrease in the levels of virus in the infected plants. Similarly, expressing a DI-RNA of cymbidium ringspot tombusvirus (CyRSV) in transgenic *Nicotiana benthamiana* plants resulted in the suppression of symptoms and the plants subsequently produced seed, whereas, in the absence of the DI-RNA, CyRSV caused apical necrosis and death in control plants. Unfortunately, the occurrence of naturally occurring DIs is rare, so this strategy will not be very useful for engineering resistance to the large majority of viral diseases.

Pathogen-derived resistance has been applied to viruses having either positive or negative sense RNA genomes, and against a virus having a DNA genome. The viruses differ in their genome organization, particle morphology and mode of transmission, indicating the utility of the approach. In the few cases examined, PDR is effective against vector transmission of virus infection. Genetically engineered virus resistance conferred by CP or replicase genes (protein or RNA mediated) have been sufficiently developed for use in commercial agriculture.

Plant-derived Resistance Genes

Naturally occurring virus resistance genes

A large number of naturally occurring viral resistance genes have been identified in plants and the utilization of these genes by traditional breeding techniques has resulted in stable, and in many cases, durable resistance. While traditional breeding techniques will continue to be used in developing resistant cultivars, the generation of transgenic plants expressing naturally occurring viral resistance genes offers two additional benefits: (1) resistance genes can be introduced into the plant genome rapidly and the sequence and nature of the genetic material is known; and (2) transgenic plant technology can overcome problems where genetic incompatibility between plants of related species or genera is an insurmountable barrier to introgressing naturally occurring viral resistance genes into agronomically important plants.

Most viral resistance mechanisms studied in plants are genetically simple, being monogenic and represented by either a single dominant or a single recessive gene. Typically, monogenic resistance traits function by inhibiting replication, blocking cell-to-cell movement in inoculated tissue, inhibiting long-distance movement into noninoculated tissue or blocking vector transmission of virus from infected to uninfected plants.

The validity of expressing a plant-derived viral resistance gene in transgenic plants is illustrated by the dominant *N* gene of tobacco. Resistance mediated by the *N* gene to tobamoviruses (e.g. TMV) is characterized by a hypersensitive response (HR): the formation of necrotic lesions at the site of virus infection. The role of the *N* gene in the induction of

the HR was confirmed when transgenic tobacco expressing the *N* gene responded to TMV infection with necrotic lesions. The *N* gene was also transformed into TMV susceptible tomato. When transgenic tomato plants were inoculated with TMV, the role of the *N* gene in induction of the HR was confirmed by the production of necrotic lesions on the inoculated leaves. That the *N* gene from tobacco functions in tomato illustrates the conservation of components required for *N* gene-mediated resistance across species and the utility of using genetic engineering to move resistance genes across genetically incompatible species barriers for engineering crop resistance to viruses.

While the potential to rapidly and efficiently move viral resistance genes within and between species by genetic engineering is great, the ability to isolate naturally occurring virus resistant genes is difficult. However, techniques will be developed to overcome these difficulties with the considerable interest in isolating resistance genes to viruses and other plant pathogens.

Ribosome-inactivating proteins

Ribosome-inactivating proteins (RIPs) are present in different tissue types of a wide variety of plants. Ribosome inactivation is achieved by depurinating a specific adenine residue in a conserved region of the 28S ribosomal RNA (rRNA). The modified ribosomes are unable to bind the EF-2/GTP complex and protein synthesis is blocked at the translocation step. The most common RIPs (type I) are single-chain proteins which are highly active against eukaryotic ribosomes, but are not cytotoxic because they do not bind to and enter cells. Infrequently found are type II RIPs that are composed of two subunits. The A subunit modifies rRNA, while the B subunit is a lectin that can bind to and enter cells, making type II RIPs extremely cytotoxic. It is assumed that the proteins function in a defensive role to protect both plant and seed from pathogen attack because of the toxic nature of RIPs. Many RIPs have antiviral activities towards plant and animal viruses. The broad-spectrum antiviral activities of type I RIPs, along with their inability to bind to and enter cells, make them ideal candidates for genetically engineered virus resistance. RIPs present in pokeweed tissues (collectively designated pokeweed antiviral proteins, PAP) have antiviral activity to viruses in a number of host plants. Transgenic potato and tobacco plants expressing PAP show broad-spectrum resistance to mechanical infection by CMV, PVX and PVY. The plants are also resistant to PVY inoculation with aphids, the insect vector of the virus. A moderate to high level of resistance was observed using this strategy. A negative aspect of RIP-mediated resistance is that host cell ribosomes are susceptible to exogenous RIPs, so the selection of transgenic plants with virus resistance resulting from the expression of nontoxic levels of RIPs will likely be necessary to obtain virus resistant plants with normal phenotypes and acceptable agronomic traits.

Nonplant-nonpathogen-derived Resistance

Interferon-induced 2-5A system

Components of two mammalian antiviral systems have been exploited to generate genetically engineered virus resistance. Possibly the most promising for broad-based resistance is the interferon-induced 2-5A system. Interferon (a family of cytokines present in higher vertebrates) induces antiviral activities; one such activity is the 2-5A system, which is lacking in plants (at least members of the genus *Nicotiana*). Two enzymes are essential for a functional 2-5A system: a 2-5A synthetase, which in the presence of double-stranded RNA produces 2-5A (2′,5′-linked oligoadenylates), and 2-5A-dependent RNAase L. In the presence of 2-5A, RNAase L is activated and cleaves single-stranded RNAs. In the case of a virus-infected cell, the double-stranded replicative intermediates formed during replication activate 2-5A synthetase, resulting in RNA degradation by RNAase L. Transgenic tobacco plants expressing both human 2-5A synthetase and human RNAase L show a high level of resistance to TMV and members of the *Bromoviridae* (AlMV and CMV) and *Potyviridae* (TEV and PVY). The antiviral response is presumably due to degradation of viral RNA, although the response in plants is accompanied by the destruction of infected cells as indicated by the formation of necrotic lesions. Potential advantages of the 2-5A system over PDR are that there is no potential for the generation of more virulent viruses through recombination between mRNA from virus-derived transgenes and the infecting viruses, a concern that is believed by many to be highly unlikely, and the resistance is broad based in contrast to PDR, which is generally more specific to the virus from which the transgene originated or to closely related viruses.

Plantibodies

The immune system of mammals provides an extremely efficient defense against invading pathogens, including viruses. Antibodies, representing one component of the immune system, are generated against and bind to specific antigens (i.e. viral

proteins), ultimately leading to the destruction of the antigen. Transgenic plants have been generated that express both the heavy and light chains of monoclonal antibodies or single-chain antibodies (scFv; single-chain variable region), which contain the light and heavy chain variable regions derived from monoclonal antibodies. The strategy is that antibodies expressed in plants (plantibodies) will bind to viral products during infection and interfere with aspects of the virus life cycle, resulting in either a less virulent infection or an aborted infection. Upon infection with TMV, transgenic plants expressing a high-affinity monoclonal antibody directed against a surface epitope of TMV show a reduction in the number of infection sites on inoculated leaves. The reduction in the number of infection sites correlated with increased levels of antibody detected in the transgenic plants. The expression of monoclonal antibodies in plants requires cotranslational insertion into the endoplasmic reticulum for assembly and stability, an obvious limitation since most plant viruses have RNA genomes that replicate in the cytoplasm. Therefore, since the TMV antibodies are secreted into apoplastic spaces, they likely bind to virus during inoculation to block infection, possibly by inhibiting viral disassembly after the virions have entered cells.

Using a similar approach, transgenic plants expressing a cytoplasmic targeted scFv antibody directed to a conserved site of the artichoke mottled crinkle virus (AMCV) CP were less susceptible to infection. Those transgenic plants that did become infected showed a delay in symptom development. The scFv antibody binds to a CP eptitope involved in Ca^{2+} binding, which is required to stabilize coat protein interactions in the assembled virus icosahedron shell, and may block uncoating or assembly of the virus particle. One advantage of scFv antibodies is that they do not require assembly and can be directly expressed in the cytosol. Signal peptides attached to scFv antibodies can facilitate targeting of the proteins to organelles, such as the endoplasmic reticulum, or to be secreted into apoplastic spaces.

When expressing both whole antibodies and scFv antibodies in plants, conditions need to be defined to maximize accumulation of functional molecules to a level required to neutralize infection. Another aspect of increasing importance, as the technology develops, is the ability to target the functional antibodies to appropriate subcellular compartments. Research to date has focused on well-characterized monoclonal antibodies or single-chain antibodies derived from monoclonal antibodies directed towards viral CPs with the strategy of blocking assembly or disassembly. In the future, antibodies directed to nonstructural proteins, such as those involved in replication or movement, have the potential to increase the efficacy of the approach. The utility of expressing antibodies to nonstructural proteins has been demonstrated by the ability of antibody fragments, directed against the reverse transcriptase of the human immunodeficiency virus (HIV), to prevent HIV infection in cultured mammalian cells. In addition, expression of catalytic antibodies may provide another approach for disease resistance.

Concluding Remarks

Genetically-engineered virus resistance has significantly contributed to crop improvement. While it is too early to determine which approaches to genetically-engineered virus resistance will ultimately provide the most useful and durable resistance, a broad foundation, including PDR and non-PDR strategies, is in place and new examples of application continue to appear. Some degree of resistance has been achieved in nearly all plants in which the different strategies have been tried. While immunity to virus infection has been obtained through genetically engineered resistance in a few cases, and is obviously preferred, it is generally accepted that it is not required. Engineered resistance that sufficiently retards the accumulation of virus in infected plants, and/or results in a decrease in virus transmission, will likely be adequate in the absence of immunity. Indeed, many natural forms of virus resistance do not confer immunity.

Genetically-engineered virus resistance will improve as the mechanisms behind the resistance strategies are better understood and are manipulated to further exploit these strategies. In addition, development of new strategies (e.g. ribozymes and protease inhibitors) will add to the current repertoire. Continued advancements in technology to transform plants will increase the number of species that can be engineered for virus resistance and increase the transformation efficiency of species that are currently difficult to transform. Combining several genetic engineering strategies, as well as breeding virus-resistant transgenic cultivars with cultivars that have naturally occurring resistant genes, will lead to plants having a broad-spectrum of virus resistance.

See also: **Alfamovirus and Ilarviruses (*Bromoviridae*); Bromoviruses (*Bromoviridae*); Carmoviruses (*Tombusviridae*); Comoviruses (*Comoviridae*); Cucumoviruses (*Bromoviridae*): General features, Molecular biology; Geminiviruses (*Geminiviridae*); Immune response: Cell mediated immune response, General features; Interferons: General features, Therapy of aids and cancer;**

Luteovirus; Nepoviruses (*Comoviridae*); Pathogenesis: Plant viruses; Plant pararetroviruses: Badnaviruses, Plant pararetroviruses (*Caulimoviridae*): Cassava vein mosaic virus, Caulimoviruses: general features, Caulimoviruses: molecular biology, Legume caulimoviruses, Rice tungro bacilliform virus; Potexviruses; Propagation of viruses: Plant; Ribozymes; Satellite RNAs and Satellite viruses; Tobamoviruses; Tobraviruses; Tombusviruses; Tospoviruses (*Bunyaviridae*); Transformation: Plant viruses; Vectors: Plant viruses.

Further Reading

Baulcombe DC (1996) Mechanisms of pathogen-derived resistance to viruses in transgenic plants. *Plant Cell* 8: 1833.

Fitchen JH and Beachy RN (1993) Genetically engineered protection against viruses in transgenic plants. *Ann. Rev. Microbiol.* 47: 739.

Grumet R (1995) Genetic engineering for crop virus resistance. *HortScience* 30: 449.

Hartley MR, Chaddock JA and Bonness MS (1996) The structure and function of ribosome-inactivating proteins. *Trends Plant Sci.* 1: 254.

Kavanagh TA and Spillane C (1995) Strategies for engineering virus resistance in transgenic plants. *Euphytica* 85: 149.

Whitelam GC and Cockburn W (1996) Antibody expression in transgenic plants. *Trends Plant Sci.* 1: 268.

PLANT RETROELEMENTS

Roger Hull, John Innes Centre, Norwich Research Park, Colney, Norwich, UK

Introduction

The replication of most nucleic acids is mainly either DNA to DNA (chromosomal and viral nucleic acids) or RNA to RNA (viral genomes). However, there is an increasing number of nucleic acid sequences which involve a combination of transcription of RNA from DNA and reverse transcription of DNA from RNA. These are termed retroelements and they are found in many taxonomic groups, including higher animals and plants, fungi, insects and bacteria. Retroelements comprise three major groups, viral retroelements, eucaryotic nonviral retroelements and bacterial elements. These major groups have been subdivided on structural and biological criteria (**Table 1**) but recent findings are blurring some of the boundaries between these groups.

The main features that distinguish these groups and subgroups are that viruses have properties which potentiate horizontal transmission; some may also be vertically transmitted. Nonviral retroelements are generally considered to be only vertically transmitted but there may be instances of horizontal transmission, say between plants by pathogenic fungi. Retroviruses and the nonviral retroelements integrate into the 'host's' genome, whereas pararetroviruses are episomal. The genomes of retroviruses, retrotransposons and retroposons encode an integrase activity, and those of retroviruses and retrotransposons have long terminal repeats, both features involved in integration. All, except for retrosequences, which are cDNAs converted from cellular RNAs by endogenous reverse transcriptase activity, encode for reverse transcriptase and its associated RNaseH.

In plants the retroelements currently described fall into the pararetrovirus, retrotransposon, retroposon and retrosequence groups. The reason for there being no known examples of plant retroviruses *sensu stricto* is unknown.

Plant Pararetroviruses

There are two genera of plant pararetroviruses, the Caulimovirus and the Badnavirus in the *Caulimoviridae* family. The genome organization can be considered as comprising the core replicon of polymerase (reverse transcriptase + RNaseH) and coat protein which is considered to organize the replication complex together with genes which adapt the replicon to the plant viral situation. These additional genes are best characterized in cauliflower mosaic virus (CaMV) in which the core replicon is encoded by open reading frame (ORF) IV giving the coat protein and ORF V the polymerase. The plant-adapting genes include the ORF I product which facilitates the movement of the virus from cell to cell through plasmodesmata, and the ORF II product which is

disease epidemiology, RTBV-induced disease in rice is amenable to control strategies not relevant to the other badnavirus diseases. Breeding and selection of virus- or vector-resistant varieties is a prime option in the case of rice, but is less readily accomplished in clonally propagated crop plant hosts of other badnaviruses. Control of vector populations by cultural or chemical methods is also far more relevant to RTBV than to other badnaviruses.

See also: **Plant pararetroviruses (*Caulimoviridae*): Caulimoviruses: general features, Caulimoviruses: molecular biology.**

Further Reading

Cheng C-P, Lockhart, BEL and Olszewski NE (1996) The ORF I and II proteins of *Commelina* yellow mottle virus are virion-associated. *Virology* 223: 263.

Hay J, Grieco F, Druka A, Pinner M, Lee S-C and Hull R (1994) Detection of rice tungro bacilliform virus gene products *in vivo*. *Virology* 205: 430.

Hibino H (1983) Relation of rice tungro bacilliform and rice tungro spherical virus with their vector *Nephotettix virescens*. *Ann. Phytopathol. Soc. Jpn* 49: 545.

Hohn T and Futterer J (1997) The proteins and functions of plant pararetroviruses: knowns and unknowns. *Crit. Rev. Plant Sci.* 16: 133.

Jacquot E, Keller M and Yot P (1997) A short basic region supports nucleic acid binding activity in the rice tungro bacilliform virus open reading frame 2 product. *Virology* 239: 352.

Tzafrir I, Ayala-Navarrette L, Lockhart BEL and Olszewski NE (1997) The N-terminal portion of the 216-kDa polyprotein of *Commelina* yellow mottle badnavirus is required for virus movement but not for replication. *Virology* 232: 359.

PLANT RESISTANCE TO VIRUSES

Contents

Natural Resistance

RSS Fraser, Society for General Microbiology, Reading, UK

Importance of Resistance in Disease Control

Plant viruses cause serious economic losses in many crops, in three main ways. Infection normally reduces yield biomass; an important example is the effect of the complex of related viruses known as barley yellow dwarf on all major cereal crops. In crops which are sold on the basis of appearance, such as ornamentals and many fruits and vegetables, blemishing and distortion as a result of virus infection can lead to complete loss of marketability, even in cases where yield biomass is only slightly reduced. Finally, costs are incurred if eradication or avoidance programs are enforced, for example in production and certification of virus-tested seed potatoes.

In contrast to the control of fungal diseases of plants, no chemical pesticides are available for routine use as direct antiviral agents. It has, therefore, been necessary to develop a number of alternative control strategies. These include control of the biotic vectors of particular plant viruses, use of virus-free seed or planting material of vegetatively propagated crops, good cultural practices to minimize transmission, and deployment of a number of different mechanisms of plant resistance. Naturally occurring resistance mechanisms, the topic of this article, have been of most use in crop protection until now, but several types of genetically engineered resistance mechanisms, using viral and nonviral genes, are now becoming available. In both types of resistance, it is important to study the mechanisms involved, to increase the effectiveness with which resistance can be used in crop protection, and to understand more fundamental questions about the molecular interactions between plants and viruses.

Types of Natural Resistance Mechanism

Plant viruses are very diverse in their structure, mode of replication and transmission, and in their pathogenic effects on their hosts. Perhaps not surprisingly,

Table 1 Resistance at different levels of plant population complexity

Plant population	*Type of resistance*	*Features and questions*
Species	Nonhost immunity	What determines the host range of a virus?
Cultivar	Cultivar resistance	As used in plant breeding. Resistance overcome by virulent isolates
Normally susceptible individual plant	Acquired or induced resistance	A number of mechanisms of varying usefulness in crop protection

plants have evolved a correspondingly diverse array of resistance mechanisms, and a number of examples are considered here. However, it will first be useful to provide a framework for this diversity, by considering resistance mechanisms in a more conceptual manner. This is approached in two ways: in relation to the stages of the virus replicative and transmission cycle in a model susceptible plant which might form potential targets for different types of resistance mechanism (**Fig. 1**), and in relation to differences in the complexity of the plant populations in which these resistance mechanisms might operate (**Table 1**).

Plant viruses initiate infection of susceptible hosts through wounds, either caused mechanically by abrasion or by plant-feeding virus vectors such as insects and nematodes. There is no evidence for virus-specific cell surface receptors such as exist for many animal viruses and bacteriophages. Once within a cell of a susceptible plant, the virus exposes its genetic material for translation of catalytic and structural viral proteins, and production of further copies of the genome, which are then assembled to form progeny virus particles. Virus multiplication requires a number of host-provided 'helper' functions. These may be nonspecific 'housekeeping' functions such as the provision of ribosomes, amino acids, nucleoside triphosphates and an energy supply, or specific to the particular virus. For example, there is evidence that the complex enzymes responsible for replicating the tobamovirus and bromovirus genomes, and expressing subgenomic viral messenger RNAs, involve not only virus-coded components, but also one or more polypeptides of host origin.

Here it is useful to introduce the concepts of *positive* and *negative* mechanisms of resistance, alternatively referred to by some authors as *active* and *passive* mechanisms. In positive mechanisms a plant is resistant because it produces an inhibitor which interferes with some stage of the virus replicative cycle, or contains a factor which recognizes some virus-coded molecule and switches on a resistance response. In negative mechanisms a plant is resistant because it lacks the specific helper functions required by the virus, or possesses them in mutated form unable to operate in the virus replicative cycle. The host-coded component of the virus replicase mentioned above would fall into this category.

From the initially infected cell, the virus spreads to establish further cycles of infection in adjoining cells. The nature of the infectious entity that moves from cell to cell differs for different types of plant virus. For tobamoviruses and potyviruses, the entity is the virus genomic RNA, in association with one or more virus-coded proteins known as movement proteins. These have a number of functions, including binding to the viral RNA and unfolding it; modifying the plasmodesmata which form cytoplasmic connections between adjoining cells, to permit the passage of larger molecules than in healthy plants; and possible interactions with the cytoskeleton, all of which can be seen as facilitating cell-to-cell movement. Viruses in other groups, such as the comoviruses, appear to move from cell to cell as assembled virions, through virus-induced tubular structures which contain virus-coded movement proteins also involved in modification of plasmodesmata. Clearly, both types of movement mechanism, with the likelihood of complex interactions between virus- and host-coded components, could be potential targets for resistance mechanisms of positive or negative types. The evidence, to be considered in detail later in this article, indicates that cell-to-cell movement is indeed the critical stage for a number of different resistance mechanisms.

Long-distance movement of infection is via the phloem. The evidence suggests that the mechanisms of passage of the virus into and out of the sieve tubes may be in part different from those involved in cell-to-cell movement. Thus for tobamoviruses, coat protein is required for long-distance transport but not for cell-to-cell movement. One of several mechanisms of resistance to bean common mosaic virus (BCMV) in *Phaseolus vulgaris* appears to operate by restricting virus movement from the initially infected leaf. At the other end, lettuce cultivars differ in the extent to which lettuce mosaic virus is seed transmitted, and this may reflect genetic differences in virus transport into the developing seed.

During the course of accumulation of progeny virus

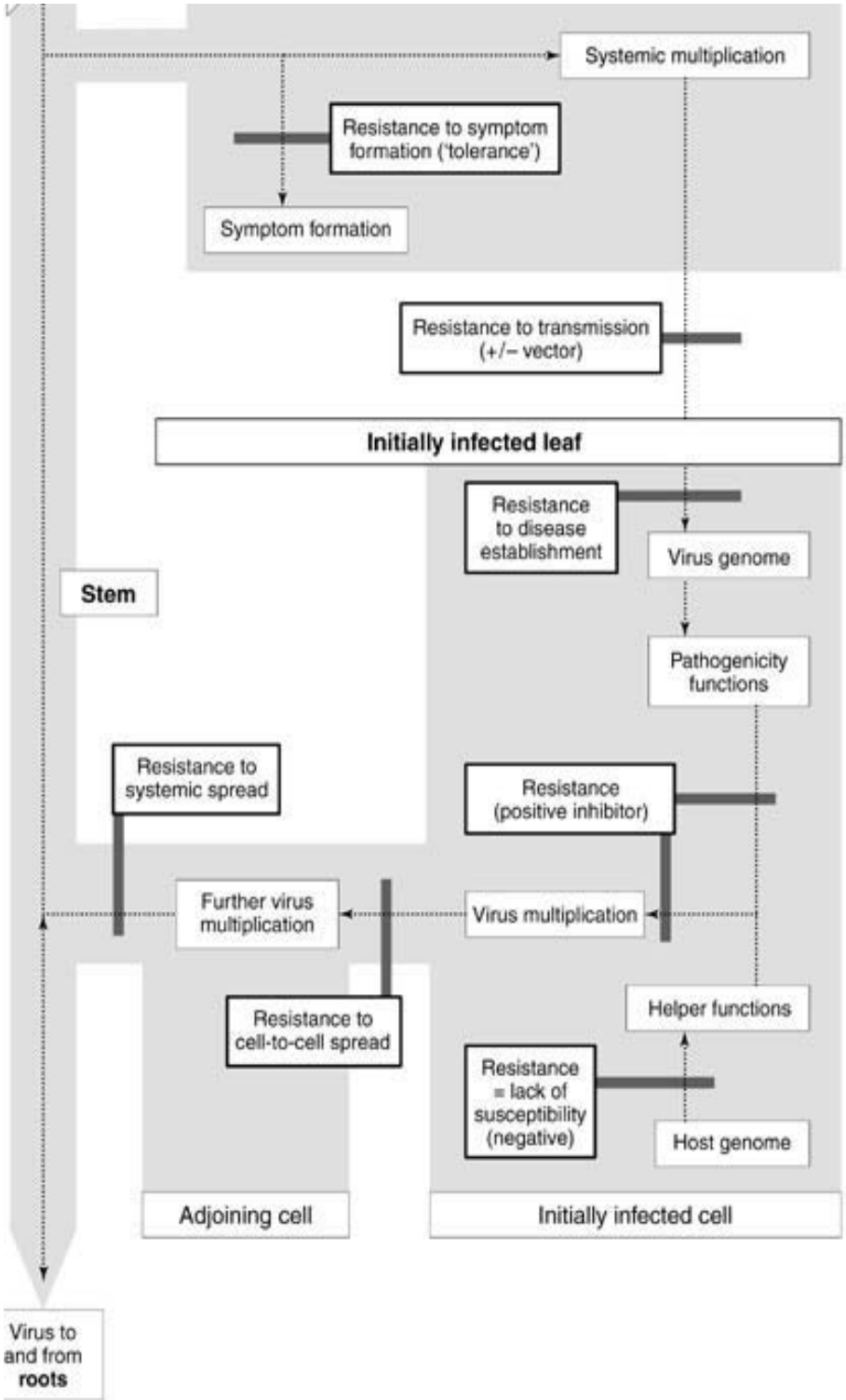


Figure 1 A model replicative cycle of a typical plant virus, showing various possible targets of resistance mechanisms. The track of the virus replicative cycle within the plant and from plant-to-plant is shown by the dotted line.

particles and colonization of a susceptible plant, the virus usually causes formation of visible symptoms such as mosaic or yellowing, as well as reduced and distorted growth. A type of resistance mechanism which appears to operate primarily against symptom formation is referred to as *tolerance*. However, the

term is rather loosely used and some plant virologists prefer to avoid it. Infectious virus can be detected in tolerant plants, but most studies have not measured virus concentration by quantitative methods to determine whether inhibition of multiplication, with a possible indirect effect on symptom severity, does or does not occur.

Progeny viruses are eventually released into the environment, by plant death and decay, mechanical contact, or into a biotic vector such as a phloem-feeding aphid. These routes eventually allow some of the progeny to establish fresh cycles of infection in further plants. There are potential targets for resistance mechanisms in the processes of transmission and establishment, which may operate directly against the virus or indirectly against the vector. An example of the former is a tobacco cultivar partly resistant to mechanical inoculation by a number of viruses because its epidermal cells have a reduced number of ectodesmata, thought to be an important initial channel of infection by wounding. A good example of the latter is the secretion of the aphid alarm pheromone (E)-β-farnesene by glandular hairs on the leaf surfaces of the wild potato species *Solanum berthaultii*, which causes aphids to avoid the plant and reduces transmission of potato virus Y and beet yellows virus.

In summary, there are clearly many and varied potential targets for resistance mechanisms in the virus replicative cycle; it is now appropriate to examine their operation in more detail, in the different types of plant population described in **Table 1**.

Nonhost Resistance

Effectively, most plant species appear to be nonhosts for most plant viruses. In the natural or cultivated environment they do not come into contact because of geographical separation or lack of suitable vectors. However, in the laboratory, experimental infections can often be established. Some viruses such as tobacco mosaic virus (TMV), cucumber mosaic virus (CMV) and tomato spotted wilt virus have been found to infect hundreds of species naturally and experimentally. In contrast, some other viruses have very limited natural host ranges, which cannot easily be extended under experimental conditions. Thus, BCMV is almost entirely restricted to *Phaseolus vulgaris*. What determines host range, and how is it much wider for some viruses than others? What are the mechanisms involved in resistance by nonhost species to different viruses, and could they be further exploited in plant breeding for virus disease control?

Two main types of mechanism have been proposed. The first centers on the interaction of virus-coded pathogenicity factors with host-coded 'helper' functions: nonhost species would simply be those lacking the necessary specific helper functions, a prime example of a negative-type resistance mechanism and perhaps difficult to exploit further in breeding for that reason. At the time of writing, the molecular biology of host components required by viruses – such as replicase subunits, or plasmodesmatal components able to interact with virus-coded movement proteins – is just beginning to be understood. This should lead to an improved ability to evaluate the importance of this model, which conceptually seems a probable one.

The available evidence suggests that cell-to-cell movement of infection is a critical stage in some cases of nonhost resistance. In so-called 'subliminal' infections, plant species previously thought to be completely immune to infection by particular viruses were shown to contain a very small number of cells in the inoculated leaf in which virus multiplication had occurred: presumably those directly infected by the inoculation. Further work with protoplasts prepared from apparent nonhost species for particular viruses showed that they too supported virus multiplication when inoculated. It will be intriguing to discover the basis for the failure of cell-to-cell spread in these situations, but absence of a host component able to interact with the virus movement proteins would seem a possibility. Information is available on too few plant–virus combinations to say whether this is a predominant mechanism in nonhost resistance. As others have pointed out, the data may be skewed, in that reports of failure of viruses to multiply in protoplasts of nonhost species would be difficult to publish. Alternative mechanisms such as lack of a suitable host-coded replicase subunit must remain as attractive possibilities.

The second type of mechanism suggests that plant species may be nonhosts of particular viruses for much less specific reasons, for example because of their content of chemicals such as tannins or phenolics generally antagonistic to viruses, or unsuitable chemical conditions such as cytoplasmic pH. The former may explain the scarcity of reports of virus infections in plant groups such as the Gymnosperms and Pteridophytes. As a possible example of the latter, TMV is known to require a cytoplasmic region with a pH of around 8 and low Ca^{2+} concentration to remove the coat protein subunits from the 5′ end of its genomic RNA, and thus permit a host ribosome to initiate expression of the virus replicase genes and the processes of virus multiplication. A plant species with lower cytoplasmic pH or generally higher Ca^{2+} concentration might not permit TMV to initiate infection.

Table 2 The gene-for-gene relationship between resistance in tomato and virulence in tomato mosaic virus (ToMV)

	ToMV strain							
Host genotype	*0*	*1*	*2*	*2^2*	*1.2*	*1.2^2*	*2.2^2(rare)*	*$1.2.2^2$(rare)*
+/+	M	M	M	M	M	M	M	M
Tm-1	R	M	R	R	M	M	R	M
Tm-2	R	R	M	R	M	R	M	M
Tm-2^2	R	R	R	M	R	M	M	M
Tm-1/Tm-2	R	R	R	R	M	R	R	M
Tm-1/Tm-2^2	R	R	R	R	R	M	R	M
Tm-1/Tm-2/Tm-2^2	R	R	R	R	R	R	R	M

R, resistant, no symptoms; M, susceptible, systemic mosaic and stunting.

Cultivar Resistance

In a crop species normally susceptible to a particular virus, some cultivars may be resistant because of possession of one or more genes for resistance to that virus. The resistance genes have either been detected by screening a wide range of germplasm of the species, including landraces, or transferred from closely related wild species.

The review articles cited in the Further Reading section list well over one hundred examples of virus resistance in crop species where the resistance has been characterized genetically, where something may be known about the mechanism of resistance, and where the response of the virus to the challenge of resistance is known. Strains of virus which are controlled by a resistance mechanism are referred to as *avirulent* against that mechanism. However, many viruses either contain strains with the ability to overcome particular resistance genes, or have evolved this ability in the face of the selection pressure stemming from resistance. These resistance-breaking isolates are referred to as *virulent*. Where a host contains several genes for resistance to a particular virus, and the virus contains one or more virulence determinants against these genes, a *gene-for-gene relationship* is said to exist between the plant and virus. An example is shown in **Table 2**. The study of virulence has given some insights into the mechanisms of resistance, and is clearly relevant to the effectiveness of resistance gene deployment in practical crop protection.

The great majority of the resistances in these review surveys involve genes at a single locus. There are a few well-attested examples of resistance mechanisms which require cooperative (as opposed to merely additive) effects of genes at two loci. Thus resistance to BCMV in *Phaseolus vulgaris* conferred by any one of the genes referred to as *bc-1, bc-2* or *bc-3* requires the presence of the *bc-u* gene as well. The vast majority of genes confer resistance against a single virus or a closely related group. A possible exception is the *I* gene in *Phaseolus vulgaris* which confers resistance against nine different viruses within the potyvirus group, presumably by recognizing some strongly conserved structural element common to all.

About half the resistance genes in the survey are classified genetically as dominant over susceptibility. About one-quarter are incompletely dominant, that is they show a clear effect of resistance allele dosage, with more effective resistance in plants homozygous for the resistance allele than in those that are heterozygous. The remainder of the resistance mechanisms appear to involve recessive alleles. This distribution from dominance to recessiveness is interesting in its diversity; it contrasts with resistance to fungal and bacterial pathogens of plants, which is much more strongly associated with dominance.

In terms of mechanism, dominant resistance is strongly associated with the hypersensitive response (HR), in which the virus initially multiplies unhindered and spreads from the primarily infected cell to a small group of several hundred. However, within a few days, virus spread ceases and the infected area forms a necrotic local lesion (**Fig. 2**). The best-known example of this type of resistance is the *N* gene for TMV resistance in tobacco. This originated in the wild species *Nicotiana glutinosa* and was transferred to the cultivated tobacco *N. tabacum* via an interspecific synthetic hybrid as early as 1925, and was characterized genetically by F. O. Holmes by 1938. This gene has been introduced into a number of commercial cultivars – cigar smokers may have noticed its presence as betrayed by the occasional lesion on the cigar outer leaf.

The *N* gene is the first (and at the time of writing the only) plant virus resistance gene to have been isolated, using a transposon-tagging technique. The isolated gene was shown to be the *N* gene by transformation into susceptible tobacco, which subse-

quently exhibited the hypersensitive response when inoculated with TMV. Further confirmation that this single gene was enough to switch on HR came from the demonstration that it could also confer HR in transformed TMV-susceptible tomato plants.

Sequence analysis of the *N* gene cDNA and genomic clones indicated that it contains five exons which could be spliced to give a protein with 1144 amino acids and a molecular mass of 131 kDa. Comparison of the deduced amino acid sequence of the *N* gene product shows close structural similarities to a number of other genes for resistance to bacterial and fungal pathogens, with common elements indicating a cytoplasmic location, nucleotide binding sites and a region of leucine-rich repeats. The main structural motifs in these resistance genes also occur in a number of other proteins whose function is more clearly understood, for example in the activity of mammalian hormones, and control of insect development. These similarities may give some clues as to how the *N* gene product specifically recognizes TMV, then signals a series of metabolic changes which include the mechanisms responsible for inhibition of virus multiplication or spread. A tobamovirus closely related to TMV, *Solanum dulcamare* yellow fleck virus, has the unique property for this group of being able to overcome *N*-gene resistance. This has been shown to be determined by a single base change in the 126 kDa viral replicase gene, which may alter the recognition or inhibition components of the resistance mechanism.

A great variety of metabolic changes occur during the formation of local lesions, and most probably represent a generalized activation of defense responses rather than specific antivirus mechanisms only. They include induction of synthesis of the pathogenesis-related proteins, which are potentially involved in resistance to fungal and bacterial pathogens, synthesis of phytoalexins with antifungal activity, increased activities of certain enzymes such as phenylalanine ammonia lyase and chalcone synthase, alterations in cell wall structure by lignification and callose deposition, stimulated production of activated oxygen species, and synthesis of various putative inhibitors of virus multiplication. Of these, cell wall modification to restrict virus movement through plasmodesmata, and multiplication inhibitors, would seem attractive suggestions for the means of expression of the ultimate antiviral activity conferred by the *N* gene, but the case for either has not yet been fully made. What is clear is that necrosis itself is not the primary antiviral mechanism, but a secondary consequence of the other metabolic changes.

Another type of localizing resistance is exemplified by the *Tm-2* and *Tm-2*^{2} genes in tomato, which confine avirulent isolates of tomato mosaic virus (ToMV) to the initially infected cells, in which, however, they multiply normally. It appears therefore that cell-to-cell movement of infection is the target of these resistances. It is entirely consistent with this that virulent isolates of ToMV, which spread unhindered, have been shown to have critical point mutations in their movement protein.

Resistance mechanisms involving alleles that show dosage dependence may be associated with mechanisms that inhibit virus multiplication and symptom development, while allowing some virus spread locally or systemically. A good example is provided by the *Tm-1* gene for ToMV resistance in tomato. This inhibits virus multiplication by 70% in heterozygous form and by 95% when homozygous. Virulent ToMV isolates overcoming the *Tm-1* gene have been shown to have a mutation in the viral replicase gene. The implication is that resistant plants contain an inhibitor of replicase activity, and that the replicase of virulent isolates does not interact with the inhibitor.

Very little appears to be known about the mechanisms of action of resistance controlled by recessive alleles; the recessiveness would certainly be consistent with the model involving lack of a required host-specified helper function.

Two further specific mechanisms of resistance deserve mention because their biochemistry is comparatively well understood. In cowpea, resistance to cowpea mosaic virus (CPMV) is conferred by a specific protease inhibitor. Comoviruses such as CPMV express their genomic information initially as two polyproteins transcribed from the two genomic RNAs. The polyproteins are then cleaved to mature products such as coat protein and replicase components by a virus-coded protease, which is the target of this resistance mechanism.

Ribosome-inactivating proteins (RIPs) occur in numerous plant species, and inhibit the translocation step of translation by removing a specific adenine base from the larger ribosomal RNA. Many RIPs, such as that from *Phytolacca americana* (pokeweed antiviral protein, PAP), have been shown to have broad-spectrum antiviral activity. PAP is normally sequestered in the cell walls in healthy plants, and thus does not inactivate their ribosomes. This led to the 'local suicide' hypothesis for the observed antiviral effects: when a virus enters a cell by wounding or insect feeding, PAP is also allowed to enter the cytosol where it inhibits protein synthesis and thus prevents expression of viral functions. However, more recent results suggest that the antiviral activity of PAP may be even more specific than a general, if highly localized, inhibition of total protein synthesis.

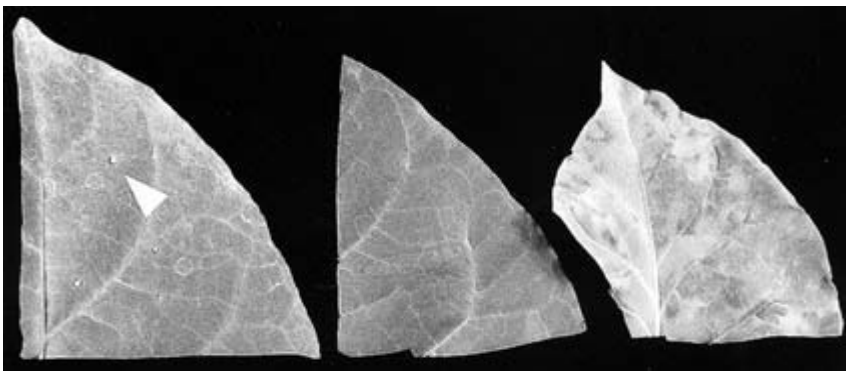


Figure 2 Reaction of tobacco to inoculation with tobacco mosaic virus (TMV). Center: healthy, uninoculated leaf. Right: systemically infected leaf of a susceptible cultivar, showing light-green/dark-green mosaic symptoms of virus infection. Left: an inoculated leaf of a resistant variety carrying the *N* gene, showing restriction of the virus to necrotic local lesions formed around each site of infection (arrowed).

Acquired or Induced Resistance

This heading covers a diverse collection of resistance or resistance-like mechanisms. Some are as yet of academic interest only, whereas a few have found application in practical crop protection.

In susceptible plants showing the common light-green/dark-green mosaic symptoms in upper leaves infected at an early stage in their development by systemic spread of virus (**Fig. 2**), the dark green areas contain little or no virus, and are partly resistant to challenge inoculation. The mechanism is not understood, but one possibility is that the plasmodesmata have remained as the primary forms characteristic of meristematic tissue, and less susceptible to modification by viruses. This may also explain the fact that meristems of virus-infected plants are usually virus-free. Dark green areas of TMV-infected leaves have been shown to have significantly altered levels of the plant hormones abscisic acid and zeatin, and their metabolites, possible indicators of a maintained juvenile state.

Local and systemic acquired resistances (LAR, SAR) are phenomena occurring in plants showing a hypersensitive resistance response to a particular virus. If plants have formed lesions as a result of a first inoculation, the lesions formed after a second inoculation on the previously inoculated leaf (LAR), or on previously uninoculated upper leaves (SAR), are smaller and often fewer in number than those formed on previously uninoculated plants. The first HR induces synthesis of numerous pathogenesis related (PR) proteins, which have potential defense functions against fungal and bacterial pathogens, and insect pests. None has so far been shown to have antiviral activity. LAR and SAR are best regarded as modulations of the primary (and highly effective) resistance response; the additional effects if any have not been exploited in crop protection.

Crossprotection occurs when a plant is systemically infected by a mild (attenuated) strain of a virus, and becomes resistant to infection or disease caused by a severe strain of the same virus. It is used in agriculture for control of a small number of viruses, especially where cultivar resistance is not available. Examples include protection of tomato cultivars lacking the *Tm-2*^{2} gene for ToMV resistance, and citrus fruits against citrus tristeza virus. The mechanism of protection is probably similar to that operating in coat protein transgenic plants, described in a separate article.

The severity of disease caused by cucumoviruses and a small number of other groups can be reduced by the presence of satellite RNAs, which depend on the virus for their multiplication. Tomato and cucurbit crops in the People's Republic of China have been extensively inoculated with such benign satellites to protect against CMV.

Use of Resistance in Plant Breeding

Conventional breeding for virus resistance has had some notable successes and some unfortunate failures. The latter are largely related to the ease and speed with which the target virus has succeeded in producing resistance-breaking isolates; more than half of the resistance genes in the review surveys referred to earlier are reported as having been overcome by virulent strains of virus. Thus in tomato, the *Tm-1* gene for ToMV resistance was overcome within 1 year of its introduction; resistance-breaking isolates quickly became widespread. In contrast, the *Tm-2*^{2} gene has proved outstandingly durable, and has been the mainstay of protection for many years. The

breeder should attempt to predict the likely durability of a resistance gene, by testing it against as many isolates of the virus as possible, at an early stage of the breeding program.

However, another problem is that the genetic base of resistance to plant viruses is rather limited, and in many crops no resistance to particular diseases is known. Screening of wild relatives and gene bank collections will undoubtedly reveal further natural resistance genes, although at a cost. The various techniques for developing resistance by introduction of transgenes offers an excellent prospect of broadening the basis of control.

See also: **Vector transmission of plant viruses; Pathogenesis: Plant viruses; Plant resistance to viruses: Engineered resistance; Plant virus disease – economic aspects; Transformation: Plant viruses; Vectors: Plant viruses.**

Further Reading

Brunt AA, Crabtree K, Dallwitz MJ, Gibbs AJ and Watson L (1996) *Viruses of Plants: Descriptions and Lists from the VIDE Database*. Wallingford: CAB International.

Fraser RSS (1990) Genetics of resistance to viruses. *Annu. Rev. Phytopathol.* 28: 179.

Hadidi A, Khetarpal RK and Koganezawa H (eds) (1998) *Plant Virus Disease Control*. St Paul: American Phytopathological Society Press.

Lartey R, Ghoshroy S, Sheng J and Citovsky V (1997) Transport through plasmodesmata and nuclear pores: cell-to-cell movement of plant viruses and nuclear import of *Agrobacterium* T-DNA. In: McCrae MA, Saunders JR, Smyth CJ and Stow ND (eds) *Molecular Aspects of Host–Pathogen Interactions*. Society for General Microbiology Symposium Series, Vol. 55, p. 253. Cambridge: Cambridge University Press.

Matthews REF (1991) *Plant Virology*, 3rd edn. San Diego and London: Academic Press.

Slusarenko A, Fraser RSS and Van Loon LC (eds) (1999) *Mechanisms of Resistance to Plant Diseases*, 2nd edn. Dordrecht: Kluwer.

Engineered Resistance

C Michael Deom, University of Georgia, Athens, Georgia, USA

Introduction

Engineered resistance (or genetically engineered resistance) to viral pathogens refers to the introduction and expression of foreign genes, termed transgenes, in plants such that expression of a transgene interferes with the virus life cycle and thereby confers resistance to the virus. Advances in plant transformation technologies for a variety of plant species have resulted in the introduction of transgenes from a variety of sources that confer resistance to viral pathogens. The transgenes are stably integrated into the plant genomes, are heritable, and resistance segregates with the transgenes. In many cases, transgenic plants have been generated that have resistance to viruses by mechanisms that do not occur naturally and could not have been introduced into the plants by traditional breeding techniques. Genetically engineered virus resistance in crop plants shows great promise in efficiently reducing losses due to virus disease and complements traditional plant breeding.

Transgenes are designed as chimeric genes under the control of a promoter and contain nonviral sequences at the 3′ end to provide transcription termination and polyadenylation signaling. With few exceptions the preferred promoter has been the 35S promoter from the plant DNA virus cauliflower mosaic virus, which is constitutively expressed and drives high levels of expression of transgenes in most plant tissues. In some cases, the transgene is placed under the control of a tissue-specific promoter to localize expression of the transgene.

Pathogen-derived Resistance

The transformation of plants to confer virus resistance was an initial focus of plant biotechnology. The most widely applied and effective strategy presently used for this purpose is pathogen-derived resistance (PDR): resistance obtained by expressing viral-derived transgenes (genes or genomic sequences) in transgenic plants (**Table 1**). This strategy has provided some degree of virus resistance in almost all hosts to which it has been applied. In all cases, the level of PDR is variable, depending on both the type of virus from which the transgene sequence is derived and on the individual transgenic plant line. In some instances, transgenic plants have been developed that are essentially immune to virus infection. Therefore, biologically screening a number of transgenic plant lines is required to identify those having the highest levels of genetically engineered virus resistance and acceptable agronomic traits. Unfortunately, the resistance mechanisms are poorly understood at the cellular and molecular levels. PDR can be divided into two broad categories, depending on whether the resistance mechanism is mediated by protein or nucleic acid. In some instances it is not clear whether the resistance is conferred by expression of the protein or the nucleic acid encoded by the transgene and in

Luteovirus; Nepoviruses (*Comoviridae*); Pathogenesis: Plant viruses; Plant pararetroviruses: Badnaviruses, Plant pararetroviruses (*Caulimoviridae*): Cassava vein mosaic virus, Caulimoviruses: general features, Caulimoviruses: molecular biology, Legume caulimoviruses, Rice tungro bacilliform virus; Potexviruses; Propagation of viruses: Plant; Ribozymes; Satellite RNAs and Satellite viruses; Tobamoviruses; Tobraviruses; Tombusviruses; Tospoviruses (*Bunyaviridae*); Transformation: Plant viruses; Vectors: Plant viruses.

Further Reading

Baulcombe DC (1996) Mechanisms of pathogen-derived resistance to viruses in transgenic plants. *Plant Cell* 8: 1833.

Fitchen JH and Beachy RN (1993) Genetically engineered protection against viruses in transgenic plants. *Ann. Rev. Microbiol.* 47: 739.

Grumet R (1995) Genetic engineering for crop virus resistance. *HortScience* 30: 449.

Hartley MR, Chaddock JA and Bonness MS (1996) The structure and function of ribosome-inactivating proteins. *Trends Plant Sci.* 1: 254.

Kavanagh TA and Spillane C (1995) Strategies for engineering virus resistance in transgenic plants. *Euphytica* 85: 149.

Whitelam GC and Cockburn W (1996) Antibody expression in transgenic plants. *Trends Plant Sci.* 1: 268.

PLANT RETROELEMENTS

Roger Hull, John Innes Centre, Norwich Research Park, Colney, Norwich, UK

Introduction

The replication of most nucleic acids is mainly either DNA to DNA (chromosomal and viral nucleic acids) or RNA to RNA (viral genomes). However, there is an increasing number of nucleic acid sequences which involve a combination of transcription of RNA from DNA and reverse transcription of DNA from RNA. These are termed retroelements and they are found in many taxonomic groups, including higher animals and plants, fungi, insects and bacteria. Retroelements comprise three major groups, viral retroelements, eucaryotic nonviral retroelements and bacterial elements. These major groups have been subdivided on structural and biological criteria (**Table 1**) but recent findings are blurring some of the boundaries between these groups.

The main features that distinguish these groups and subgroups are that viruses have properties which potentiate horizontal transmission; some may also be vertically transmitted. Nonviral retroelements are generally considered to be only vertically transmitted but there may be instances of horizontal transmission, say between plants by pathogenic fungi. Retroviruses and the nonviral retroelements integrate into the 'host's' genome, whereas pararetroviruses are episomal. The genomes of retroviruses, retrotransposons and retroposons encode an integrase activity, and those of retroviruses and retrotransposons have long terminal repeats, both features involved in integration. All, except for retrosequences, which are cDNAs converted from cellular RNAs by endogenous reverse transcriptase activity, encode for reverse transcriptase and its associated RNaseH.

In plants the retroelements currently described fall into the pararetrovirus, retrotransposon, retroposon and retrosequence groups. The reason for there being no known examples of plant retroviruses *sensu stricto* is unknown.

Plant Pararetroviruses

There are two genera of plant pararetroviruses, the Caulimovirus and the Badnavirus in the *Caulimoviridae* family. The genome organization can be considered as comprising the core replicon of polymerase (reverse transcriptase + RNaseH) and coat protein which is considered to organize the replication complex together with genes which adapt the replicon to the plant viral situation. These additional genes are best characterized in cauliflower mosaic virus (CaMV) in which the core replicon is encoded by open reading frame (ORF) IV giving the coat protein and ORF V the polymerase. The plant-adapting genes include the ORF I product which facilitates the movement of the virus from cell to cell through plasmodesmata, and the ORF II product which is

Table 1 Classes of retroelements

A. Viral retroelements				
		1. Retroviruses	*2. Pararetroviruses*	
Viral genome		RNA	DNA	
LTR		+	−	
RT		+	+	
INT		+	−	
Replication		By integration	Episomal	
Examples		Oncoviruses Lentiviruses Spumaretroviruses	Hepadnaviruses Caulimoviruses Badnaviruses	
B. Eukaryotic nonviral retroelements				
	1. Retrotransposon	*2. Retroposon*	*3. Retrosequence*	
LTR	+		−	−
RT	+		+	−
INT	+		+	−
Examples	Ty, copia, gypsy Tnt1, Tos1, Intercisternal A particles		LINE, Mitochondrial introns Plasmids	cDNA gene
C. Bacterial retroelements				
	Retron			
LTR	−			
RT	+			
INT	−			
Example	msDNA			

LTR, long terminal repeat; RT, reverse transcriptase; INT, integrase; LINE, long interspersed nuclear element.

involved in interactions with the aphid vector. The ORF III product probably assists with particle assembly and/or stabilization, and the ORF VI product, as well as being associated with the replication complex, has a transactivating activity associated with expression of the virus genome. It is likely that the gene products of most, if not all, the other caulimoviruses and badnaviruses have activities similar to those of CaMV.

Plant Retrotransposons

The plant retrotransposons are also termed LTR (long terminal repeat) retrotransposons to contrast them with retroposons or non-LTR retrotransposons. Retrotransposon sequences are found incorporated in the chromosomal DNAs of most, if not all, higher plant species, and have recently been found in the mitochondrial DNA of *Arabidopsis thaliana*. Retrotransposons encode various proteins, including the *gag* protein which is analogous to the retroviral coat protein, an aspartate protease responsible for cleaving various proteins into functional forms, the replication proteins which are reverse transcriptase and ribonuclease H and the integrase which is involved in integration of the retrotransposon into the host chromosome. There are two major groups of plant retrotransposons, the *copia*-like and the *gypsy*-like, which are so named because they have genome organizations (see below) similar to those of *Ty1/Copia* and *Ty3/Gypsy* retrotransposons of yeast and *Drosophila*. Most of these have been recognized by polymerase chain reaction (PCR) of total DNA from plants using specific primers.

Copia-like retrotransposons

The *copia*-like retrotransposons have been the most studied and are considered to be the most widespread in plants. However, this may be a reflection of the searches being targeted with PCR primers directed towards this class of retrotransposon. They can occur in very high copy number, for instance there are about 50 000 copies of *BARE1* retrotransposon in the barley genome, and the *Bis-1* retrotransposon is estimated to make up about 5% of the wheat genome. On the other hand some are present in low copy number, e.g. *Ta1* from *A. thaliana* and *Tst1* from potato, which have

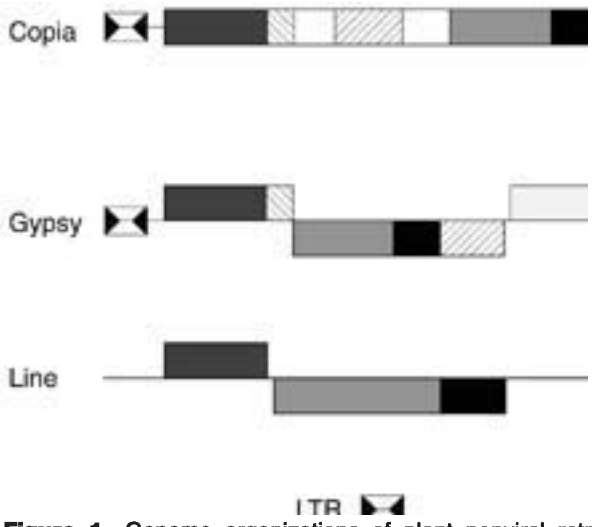


Figure 1 Genome organizations of plant nonviral retroelements. The thin line represents the DNA with the long terminal repeats (LTRs) and coding sequences shown as boxes with different infills; $(AAA)_n$ indicates the polyadenylate sequence.

one to three copies per haploid genome. Overall, *copia*-like retrotransposons appear to be in lower copy number than the *gypsy*-like elements.

The full genome of a *copia*-like retrotransposon codes for a polyprotein which is processed to give the *gag* protein, the aspartate protease, the endonuclease, the reverse transcriptase and the RNaseH (**Fig. 1**). It is thought that, as with the yeast *copia* element, the gene products downstream of the *gag* gene are expressed from a spliced mRNA, which would control the relative proportions of *gag* and *pol*.

In the plant species that have been examined most extensively, there are several to many families of *copia*-like retrotransposons which differ from each other in sequence. In rice at least 20 families (*Tos1* to *Tos20*) and in maize ten families (each with a different distinct name) have been recognized. Sequencing shows that most of these are potentially inactive as they contain point mutations, insertions or deletions which would prevent the full expression of the coding sequences essential for expression and transposition. Only a small number of *copia*-like retrotransposons (e.g. *Tnt1*, *Tto1* and *Tto2* from tobacco and *Tos10*, *Tos17* and *Tos19* from rice) have been shown to be capable of transposition. The unequivocal evidence for full transposition is limited to the detection of an insertion into a nitrate reductase gene by *Tnt1* and the increase in copy number of the other elements. However, this must be associated with the transcription of the RNA replication intermediates, which have provided useful probes for such activity. Searches for these RNA intermediates showed that they are produced under conditions of stress, such as tissue culture, bacterial and viral infections and wounding.

Gypsy-like retrotransposons

Although many fewer *gypsy*-like retrotransposons have been recognized in plants, in some species there can be many copies. For instance *del-1* makes up 0.4% of the genome of *Lilium henryi*.

The genome organization of *gypsy*-like retrotransposons (**Fig. 1**) more closely resembles that of some of the true retroviruses than does that of the *copia*-like elements. The *gag* and aspartate protease are encoded on one ORF, which can frameshift into a second ORF encoding the reverse transcriptase, RNaseH and endonuclease. However, these retrotransposons do not encode an envelope protein, which in retroviruses is on a separate ORF and is expressed from a spliced mRNA. Thus, the *gypsy*-like retrotransposon differs from the *copia*-like element by encoding the proteins in several ORFs and in the gene position of the endonuclease in relation to the reverse transcriptase and RNAse H.

Replication of retrotransposons

The replication mechanism of both groups of plant retrotransposons is considered essentially the same as that of retroviruses. A more-than-genome length RNA is transcribed from the integrated element using the promoter and termination sequences in the LTR and catalyzed by the host DNA-dependent RNA polymerase II. This acts as both the mRNA for expression of the gene products (sometimes involving splicing) and the template for reverse transcription. The formation of (−)-strand DNA is primed by the 3′ end of a tRNA whose complementary sequence is located in the transcript just downstream of the 5′ LTR. Until recently it was thought that all plant retroelements required $tRNA^{met\ init}$ for this priming but recently some other tRNA requirements have been recognized. Template strand switching, priming of (+)-strand DNA synthesis, formation of LTRs and integration of the newly synthesized retroelement are considered to be essentially as in retroviruses.

Plant Retroposons

Until relatively recently it was considered that LINEs (long interspersed nuclear elements) were exclusive to animals; however, there have been an increasing number of reports of these elements being found in plants, both in the nuclear and in the mitochondrial genomes. As with *gypsy*-like elements, this lack of their recognition is probably a reflection of targeted PCR-based searches.

The genomes of these retroposons lack LTRs but

have a 3′-terminal polyadenylate sequence. They contain two ORFs, one encoding the analogue of the *gag* gene and the other the polymerase (reverse transcriptase, RNaseH) gene (**Fig. 1**). Their replication mechanism is not yet understood. As with retrotransposons, plant species in which they have been found contain several families of LINEs, e.g. *Tal1-1* to *Tal1-17* in *A. thaliana*. Also, like retrotransposons, most of these would appear to be inactive. There is little information on activation and transposition by these elements. Fluorescent *in situ* hybridization showed that the BNR1 family of LINEs was present on all sugarbeet chromosomes but was largely excluded from the chromosomal regions harboring the 18S–5.8S–25S ribosomal RNA genes.

Evolutionary Significance of Nonviral Retroelements

There are two major aspects to the evolutionary significance of retroelements, the evolution of the elements themselves and the impact that the presence and retrotransposition of the elements has on the host plant. It has been suggested that the different types of element could occupy different 'niches' within the cell and have specific adaptation to the 'niche'. It would also appear that the relatively low proportion of active elements indicates that they are not particularly well adapted to survival in their host environment, although it may be that the inactive elements have some, as yet undetermined, positive function.

Retroelements often make up a significant proportion of the plant's genome. Although the majority of them are considered to be inactive they must represent a considerable accumulation of retrotransposition events over a long period. Various questions arise, and are currently under discussion, on the impact that these have on plant evolution. These include:

- Where did these elements come from? Do they have anything to do with retroviruses? As noted above, no true retroviruses have been found in plants. However, it is frequently overlooked that plant viruses do not require an analogue for the retroviral *env* gene that is essential for the targeting and entry of animal host cells. Plant viruses are introduced into the cytoplasm of their hosts by mechanical damage caused by their vector (often an insect feeding) and thus do not have to recognize and cross a plasmamembrane. They usually need a gene product to facilitate movement from cell to cell via cytoplasmic connections (plasmodesmata). Thus, one could envisage a plant retrovirus with the *gag* analogue, replicase, integrase and gene(s) adapting them to plants.
- Retrotransposons are widespread, if not ubiquitous, in higher plants, raising the question of whether retrotransposons move between plants. Most transmission of retrotransposons is vertical, although there is some evidence for possible horizontal transmission in association with RNA viral and fungal infections.
- What is the significance of retrotransposons to plant evolution? It is obvious that transposition into important plant genes would be detrimental to the host and most retrotransposons are found outside host coding sequences. Recent analyses have shown that, at least in some plant species, e.g. maize, retrotransposons and retroposons are grouped together, leading to the concept of 'retrotransposon landing pads'. The fact that most are 'dead', with only a small proportion being active, has led to the suggestion that they have been involved in the creation of variation in plants which is the basis for evolution. This is further supported by the recognition that activation is often in response to stress.

See also: **Plant pararetroviruses (*Caulimoviridae*): Caulimoviruses: general features, Caulimoviruses: molecular biology, Cassava vein mosaic virus, Legume caulimoviruses; Plant pararetroviruses: Badnaviruses, Plant pararetroviruses: Rice tungro bacilliform virus; Retrotransposons of fungi; Retroviruses – type D (*Retroviridae*); Retroviruses of drosophila: The gypsy paradigm.**

Further Reading

Bennetzen JL (1996) The contributions of retroelements to plant genome organization, function and evolution. *Trends Microbiol.* 4: 347.

Hirochika H, Sugimoto K, Otsuki Y, Tsugawa H and Kanda M (1996) Retrotransposons of rice involved in mutations induced by tissue culture. *Proc. Natl Acad. Sci. USA* 93: 7783.

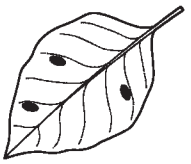
Hull R and Covey SN (1996) Retroelements: propagation and adaptation. *Virus Genes* 11: 105.

Schmidt T, Kubis S and Heslop-Harrison JS (1995). Analysis and chromosomal localization of retrotransposons in sugar beet (*Beta vulgaris* L.): LINEs and Tyl-copia-like elements as a major component of the genome. *Chromosome Res.* 3: 335.

Wessler SR (1996) Plant retrotransposons: turned on by stress. *Curr. Biol.* 6: 959.

Wright DA, Ke N, Smalle J *et al* (1996) Multiple non-LTR retrotransposons in the genome of *Arabidopsis thaliana*. *Genetics* 142: 569.

PLANT VIRUS DISEASE – ECONOMIC ASPECTS



OW Barnett and **Charles E Main**, Department of Plant Pathology, North Carolina State University, Raleigh, North Carolina, USA

Introduction

Everyone has seen some of the effects of viruses on plants, perhaps without knowing it. If you are interested in art, you probably have seen the still-life paintings from the seventeenth century Dutch school, which depict tulip flowers with beautiful stripes of color induced by tulip breaking virus. Perhaps the color variegations of certain ornamental plants have imparted their beauty to you. For example, in the woody ornamentals, velvetleaf and flowering maple (*Abutilon* spp.), which have variegated leaf colorations, the beauty of the plants is enhanced by the viral infection, increasing their economic value (**Fig. 1**).

The term 'economic aspects' can be broadly interpreted to mean not only monetary returns from crops but also the esthetic value of plants and the value of plants as members of the biosphere. Anything that affects our world will eventually have an economic effect.

There are no accurate estimates of the economic impact of viral diseases on a global basis. Among plant microbial pathogens, reduced crop production resulting from viral infections is thought to be second only to that caused by fungi. Crop production reduced from its full potential as a result of certain viral diseases varies among countries, regions and even within a local area. Although reduced production on one farm can reduce income for that farmer, a farmer in another location who has a good crop may have a higher income because increased crop prices result from the lower supply (**Fig. 2**).

A single viral disease can affect an entire country's economy. For instance, in Ghana the cacao industry provided an extremely important export commodity until cacao swollen shoot viral infections caused a decline in cocoa production by over 65% from 1936 to 1956 in the eastern region of the country. Plant viruses infect many species of Angiosperms but only a few species of Gymnosperms, Pteridophytes and Algae. In contrast to viral infections of animals, once a plant is infected with a virus it usually remains infected for life. In most plant/virus combinations, the virus invades all parts of the plant, although shoot and root meristem regions may not be invaded by some viruses. In most plant/virus combinations, viruses are not transmitted through true seed to the next generation. To understand the economic aspects of plant viral diseases, the range of effects (symptoms) of viruses on growth and development of plants need to be summarized.

Symptoms Caused by Viral Infection

Viral infections are usually obvious when a disease condition occurs, but infections can also be latent (inapparent). The diseased condition of the plant may be expressed in many ways. Reduced growth or stunting is probably the most universal symptom of a virus-infected plant. Even latent infections may result in reduced growth. Changes in color are the most obvious symptoms of viral infection for most plant/virus combinations. Decreases in green pigments of leaves lead to the development of light green and yellow areas and result in patterns of color such as mosaic, mottling, ringspots, streaking, striping or line patterns. Necrosis or death of tissue is sometimes classified as a color deviation; for instance, necrotic streaks on plant stems are common symptoms of some viral infections. Color deviations are most obvious in leaves (e.g. tobacco infected with tobacco mosaic virus), but also occur in stems (e.g. young apple twigs infected with apple mosaic virus), flowers (e.g. tulip color breaking caused by tulip breaking virus), seed pods (e.g. edible pods of French bean infected with bean pod mottle virus), fruits (e.g. tomatoes with rings and line patterns caused by tomato spotted wilt virus) and seeds (soybean seeds mottled due to infection with soybean mosaic virus).

Some viral infections interfere with water relationships, which results in wilting or desiccation leading to tissue necrosis. Tabasco pepper infected with tobacco etch virus is a classic example of a viral infection that leads to wilting. Cucumber mosaic virus infection can cause wilting of cucumber plants in certain environmental conditions.

Plant morphology can be changed by viral infection. Abnormal leaf morphology commonly occurs with viral infections that cause mosaic or mottling. The leaf lamina may be crinkled, curled, blistered, narrowed or have altered margins. Abnormal growths called enations may develop from the lamina.

Figure 1 Examples of the effects of virus infection in plants. (**A**) Tulip flower with color variegation caused by tulip breaking virus; (**B**) *Abutilon* sp. with mosaic caused by Abutilon mosaic virus.

Stems may be distorted or stem internodes shorter. Whole plants may have uncharacteristic growth patterns, such as bushy growth or a more open pattern of branching because fewer stems are produced.

Plant viral symptoms are usually not characteristic enough for identification of the virus because different viruses in the same plant can cause similar symptoms and different strains of the same virus may cause very different symptoms. Symptoms caused by viral infection may also differ with environmental conditions, plant variety and time infected. In fact, symptoms are not static and the expression of symptoms at different times after infection (symptom syndrome) must be considered when the effects of viruses on plants are studied. When viruses induce visible symptoms in

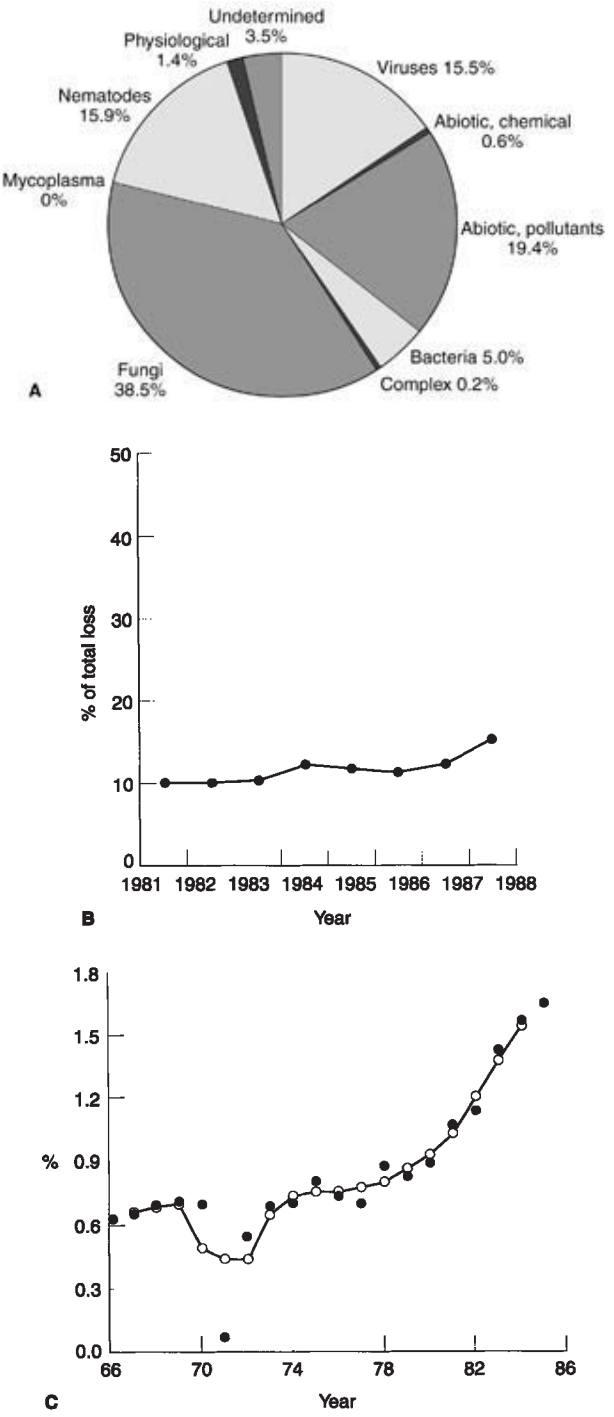


Figure 2 Relative crop losses in North Carolina, USA, due to biotic and abiotic causes and variation in crop losses due to viruses over time. (**A**) Losses from all causes in 1988 by pathogen category in 189 crop and ornamental species. Vegetables suffered a 25% reduction in yield, fruits and nuts 23%, and field crops 15% on a total crop value of $3.3 billion. (**B**) Reductions in crop value caused by viruses ranged from 9 to 15% between 1981 and 1988. Viruses reduced the value of all crops in 1988 by an average of 3.6%, a figure that is 16% of all losses. (**C**) Tobacco mosaic virus reduced the value of tobacco from 0.4% to 1.8% during 1966 to 1986; ○, moving average, ●, original values. Main CE and Gurtz SK (1989) *1988 Estimates of Crop Losses in North Carolina Due to Plant Diseases and Nematodes.* Department of Plant Pathology, Special Publication No. 8, NC State University, NC, 209pp.

plants, the initial sites of infection lead to local symptoms in 3–5 days. Upon reaching the vascular tissue, the virus is usually translocated and causes systemic symptoms. Systemic symptoms that develop first are often very severe. This acute phase is followed by a chronic phase in which symptoms are less severe or are absent even though the tissues remain infected. In some perennial plants, progressive deterioration leads to a condition called a decline. In general, viral infections cause premature senescence of plants.

Damage from Viral Infections

Viral infections of plants usually lead to symptoms of the plant disease. Symptoms result from biochemical and metabolic changes induced in the host which cause various categories of direct damage. Indirect damage from viral diseases results from procedures undertaken to maintain healthy plants. Direct damage can be categorized (Lute Bos, Research Institute for Plant Protection, Wageningen, The Netherlands) as reductions in growth, vigor, or quality and market value.

Reductions in growth

Reductions in growth due to viral infections are manifested in many ways, including crop failure, yield reductions, internode or stem length changes, reduced rooting and root growth, reduced flower and seed production and reduced seed weight (**Table 1**).

Crop failures, although not the most frequent effect, can occur with viral diseases. Sugarbeet production in the western USA was sporadic until 1940 when varieties resistant to curly top virus were introduced. In 1925, one-third of the sugarbeet crop was destroyed by the curly top disease in the Sacramento Valley of California and all of the late plantings were destroyed in the San Joaquin Valley and southern part of the Salinas Valley. When severe infections (100%) of sugarcane with Fiji disease virus occurred in Australia, growth of the canes was reduced to only 5–10% of a healthy crop, and complete fields were destroyed by growers prior to harvest because their maintenance and harvest were not economical. In The Netherlands, fields of the lily cultivar Enchantment can be 100% infected with tulip breaking virus plus lily symptomless virus. This double infection leads to leaf and stem necrosis and eventual death of the plants.

Usually even 100% viral infection does not result in total loss of the crop. For example, the complete stock of many potato varieties were once infected with one or more viruses. The production limits that viruses imposed on the genetic potential of the cultivar could not be determined until parts of the crop could be freed of virus. Potato virus M (potato paracrinkle virus) infected the total stock of King Edward potato producing only mild symptoms, but, when virus-free potatoes became available, the virus was shown to reduce the yield of tubers by around 10%. The infected plants also produced tubers of less uniform size. Potato virus X (PVX) was known as the healthy potato virus because North American potato stocks were so generally infected, with few or no symptoms. The healthiest potatoes contained only this virus. PVX was found to reduce potato yields by 9–22%, depending on viral strain and potato variety.

Potato crops have been grown in many countries for many years and cover large acreages. Viral damage which most agriculturists would consider insignificant is thus overall very extensive. The potato crop in the USA in 1989 was 370.5 million hundred weight (18.9 million tonnes) valued at $2.5 billion. A 10% reduction in yield is 41.2 million hundred weight (2.1 million tonne), or $278 million in reduced sales for 1 year.

Years ago, Holmes recognized that small yield reductions of crops having a high dollar value and grown worldwide are probably the most important economically. The mosaic disease of tobacco caused by tobacco mosaic virus (TMV) is an example of a virus that causes readily visible symptoms. Growers may not notice symptoms on plants widely scattered in a field of healthy plants. In North Carolina and South Carolina USA flue-cured tobacco, this disease causes the second or third highest losses year after year because the gene for hypersensitive resistance has not been incorporated into a flue-cured cultivar having the same high quality and yield as cultivars without this gene. For instance, when 15, 30, 60 and 100% of flue-cured tobacco plants are infected with TMV, yield is reduced by 7, 14, 21 and 30%, respectively. In 1988, TMV caused a value loss of $12.5 million with an average of 1.3% infection for North Carolina. Ironically, tobacco mosaic virus was the first plant virus described and is probably the most studied of all plant viruses. Viruses of forage legumes reduce biomass of forage from very little up to 55%. Forages are thought of as low-value crops, but they cover large areas of land so that, from a global perspective, the reduction is large.

Other viruses cause dramatic epidemics as they spread into a crop. In Ghana, cacao swollen shoot virus infected native vegetation, but soon after cacao production began the virus spread into new plantings and caused severe economic losses. Cadang-cadang is a devastating lethal disease of coconut palms, which was epidemic in the Philippines in the 1930s. Over 30 million coconut trees are estimated to have been killed

Table 1 Effect of viruses under field conditions and measured components of yield. Some examples from the literature

Field incidence or reduction		
1988	Rice stripe virus on rice 1st crop yield losses earlier infection 54–63% late infection 22–32% 2nd crop yield losses earlier infection 41–66%	Chen CC and Ko WH (1988) Effects of the stripe virus on rice yield and yield components. *Plant Protect. Bull.* 30: 259.
1992	Cucumber mosaic virus on garlic cloves Yield reduced 35–54%	Stefanac Z and Milicic D (1992) Observations on infection of garlic (*Allium sativum* L.) with cucumber mosaic virus. *Acta Bot. Croatica* 51: 1.
1993	Potyvirus on hot pepper Virus incidence 40–100% Yield reduction 15–50%	Agranovsky AA (1993) Virus diseases of pepper (*Capsicum annuum* L.) in Ethiopia. *J. Phytopathol.* (Berl.) 138: 89.
Effects of virus infections (% reduction)		
1984	Maize dwarf mosaic virus of maize Decreased yields 2.4% for each 10% increase in virus incidence	Scott GE *et al* (1988) Yield losses caused by maize dwarf mosaic virus in maize. *Crop Sci.* 28: 691.
1992	Wheat spindle streak mosaic virus on wheat Grain yield 32% susceptible cultivars 14 kg ha^{-1} reduction per 1% increase in virus incidence Yield decrease of 0.45% per net days (20–45) with temperature between 2–11°C	Miller NR, Bergstrom GC and Sorrells ME (1992) Effect of wheat spindle streak mosaic virus on yield of winter wheat in New York. *Phytopathology* 82: 852.
1992	Alfalfa mosaic virus on subterranean clover Herbage and root production 20–49% Seed yield 71% Cucumber mosaic virus on murex medic Herbage and root production 78–90% Cucumber mosaic virus on burr medic Herbage and root production 56–82% Seed yield 94% Bean yellow mosaic virus on subterranean clover Herbage and root production 78–90% Seed yield 58–76% Bean yellow mosaic virus on burr medic Herbage and root production 38–61%	Jones RAC (1992) Further studies on losses in productivity caused by infection of annual pasture legumes with three viruses. *Aust. J. Agric. Res.* 43: 1229.
1992	Maize streak virus on maize hybrids Yield: most resistant hybrids 6% Yield: most susceptible hybrids 94%	Barrow MR (1992) Development of maize hybrids resistant to maize streak virus. *Crop Protect.* 11: 267.
1993	Soybean mosaic virus on soybeans Plant height 4–17% Number of pods 9–33% Pod length 10–26% Seed per pod 6–15% Seed weight 7–18%	Haque GU, Arif M, Hassan S, Ali A and Khan M (1993) Assessment of yield losses in soybean due to soybean mosaic virus. *Sarhad J. Agric.* 9: 227.
1993	Beet soilborne mosaic virus on sugar beet Tap-root weight 20%	Kaufmann A, Koenig R and Rohloff H (1993) Influence of beet soil-borne virus on mechanically inoculated sugar beet. *Plant Pathol.* 42: 413.
1996	Lily symptomless virus on Asiatic and Oriental *Lilium* hybrids Stem lengths 8–10% Fresh weight 18–23% Bud size 12% Petal length 9%	Blake MR and Wilson CR (1996) Significance of secondary infections with lily symptomless carlavirus to cut-flower production of *Lilium*. *Ann. Appl. Biol.* 129: 39.

by this viroid disease since it was first recognized. The loss in production for each planting site with an infected tree has been valued at US$80–100, based on average yield and copra prices. The economic impact becomes even more vivid when it is realized that 96% of the world copra production occurs on small land holdings where coconut is both a subsistence and a cash crop. A similar disease, 'tenangaja', occurs on Guam.

Reductions in growth can be related to reduction in vigor or quality. In ornamental production, tobacco ringspot virus in *Jasminum*, a woody ornamental, causes fewer cuttings to root than from uninfected plants. Cuttings that do root are of poorer quality, produce 50% less growth, and have a higher mortality rate. Dasheen mosaic virus-infected *Dieffenbachia* plants produce only 39% as many cuttings per plant as do virus-free plants. The average weight of the cuttings was not reduced by virus infection, but only plants produced from virus-free plants were marketable. Virus-free narcissi not only produce twice as many flowers but also larger ones.

Reductions in vigor

This can be manifested in many ways. Seedlings are usually larger and better able to escape disease when derived from larger seeds. Virus-infected white clover produces fewer and smaller seeds, which produce smaller, slower growing seedlings. Infection of perennial crops may lead to reduced cold-hardiness or drought tolerance. Wheat or oat plants infected with barley yellow dwarf virus do not survive encasement in ice as well as noninfected plants. Red and white clover plants infected with clover yellow mosaic virus are less winter-hardy and more susceptible to drought.

Many perennial or vegetatively propagated plants develop conditions given various names such as 'degeneration', 'running out' and 'senility'. Many of these situations are related to the increasing prevalence of one or more viruses in the crop or stock plants. For example, from 1770 into the 1800s, potato cultivars introduced into Europe from true seed gradually became less productive and produced less vigorous growth. Eventually, this condition was found to result from mild infection of potatoes with a number of viruses.

White clover is another perennial that can be severely affected by viral infection. Individual plants infected simultaneously with as many as four viruses will not survive competition in the field. Grasses usually become predominant in a sward because the infected clover is slower growing and shaded by more vigorous uninfected clover and grasses. Infected clover is also more susceptible to root rot, and fewer and less efficient nitrogen-fixing nodules are produced. White clover–grass pastures should last 10–20 years, but, in the southeastern USA, the white clover component of pastures is often insignificant after 3–6 years. Although other factors are important, high viral incidence often precedes clover disappearance.

Early leaf fall occurs on rose bushes infected with *Prunus* necrotic ringspot virus. Bushes lack vigor, are more sensitive to winter kill, and reestablish with difficulty after transplantation.

In general, viral infection increases susceptibility of plants to infection by fungi. For instance, sugarbeet plants infected by beet mild yellowing virus are more susceptible to infection by *Alternaria*. Corn leaves infected with maize dwarf mosaic virus produce more lesions and earlier sporulation of *Helminthosporium maydis*. Wheat and barley infected with barley yellow dwarf virus are more susceptible to *Cladosporium* and *Verticillum* species.

In contrast, plants in which viral infection causes necrosis are protected from certain fungal infections. Cucumber plants artificially inoculated with tobacco mosaic virus are resistant to anthracnose, and similarly inoculated tobacco is resistant to several fungi.

Virus-infected plants generally tolerate air pollution better than uninfected plants. For example, tobacco infected with tobacco mosaic virus had 6% less leaf damage due to ozone. Virus-infected plants may be more attractive to aphids, aphid multiplication may be enhanced, and maturation of flying aphids may be more rapid, but sometimes the longevity of aphids is less than on healthy plants.

These examples are effects of viruses on plants in relation to additional abiotic and biotic stress. In general, virus-infected plants are damaged more by other stress factors but in some cases viral infection actually allows the plant to tolerate additional stress. Thus virus-infected plants may be more or less vigorous depending on other environmental and biotic factors.

Reduction of quality and market value

Quality and market value are reduced when plants are infected with viruses. Yellow summer squash from plants infected with several viruses have green markings on the fruit. Even a low percentage of green fruit means rejection of the truckload of squash at a processing plant. Snap beans for fresh market will also be rejected if they are mottled as a result of bean pod mottle virus infection, which causes pod symptoms. Sweetpotatoes infected with sweetpotato feathery mottle virus may be harvested with apparently high-quality tubers; but after storage many tubers will

have internal cork which severely reduces market quality. Tomato fruits from plants with late infections by tobacco mosaic virus develop internal necrosis, which reduces marketability.

Tulip flowers with color breaking were once highly valued. Now growers realize that flower breaking results from viral infections that lead to degeneration of bulb stocks. This often cited example of a 'beneficial viral infection' does not impress modern bulb growers. Grades of flower bulbs and quality of flowers are deleteriously affected by viral infection; color deviations intensify and the size of plants and flowers and yield of bulbs are reduced.

In roses, the onset of flowering is delayed, flower size decreases, and the percentage of deformed flowers is greater from virus-infected than from healthy bushes. Pelargoniums with ringspot symptoms, which can be caused by several viruses, exhibit delayed flowering, greater flower abortion, decreased flower and floret numbers, and reduced flower stem length. Orchids infected with one of several viruses may develop necrotic streaks on the flowers about a week after opening. Acceptable flowers are sometimes shipped to wholesale houses prior to symptom appearance. Foliage ornamentals may be deformed by viral infection; for example, *Dieffenbachia* infected with dasheen mosaic virus.

Quality factors such as these are of economic importance because they affect the growers' reputation. Commercial producers must establish a good relationship with their clientele. Viral diseases can adversely affect these relationships; quality may be acceptable when shipped, but upon or shortly after receipt the quality deteriorates. Producer/client relationships then suffer.

An unusual situation occurs with rhubarb. Virus-free rhubarb requires more chilling hours (20–50%) than virus-infected rhubarb. The virus-free rhubarb can not be forced as early in the season as the virus-infected rhubarb and was thus of lower commercial value. Some quality factors caused by viruses may thus be beneficial!

Indirect Effects of Viral Diseases: The Cost of Maintaining Plant Health

Bawden, in a discussion of the degenerative disease of potatoes in the late 1700s, mentioned that potato viruses prevalent elsewhere were rare in the UK. In 1963 he wrote: 'This does not mean that they (potato viruses) are unimportant; they are rare only because they are controlled, at a cost to the English potato growers of about £10,000,000 a year.' Potato viruses are controlled by systems for production and certification of virus-tested seed potatoes. The virus-tested planting material allows commercial growers to produce high-quality potatoes reliably. Although planting material may be more expensive, high-quality potatoes can be produced, perhaps sold at a higher price and with lower production costs because less land is required. Even though control is expensive, return must be greater than the cost or farmers would not have adopted the practice. Other economic aspects relate to programs that produce the virus-tested planting stock. Research scientists must monitor the crop for new viral diseases, mother potato stocks must be maintained free of virus and tested regularly, and stock increases require that inspectors monitor the process. Some growers in areas where virus spread is low will produce high-value seed potato crops. Thus, control of viral diseases means more jobs, another industry and more governmental agencies. A simple balance sheet really tells only a portion of the story about viral disease control.

When individual growers cannot solve a disease problem, governments may assist. Control of swollen shoot of cacao in Ghana included several approaches, but the major procedure was eradication of infected plants. The disease was recognized in 1936, the eradication program began in 1946 and by 1989 had removed over 188.7 million infected trees. Farmers resisted eradication of infected trees, especially those without symptoms surrounding infected trees, even though compensation was paid for trees cut down and grants were made for replanting. Political concerns sometimes override control programs for this viral disease; war, farmer opposition, political intervention, lack of trained manpower and financial resources all affected the eradication program. It is interesting that when compulsory eradication was abandoned, eradication continued on a voluntary or consent basis, but when officials announced that the compensation program might resume, farmers discontinued voluntary eradication and less effective control resulted. Despite the control efforts, this disease is still prevalent, especially in the eastern growing region.

Zadoks contends that 'most researchers have little understanding of farmers' needs' as the farmer 'has more – and often more important – concerns than crop protection.' However, successful modern agriculturists must make crop protection in general, and viral disease control in particular, an important management concern. Profit margins are so low that growers cannot afford to make many mistakes.

Assessment of Damage

Observations by trained agriculturists reveal that viruses occur commonly on many plants. Clinic

Table 2 Percentage virus infection in plant clinic samples from 1990 to 1996

Plant type	*1990*	*1991*	*1992*	*1993*	*1994*	*1995*	*1996*	*Average*
Ornamentals								
Herbaceous	7%/503[a]	3%/497	3%/501	4%/477	4%/453	4%/537	3%/419	4%/422
Interior	9%/351	4%/326	3%/398	4%/374	4%/257	4%/281	2%/279	4%/324
Field crops	12%/522	13%/581	16%/842	18%/591	9%/597	12%/481	15%/522	14%/591
Small grain	10%/156	26%/91	24%/72	22%/86	35%/52	13%/45	30%/53	23%/79
Vegetables	4%/642	5%/673	5%/679	5%/674	5%/669	3%/731	7%/807	5%/696
Average/number	8%/2174	10%/2168	10%/2492	11%/2202	11%/2028	7%/2075	11%/2080	

[a] Percentage virus infected samples/total sample numbers of plant categories submitted to the Plant Disease and Insect Clinic at North Carolina State University (unpublished).

records confirm that viruses occur consistently in various crops (**Table 2**). Assessment of damage caused by a virus requires more than knowing that a plant is infected, but this is an important first step. Diagnosis (detection and identification) often requires application of serological, nucleic acid hybridization or biological assays. Mixed infections with two or more viruses commonly occur, and these plants may have symptoms similar to those of a single infection. Effects may be additive, or a synergistic reaction may occur.

Experimental measurement of damage should be carried out in several environments, with plants inoculated at different ages, in different cultivars, with different viral strains and at various plant densities. Ideally these measurements should be made in the field, but viral spread into uninfected control plants may make this impossible. Greenhouse or growth chamber experiments allow use of more defined conditions but do not mimic field conditions.

Next, viral incidence in the crop must be determined. Surveys for viral incidence may be intensive (accurate knowledge of viral incidence but of only a small area) or extensive (less accurate viral incidence but over larger areas). Field surveys may be needed several times during crop growth to determine the duration of infection or increase in incidence. Knowing how much damage an infected plant incurs and how many plants are infected allows calculation of yield reductions and even modeling of the disease for forecasting of what will happen.

The best example of predicting viral spread, viral incidence and potential loss due to viral infection and developing spray warning schedules for control of spread by vectors is for yellows disease of sugarbeet in the UK caused by a complex of aphid-borne viruses. Yield reductions can be large and are related to the length of time sugar beet plants are infected (3–5% potential sugar yield reduction per week infected). Predictions of viral incidence for the coming growing season can be made on the basis of vector numbers and winter severity. Spray warnings are usually based on aphid numbers and viral incidence.

Concluding Remarks

Viruses are very important economically because of the diseases that they cause; they are also important tools for scientific study leading to better understanding of protein structure, antibody/antigen reactions and plant functions at the molecular level. Plant viruses have also invoked curiosity, as well as inspired poetry and art. They have been present throughout recorded history and have been a part of natural ecosystems for much longer. They are a normal part of our environment and become disruptive forces in situations where humans have managed crops for human gain.

The use of resistant varieties, if available, is the most reliable means of control. However, many strains of each virus occur and resistance is often strain specific. Biotechnology offers the means of engineering plants with novel types of viral resistance, which may be useful against a range of viruses. Transgenic plants with genes coding for the viral replicase, antisense RNA and satellite RNA may allow control of some viruses. Biotechnology has stimulated development of sensitive methods for identification and detection of viruses with such tools as monoclonal antibodies and DNA probes. Virology is an exciting and dynamic science, which promises better control methods for viral diseases.

See also: **Pathogenesis: Plant viruses; Plant resistance to viruses: Engineered resistance, Natural resistance.**

Further Reading

Agrios GN (1990) Economic considerations. In: Agrios GN

and Mandahar CL (eds), *Plant Viruses*, Vol. II, *Pathology*, p.1. Boca Raton, FL: CRC Press.
Bawden FC (1964) *Plant Viruses and Virus Diseases*, 4th edn. New York: The Ronald Press.
Bos L (1978) *Symptoms of Virus Diseases in Plants*, 3rd edn. Wageningen, The Netherlands: Pudoc.
Heathcote GD (1986) Virus yellows of sugar beet. In: McLean GD, Garrett RG and Ruesink WR (eds) *Plant Virus Epidemics. Monitoring, Modelling and Predicting Outbreaks*, p. 399. Sydney: Academic Press.
Matthews REF (1991) *Plant Virology*, 3rd edn. New York: Academic Press.
Zadoks JC (1985) On the conceptual basis of crop loss assessment: the threshold theory. *Annu. Rev. Phytopathol.* 23: 455.

POLIOVIRUSES (*PICORNAVIRIDAE*)

Contents

General Features

Philip D. Minor, National Institute for Biological Standards and Control, South Mimms, Potters Bar, Hertfordshire, UK

History

A funerary stele of a Middle Kingdom Egyptian scribe from about 1500 BC bears a carving of an individual with the withered leg and foot drop deformity characteristic of paralytic poliomyelitis. If the diagnosis is correct, this is the earliest record of a virus disease. Poliomyelitis, which is caused by poliovirus, became a well-recognized disease in Europe and North America in the first decade of the twentieth century as a result of serious epidemics which replaced the endemic pattern of disease that had occurred previously.

Poliomyelitis was transmitted to monkeys by the inoculation of extracts of spinal cord obtained from fatal human cases by Landsteiner and Popper in 1909. Enders and coworkers demonstrated that the virus could be grown in tissue culture in cells of nonneural origin in 1949, and this encouraged the development of formalin-inactivated vaccines by Salk in the mid-1950s. The difficulties of producing sufficient safe and potent vaccine led to the use of the live attenuated vaccines developed by Sabin in the late 1950s and the early 1960s. The World Health Organization has declared its intention of eliminating the disease from the world by the year 2000 and has made substantial progress towards this goal. The first complete sequence of poliovirus genomic RNA was published by Wimmer and coworkers in 1981, and the first atomic structure in 1985 by Hogle.

Taxonomy and Classification

Poliovirus is a member of the family *Picornaviridae* genus *Enterovirus*. Shown by electron microscopy to be icosahedral viruses about 28 nm in diameter, particles lack a lipid membrane. Current classification of the *Picornaviridae* divides them into six genera: *Enterovirus* of which poliovirus is the type member; *Rhinovirus* which is the causative agent of the common cold; *Hepatovirus* which causes hepatitis A; *Cardiovirus* of mice; and *Aphthovirus* or foot-and-mouth disease viruses. A sixth genus (*Parechovirus*) composed of echoviruses 22 and 23 has been recently added as the genomic sequences of these viruses are strikingly different from that of the enteroviruses with which they were previously grouped.

Classically the picornaviruses have been assigned to genera largely on the basis of buoyant density and sensitivity to acid pH, although increasingly sequence comparisons have been used. Polioviruses exist in three distinct serotypes designated type 1, type 2 and type 3. Strains may be distinguished by comparison of the sequence of small regions of the genomic RNA and are identified by serotype, name, country of isolation and year of isolation, e.g. P3/Leon/USA/1937.

Geographic and Seasonal Distribution

Polioviruses are found worldwide, although less commonly in areas where hygiene is good, and inactivated rather than live attenuated vaccines are used. Isolates are thus rare in countries such as Sweden, but common in developing countries or in

and Mandahar CL (eds), *Plant Viruses*, Vol. II, *Pathology*, p.1. Boca Raton, FL: CRC Press.
Bawden FC (1964) *Plant Viruses and Virus Diseases*, 4th edn. New York: The Ronald Press.
Bos L (1978) *Symptoms of Virus Diseases in Plants*, 3rd edn. Wageningen, The Netherlands: Pudoc.
Heathcote GD (1986) Virus yellows of sugar beet. In: McLean GD, Garrett RG and Ruesink WR (eds) *Plant Virus Epidemics. Monitoring, Modelling and Predicting Outbreaks*, p. 399. Sydney: Academic Press.
Matthews REF (1991) *Plant Virology*, 3rd edn. New York: Academic Press.
Zadoks JC (1985) On the conceptual basis of crop loss assessment: the threshold theory. *Annu. Rev. Phytopathol.* 23: 455.

POLIOVIRUSES (*PICORNAVIRIDAE*)

Contents

General Features

Philip D. Minor, National Institute for Biological Standards and Control, South Mimms, Potters Bar, Hertfordshire, UK

History

A funerary stele of a Middle Kingdom Egyptian scribe from about 1500 BC bears a carving of an individual with the withered leg and foot drop deformity characteristic of paralytic poliomyelitis. If the diagnosis is correct, this is the earliest record of a virus disease. Poliomyelitis, which is caused by poliovirus, became a well-recognized disease in Europe and North America in the first decade of the twentieth century as a result of serious epidemics which replaced the endemic pattern of disease that had occurred previously.

Poliomyelitis was transmitted to monkeys by the inoculation of extracts of spinal cord obtained from fatal human cases by Landsteiner and Popper in 1909. Enders and coworkers demonstrated that the virus could be grown in tissue culture in cells of nonneural origin in 1949, and this encouraged the development of formalin-inactivated vaccines by Salk in the mid-1950s. The difficulties of producing sufficient safe and potent vaccine led to the use of the live attenuated vaccines developed by Sabin in the late 1950s and the early 1960s. The World Health Organization has declared its intention of eliminating the disease from the world by the year 2000 and has made substantial progress towards this goal. The first complete sequence of poliovirus genomic RNA was published by Wimmer and coworkers in 1981, and the first atomic structure in 1985 by Hogle.

Taxonomy and Classification

Poliovirus is a member of the family *Picornaviridae* genus *Enterovirus*. Shown by electron microscopy to be icosahedral viruses about 28 nm in diameter, particles lack a lipid membrane. Current classification of the *Picornaviridae* divides them into six genera: *Enterovirus* of which poliovirus is the type member; *Rhinovirus* which is the causative agent of the common cold; *Hepatovirus* which causes hepatitis A; *Cardiovirus* of mice; and *Aphthovirus* or foot-and-mouth disease viruses. A sixth genus (*Parechovirus*) composed of echoviruses 22 and 23 has been recently added as the genomic sequences of these viruses are strikingly different from that of the enteroviruses with which they were previously grouped.

Classically the picornaviruses have been assigned to genera largely on the basis of buoyant density and sensitivity to acid pH, although increasingly sequence comparisons have been used. Polioviruses exist in three distinct serotypes designated type 1, type 2 and type 3. Strains may be distinguished by comparison of the sequence of small regions of the genomic RNA and are identified by serotype, name, country of isolation and year of isolation, e.g. P3/Leon/USA/1937.

Geographic and Seasonal Distribution

Polioviruses are found worldwide, although less commonly in areas where hygiene is good, and inactivated rather than live attenuated vaccines are used. Isolates are thus rare in countries such as Sweden, but common in developing countries or in

countries where live vaccines are used extensively. Strains may be indigenous to the country of origin, imported from other areas or derived from live attenuated vaccines. It has been found that the sequence similarities between indigenous strains are surprisingly well conserved over time, and that specific genotypes (defined as those differing by less than 15% at the nucleotide level) are confined to particular geographic areas, thus making it possible to identify the probable source of epidemics. This has great value in vaccination and eradication programs, as it provides a clear measure of indigenous viral transmission and its interruption by vaccination. Viral prevalence is favored by warm wet conditions and is thus high in temperate climates in summer and autumn. In tropical climates the virus is believed to be endemic.

Host Range and Virus Propagation

Poliovirus grows naturally only in humans and chimpanzees, which may both be infected by the oral route. Old World monkeys may be infected parenterally and less readily by mouth. Certain strains of virus, notably of serotype 2, can cause a paralytic disease very similar to poliomyelitis following parenteral inoculation of mice although murine cells *in vitro* are not susceptible to infection. Host restriction has been shown to be attributable to a specific receptor. Transgenic mice carrying the gene for the human receptor have been prepared, and shown to be susceptible to infection with all three serotypes, although not readily by the oral route.

Virus can be propagated in cells of human or primate origin, such as primary monkey kidney cells, human diploid cells such as MRC5 or WI38, continuous cell lines of human origin such as HeLa or Hep2c, or continuous cell lines of simian origin such as Vero. Mouse cells stably transfected with the gene for the human receptor for poliovirus are also able to support virus infection.

Genetics

Polioviruses of all three serotypes rapidly mutate on replication in the human gut, accumulating point changes during an epidemic at an estimated rate of about 10 per genome per month. Point mutations are readily selected *in vitro*. Deletions and insertions may be less common because of the strategy of translation of the viral proteins, which involves a single large open reading frame encoding a polyprotein which is then subjected to proteolytic processing. Mutations must, therefore, conserve the reading frame to give viable virus. Recombination between viruses of different serotypes is extremely common in recipients of the live vaccines and recombination *in vitro* is well documented. It is therefore likely to occur in the wild, both between and within serotypes, although this may be difficult to prove in practice. The relative stability of the sequences associated with isolates from a given geographical region is surprising in view of the high potential variability of the genome.

Serologic Relationships and Variability

Polioviruses of different serotypes are antigenically distinct and immunity to one serotype is believed not to confer significant immunity to the other two. There is evidence for antibodies able to neutralize both type 1 and type 2, however, and partial denaturation of the virus exposes antigenic sites which are crossreactive between viruses of different serotypes and other enteroviruses. Point mutations in known antigenic sites are common during infections, but the viruses can still be neutralized by polyclonal type-specific sera. Significant antigenic drift is therefore not observed, as illustrated by the fact that the vaccine strains of virus in use since 1955 have remained able to induce protective immunity against wild strains. The continuing efficacy of vaccines is also evidence that no serotype 4 poliovirus exists. It is possible that the pathology associated with poliovirus is linked to its use of a particular cellular receptor site which is determined by its structural proteins. A virus whose structural proteins have drifted to such an extent that the virus is no longer antigenically recognizable as one of the three serotypes may therefore have concomitantly changed receptor site and pathology.

Epidemiology

Young children form a reservoir of infection and high frequencies of transmission are associated with poor living conditions and low socioeconomic status. Improvements in hygiene result in less exposure in extreme infancy, when the child is protected from disease in infants by maternally acquired antibody and exposure at slightly greater ages, leading to disease, hence infantile paralysis as a term for poliomyelitis.

Transmission and Tissue Tropism

Polioviruses are generally transmitted by the fecal–oral route, although transmission from the nasopharynx also occurs. A transient viremia may follow infection but the major site of virus growth is the gut, where replication may be expected to persist for about 5 weeks in half of infected individuals. The restriction of replication to the intestine, the central

nervous system (CNS) and lymphoid tissues, such as Peyer's patches and the tonsils, cannot be explained by the exclusive expression of the cellular receptor in those tissues as it is also found in such tissues as kidneys, which are thought not to be susceptible. Virus can be excreted in stool at a level of 10^5 infectious units g^{-1}.

Pathogenicity

Most poliovirus infections are asymptomatic, with generally no more than 1 in 100 resulting in disease. Infections with type 1 polioviruses are more likely to lead to disease than those with type 3, whereas infections with type 2 are the least likely to have clinical effects. The circulation of the three serotypes occurs at a similar rate, as shown by the similar age of seroconversion to each type, and it is believed that the differences in morbidity rates reflect general differences of pathogenicity between the serotypes. Within a type, however, strains may also differ widely in pathogenicity. Although the molecular basis for the lack of pathogenesis of the live Sabin vaccine strains is at least partially understood for each of the three strains, the variation in the virulence of the wild strains has not been systematically examined and could have a number of different molecular explanations.

Clinical Features of Infection

Infection with poliovirus may initially be associated with slight gastrointestinal symptoms. This may be followed by fever, sore throat or influenza-like illness, from which the patient recovers within a few days. This pattern has been termed abortive poliomyelitis. Nonparalytic poliomyelitis may occur in 1–2% of infections, and is associated with the symptoms of abortive poliomyelitis, followed by invasion of the CNS leading to aseptic meningitis, often accompanied by back pain and muscle spasm. The illness lasts 2–10 days and recovery is usually complete. Paralytic poliomyelitis occurs in 0.1–2% of infections approximately 7–30 days after infection and usually begins with the symptoms of abortive poliomyelitis, progressing to flaccid paralysis resulting from lower motor neuron damage. Paralysis is defined as spinal or bulbar if it affects the brainstem. Encephalitis also occurs. Paralyzed patients recover wholly or to a significant degree in 10% of cases, up to 10% of cases are likely to be fatal, and in 80% of patients there is significant residual paralysis.

Pathology and Histopathology

Poliovirus naturally infects via the oral route and multiplies either in the mucosa of the intestinal tract as proposed by Sabin, or in lymphatic tissues including tonsils, lymph nodes of the neck and Peyer's patches of the small intestine as proposed by Bodian. Infection of the tonsils may occur after replication is established in the gut following a brief viremic phase, or in the initial infection by mouth. Low levels of circulating antibody, including passively administered antibody are able to prevent paralytic poliomyelitis suggesting that CNS infection requires a viremic phase. However, the clinical development of some cases suggests that infection of the CNS may occur directly from nerves associated with the intestine. Virus growth in nerve cells is restricted to motor neurons, usually the anterior horn cells of the spinal cord although in severe cases intermediate gray ganglia and posterior horn and dorsal root ganglia may be affected. Damage appears to be by replication in the neurons themselves rather than in supportive tissues, as shown by the histological location of viral genomes and antigens. In the brain, affected areas may include the reticular formation, the vestibular nuclei, the cerebellar vermis and the deep cerebellar nuclei. Polioviruses may also infect the myocardium.

Immune Response

The virus may be partially denatured by relatively mild treatments, such as heating at 56°C for 10 min or UV irradiation, changing in antigenic properties as a result from the N (or D) form found in the infectious virus to an H (or C) form. This change is also associated with the early stages of virus uncoating and is sufficiently drastic to be readily demonstrated with polyclonal sera. Individuals in the acute phase of poliomyelitis mount a humoral immune response predominantly directed against H (or C) antigen, whereas those in the convalescent phase have antibodies to the N (or D) form. Immune serum with antibodies specific for the N form is protective. Moreover, individuals suffering from primary immune deficiencies associated with defects in the humoral but not cellular arms of the immune response are particularly susceptible to infection and disease caused by enteroviruses in general and poliovirus in particular. The predominant protective immune response is thus believed to be humoral. The significance of cellular immunity to protection is not clear. However, infection or immunization with type 2 poliovirus is believed to be able to prime for a secondary response to type 1 and type 3 and this may be due to crossreactive T helper cells. Although an immune response may be detected in sera by 1–2 weeks postinfection, virus excretion persists for much longer periods. There is evidence for a mucosal

immune response and the production of immunoglobulin (Ig)A antibodies. Infection with the live attenuated vaccines produces immunity to reinfection in the gut, which although real may be of short duration.

Prevention and Control of Poliomyelitis

Whereas passive immunoglobulin confers short-lived protection from disease, strategies for the prevention and control of poliomyelitis depend on the use of vaccines either based on the formalin-killed preparations developed by Salk or the live attenuated strains developed by Sabin. No antiviral chemotherapy is available.

Vaccine viruses are grown in susceptible cells, usually primary monkey kidney cells or human diploid cells. Modern Salk-type vaccines are of far greater potency and quality than those manufactured in the 1950s and are currently prepared by concentrating, purifying and filtering the harvest to remove aggregates, then treating with 3 mM formaldehyde at 37°C for 2 weeks, before a second filtration step. The prolonged and slow inactivation method conserves the antigenic properties of the virus and the filtration steps remove aggregates which may protect virus from the formalin and therefore contain live virus particles. Such infectious aggregates are believed to have been the cause of the 'Cutter incident' of the mid-1950s in which recipients of inactivated vaccine became paralyzed. It is widely believed that immunization with inactivated vaccine has little effect on the infection of the human gut by other viruses. However, circulation of wild-type viruses in several European countries appears to have been prevented by its use.

The Sabin vaccine strains were produced by passage of poliovirus isolates under a variety of regimens. The type 1 strain is designated LSc_1 the type 2 strain P2712 and the type 3 strain Leon $12a_1b$. The passage histories leading to attenuation, including *in vivo* passage in primates and *in vitro* passage under different conditions are very different for each serotype. Attenuated strains were selected on the basis of their clinical and histopathological properties when inoculated into primates by different routes, and by their growth properties and stability in the laboratory. The molecular basis of the attenuation of the three strains has been examined, and there are striking similarities between them, including the presence of comparable mutations in the 5′ noncoding portion of the genome before the single open reading frame which probably act by reducing the efficiency of initiation of translation. The viruses are usually given as a trivalent mixture, and extensive changes occur in recipients, including loss or suppression of attenuating mutations, simple or complex intertypic recombination events in the region of the genome encoding the nonstructural proteins, point mutations in known antigenic sites and other changes of unknown significance. The estimated incidence of paralytic poliomyelitis due to reversion of the vaccine strain is 1 per 530 000 primary vaccinees, and 1 per 2 million of all vaccinees.

Live vaccines eliminated poliomyelitis attributable to wild-type poliovirus from developed countries when they were administered to infants at specified ages during the first year of life. This strategy was ineffective in developing countries, probably because many children were infected before the first vaccination and coverage was probably low. A strategy of national immunization days has therefore been developed in which the aim is to vaccinate all children up to a certain age in a single day; thus all susceptible intestinal tracts are colonized with vaccine virus and transmission of wild virus is broken. This approach has eradicated wild-type polio in the Americas, which have not had a case since 1992, and nearly eliminated it in the Far East, including China. Major efforts to eradicate polio in Africa are underway.

Future Perspectives

The World Health Organization has declared its intention of eradicating poliomyelitis by the year 2000, and substantial progress has been made to this end, using the existing live vaccines. Although a number of potential difficulties may be envisaged, including the question of when it is safe to stop vaccinating as the wild-type virus is replaced by viruses derived from the live vaccine strains, it is likely that the goal will be achieved at or near that date. Poliomyelitis will thus have been a significant human disease for exactly 100 years.

See also: **Cardioviruses (*Picornaviridae*); Enteroviruses (*Picornaviridae*): Animal and related viruses; Human enteroviruses (serotypes 68–71); Foot and mouth disease viruses (*Picornaviridae*); Hepatitis A virus (*Picornaviridae*); Polioviruses (*Picornaviridae*): Molecular biology; Rhinoviruses (*Picornaviridae*).**

Further Reading

Minor PD (1997) Poliovirus. In: Nathanson N, Ahmed R, Gonzalez-Scarano F *et al* (eds) *Viral Pathogenesis*, p. 555. Philadelphia, New York: Lippincott-Raven.

Molecular Biology

Thomas Pfister, **Caroline Mirzayan** and **Eckard Wimmer**, State University of New York Stony Brook, Stony Brook, New York, USA

Introduction

Picornaviridae is the name of a large family of small (Lat. *pico*) RNA (rna) viruses that has been subdivided into six genera: *Enterovirus*, *Rhinovirus*, *Hepatovirus*, *Parechovirus*, *Cardiovirus* and *Aphthovirus*. The first four of these genera include numerous human pathogenic agents of which poliovirus (PV) is the best known and the most feared. Poliovirus belongs to the genus *Enterovirus* which is a large group of pathogens that replicate predominantly in the gastroenteric tract, spreading occasionally from there to other tissues in the human body. Poliovirus rarely (1–2% of normal infections) invades the central nervous system (CNS). However, once in the CNS, poliovirus displays a remarkable preference for replicating in motor neurons of the gray matter of the spinal cord, causing paralysis and even death. This dreaded disease has been called poliomyelitis from the Greek words: πολιὸζ (polios, gray) and μυελὸζ (myelos; the gray matter of the spinal chord). The term poliovirus is an abbreviation of 'poliomyelitis virus'.

Poliovirus is a nonenveloped icosahedral particle, 28 nm in diameter, that consists of 60 copies each of the structural proteins VP1 and VP3, 58–59 copies of VP2 and VP4, and one or two copies of VP0 (the uncleaved precursor of VP4 and VP2) (see **Table 1**). This viral capsid is remarkably stable to organic solvents, detergents and acidic conditions. It encloses a positive-sense (mRNA sense) genome of single-stranded RNA of approximately 7500 nucleotides that is polyadenylylated at its 3′ end and linked at its 5′ end to a small viral polypeptide, VPg.

There are three serotypes of poliovirus (PV1, PV2 and PV3), classified according to the ability of immune sera or monoclonal antibodies to neutralize viral infectivity. That is, immune sera against poliovirus type 1 will neutralize (inactivate) infectivity only of poliovirus type 1, but not of type 2 or type 3. Each serotype has been further subdivided into different strains that can be identified via neutralization by strain-specific antiserum or by determining nucleotide and amino acid sequences. Poliovirus type 1, strain Mahoney [PV1(M)] was the first picornavirus genome whose sequence was determined in its entirety. Representative strains of the three serotypes have subsequently been sequenced and found to be highly homologous in both nucleotide and amino acid sequence. Moreover, microsequence analysis of all viral polypeptides has led to the establishment of a complete genetic map.

Numerous studies on the molecular biology of poliovirus, together with the elucidation of the primary structure and gene organization, and the development of means for genetic manipulation of its RNA genome (infectious cDNA) have led to the recognition of many biological processes that are representative of all picornaviruses and, indeed, of numerous plus strand RNA viruses belonging to entirely different families. First, the positive-stranded RNA genome is of messenger-sense polarity and is thus immediately infectious upon entry into the cell. Second, polioviruses have evolved a cap-independent translational mechanism which, coupled to their ability to inactivate cap-dependent translation of host cellular mRNAs, selectively promotes translation of their own genome. Third, while maintaining a monocistronic genome, they encode multiple polypeptides that are liberated by proteolysis from a single translation product, the polyprotein. Limited proteolytic processing yields precursor molecules that may serve a function distinct from those of their cleavage products. Here, unique properties of poliovirus structure and proliferation will be discussed in relation to viral and host cell metabolism.

Capsid Structure and Antigenicity

The poliovirus capsid has icosahedral symmetry and consists of 60 identical asymmetric protomers (**Fig. 1a**). Each protomer is composed of a single copy of each of the three nonidentical capsid proteins VP1, VP2 and VP3 (**Fig. 1b**). Five protomers are located in a pentameric arrangement around the fivefold symmetry axis. Twelve pentamers build up the complete virus capsid. At the interface between the pentamers, twofold and threefold symmetry axes are located (**Fig. 1a**). The smallest capsid protein, VP4, is located on the inner surface of the virion and is relatively disordered. The crystal structure of PV1 (Mahoney) revealed remarkable similarity to that of human rhinoviruses and spherical RNA plant viruses.

Although VP1, VP2 and VP3 differ in size and amino acid sequence, they have similar tertiary structures. Each capsid protein presents a common structural motif, an eight-stranded antiparallel β-barrel core (**Fig. 2A**). The loop extensions protrude from the surface of the virion where they may become well exposed, serving as the major antigenic sites of the virus.

The folding of the β strands gives the barrel the shape of a triangular wedge where the thin end of the

Table 1 Properties of poliovirus, type 1 (Mahoney)[a]

Empirical formula[b]	$C_{332,652}H_{492,388}N_{98,245}O_{131,196}P_{7501}S_{2340}$
Molecular weight of particle[c]	8.25×10^6 Da
Molecular weight of RNA[c]	2.4×10^6 Da
Percentage RNA of virion	29.2%
Diameter	28–30 nm
Sedimentation coefficient	150–160S
Density at 20°C	1.34 g cm^{-3}
Partial specific volume	0.685 ml g^{-1}
Virions/mg	7.07×10^{13}
Virions/OD_{260} unit	9.4×10^{12}
Stability to pH	3–8.5
lipid solvents	ether, chloroform
denaturants	1% SDS, EDTA, 4 M urea, 4 M guanidine–HCl
heat	45°C in isotonic solution; 56°C in hypertonic solution, 1 M $MgCl_2$
Composition	Molecules per particle
VP0	1–2
VP1	60
VP2	58–59
VP3	60
VP4	58–59
VPg-RNA	1
K^+	4900
Na^+	900
Mg^{2+}	110
Putrescine^{2+}	42
Spermidine^{3+}	12
Lipids	12 (sphingosine)
Carbohydrates	not detectable

[a] Compiled from numerous publications and from Koch and Koch (1985) *The Molecular Biology of Poliovirus*. Wien–New York: Springer-Verlag.
[b] Of the organic matter (Molla *et al.*, 1991. *Science* 254: 1647).
[c] Calculated without water molecules and cations.

VP1 wedge is directed toward the fivefold axis, and the equivalent ends of VP2 and VP3 alternate around the threefold axis. The N-terminal extensions of the capsid proteins (including VP4) form an intertwined network of connections in the interior of the capsid shell which contributes largely to its stability. Of significance is the interaction of the N-termini of five subunits of VP3 around the fivefold axis to form a five-stranded twisted tube, described as a 'β cylinder' which in turn interacts with five two-stranded β-sheets formed by the N-termini of VP4. Proteolytic processing of the P1 capsid precursor is *a priori* in this assembly pathway because the N-termini of VP3 and VP1 must be freed before pentamer formation. Additional factors may favor stability of capsids in the assembly process. One notable example is a myristic acid moiety, covalently attached to the N-terminal Gly of VP4. Myristate forms an integral part of the interior protein shell of the virion. Myristate groups are thought to stabilize the association of N-termini of VP4 with the β cylinder at the fivefold axis by interacting with amino acid sidechains of VP4 and VP3.

The exterior of the virion is marked by the already mentioned protrusions, by 'broad plateaus' and 'deep crevices'. One notable surface feature is a depression or 'canyon' formed at the junction of VP1 and VP3, encircling the fivefold axis. The 'canyon' is the receptor binding site on the virion for human rhinovirus 14 (HRV14) and for polioviruses (see next section). A number of drugs known as 'WIN compounds' have been reported to inhibit attachment of HRV to the cellular receptor or uncoating, the effect depending on the type of rhinovirus. These compounds insert into a hydrophobic pocket which lies just beneath the floor of the canyon. Drug binding induces a conformational change in this pocket that inhibits virus binding to the cellular receptor. Interestingly, in PV1 (Mahoney), the hydrophobic pocket appears to be occupied by a sphingosine-like molecule. Whether this lipid is a cofactor in assembly or disassembly remains to be seen.

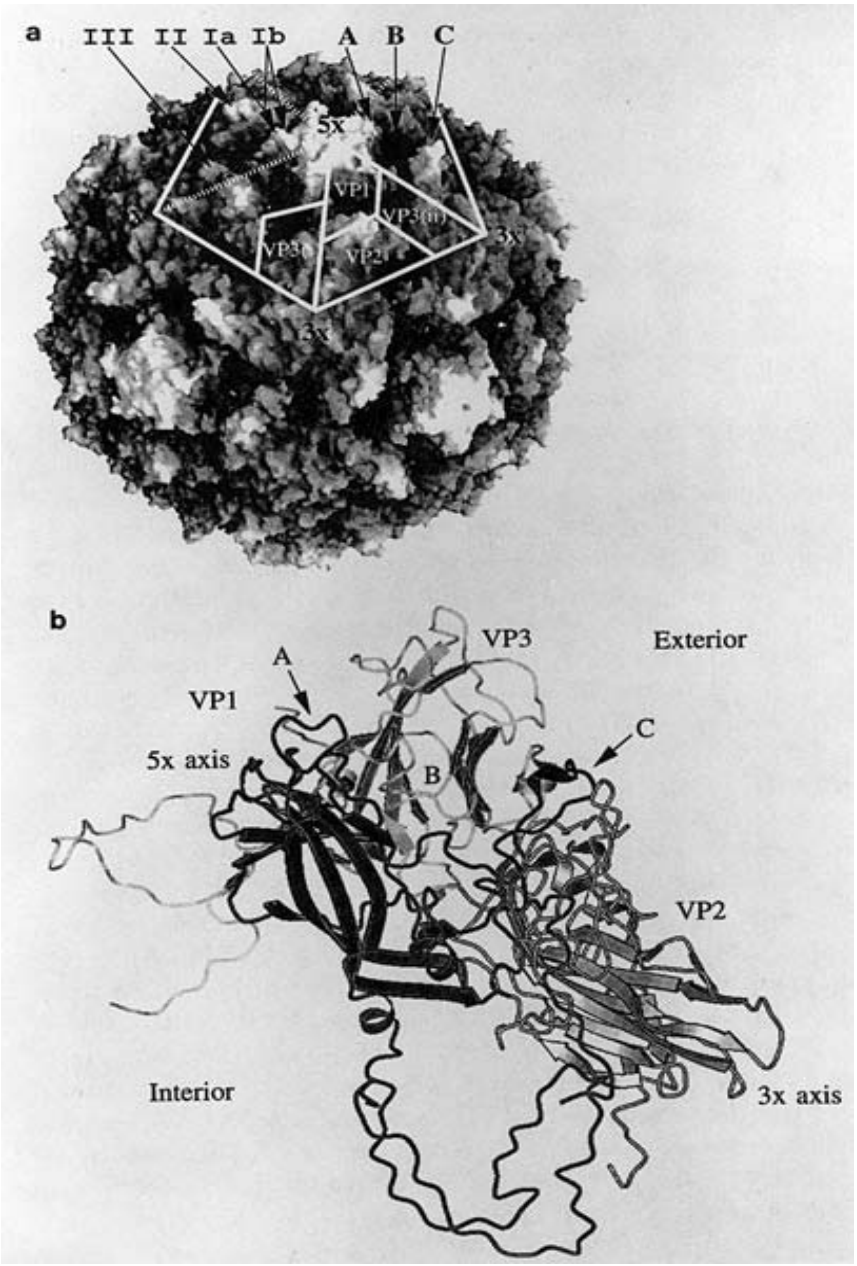


Figure 1 Structure of the poliovirion and its protomer. A complete capsid structure of PV1(M) illustrated as a water-accessible molecular surface. One of the 12 pentameric subunits of the capsid and its five constituent triangular pseudoprotomeric subunits are illustrated. The 5× and 3× labels indicate the locations of the fivefold and the threefold axes of this pentamer. The twofold axes occur at the intersection of the three adjacent pentamers. The central pseudoprotomer illustrates the subunit geometry of VP1, VP2 and VP3 (ii). The biologically relevant protomer (to viral assembly) is pear-shaped and consists of VP1, VP2, and VP3 (i). The internal VP4 protein is not visible from the surface. The canyon's north wall (A), south wall (C), and bottom (B) are indicated. The major antigenic sites to which neutralizing antibodies bind, are labeled Ia, Ib, II, III on an adjacent pseudoprotomer.

Neutralization antigenic sites (N-Ags) on the surface of the virion have been identified with neutralizing monoclonal antibodies that selected neutralization 'escape mutants' among the virus population. These escape mutants are resistant to neutralization; sequence analyses of the capsid proteins identified the site responsible for escape. The escape mutations, by definition, are located at sites to which the neutralizing monoclonal antibody would bind in the case of wild-type virus. Such mutations are found in surface

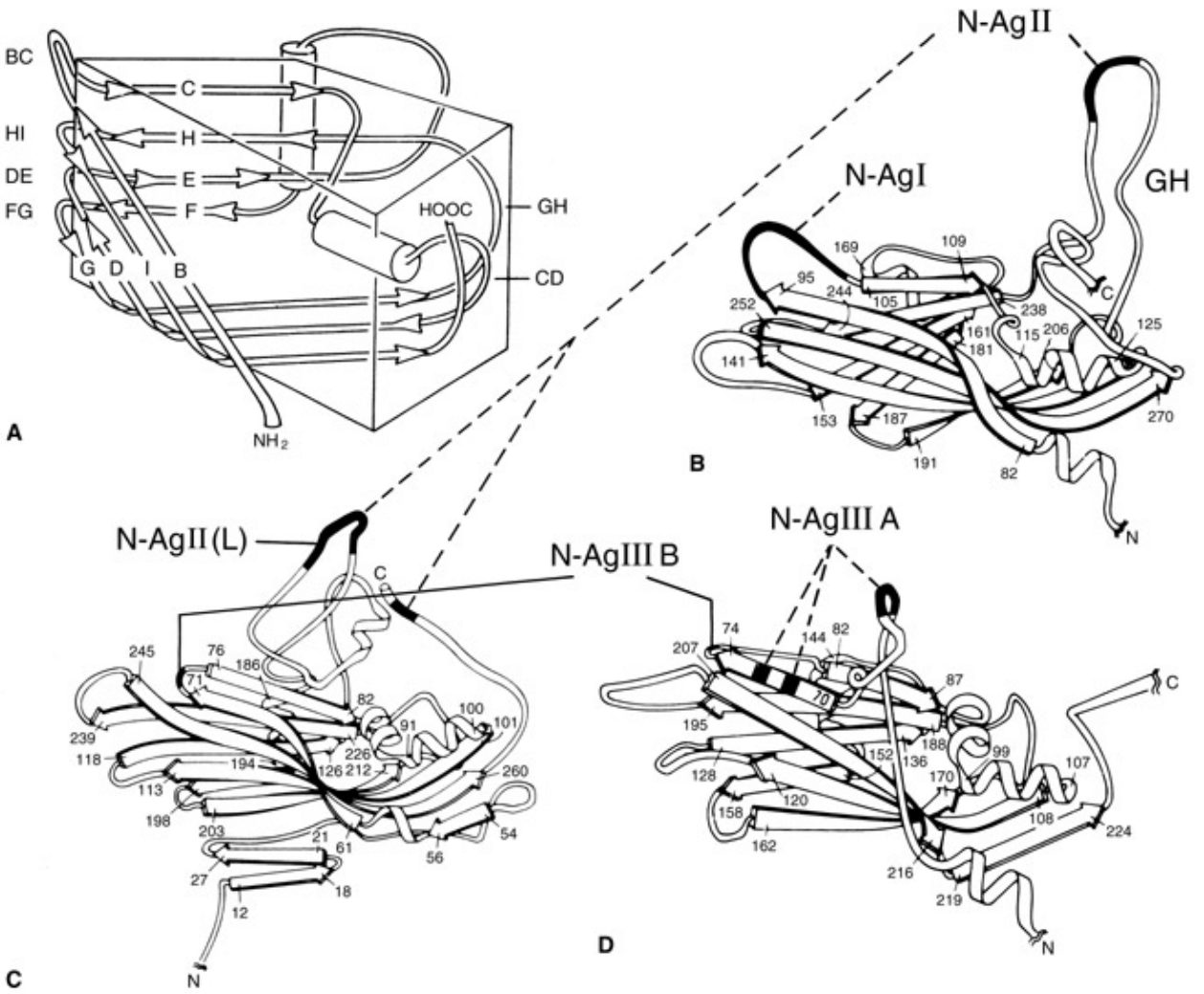


Figure 2 Schematic representation of the poliovirus capsid proteins. A common structural motif, an eight-stranded antiparallel β-barrel core, is shared among each capsid protein (**A**). Ribbon diagrams show (**B**) VP1, (**C**) VP2 and (**D**) VP3. Four neutralization antigenic sites (N-Ags) mapped to surface loop extensions are colored black. N-AgI is a continuous sequence of amino acids mapping to positions 95–105 of VP1. N-AgII is discontinuous, being composed of amino acids 221–226 of VP1 and of amino acids 164–172 and 270 of VP2. N-AgIII is composed of two independent sites, IIIA and IIIB. N-AgIIIA consists of amino acids 58–60 and 71–73 of VP3, whereas N-AgIIIB consists of amino acid 72 of VP2 and 76–79 of VP3. Note that N-AgI and N-AgII may also be subdivided, but the divisions are less distinct than those in N-AgIII.

loop structures or adjacent β strands on the exterior of the virion. Four neutralizing antigenic sites (N-Ags I, II, IIIA and IIIB) are known to map to the capsid proteins, VP1, VP2 and VP3 (**Fig. 2B, C, D**). The B–C loop of VP1 is a continuous sequence of amino acids (95–105) which maps to N-AgIA. Remarkably, this sequence can also carry determinants for poliovirus host range. A hybrid virus containing a six amino acid replacement in the N-AgIA site of PV1 (Mahoney) with that of PV2 (Lansing) can confer neurovirulence in mice. More strikingly, this sequence of ten amino acids can be replaced by heterologous amino acids; in many cases, such manipulation does not lead to loss of viral proliferation. If the heterologous sequence is that of a neutralizing antigenic determinant of another virus, a poliovirus variant has been created that can induce immune sera to foreign infectious agents. Unfortunately, this strategy has not yet led to novel vaccines, as was originally anticipated.

Recent genetic and biochemical analyses have led to the conclusion that, unexpectedly, some neutralization antigenic sites of poliovirus participate in the binding of the cognate poliovirus receptor.

The Cellular Receptor CD155 of Poliovirus

The poliovirus infectious cycle is initiated by attach-

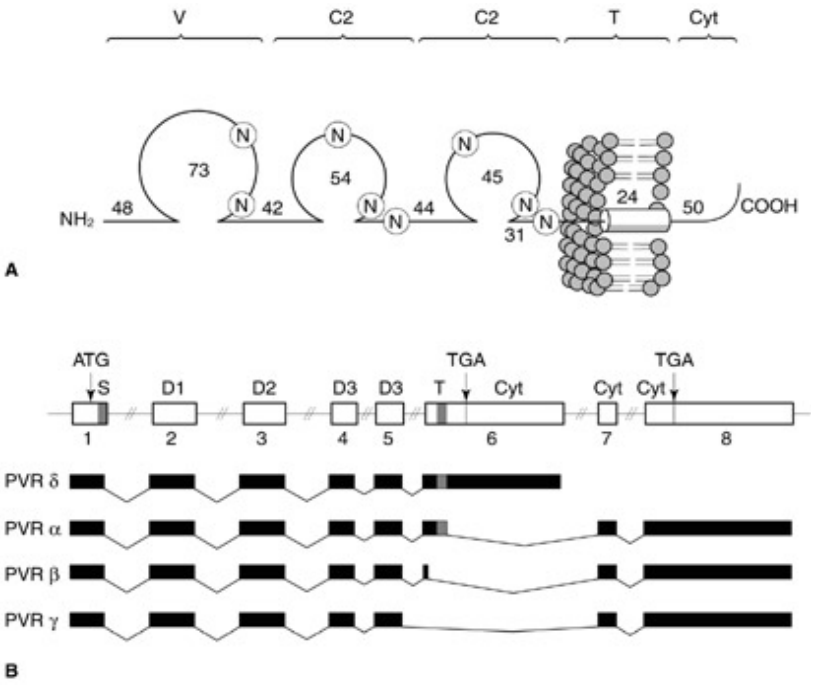


Figure 3 Structure of CD155/hPVRα and genetic organization of *CD155* isoforms α–δ. **(A)** The overall domain arrangement is indicated on top. The length of individual domains subdivided by the cysteine disulfide bonds characteristic for the immunoglobulin-like domains is indicated by numbers. Potential extracellular glycosylation sites are represented by circled 'N' symbols. **(B)**. Eight exons specifying the three extracellular domains (D1–D3), the signal peptide (S), the transmembrane region (T), and the cytoplasmic tail (Cyt) are displayed. ATG and TGA triplets determine the open reading frame for CD155 proteins. The composition according to the structure of their mRNA splice variants is indicated for each *CD155* isoform (PVRα–δ).

ment and internalization of the virus via a cellular receptor, followed by uncoating of the virus and release of the viral genome into the cytoplasm. Little is known regarding the mechanism of internalization, but the cloning and identification of the cellular receptor for poliovirus have greatly advanced knowledge of the first step of virus infection: attachment to the host cell. The cellular receptor for poliovirus (referred to as PVR and, more recently, as CD155) is a new member of the immunoglobulin (Ig) super gene family with three distinct Ig-like domains, arranged in the order, V-C2-C2, transmembrane and C-terminal cytoplasmic tail (**Fig. 3A**). The gene for CD155 has been extensively characterized; it is expressed in human tissues as four isoforms of which two are membrane-associated (and serve as poliovirus receptor), whereas the two other isoforms are secreted from cells (**Fig. 3B**). Monkeys express a homologue to CD155 that can serve as poliovirus receptor; hence, monkeys are susceptible to poliovirus infections. Mice also express a CD155 homologue. However, this protein does not bind poliovirus for which reason mice are resistant to poliovirus infection (except for some mouse-adapted virus strains that, upon injection, can kill a mouse).

All three serotypes of poliovirus compete for the same receptor; CD155, however, is not used by any other picornaviruses. Most recently, it has been reported that homologues to the human protein CD155 (*p*oliovirus *r*eceptor-*r*elated proteins PRR1 and PRR2) can serve as receptors for certain herpes viruses. The sequences of poliovirus receptor cDNAs predict protein(s) of ca. 46 kDa. However, the real splice isomers of CD155 are proteins of $\geq$80 kDa, an increase due to *N*-glycosylation of eight potential sites on the extracellular portion of the polypeptide chain. Serial deletion of the Ig-like domains of the receptor molecule has demonstrated that the V-domain is both necessary and sufficient for virus binding and infection, although the presence of the C2-domains augment this interaction. Hybrid receptor molecules have been constructed whereby the CD155 V-domain was fused to a truncated ICAM-1 molecule (the receptor for the major human rhinovirus group) or to the CD4 molecule (the receptor for the human immunodeficiency virus, type 1). These hybrid molecules are functional poliovirus receptors, confirming the V-domain as the major virus binding site. Detailed analyses of the poliovirus/CD155 interaction has identified regions in the V-domain that are likely contact points between the binding partners.

Tissue tropism, a term defining susceptibility of

specific tissues to infection with a virus, has been generally attributed to the presence or absence of receptor(s). Polioviruses display a restricted tissue tropism. They infect predominantly cells in the nasopharynx, Peyer's patches of the gut and the motor neurons in the CNS. Northern blot analyses, however, have suggested that the expression of receptor-related mRNA in human tissues is more widespread than was expected from tissues supporting poliovirus infection. Currently, this conundrum has not been solved.

As mentioned, mice are resistant to poliovirus infection (with the exception of specific mouse-adapted viral strains). Mice transgenic with the CD155 gene (CD155 *tg* mice) have been generated and they have been found to be susceptible to all three serotypes of poliovirus. However, for unknown reasons the CD155 *tg* mice cannot be infected via the oral route with poliovirus. Intramuscular, intravenous or intraspinal injections of poliovirus in the CD155 *tg* mice lead to a syndrome that is indistinguishable from human poliomyelitis (flaccid paralysis or death). Thus, although not a precise animal model for poliomyelitis, CD155 *tg* mice have served as highly valuable agents to study poliovirus pathogenesis.

The role of CD155 in docking the virus to the cell surface is undisputed. However, details of the steps subsequent to docking – uptake and uncoating – are largely obscure. Nevertheless, the interaction between cell-associated receptor and virus has a profound effect on the structure of the virion, a phenomenon that can be reproduced with purified CD155 and purified virus. Physical characteristics associated with the structural rearrangements of the virion are: altered sedimentation rate (160S to 135S), change in antigenic properties, loss of viral capsid protein VP4, and extrusion of the N-terminus of VP1. The irreversible process leads to the so-called 'A-particle'. The 'A-particle' is significantly more hydrophobic than the parental derivative and, as a result, it is able to attach to membranes. The affinity to lipids which is absent from virions, has been attributed to the N-terminal 30 amino acid residues of VP1 which may form an amphipathic helix. Currently, it is believed that endocytosis is the first step of uptake. It is possible that the hydrophobic N-termini of the VP1 molecules may make contact with the endosomal membrane, thereby building a pore through which the RNA is extruded into the cytoplasm.

Based on these considerations, it can be concluded that CD155 plays a dual role in the early steps of poliovirus infection: binding the virus to the cell surface, and 'unlocking' the integrity of the virus to allow ultimately escape of the viral genome into the cell cytoplasm. This CD155-mediated destabilization of the virus makes sense: poliovirus is extraordinarily stable to acid pH and proteases, as would be necessary for an enteric virus passing through the stomach (pH 2) on route to the intestines. Uptake into an endosomal compartment whose pH does not go below 5, would not change the virus structure and, consequently, would not lead to infection. Indeed, Fc-receptor-mediated uptake of poliovirus is nonproductive.

The Poliovirus Genome

The 'brain' of poliovirus is its genome, a single copy of single-stranded RNA, approximately 7500 nucleotides (nt) in length (**Fig. 4**). Generally, storing genetic information in RNA rather than in DNA is a poor choice since RNA is chemically less stable than DNA, even at physiological pH. Second, on entry into the cell, RNA genomes face numerous cytoplasmic and nuclear enzymes that may degrade, splice, or modify them. Third, RNA viruses cannot use cellular proofreading and editing functions to eliminate errors occurring spontaneously during genome replication at the astoundingly high rate of ca. 10^{-4} per nucleotide incorporated. RNA viruses must, therefore, live with high mutation rates. All RNA viruses, poliovirus included, have responded to these predicaments by minimizing the size of their genomes. Thus, most RNA virus genomes are $\leq$15 000 nt in length. The small genome size and the high spontaneous mutation rate has the advantage of rapid replication and swift adaptation to a new environment, respectively (for example, resistance to a drug). It has the disadvantage that these viruses must exist under conditions of extreme genetic austerity (limited coding capacity for proteins).

The heteropolymeric region of the PV1, PV2 and PV3 genomes is 7440 nt, 7439 nt and 7434 nt long, respectively. All serotypes carry at the 3′ end polyadenylic acid on the average 60 nt long. The 5′ end is covalently linked to the small, virus-encoded protein VPg. VPg is only 22 amino acids long; its third amino acid is tyrosine whose O^4 hydroxyl group is esterified to the 5′ terminal phosphate of the genome [O^4-(5′-uridylyl)tyrosine bond) such that the general structure of the 5′ end is VPg-pUU... This terminus is invariant among all picornavirus genomes.

The first 88 nucleotides of the genome form a cloverleaf structure that is an essential *cis*-acting signal for genome replication. It is followed by the *i*nternal *r*ibosomal *e*ntry *s*ite (IRES) of roughly 400 nt, a complex structure regulating translation. The IRES is separated from the long open reading frame (ORF) of the polyprotein by a spacer of 154 nt. Poliovirus

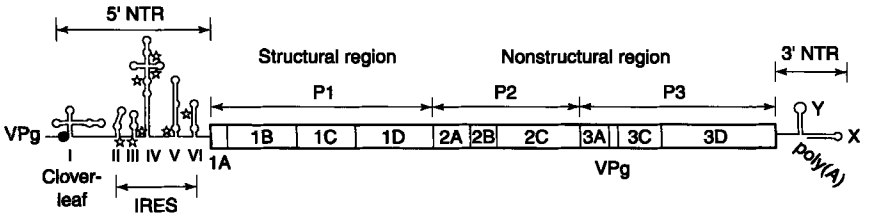


Figure 4 Genomic structure of poliovirus. The single-stranded RNA of poliovirus is shown with the genome-linked protein VPg (3B) at the 5′ end of the nontranslated region (5′ NTR, single line) and the 3′ NTR (single line) with the poly(A) tail. The boxed region shows the ORF and vertical lines within the box indicate proteinase cleavage sites within the polyprotein. The locations of the structural (P1) and nonstructural (P2, P3) region is shown on top. RNA structural domains within the 5′ nontranslated region are shown by Roman numerals; cloverleaf (I), IRES (II–VI). Stars indicate the positions of noninitiating AUG triplets. The stem–loop structures within the 3′ NTR are labeled with the letters X and Y.

encodes only one polypeptide, the polyprotein. The ORF is followed by the 3′ nontranslated region (3′NTR) that consists of 65 heteropolymeric nucleotides forming a complex double stem–loop structure, and, finally, the poly(A) tail (**Fig. 4**). Details of the function of the different sections of the viral genome are presented below.

Translation

Following uncoating of the virus in the cytoplasm, the plus-stranded RNA serves as mRNA to translate its single ORF. However, the elucidation of the genome structure of poliovirus led to the surprising discovery that poliovirus mRNA lacks some essential *cis*-acting signals common to all eukaryotic cellular mRNAs.

All eukaryotic mRNAs carry at their 5′ end a 'cap' which consists of a methylated guanosine nucleotide (m^7GpppNp...). Initiation of translation depends on recognition of the cap by, and binding of, a specific initiation factor (eIF-4) that subsequently recruits the 40S ribosomal subunit. The 40S ribosomal subunit, once associated with the mRNA, is then thought to 'scan' along the 5′ nontranslated region (5′NTR) of the mRNA until the first AUG triplet in a 'favorable context' is encountered where it pauses to assemble to a mature initiation complex. Poliovirus mRNA, however, displays a number of unique features that differ from the paradigm of the eukaryotic mRNA. In place of the cap structure, the 5′ end of poliovirus genomic RNA is covalently bound to VPg that, in fact, is removed by an unknown cellular enzyme from those viral RNA molecules destined to engage in protein synthesis. Thus, poliovirus mRNAs found in polysomes have a pUp.... 5′ terminus (note that poliovirus genomic RNA and mRNA are identical except for the presence or absence of VPg, respectively).

In addition to its unconventional pUp... terminus, poliovirus genomic RNA (= mRNA) also contains an unusually long 5′NTR of 743 nt whereas the 5′NTRs of its eukaryotic counterparts are on the average only 50–100 nt in length. In view of the fact that viral genomes tend to be small for reasons discussed above, such a long segment of noncoding viral RNA is highly unusual. Moreover, the poliovirus 5′NTR contains secondary structures that are thought to be sufficiently stable to obstruct ribosome scanning toward the initiation codon (**Fig. 4**). Finally, eight AUG codons are scattered throughout the 5′NTR preceding the initiating AUG at nucleotide 743. At least one of these AUGs is in a correct nucleotide context for efficient translation by the criterion of Kozak yet it appears to be silent.

Given these observations, it appeared likely that polioviruses (and, by analogy, all picornaviruses) use a method for initiation of protein synthesis that is radically different from that of cap-dependent eukaryotic mRNAs. Since polioviruses translate their RNA under conditions where cap-dependent translation is inactivated (see later), it was speculated that translational initiation is independent of the viral 5′ end altogether. Evidence in support of this hypothesis was obtained by constructing dicistronic mRNAs in which the first and second cistron were separated by a segment of the 5′NTR of poliovirus or EMCV (cellular 5′NTR-CISTRON 1-picornavirus 5′NTR-CISTRON 2). It was found that translation of the second cistron occurred independently of the first cistron, an observation suggesting that ribosomes could bind to a region of the viral 5′NTR placed downstream of the first cistron, and direct translation of the second cistron.

The genetic element in the 5′NTR of picornaviruses that was discovered to be necessary and sufficient to direct internal ribosomal binding was termed 'IRES', an acronym for *i*nternal *r*ibosomal *e*ntry *s*ite. All picornaviruses contain IRES elements in their respective 5′NTRs. Indeed, even the genome of hepatitis C virus, a flavivirus, contains an IRES

element. Although the function of IRES element as an internal ribosomal entry site *in vivo* was convincingly shown by expression of suitable dicistronic mRNAs in COS-I cells, final proof for 5′-independent initiation of translation was provided by an analysis of a dicistronic poliovirus containing two distinct IRES elements, one of poliovirus, the other of encephalomyocarditis virus (EMCV), a picornavirus belonging to the genus *Cardiovirus* (**Fig. 5**). Remarkably, IRES elements from different picornaviruses, or that of HCV, may not show any apparent homology in sequence and structure (**Fig. 5**). Yet, these elements function in a similar manner, a phenomenon which is demonstrated best by the fact that the cognate IRES of poliovirus can be replaced with that of EMCV, thereby generating a virus that replicates with near wt kinetics. Thus, the term IRES refers to function, not to specific structural features.

Indeed, small deletions and point mutations have profound effects on the efficiency of translation indicating that the structural integrity of the IRES element is of paramount importance. The identification of a large region of nucleotides defining the IRES elements argues for the occurrence of long-range tertiary interactions between *cis*-acting nucleotide elements and *trans*-acting factors.

The mechanism by which IRES elements function is still obscure. The minimal length for efficient function is roughly 400 nt, and some of the domains of the overall IRES structure are more sensitive to mutation than others. *Cis*-acting sequence elements have been identified that are required for IRES function. For example, all picornavirus IRESes contain an essential Y_n-X_m-AUG motif where Y is pyrimidine, n = 6–8, X is any nucleotide and m = 18–20 nucleotides). This Y_n-X_m-AUG motif may play an important role in ribosome entry. In the poliovirus IRES the Y_n-X_m-AUG motif is separated from the downstream-initiating AUG by a spacer sequence of 154 nucleotides, whereas in the EMCV IRES, the AUG of the Y_n-X_m-AUG motif is the codon from which polyprotein synthesis is initiated (see **Fig. 5**).

Depending on the origin of the element, specific sets of canonical cellular initiation factors for protein synthesis are required for IRES function. Surprisingly, cellular proteins not known to be involved in cellular protein synthesis are required as *trans*-acting factors for IRES function. The number of these cellular factors seems to be rising and their role in IRES translation has been deduced only by biochemical approaches. Genetic evidence for absolute requirement of these cellular proteins is as yet lacking. For polio- and rhinoviruses, the list of cellular factors includes: *PTB* (p57, polypyrimidine track binding protein); *PCBP1, 2* (p38, polycytidine binding protein 1 and 2); *unr* (p96; *u*pstream of *N-r*as) that is complexed to *unrip* (p38; *unr*-interacting protein), and *La* (p52, *La* auto antigen).

Little is known about tissue-specific expression of cistrons under the translational control of IRES elements. However, it has been known for some time that the IRES elements of entero- and rhinoviruses do not function well in cell-free lysates of rabbit reticulocytes (RRL). This property has contributed to the discovery of IRES elements. Presumably, RRL lacks (or is in short supply of) cellular factors for efficient function, and *unr* and *unrip* have been suggested to be responsible for this *in vitro* 'tropism' effect.

Two other cases are noteworthy. First, the attenuated vaccine strains of poliovirus (the Sabin strains) have been shown to carry point mutations in the IRES that contribute to a decrease of these strains to proliferate in cells of neuronal origin. Specifically, each vaccine strain harbors a single attenuating point mutation in domain V of the poliovirus IRES. Whereas there is no doubt about the significance of these mutations it is important to realize that the genomes of the vaccine strains contain many more mutations important for the attenuation phenotype. Second, a virus hybrid in which the cognate IRES element has been replaced by that of human rhinovirus type 2 (HRV2), has been found to be highly attenuated in *CD155 tg* mice and in *Cynomolgus* monkeys. On the other hand, the hybrid virus, called PV1(RIPO), replicates in human transformed cell lines, such as HeLa cells, with *wt* kinetics. The locus for this host range effect has been mapped to domains V and VI of the poliovirus IRES.

Proteolytic Processing of the Poliovirus Polyprotein

The poliovirus genome is monocistronic encoding a single polyprotein of 247 kDa. The polyprotein is highly unstable and will undergo multiple steps of proteolytic cleavages to yield functional structural and nonstructural proteins (**Fig. 6**). This strategy of gene expression is characteristic for most positive-strand RNA viruses and for all retroviruses. It allows maximal exploitation of genetic information under conditions of genetic austerity.

The poliovirus structural proteins encoded in the N-terminal half of the genome are four nonidentical polypeptides encoded in the order VP4-VP2-VP3-VP1. They are products of proteolytic processing of the capsid precursor P1. VP4 and its precursors VP0 and P1 are myristoylated at the N-terminus. Apart from VPg uridylylation (see below), myristoylation is the only other known modification of a polioviral

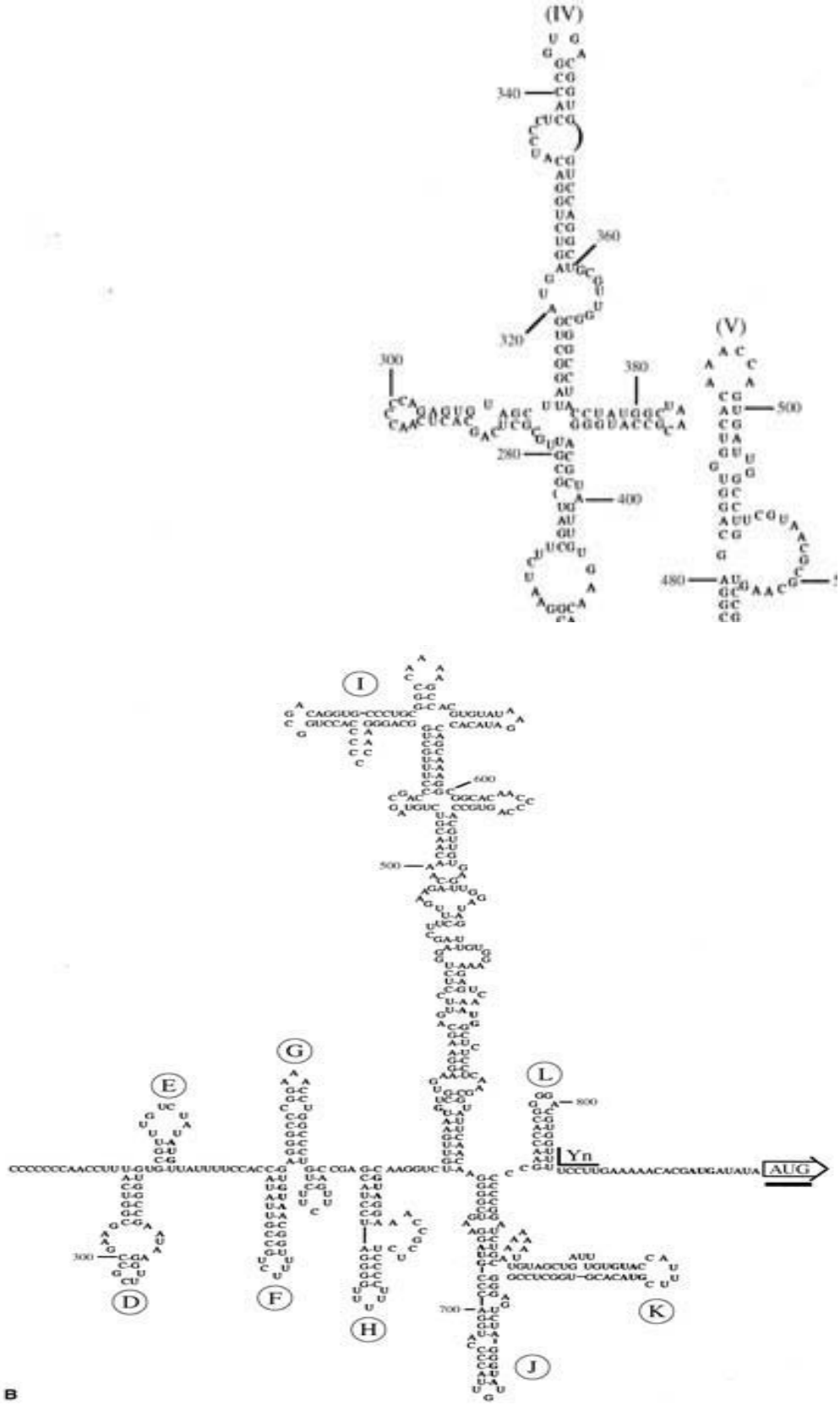
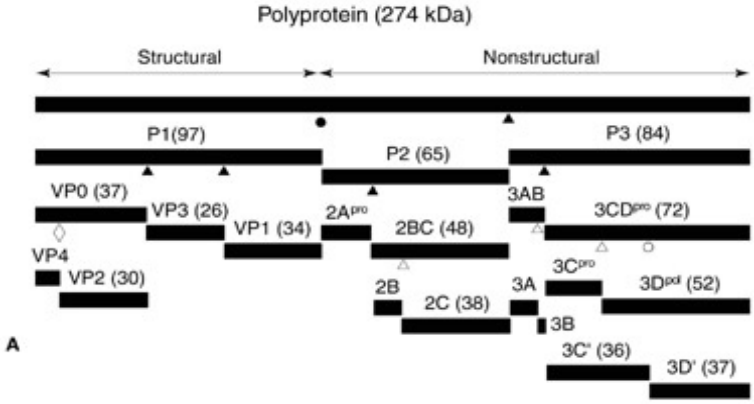


Figure 5 Comparison of the secondary structures of (**A**) poliovirus type 1 IRES element and (**B**) EMCV type 2 IRES element. Stem–loop structures are labeled with Roman numerals and letters. Yn and AUG of the YnXmAUG motif are indicated by thin and thick bars, respectively. The position of the start codons are indicated by arrows. The mutation at nt 480 (A to G) in PV1 IRES confers attenuation in the Sabin strains of PV1.



	Site	P6	P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'	P5'
B	VP0/VP3	T	L	P	R	L	Q	G	L	P	V	M
	VP3/VP1	Q	K	A	L	A	Q	G	L	G	Q	M
	2A/2B	E	E	A	M	E	Q	G	I	T	N	Y
	2B/2C	P	Y	V	I	K	Q	G	D	S	W	L
	2C/3A	M	E	A	L	F	Q	G	P	L	Q	Y
	3A/3B	L	F	A	G	H	Q	G	A	Y	T	G
	3B/3C	R	T	A	K	V	Q	G	P	G	F	D
	3C/3D	Y	F	T	Q	S	Q	G	E	I	Q	W
C	VP1/2A	K	D	L	T	T	Y	G	F	G	H	Q
	3C'/3D'	K	L	L	D	T	Y	G	I	N	L	P
D	VP4/VP2	T	A	P	M	L	N	S	P	N	I	E

Figure 6 Processing scheme and cleavage sites of the poliovirus polyprotein. (**A**) Proteolytic cleavages of the polyprotein. Triangles indicate cleavage by $3C^{pro}$ and/or $3CD^{pro}$. Solid triangles represent efficient cleavage sites, whereas open triangles represent slowly cleaved sites resulting in stable precursor proteins. The $2A^{pro}$-mediated cleavages are depicted with circles. Only the cleavage between P1 and P2–P3 (solid circle) is essential, whereas the cleavage of $3CD^{pro}$ in 3C′ and 3D′ is dispensable (open circle). The maturation cleavage is indicated by the open diamond. Numbers in brackets indicate the molecular weight in kDa. The amino acid residues at sites cleaved (**B**) by $3C^{pro}$ and/or $3CD^{pro}$, (**C**) by $2A^{pro}$ and (**D**) by an unknown mechanism (maturation cleavage) are shown by single-letter code. The positions of the amino acid residues are designated P1, P2, P3... at the newly generated C-termini, or P1′, P2′, P3′... at the newly generated N-termini.

polypeptide. The downstream P2 and P3 precursors encode the nonstructural proteins of which all are involved in genome replication and/or processing. As endproducts of processing, the P2 region yields polypeptides $2A^{pro}$ (a proteinase), 2B, and $2C^{ATPase}$, whereas the P3 region yields polypeptides 3A, $3B^{VPg}$, $3C^{pro}$ (a proteinase) and $3D^{pol}$, the RNA polymerase (**Fig. 6**). However, intermediates in the processing pathway fulfill important functions on their own, such as 2BC, 3AB, and $3CD^{pro}$.

Proteolysis of the polyprotein can be divided into three steps. The first step is to cleave the P1 capsid protein precursor from the nascent polyprotein. This primary cleavage event is catalyzed by the viral proteinase $2A^{pro}$. The second step is to process P1 into the structural proteins and to process the P2-P3 precursor into the nonstructural proteins. These reactions are catalyzed by $3C^{pro}$ and its precursor $3CD^{pro}$. The third step is the processing of VP0 into VP4 and VP2 ('maturation cleavage') which takes place at the time of encapsidation of the viral RNA. The catalyst for this event has not been identified, but it is unlikely to involve any of the aforementioned viral proteinases.

Proteolysis of the polioviral polypeptide occurs in a temporally controlled fashion. Moreover, precursor polypeptides liberated first may execute specific functions early in the viral life cycle, but are subsequently cleaved to mature proteins that have separate functions. Polypeptide $3CD^{pro}$, an RNA binding protein and important proteinase, is an example of this dual function that extends the genetic

content of the virus (see later). $3CD^{pro}$ that itself has no RNA polymerase activity, is slowly cleaved to $3C^{pro}$ and the all-important RNA polymerase $3D^{pol}$.

Substrate recognition by the poliovirus proteinases is highly restricted to viral proteins and only a few heterologous polypeptides of the cell are cleaved (see later). Proteinase $2A^{pro}$ cleaves at only two of the 10 Tyr-Gly dipeptides in the polyprotein. The first site is at the junction of VP1 and 2A and is cleaved in *cis*, whereas the second site (within the RNA polymerase, $3D^{pol}$) is cleaved in *trans* yielding products 3C′ and 3D′. This latter cleavage may be adventitious, since these cleavage products are not necessary for viral replication or proliferation. Alignment of $2A^{pro}$ cleavage sites (**Fig. 6**) shows the prevalence of a Thr residue in the P_2 position and a Leu in the P_4 position (the newly generated C terminus of a peptide bond is designated P_1, followed by the P_2, P_3, etc. residues; the newly generated N-terminus is designed P_1, followed by P_2', P_3' etc. residues). Site-directed mutagenesis of cleavage site residues suggests that the requirements for recognition of a cleavage site by 2A in *trans* are more stringent than for recognition in *cis*.

Cleavage by $3C^{pro}$ or $3CD^{pro}$ in the poliovirus polyprotein occurs exclusively at Gln-Gly dipeptides (a single exception occurs in a mouse-adapted W2 strain), but residues at positions flanking the scissile bond are mostly heterogeneous (**Fig. 6b**). Exceptions are found in the P_4 position where an Ala or other small aliphatic amino acids prevail, and to a lesser extent the P_2' position of efficiently cleaved sites, where Pro residues prevail. Only nine of 13 Gln-Gly dipeptides in the polyprotein are cleaved, an observation indicating that there must be additional determinants of cleavage site recognition. In principle, the sequence of the cleavage site determines the kinetics of cleavage. This, in turn, determines the half-life of precursor polypeptides which is very important if the precursors are to be used for special functions (see below).

Oligopeptide substrates that mimic dipeptide $3C^{pro}$ or $3CD^{pro}$ cleavage sites are cleaved *in vitro* with an efficiency that parallels the efficiency of cleavage *in vivo*. Peptides corresponding to the 2B/2C site were cleaved rapidly, whereas those corresponding to the 3A/3B and 3C/3D sites were hydrolyzed slowly or not at all. The contribution of residues flanking the scissile bond to recognition by $3C^{pro}$ or $3CD^{pro}$ has been assessed by site-directed mutagenesis of polyprotein substrates and by varying the length and composition of peptide substrates. There is a minimum substrate requirement of six or seven residues for peptide substrates to ensure cleavage; although the efficiency of cleavage increases as substrate length increases further. Experimental evidence obtained both *in vitro* and *in vivo* indicates that the P4 position regulates the efficiency of cleavage. Thus, a peptide mimicking the 3CD site was cleaved at least 100 times more efficiently following substitution of the wild-type Thr residue by Ala. The inefficient cleavage at specific sites leading to accumulation of stable precursors *in vivo* is significant, since some precursor polypeptides such as $3CD^{pro}$ have been shown to have roles (proteinase and RNA binding protein) that are distinct from those played by their constituent moieties (i.e. RNA polymerase $3D^{pol}$) after proteolytic scission. In addition to specificity at the primary amino acid level, additional determinants are dictated at the structural level. Experimental evidence supports that the entire P1 precursor is required for cleavage at Gln-Gly pairs within the structural precursor P1. Furthermore, the proper folding of the P1 (barrel structure) is required for efficient cleavage at Gln-Gly pairs. Therefore, it is likely that primary substrate recognition by $3C^{pro}$ leads to the specificity for the Gln-Gly pair whereas differences in residues flanking this dipeptide in concert with structural determinants mediate efficiency of cleavage.

Both $2A^{pro}$ and $3C^{pro}$ have been classified as cysteine proteinases since they are inhibited by iodoacetamide and *N*-ethylmaleimide, two agents blocking proteases with an active-site thiol group. On the other hand, sequence alignment and tertiary structure modeling have suggested that the picornavirus proteinases are structurally related to the cellular trypsin-like serine proteinases. On this basis, an evolutionary relationship has been proposed whereby the nucleophilic Ser residue in the catalytic triad of the cellular enzyme is replaced by a Cys residue in the equivalent viral proteinase. Site-directed mutagenesis has confirmed the proposed constituents of the catalytic triad of the poliovirus proteinases as His20, Asp38 and Cys109 for $2A^{pro}$ and His40, Glu71 and Cys147 for $3C^{pro}$. The predictions have been confirmed by the solution of the crystal structure of several picornavirus $3C^{pro}$ proteinases.

The third and final proteolytic processing step in the poliovirus replicative cycle is the cleavage of VP0 into VP4 and VP2 at an Asn-Ser site. It occurs during the final stages of virion assembly, probably after RNA encapsidation. It is not yet known what catalyzes this cleavage event, although the participation of $2A^{pro}$ or $3C^{pro}$ is unlikely since the cleavage site specificity is different from those recognized by either proteinase. It has been suggested that cleavage of VP0 may occur autocatalytically.

Finally, it has been possible to construct a dicistronic poliovirus in which the ORF of the polyprotein has been genetically disrupted at the P1*P2 junction by 602 nucleotides containing the

EMCV IRES element. This poliovirus whose genotype is (*PV*)5′NTR-P1-(*EMCV*)IRES-(*PV*)P2-P3-3′NTR grows well in HeLa cells albeit with a small plaque phenotype. Clearly, the primary cleavage is no longer required in this viral construct as the capsid precursor P1 has been genetically separated from the rest of the viral polyprotein by engineering a termination codon into the C-terminal region immediately after the C-terminal Gln residues of P1. The EMCV IRES, in turn, allows initiation at the beginning of the $2A^{pro}$ coding sequences by the use of an engineered AUG codon. This dicistronic virus is interesting for several reasons. First, as mentioned before, it provided the first conclusive evidence that IRES elements function *in vivo* as predicted from biochemical studies. Second, it represented a truly dicistronic plus strand RNA virus for which there is no other example (since mammalian mRNAs are for the most part, monocistronic, the dicistronic mRNA is a biological oddity). Third, the general gene organization of the dicistronic virus is reminiscent of that of the comoviruses. These plant viruses are closely related to the picornaviruses except that there P1 coding sequence is encoded by a separate gene segment (dipartite virus). Fourth, the viability of the dicistronic poliovirus described above has encouraged experiments in which the polyprotein was interrupted with the EMCV IRES at every site of proteolytic processing of the poliovirus polyprotein (IRES scanning). Interestingly, only two IRES insertions are tolerated by the virus: that between P1*P2 and that between P1-2A*2B-2C-P3. This result has been interpreted to mean that precursors (e.g. 2BC) are absolutely required for viral proliferation. However, in some cases, the IRES insertion led to totally aberrant proteolytic processing products, a result indicating that proper folding of the polyprotein did not occur.

RNA Replication

The replication of the poliovirus RNA genome is exceedingly complex. Any model describing the mechanism of replication must accommodate the following facts.

1. The viral genome has two different termini (see **Fig. 4**) that may suggest different mechanisms for initiation of transcription.
2. The viral RNA polymerase $3D^{pol}$ is primer dependent.
3. The genome and even minus-stranded viral RNA (the template for genomic RNA synthesis) are VPg linked (see above).
4. Genome replication occurs in a membranous environment.

Numerous biochemical and genetic studies have shown that all nonstructural proteins are involved in poliovirus genome replication. Whether cellular polypeptides ('host factors') also play an essential role in this process has not yet been proven but there is a candidate protein that may qualify for such a task (see below).

The functions of the P2-encoded polypeptides $2A^{pro}$, 2BC, 2B, and $2C^{ATPase}$ are the least understood in poliovirus replication. Genetic evidence suggested that these proteins are essential for RNA replication. Their precise function on RNA replication is yet unknown. 2BC on its own has been shown to induce membrane rearrangements in the host cell that resemble those observed in poliovirus infected cells. $2C^{ATPase}$ contains a motif (motif 'C') that is conserved among superfamily III helicases. Whereas no helicase activity has been associated with $2C^{ATPase}$, an ATPase activity and specific and unspecific RNA binding abilities have been reported. The ATPase activity of $2C^{ATPase}$ has been identified as a function that is inhibited by low concentrations of guanidine hydrochloride (Gua-HCl; $\leq$2 mM). These low concentrations of Gua-HCl were known for decades to inhibit picornavirus RNA replication. By growing poliovirus in the presence of inhibiting levels of Gua-HCl, it was possible to select viral variants that displayed guanidine resistance (g^r) or dependence (g^d). Characterization of resistant mutants identified a single mutation, Asn179-Gly, in $2C^{ATPase}$ as sufficient to render a guanidine-sensitive (g^s) virus g^r. $2C^{ATPase}$ is an example for the multifunctionality of many polioviral proteins as a consequence of genetic austerity: $2C^{ATPase}$ contains the determinants for vesicle induction (see below), is involved in initiation and elongation of RNA synthesis, and functions during RNA encapsidation and virus uncoating.

Biochemical and genetic studies of the P3 polypeptides in genome replication have yielded more information than those of P2 protein. 3AB is a nonspecific RNA binding protein that interacts specifically with both $3CD^{pro}$ and $3D^{pol}$. 3AB stimulates the polymerase activity of $3D^{pol}$ *in vitro* and may thus be a cofactor during RNA synthesis. VPg (=3B) is the primer for the RNA polymerase (see below). $3C^{pro}$ is a proteinase whose function in RNA replication may only be expressed as $3CD^{pro}$. The latter is an RNA binding protein in conjunction with 3AB (or a 'host factor', see below) and the most active proteinase encoded by the virus. $3D^{pol}$ is an RNA polymerase, that is dependent on a primer and an

RNA template. In the process of RNA elongation, $3D^{pol}$ has unwinding activity.

A hallmark of all RNA molecules synthesized by the virus is that they are covalently attached to the genome-linked protein, VPg (3B) at their 5′ end, an observation suggesting that VPg may be a primer for initiation of both plus-strand and minus-strand RNA synthesis. Biochemical studies using membrane-associated replication complexes isolated from infected cells, and genetic data have led strong support for this model. Moreover, genetic data implicated the RNA polymerase $3D^{pol}$ in the first step of initiation: the synthesis of uridylylated VPg. This biochemical reaction has been reproduced recently in a reconstituted system using purified components. Uridylylation of VPg to VPg-pU, and elongation of this nucleotidylyl-peptide to VPg-poly(U) required synthetic VPg, bacterially expressed $3D^{pol}$, poly(A), UTP, and Mn^{2+} or Mg^{2+} cations. It has been proposed that this reaction occurs at the 3′-terminal poly(A) of poliovirus, leading to the synthesis of minus strands whose 5′ termini are indeed poly(U). The 3′ end of poliovirus [the stem–loop structures together with the poly(A)] has, therefore, been termed *oriR*, the origin of synthesis of minus-strand RNA.

What is the nature of the origin of synthesis of plus strand RNA which we will call *oriL*? Clearly, it was anticipated that the 3′ end of minus strands would constitute the *oriL*. It came as a big surprise when it was discovered that the 5′ end of plus strands, the 'cloverleaf', is an essential component of the *oriL*.

The 5′NTR of poliovirus stands out due to linkage to VPg and the complex structures of cloverleaf and IRES. Genetic evidence, supported by biochemical data, has revealed that the integrity of the cloverleaf of the plus strand plays a crucial role in plus-strand RNA synthesis. Computer-aided folding, phylogenetic studies, and biochemical probing has led to a model of the cloverleaf consisting of four domains Ia–d. The integrity of these domains is important for plus-strand RNA synthesis to occur.

Studies of the role of the cloverleaf were greatly advanced when a suppresser mutation to a linker insertion at nucleotide 68 in domain d of the cloverleaf was found to map to the coding region of $3C^{pro}$. This led to the important discovery that proteinase $3CD^{pro}$, in conjunction with a second protein, has the ability to form an RNP with the cloverleaf, a process that has been proven to be essential in poliovirus RNA synthesis.

Purified poliovirus $3CD^{pro}$ or $3C^{pro}$ do not, or only weakly, bind to RNA. However, in conjunction with an RNA binding protein, $3CD^{pro}$ will bind strongly to the cloverleaf. Currently, there are two candidate polypeptides that can augment the $3CD^{pro}$ RNA binding activity: viral protein 3AB and cellular protein PCBP2, the latter being a protein that also binds specifically to a segment within the IRES. Biochemical and genetic evidence carried out at the level of RNA and $3CD^{pro}$ support the role of either protein. Genetic analyses of 3AB revealed that this protein is crucial in the formation of the cloverleaf RNP involved in plus-strand RNA synthesis.

Semiconservative nucleic acid synthesis requires base pairing of the nascent strand to the template. Therefore, synthesis of minus-strand RNA leads to the formation of double-stranded (ds) RNA (**Fig. 7**). It has not yet been determined whether dsRNA is unwound immediately following its synthesis resulting in a predominantly single-stranded (ss) RNA of negative polarity, or whether dsRNA is stable until the next round of replication. Available evidence argues for the latter scenario. First, dsRNA (replicative form, RF) has been isolated from infected cells, and its role as an intermediate in RNA replication has been shown in kinetic experiments. Second, free minus-stranded RNA has not been detected in infected cells. Thus, only RF and RI contain minus strands in infected cells. Third, $3D^{pol}$ has unwinding activity during elongation indicating that the RNA replication mechanism encounters dsRNA. In contrast, helicase activity, that would unwind dsRNA in a polymerase-independent fashion, has not been found to be associated with any of the viral and cellular proteins involved in polioviral RNA replication.

There are two possible mechanisms for the initiation of plus-strand RNA synthesis. First, a preformed VPg-pU primer may be translocated from the 3′ end of the plus strand to the 3′ end of the minus strand. Second, uridylylation of VPg may occur with the 3′ end of minus strand as template. Initiation of plus strand RNA synthesis is expected to commence on the single-stranded 3′ termini of minus strands. It is possible that the left end of the RF (**Fig. 7**) serves as *oriL* whose heteroduplex structure, after recognition by the viral replication machinery, is destabilized by viral and cellular proteins. This would allow formation of the plus-sense cloverleaf RNP which, in turn, would liberate a free 3′ end of minus strands for initiation of plus-strand synthesis. This scenario is persuasively similar to the events leading to initiation of DNA synthesis in the replication of DNA viruses with protein-linked genomes, such as adenovirus. For example in the replication of adenovirus one viral (DNA binding protein) and three cellular polypeptides (two transcription factors and one topoisomerase) stimulate protein-primed DNA synthesis on a heteroduplex template. By analogy, the *oriL* of poliovirus RF could be destabilized by several viral

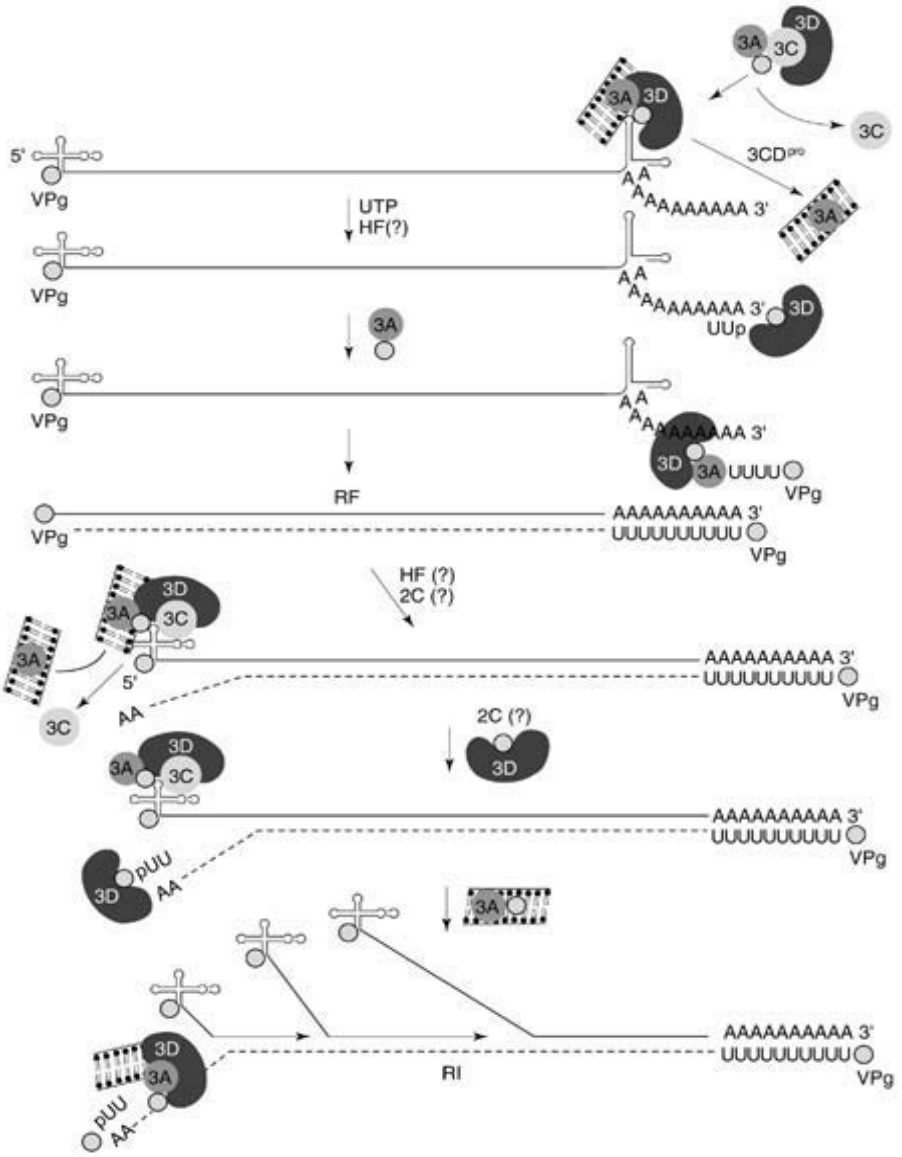


Figure 7 Models of poliovirus minus and plus-strand RNA synthesis. Plus-strand is shown with solid line, minus-strand with dashed line. The viral proteins involved in RNA synthesis and the details of the process are described in the text. The association of 3A and 3AB with membranes is indicated. RF, replicative form; RI, replicative intermediate; HF, putative host factor.

and cellular proteins, presumably those proteins that bind subsequently to the 5′ cloverleaf of plus-strand RNA or to the complementary 3′ cloverleaf of the minus-strand RNA. This hypothesis also provides an explanation for the bizarre requirement of the 3AB/3CD^{pro}/cloverleaf RNP at the 5′ end of plus-strand RNA for initiation of positive-stranded RNA. Apart from preventing annealing of the strands, the RNP may also aid in the recruitment of VPg and 3D^{pol} for uridylylation to occur at the 3′ end of minus strands, and for an influx of 3AB for stimulation of the elongation reaction. This speculation is based on the observation that many of the viral nonstructural proteins display homo- and heterobinding affinities to each other.

If the RF is the template for plus-strand synthesis, strand elongation and multiple rounds of initiation on the same minus-strand template will lead to the multistranded RI. What factors are responsible for the separation of the two RNA strands of the RF at the replication fork? As mentioned before, 3D^{pol} has RNA duplex unwinding activity while in the process of strand elongation. Thus, a requirement for a helicase in the replication fork, originally assigned

to $2C^{ATPase}$, may not exist. It should be noted that no helicase activity is used at any stage of protein-primed DNA synthesis on dsDNA templates by the polymerases of the DNA viruses. If this scenario is correct, it follows that the structure involved in the synthesis of plus strands may consist of a double-stranded backbone from which newly synthesized RNA strands emerge (**Fig. 7**). Such structure has been called 'replicative intermediate' (RI).

Considering the available information, the model of poliovirus genome replication that emerges is described in **Fig. 7**. The *oriR* is recognized by the membrane bound complex of $3AB/3D^{pol}$, and/or by $3AB/3CD^{pro}$, that leads to cleavage of 3AB and template-dependent uridylylation of VPg (3B). The template at the *oriR* is poly(A). Chain elongation leads to the synthesis of VPg-poly(U) and, subsequently, to the formation of RF. It is possible that this mechanism of initiation can only occur on single-stranded plus strands; hence, there would be no reinitiation on a template strand, and no formation of 'negative' RI molecules. The double-stranded *oriL* of the RF then is being recognized by viral proteins such as 3AB, $3CD^{pro}$, and perhaps $2C^{ATPase}$ and cellular proteins, leading to the formation of the plus-strand cloverleaf RNP. Processing of 3AB and release of the $VPg/3D^{pol}$ complex from the membranes is followed by uridylylation of VPg and synthesis of plus strands (VPg-pUUAAAACAG...). Reinitiation at the *oriL* may not only be possible but also efficient, hence the formation of RI molecules and excess synthesis of plus strands over minus strands during genome replication.

Morphogenesis

Morphogenesis of viral particles from isolated components has not been achieved to date. Morphogenesis involves proteolytic cleavage of P1 to yield the protomer (VP0, VP3, VP1), a complex that does not dissociate but forms pentamers $(VP0, VP3, VP1)_5$ that may aggregate to form empty capsids ('procapsids') $[VP0, VP3, VP1)_5]_{12}$. Crystallographic studies indicate that the processing of P1 leads to extensive 'migration' of newly formed polypeptide termini that, as noted earlier, result in stable interactions within protomers and pentamers. Whether empty capsids are biologically significant or merely an experimental artifact is uncertain. In contrast, pentamers have been shown to be associated with RNA *in vivo* and to possess RNA binding activities *in vitro*. Thus, morphogenesis may be triggered by binding of pentamers to progeny RNA. This first step in virus morphogenesis may be relatively uncoordinated, since no encapsidation signal has been identified so far. Binding of pentamers to the RNA may be followed by condensation of the RNA and capsid assembly. These events may require functions of $2C^{ATPase}$ and membranes. The ultimate particle is the provirion that contains uncleaved VP0. The existence of the provirion has been supported by biochemical and genetic analyses. The subsequent cleavage of VP0 into VP2 and VP4 marks the final step in virus maturation and renders the particle infectious.

Cellular Responses to Poliovirus Infection

Poliovirus replication takes place in the cytoplasm of the host cell and can occur in enucleated cells. It has been shown that concomitant with poliovirus infection is a rapid shut-off of host cell protein synthesis. This event is accompanied by the proteolytic cleavage of a polypeptide of 220 kDa, termed eIF-4G (formerly p220). This protein is part of the cap-binding complex eIF-4 (consisting, in addition to eIF-4G, of the cap binding protein and the initiation factor eIF-4A) that recognizes the capped 5′ end of eukaryotic mRNAs in initiation of translation. A poliovirus mutant was reported with a single amino acid insertion in $2A^{pro}$ (nucleotide 3690) that was deficient in inhibition of host cell protein synthesis. Moreover, cleavage of eIF-4G did not occur in cells infected with this mutant. Indeed, $2A^{pro}$ synthesized *in vitro* can induce cleavage of eIF-4G *in vitro*. Although purified $2A^{pro}$ can cleave eIF-4G directly, the slow kinetics of processing suggests that cellular factors are involved in this reaction. EIF-4G is a component of the cap-recognition factor eIF-4. It follows, that degradation of the eIF-4G leads to a decline of cap-dependent translation. Indeed, poliovirus uses its $2A^{pro}$ proteinase to induce the total shut-off of cellular protein synthesis while using its IRES to proceed with cap-independent translation.

More recently, genetic and biochemical evidence have implicated $3C^{pro}$ in the inhibition of the polymerase III transcription system, specifically, in the cleavage of a TFIIIC-containing complex. Whether $3C^{pro}$ directly or indirectly cleaves TFIIIC, or a component of this complex is unclear.

Electron microscopical studies of poliovirus-infected cells has revealed the emergence of an enormous body of virus-induced vesicles (viroplasm) that occupies almost the entire cytoplasmic space of the infected cell. Vesiculation of the cytoplasm coincides with RNA replication and with the occurrence of cytopathic effect (CPE) of the infected cells. On an ultrastructural level, the vesicles have been shown to bud from the endoplasmatic reticulum (ER), but other organelles like Golgi apparatus and

lysosomes may also contribute to vesicle formation which results in the disappearance of the morphological features of ER and Golgi. The expression of protein 2BC in yeast and mammalian cells in the absence of other viral proteins leads to the induction of vesicles indistinguishable from those observed in poliovirus-infected cells. The determinants of vesicle induction are located in the 2C moiety of 2BC, whereas the 2B moiety contains determinants that control the morphology of the vesicles. Protein trafficking along the secretory pathway is severely inhibited in the host cell. Inhibition is mediated by the viral proteins 2B, 2BC and 3A. Interestingly, the fungal metabolite brefeldin A (BFA) that also interferes with the secretory pathway, inhibits poliovirus replication. This observation suggests that certain steps of the secretory pathway are required for virus replication whereas other steps are disadvantageous and blocked by the viral gene products.

Virus-induced vesicles have been isolated and their morphology and biochemical activities have been studied. The isolated membranous fractions ('crude replication complex'; CRC) contain single-stranded, RI and RF RNA as well as structural and nonstructural proteins. In the presence of nucleoside-triphosphates, VPg is uridylylated, RF is chased into RI and plus-stranded RNA molecules are released. Detergent treatment of CRC prevents the release of ssRNA and abolishes uridylylation of VPg. This latter observation contrasts with the aforementioned ability of $3D^{pol}$ to uridylylate VPg in the absence of membranes in a reconstituted system.

Electron microscopy combined with immunocytochemistry, shed light on the structural organization of the RC. The RCs appear to be surrounded by virus-induced vesicles with a diameter of 100–500 nm, giving the complex the shape of a rosette with the RC in its center. The RC itself harbors numerous smaller vesicles. Both types of vesicle carry the proteins 2B, 2BC and $2C^{ATPase}$ on their surface. It has been proposed that these proteins mediate the structural organization of the RC. 3AB, the precursor of VPg, is also associated with the membranes by virtue of a hydrophobic domain in the 3A moiety. Binding of 3AB to $3D^{pol}$ and $3CD^{pro}$ recruits these proteins to the membranous RC, since $3D^{pol}$ and $3CD^{pro}$ do not associate with membranes by themselves. In addition, capsid precursors, antigenically related to protomers and pentamers are located on the surface of the virus-induced vesicles and within the RC, where they associate with RNA. It was therefore concluded that the RC is not only the site of RNA replication but also the site of early events in RNA encapsidation.

All *in vivo* RNA replicating systems known today function in close association with cellular membranes. However, the role of membranes in RNA replication is not clear. One can imagine membranes to act as a scaffolding for the replication complex, analogous to the role of the nuclear matrix in DNA replication and transcription. Membranes may also act to concentrate the viral proteins at the site of replication. Alternatively, since the viral RNA polymerase ($3D^{pol}$) is promiscuous in the choice of template, membranes may serve to separate the vRNA from contaminating cellular RNAs during replication. In addition, the virus-induced vesicles may also protect the viral replication machinery from a hostile cellular environment.

The Replication of Poliovirus: An Overview

Poliovirus is an extremely efficient parasite. Immediately upon entry and uncoating of the virion, its messenger-sense RNA is translated. Protein synthesis peaks between 2.5 and 3.5 h post infection (pi). In the same period, virus-induced vesicles start to appear. Maximal RNA synthesis is delayed by 1 h compared to protein synthesis. The onset of infectious virus correlates well with RNA replication. One infectious cycle takes only 6–7 h and, under optimal conditions in suitable host cells (e.g. HeLa cells, a human tumor cell line), up to 10^5 new virus particles may be synthesized per cell.

The replicative cycle of poliovirus is shown schematically in **Fig. 8**, and can be summarized as follows.

1. Poliovirus attaches to and is destabilized by the cellular receptor CD155.
2. Uptake and uncoating occurs presumably via the endocytotic pathway.
3. Genomic RNA is immediately translated into one polyprotein that is proteolytically cleaved by three virus-encoded proteinases ($2A^{pro}$, $3C^{pro}$ and $3CD^{pro}$) to yield functional proteins. The initiation of translation is controlled by the IRES element. Processing, in turn, is controlled by specific amino acid sequences surrounding the scissile bonds.
4. The production of protein 2BC leads to the proliferation of membranous vesicles that are involved in the membrane-associated RNA replication. Host cell protein synthesis is shut off by the action of the proteinase $2A^{pro}$ that induces the cleavage of eIF-4G.
5. RNA replication follows the common pathway of plus strand–minus strand–plus strand synthesis.

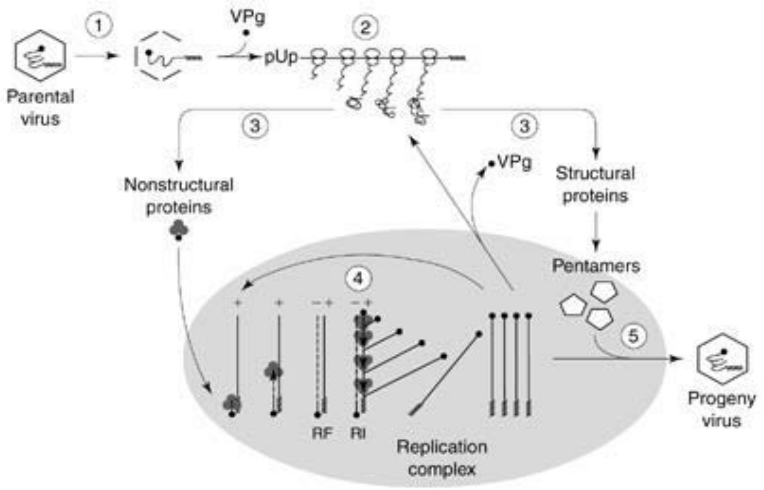


Figure 8 The replicative cycle of poliovirus. The replicative cycle of poliovirus is located in the cytoplasm of the host cell. The major steps are (1) virus binding to the receptor and uncoating, (2) translation, (3) processing of the polyprotein into functional proteins, (4) RNA replication, (5) encapsidation and virus morphogenesis. Newly synthesized plus-stranded RNA molecules may have three destinies: translation, template for RNA transcription, or encapsidation. RNA replication and early events in encapsidation are confined in a structure called replication complex.

Initiation of RNA synthesis by $3D^{pol}$ is protein (VPg)-primed.

6. Newly synthesized plus-strand RNA can enter the RNA synthesis cycle, or it can become mRNA (whereupon its 5′ terminal VPg is cleaved off), or it can be encapsidated once enough capsid proteins have been synthesized.
7. Virus assembly is poorly understood. Progeny RNA may be encapsidated by 12 pentamers. The mechanism of the maturation cleavage is obscure.

Molecular Genetics

The genetics of poliovirus is dominated by an average mutation rate of 6.3×10^{-4} per nucleotide incorporated. This high rate of mutation, a phenomenon common to all RNA viruses, results from the fact that, during evolution, RNA viruses have made the choice not to develop proof reading and editing functions (DNA and RNA polymerases operate with a similar error frequency of roughly 10^{-4}). Consequently, virions of an RNA virus isolate constitute a vast population of different genotypes replicating at the threshold of error catastrophe – the hallmark of a quasi-species. For quasi-species, a *wild-type* isolate is defined as (1) a population of viruses not having a single sequence, and (2) a population of viruses having a consensus sequence.

The high error rate in genome replication may be the main reason for the small size of the genome. An advantage of small ($<10^4$ bases) over large genome size is that more progeny RNAs can be generated from input RNA per unit time and thus more replicas are likely to survive in a hostile environment. This, combined with rampant genetic variation (mutation and recombination) allows for the rapid genetic adaptation to a new environment (host cell adaptation, drug resistance, etc). A harsh consequence of the small genome size, however, is that poliovirus must live under conditions of severe genetic austerity. That is, the virus encodes just enough proteins to survive. No viral gene products, and hardly any viral genome sequence, can be deleted without lethal consequence to the virus.

The extent of genome compression due to the high error frequency in replication has led to the elimination of sequence motifs that could effect temporal expression of poliovirus genes. This, in turn has led to the evolution of the viral polyprotein, the only translation product of the viral genome-length mRNA. This polyprotein, however, is not very stable as functional polypeptides are generated rapidly by proteolytic processing of the polyprotein, catalyzed in *cis* and *trans* by virus-encoded proteinases. As mentioned before, some precursor polypeptides in the processing cascade have functions distinct from that of their endproducts, and several processing products may have multiple distinct functions (e.g. 3AB, $3C^{pro}$, $3CD^{pro}$ and $3D^{pol}$). This strategy has provided the means to expand the number of tools available for virus replication. It also serves to explain why experiments of genetic complementation, although well documented for some viral mutants

but not for others, have not allowed genetic complementation groups to be deduced.

Genetic variation of the poliovirus genome, through misincorporation of nucleotides, is augmented by genetic recombination occurring with astounding high frequencies ranging from 10^{-2} (intratypic) to 10^{-5} (between different serotypes). Homologous recombination takes place by a mechanism of copy choice during minus-strand synthesis, and it occurs between heterologous genomes as well as, preferentially, between sibling genomes. Using the HeLa cell-free system of *de novo* poliovirus growth *in vitro*, genetic recombination has been achieved also *in vitro* by simultaneously growing genetically different poliovirus strains (starting with isolated viral RNAs) in extracts of uninfected HeLa cells.

Considering the genetic 'plasticity' of the poliovirus genome, the restriction of poliovirus to only three serotypes is surprising. A hypothesis has been developed that suggests that the serotype restriction is related to the specific manner in which poliovirus capsid interacts with the cognate cellular receptor.

The study of the genetics of poliovirus was revolutionized by the generation of infectious poliovirus cDNA. The viral RNA genome that was converted into double-stranded DNA was suddenly available for genetic manipulations hitherto unheard of. Due to the cellular transcription machinery, transfection of suitable cells with the virus-specific double-stranded DNA then yielded poliovirus at low frequency. The efficiency of this process was greatly enhanced when it was shown that the virus-specific cDNA could be transcribed with phage T7 RNA polymerase to yield unlimited amounts of viral RNA that was nearly as infectious as RNA isolated from virions.

Cell-free, *De Novo* Synthesis of Poliovirus

Viruses, lacking the genetic information as well as the tools to provide most of the essential components to replicate, are obligatory intracellular parasites. The complexity of viral proliferation – macromolecular synthesis of polypeptides and genomic nucleic acid, and encapsidation – has led to the text book wisdom that viruses are obligatory intracellular parasites unable to proliferate outside living cells.

However, poliovirus RNA (either obtained from virions or by transcription from plasmid DNA), when incubated in an extract of uninfected HeLa cells void of nuclei, mitochondria and cellular mRNA, will direct translation, genome replication, and genome encapsidation such that infectious particles are formed that are indistinguishable from poliovirus isolated from tissue cultures. Thus, poliovirus is the first virus that has been synthesized *de novo* in a cell-free extract of mammalian cells.

This experiment that has been repeated in several laboratories, has nullified the notion that viruses can proliferate exclusively in living cells. Moreover, the novel approach can be used to study individual steps of viral replication in the absence of cell membrane barriers. Since *de novo* RNA synthesis was found to be a prerequisite for production of infectious particles in the cell-free extract, this system has provided a unique tool for the study of RNA replication, recombination and morphogenesis *in vitro*. Indeed, two reports have recently described experiments of genetic recombination of poliovirus in the cell-free environment.

Concluding Remarks

Poliovirus is a virus with a brilliant past and a fascinating presence, but with no future. Poliovirus, the etiologic agent of poliomyelitis, was one of the most feared and, therefore, is one of the most thoroughly studied infectious agents today. Ninety years of investigations of poliovirus biology and pathology have unraveled numerous secrets in virology and cell biology. These efforts produced two superb vaccines that, as a triumph of medical research, will ultimately lead to a global eradication of poliovirus.

The nature of the causative agent of poliomyelitis was identified 90 years ago by Karl Landsteiner and Erwin Popper. On 18 December 1908, they reported to the k.k. Society of Physicians in Vienna the successful transfer of poliomyelitis from a deceased victim to *Cynocephalus hamadryas* monkeys. Since the human specimen from the spinal cord was free of bacteria, Landsteiner and Popper concluded correctly that 'a so-called "invisibles" e.g. a virus belonging to the class of Protozoa, must have caused the disease'.

Chemical and molecular studies have shown that poliovirus is a remarkably simple biological entity: an aggregate consisting of only five different species of macromolecules (four proteins and one single-stranded RNA) that form an icosahedral particle of 241 macromolecules altogether. This 'chemical' can be crystallized, just as has been done with tobacco mosaic virus before, and the empirical formula of its organic matter can be calculated ($C_{332\,662}$; $H_{492\,388}$; $N_{98\,245}$; $O_{131\,196}$; P_{7500}; S_{2340}). Spectacularly, the information stored in the genomic RNA is sufficient for replication and *de novo* poliovirus synthesis, provided an extract of mammalian cells. The 'chemical' poliovirus will now reveal properties of a living entity: the ability to proliferate. These studies have

demonstrated that the life cycle of a virus can be studied outside living cells, a new strategy in virus research as yet unique to poliovirus.

In nature, poliovirus will only replicate if it collides with a suitable host cell that provides the means of entry and the environment for virus-specific synthesis. Infection of host cells reveals the second face of the particle: that of a pathogen leading to the destruction of the host cell, if not incapacitation or death of the host organism.

During the last 40 years, many aspects of the genetics and molecular biology of poliovirus have been extensively studied. A model of replication has emerged, as yet incomplete, that describes the most important steps in proliferation. Hallmarks are the mode of initiation of protein synthesis by an IRES element, the processing of the single polyprotein in a variety of functional proteins, the initiation of RNA synthesis by protein priming, and the replication of RNA in the absence of a DNA intermediate.

Viral pathogenicity is governed by tissue tropism which, in turn, is determined by the distribution of the viral receptor and cell-internal conditions for replication. The poliovirus receptor (CD155), an immunoglobulin-like molecule of unknown non-pathological function, has been identified, but its distribution in human cells does not seem to match the targets of poliovirus infection. Poliovirus normally growths in the gastrointestinal tract. Serious pathogenic properties become apparent only when the virus, by a 1% chance, invades the CNS. There, the virus has a unique and as yet unexplained preference to attacking and destroying motor neurons, the reason for the flaccid paralysis characteristic of poliomyelitis. The spread of the virus from the gastrointestinal tract to the CNS can occur either by passage through the blood–brain barrier or by retrograde axonal transport. The IRES element of poliovirus functions well in cells of the CNS; this is not true for IRES elements of some of the other picornaviruses. Thus, exchanges of IRES elements can lead to highly attenuated poliovirus strains.

Following the success in the global eradication of human smallpox virus, the 42nd World Health Assembly of the World Health Organization adopted an action plan (WHA42.22) to eradicate poliovirus by the year 2000. Progress is impressive as at the time of the writing of this review, *wt* polioviruses have vanished from most parts of the world. Eventually, poliovirus will be history. Although poliovirus might be gone, the lessons it told us in molecular biology and viral pathogenesis will survive and help to unravel the secrets of the remaining viral pathogens belonging to the *Picornaviridae* and other plus-strand RNA virus families.

See also: **Coxsackieviruses (*Picornaviridae*); Immune response: Cell mediated immune response; General features; Pathogenesis: Animal viruses; Polioviruses (*Picornaviridae*): General features.**

Further Reading

Hogle HM, Chow M, Filman DJ (1985) Three-dimensional structure of poliovirus at 2.9 Å resolution. *Science* 229: 1358.

Kitamura N, Semler BL, Rothberg PG *et al* (1981) Primary structure, gene organization and polypeptide expression of poliovirus RNA. *Nature* 291: 547.

Lawson MA, Semler BL (1990) Picornavirus protein processing: enzymes, substrates and genetic regulation. *Curr. Top. Microbiol. Immunol.* 161: 49.

Minor PD (1991) Antigenic structure of picornaviruses. In: Racaniello VR (ed.) *Current Topics in Microbiology and Immunology: Picornaviruses*. Berlin: Springer-Verlag.

Molla A, Paul AV, Wimmer E (1991) Cell-free, de novo synthesis of poliovirus. *Science* 254: 1647.

Paul AV, van Boom JH, Filippov D, Wimmer E (1998) Protein-primed RNA synthesis by purified poliovirus RNA polymerase. *Nature* 393: 280.

Rueckert RR (1996) *Picornaviridae:* the viruses and their replication, pp. 609–654. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology,* 3rd edn. Philadelphia: Lippincott-Raven.

Wimmer E, Harber JJ, Bibb JA, *et al* (1994) Poliovirus receptors. In: E. Wimmer (ed.) *Cellular Receptors for Animal Viruses,* p. 101. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Wimmer E, Hellen CUT, Cao X-M (1993) Genetics of poliovirus. *Annu. Rev. Genet* 27: 353.

POLYDNAVIRUSES (*POLYDNAVIRIDAE*)

Don Stoltz, Department of Microbiology and Immunology, Dalhousie University, Halifax, Nova Scotia, Canada

Taxonomy and Classification

The polydnaviruses are defined primarily on the basis of genome structure. The polydnavirus genome is segmented, consisting of a population of double-stranded, circular DNA molecules of variable molecular mass (the name 'polydnavirus' is derived from: *poly*disperse *DNA virus*); it should be noted that no other dsDNA virus is known to have a segmented genome. Two distinct genera of polydnaviruses are recognized: the bracoviruses, found only in the parasitoid (wasp) family *Braconidae,* and the ichnoviruses, found only in the family *Ichneumonidae.* In addition to having distinct host ranges, the braco- and ichnoviruses are quite different in terms of at least one other important parameter: morphology.

Virion Structure and Morphogenesis

Bracovirus particles consist of cylindrical nucleocapsids of uniform diameter (40 nm) but variable length (30–150 nm); these are surrounded either individually or in groups by a single unit membrane (**Fig. 1**). Ichnovirus particles possess considerably larger nucleocapsids (approximately 85 × 330 nm) which are typically uniform in size, having the form of a prolate ellipsoid; ichnovirus nucleocapsids are commonly, but not always, uniform in length. Typically, nucleocapsids are individually surrounded by *two* unit membranes (**Fig. 2**).

Morphogenesis occurs only in the wasp ovary, specifically in a region referred to as the calyx, which lies between the ovarioles and the oviducts; it begins during the late pupal stages of parasitoid development, with the *de novo* appearance within the nucleus of membranes destined to form the envelope and the inner membrane, respectively, of braco- and ichnoviruses. In both braconid and ichneumonid parasitoids, mature virions enter the lumen of the oviduct, where in large numbers they comprise what is generally referred to as calyx fluid, which, like typical virus suspensions at high concentration, is opalescent. Exit of mature bracovirus particles appears to involve sloughing and lysis of calyx epithelial cells; ichnovirus particles, on the other hand, bud through the plasma membrane, thereby acquiring a second envelope. It is known that the ichnoviruses bud through the nuclear envelope, but how they traverse the cytoplasm prior to budding through the plasma membrane has not as yet been determined.

The Polydnavirus Genome

The polydnavirus genome, as extracted from purified virions, consists of double-stranded circular DNA molecules of variable molecular mass. A single female wasp ovary will often contain virus sufficient for most

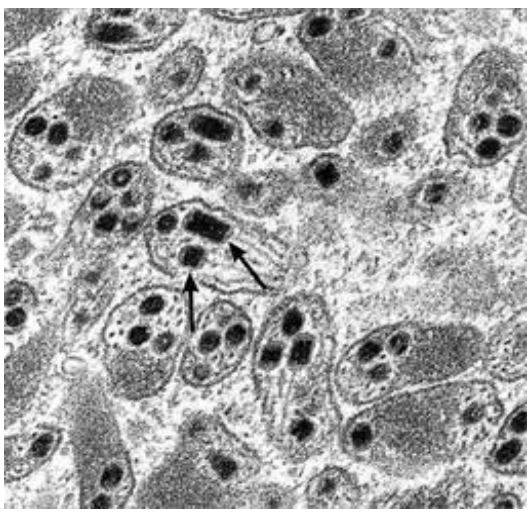


Figure 1 Bracovirus from *Cotesia paleacritae*; note that each viral envelope encloses several nucleocapsids. Nucleocapsids are indicated by arrows.

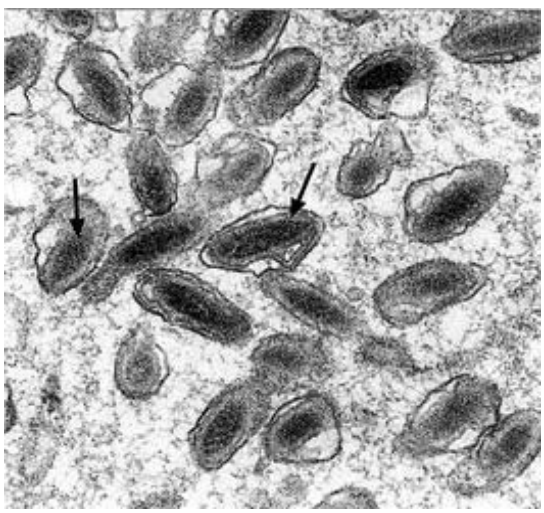


Figure 2 Ichnovirus from *Hyposoter exiguae*; note that each nucleocapsid is surrounded by two unit membranes. Nucleocapsids are indicated by arrows.

of the virus genome to be visualized following agarose gel electrophoresis and ethidium bromide staining. Ichnovirus genomes (which have been most fully characterized) appear to consist of 20–40 different molecules (genome segments), with an aggregate molecular mass of 300 kbp or more. Genetic complexity may be less than this might suggest, however, since a number of genes have been observed to be present on more than one genome segment; indeed, in some cases, smaller genome segments are repeated in their entirety within one or more larger ones. There thus exist families of both genes and genome segments. Smaller repetitive motifs have also been described. Bracovirus genomes have not as yet been investigated to the same extent; however, preliminary observations would suggest that some bracovirus genome segments are larger than those typically associated with the ichnoviruses. No convincing rationale for the segmentation of polydnavirus genomes has not as yet been proposed.

Almost nothing is known concerning how polydnavirus genomes are packaged. However, it is reasonable to suspect that the relatively large ichnovirus particle would contain a complete genome (i.e. all genome segments packaged within each nucleocapsid); on the other hand, the variable length of bracovirus nucleocapsids likely reflects packaging of DNA molecules of different sizes (i.e. one genome segment/nucleocapsid).

It is now clear that there exist within the parasitoid genome stretches of DNA which are homologous to, and colinear with, each encapsidated viral DNA molecule. Common sequences, then, are represented in both linear (chromosomal) and circular (viral) form.

Life Cycle

It is now evident that *all* individuals of *all* affected species carry polydnavirus. In keeping with this observation, it now seems clear that polydnavirus genomes are chromosomally transmitted in both egg and sperm cells in accordance with simple mendelian rules; transmission then, occurs with 100% efficiency. Recent work suggests that viral DNA replication is preceded by a series of excision events, which serve to release proviral sequences from the wasp genome; a site-specific recombinase (Hin-like) may be involved. After being excised, viral genome segments are amplified, possibly by a rolling circle mechanism (not proven yet), prior to encapsidation into virions. Preliminary evidence suggests that DNA replication may be triggered by the molting hormone, ecdysone.

Electron microscopy has established that virus particles are exported, initially into the lumen of the oviduct; during oviposition, both parasitoid eggs and polydnavirus particles are injected into the larvae of insects destined to serve as hosts for the next generation of parasitoids. Considerable experimentation has shown that in the absence of polydnavirus, parasitoid eggs/larvae are typically destroyed by an immune response. Polydnaviruses are in fact responsible for a variety of physiological changes which are induced in the parasitized host; in addition to immunosuppression, these include growth inhibition, hormonal perturbation, the inhibition of phenoloxidase activity, and so on. Many of the observed physiological changes are assumed to be relevant to successful parasitism; this raises the very important question of how polydnaviruses mediate these myriad activities.

Following oviposition, polydnavirus particles gain entry to a variety of host tissues. Nucleocapsids reach the nuclei and become uncoated either at nuclear pores (bracoviruses) or within the nucleus itself (ichnoviruses); following this, viral DNA apparently persists within host cells throughout the natural course of parasitism (parasitoid larvae usually complete development within one to two weeks.) Virus-specific transcription occurs in parasitized host animals, and would appear to be required for successful parasitism; again, note that transcription occurs here in the absence of viral morphogenesis. Viral gene products are assumed to be responsible for some or all of the observed changes associated with parasitism.

The polydnavirus life cycle, then, may be envisioned as consisting of two 'arms'. One of these, responsible for virus transmission and replication, is mediated by linear, chromosomal DNA. The second arm, mediated by circular, encapsidated DNA molecules, is responsible for a genetic colonization of the parasitized host, ultimately ensuring successful parasitism, and indeed a candidate immunosuppressive gene product has now been identified. Other virus-specific gene products have been identified, but not as yet associated with a specific biological function.

Origin

The polydnavirus phenomenon compels us to ponder the meaning of the word 'virus', since these agents could just as well be referred to as nuclear secretions. Whether or not they are viruses depends, in the first instance, on how one defines a virus. Certainly, the polydnaviruses must at the very least be described as unconventional, in the sense that there is no such thing as an uninfected host in which a productive infection can be initiated: as noted previously, all polydnavirus-carrying wasps are already infected! In

essence, each polydnavirus genome is part of a cognate wasp genome. The important question, then, is: did polydnaviruses arise from wasp genomes, or did the genes of a formerly 'classical' virus somehow become associated with certain ancestral wasp genomes.

Definitive answers to these questions are at this point in time unavailable, perhaps in part because very little sequence information is available for any polydnavirus genome. Thus, for example, we are presently unable to determine whether homologies with other viral genes exist. Although the evidence for a viral origin for the polydnaviruses is only indirect, it is nonetheless compelling: wasp ovaries appear to be unusually permissive to the apparently noncytocidal replication of a wide variety of viruses (a recent compilation included at least one member of each of the following: baculoviruses, poxviruses and rhabdoviruses, to which we may now add reoviruses and ascoviruses) and a number as of yet unclassified agents. Such observations strongly suggest that opportunities have existed (and maybe still do) for the establishment of symbioses between viruses and parasitic wasps; it may reasonably be argued that two such symbioses gave rise to the braco- and ichnoviruses.

Significance

Polydnaviruses have obvious significance in the sphere of ecology. These viruses are carried by hundreds, perhaps thousands, of species of parasitic Hymenoptera, many of which parasitize more than one species of insect (indeed, it may be argued that this is the largest group of viruses known to exist). Many host species are important pests, populations of which are often maintained at acceptably low densities as a consequence of parasitism by wasp species which carry polydnaviruses. It may be assumed that some of the virus-specific gene products expressed in parasitized host insects, particularly those having immunosuppressive properties, will ultimately have potential in the biopesticide industry.

What is perhaps most significant about polydnaviruses, however, is the degree to which these viruses may modify our concept of the range of possible virus/host interaction. Arising, one assumes, from originally 'typical' viruses (i.e. those capable of initiating a fully productive infection), the polydnaviruses would appear to have achieved considerable success in terms of the degree to which their life cycles have become integrated with those of their hosts (and see above). Since all individuals belonging to any given affected species carry virus, the acquisition of a polydnavirus genome must presumably have preceded speciation. Perhaps in keeping with this observation, no other viruses have been shown to be required for the survival of the organisms which carry them. An investigation into the coevolution of parasitoids and polydnaviruses – insofar as this is possible – will surely provide interesting reading for years to come!

Further Reading

Fleming JGW and Krell PJ (1993) Polydnavirus genome organization. In: Beckage NE, Federici BA and Thompson SN (eds) *Parasites and Pathogens of Insects*. New York: Academic Press.

Stoltz DB (1993) The polydnavirus life cycle. In: Beckage NE, Federici BA and Thompson SN (eds) *Parasites and Pathogens of Insects*. San Diego: Academic Press.

Stoltz DB and Vinson SB (1979) Viruses and parasitism in insects. *Adv. Virus Res.* 24: 125.

Stoltz DB and Whitfield GJ (1992) Viruses and virus-like entities in the parasitic Hymenoptera. *J. Hymenopt Res.* 1: 125.

Strand MR and Pech LL (1995) Immunological basis for compatibility in parasitoid/host relationships. *Annu. Rev. Entomol.* 40: 31.

POLYOMAVIRUSES – MURINE (*PAPOVAVIRIDAE*)

Contents

General Features

James M Pipas, Department of Biological Sciences, University of Pittsburgh, Pittsburgh, USA

History

Polyomavirus was discovered by Ludwig Gross in 1953 by virtue of its ability to induce tumors in inoculated newborn mice. While studying murine leukemia virus (MLV), a retrovirus that induced chronic leukemia when injected into newborn mice, Gross observed that some animals inoculated with MLV preparations developed adenocarcinomas of the parotid gland. Extracts of the tumor contained an activity, termed parotid virus, that passed the tumor to newly inoculated animals. A variety of physical techniques demonstrated that parotid virus was a different virus from MLV. Later it was shown that the virus could induce the formation of a variety of tumor types in newborn animals, and hence was given the name polyomavirus.

In 1957 Stewart, Eddy and colleagues reported that polyomavirus could be propagated in cultures of mouse embryo cells. Thus, for the first time the viral productive cycle could be analyzed in cell culture. This was followed by the development of a viral plaque assay. With these tools in hand, an exploration of the genetics of polyomavirus could begin.

Classification

Murine polyomavirus is a member of the *Paporaviridae* family which consists of two genera, *Papillomavirus* and *Polyomavirus*. The prototype members of the *Polyomavirus* genus are simian virus 40 (SV40) and murine polyomavirus itself. Also included in this genus are the human viruses BKV and JCV, SA12 (baboon), LPV (African green monkey), bovine polyomavirus (bovine), Budgerigar fledgling disease virus (BFDV), HaPV (hamsters) and KV (mice). While representatives of the family are found in species ranging from birds to humans, each virus shows a restricted host range.

Properties of the Virion

Members of the polomavirus group are characterized by icosahedral virions, approximately 45 nm in diameter, containing the viral-encoded capsid proteins VP1, VP2 and VP3. Each virion contains a single molecule of circular duplex DNA of about 5 kb, along with the cellular histones H2A, H2B, H3 and H4. Mature virions do not contain histone H1.

Genome Organization and Properties of Proteins

The circular double-stranded genome of polyomavirus consists of 5297 bp. By convention the nucleotide at the center of an imperfect palindrome that forms part of the origin of DNA replication is designated as '1' and numbering proceeds through the early coding sequences around the circle. Sometimes positions on the genome are indicated by map units (MU). This older system divides the genome into 100 MU using the single *Eco*RI site (n1562) as 0 and proceeding through the remainder of the early region and then the late region and regulatory region to 100.

Regulatory region

The viral regulatory region consists of approximately 400 bp lying between the early and late coding regions (n5086–156) and contains the *ori,* the promoters for early and late region transcription and the transcriptional enhancers. Early region transcription initiates at position 156, with the initiation codon for all three T antigens lying at position 175. The major initiation site for the late region mRNAs is at position 5086. The *ori* and all of the known *cis*-acting sequences required for early and late transcription lie between these points. This region contains multiple binding sites for the large T antigen which is thought to recognize the consensus pentanucleotide G(A/G)GGC. There are three strong T antigen-binding sites located upstream of the start of early region transcription. These are termed sites A (n27–76), B (n86–116) and C (n121–164). Each of these contain multiple copies of the recognition pentanucleotide in various orientations and spatial arrangements. None of these sites

appear to play a major role in viral DNA replication and are perhaps involved in transcriptional control. The TATA box associated with the early region promoter lies between sites B and C.

Viral DNA replication requires a stretch of nucleotides termed the core *ori* (n45–5274) linked in *cis* to either enhancer element α or β. A stretch of DNA very rich in AT residues lies just to the late side of the core *ori*. This is followed by the β and then the α elements. The specific roles of each of these sequence motifs in viral DNA replication and transcription will be discussed in an accompanying entry.

Early region

The viral early region encodes four proteins: the tiny, small, middle and large T antigens (TT, ST, MT and LT). The mature mRNAs encoding each of these proteins are produced by differential splicing of a single primary transcript which starts at nucleotide 156. The polyadenylation signal for these mRNAs lies at position n2930. All three proteins initiate at the methionine codon at position 175, and thus share a common N-terminus. The mRNA for ST is generated by splicing a donor site at n748 to an acceptor site at n797, thus eliminating an intron of 49 nucleotides. Translation of this mRNA initiates at the ATG at n175 and terminates at n807. The mRNA encoding MT is generated by utilizing the same donor site (n748) but using an acceptor at n811. This results in loss of the termination codon used for ST and opens a new open reading frame (ORF), which terminates at position n1499. Thus, MT shares N-terminal residues with ST, but each have unique C-termini. The LT mRNA is generated by utilization of a splice donor site at n411 and an acceptor site at n797. This generates an ORF that shares the first 82 codons with MT and ST, but possessing a unique C-terminus.

The LT protein plays a central role in regulating productive infection. This 795 amino acid protein is the only viral product required for viral DNA replication. One way in which LT acts is to bind specific sequences located within *ori*. The consensus sequence for LT recognition is the pentanucleotide G(A/G)GGC, which occurs multiple times within *ori* and the viral regulatory region. LT carries an ATPase/DNA helicase function which acts to melt and unwind *ori* to allow initiation of DNA replication. LT also autoregulates early region transcription, at least partially by binding to sequences located to the early side of *ori*. In addition, LT transactivates the polyomavirus late promoter, as well as a number of heterologous promoters of both viral and cellular origin. LT also carries the ability to complex the cellular retinoblastoma (Rb) protein as discussed below, and probably additional activities involved in transformation.

MT is a 432 amino acid protein that plays a role in viral assembly by regulating the phosphorylation of the major capsid protein VP1. In addition, MT is an oncogene which is sufficient to transform a number of established cell lines, and cooperates with LT to transform primary cells. Many of the actions of MT are mediated by its direct interaction with proteins involved in signal transduction (see above).

The role of the 195 amino acid ST in productive infection is unclear. Under many conditions tested, polyomavirus mutants that do not produce ST are viable and can fully transform cells. Under other conditions, such as infection of quiescent cells, ST seems to increase the efficiency of transformation. ST of both polyomavirus and SV40 are found in complex with two cellular proteins, the α and β subunits of phosphatase pp2A. ST seems to displace the γ subunit and serves to inhibit phosphatase activity. The SV40 ST transactivates the adenovirus E2 promoter in some cell types. Whether the polyomavirus ST carries a similar transactivation activity has not been reported.

Late region

As discussed above, the three viral capsid proteins are expressed by the late transcription unit. The major capsid protein is the 385 amino acid VP1. In infected cells VP1 occurs as multiple species that differ in their states of phosphorylation. Some species appear to play a role in virus adsorption and at least one is involved in virus assembly. MT seems to play a role in inducing this species. VP2 and VP3 are proteins of 319 and 204 amino acids respectively.

Additional ORFs

In addition to the major proteins discussed above, the polyomavirus genome contains several additional ORFs that could potentially encode proteins, although in most cases whether these proteins are actually made or not has not been studied. SV40 is known to express a protein termed agnoprotein from the late mRNA leader sequence. Agnoprotein is a basic protein of 61 amino acids that seems to play a role in transport of the capsid proteins to the nucleus and possibly viral assembly. Other members of the polyomavirus group, including murine polyomavirus, possess a similar ORF that would encode proteins highly related to the SV40 agnoprotein. However, the observation of an agnoprotein from these other viruses has not been reported.

Productive Infection

The viral productive cycles have been most well characterized for SV40 and polyomavirus because of well-developed cell culture systems for propagating these viruses.

Polyomavirus was first propagated in primary mouse embryo fibroblast cell cultures. Established cell lines that supported productive infection and plaque formation were discovered later. The most commonly used is NIH3T3. Polyomavirus forms plaques on monolayer cultures of these cells visible by staining with neutral red after 8 days of incubation. Infection of these cells results in a yield of approximately 300 infectious particles, or plaque forming units (PFU), per infected cell. The ratio of physical:infectious particles is about 100:1 for polyomavirus.

The polyomavirus infectious cycle can be divided into steps typical for animal virus productive infections. First, polyomavirus virions attach to a specific receptor on the cell surface. The receptor probably contains neuraminic acid, as treatment of permissive cells with neuraminidase renders them resistant to polyomavirus infection. The mechanisms involved in penetration and uncoating are not well understood, but the result is that by approximately 4–6 h postinfection, free viral DNA is present in the nucleus of the infected cell. Soon afterwards mRNAs representing about half of the viral coding sequences (see below) are detectable, followed by synthesis of four virus-encoded proteins: LT, MT, ST TT. These are collectively referred to as the early proteins, and they are encoded by the early region genes. Early region genes are those that are expressed prior to the onset of viral DNA replication.

By about 24 h postinfection, the early proteins have reached their maximum levels and viral DNA replication begins. Replication initiates within a 65 bp segment termed the *ori* and proceeds bidirectionally around the circular genome. Viral DNA replication requires a single viral-encoded protein, LT. Much progress has been made in investigations concerning the role of the polyomavirus and SV40 LT in initiating viral DNA replication. These results are reviewed elsewhere.

Shortly after the onset of viral DNA replication, transcription of the remainder of the viral genome, termed the late region, begins. This results in the synthesis of the three viral capsid proteins, VP1, VP2 and VP3. Late region mRNA generation is very complicated, with multiple 5′ ends and numerous alternate splicing events occurring. All of the 5′ ends of late mRNAs lie between n5075 and 5170 on the viral genome. Both the early and late mRNA polyadenylation sites lie about 180° around the circular genome from the *ori*. Viral assembly begins shortly after the onset of late region protein synthesis. While all of the steps in assembly have not yet been elucidated it is known that purified VP1 expressed in *Escherichia coli* will self-assemble into pentamers. However, the complete assembly of infectious particles apparently requires the action of MT, which plays a role in post-translational modification of VP1.

As the infected cells die they exhibit a characteristic cytopathic effect. There does not seem to be any specific mechanism for viral release. Rather, progeny virions seem to be released as the cell dies, with much of the viral material remaining attached to the cellular debris.

What has just been outlined is a typical cycle following polyomavirus infection of a permissive cell. In fact, the outcome of infection depends upon the type of cell infected. Infection of cell types that are semipermissive for polyomavirus can result in a persistent infection. This is perhaps more representative of the natural course of infection with this agent. Other cell types are nonpermissive, either because they lack a viral receptor and infection is blocked at the stage of attachment, or because they lack the proper machinery for allowing viral DNA replication. These latter cell types are of interest because they allow virus penetration and expression of the early viral proteins. However, as replication is blocked these infected cells do not die, and a subset of them are transformed to neoplastic cells.

Viral Transformation

When nonpermissive cells, such as those of rat or hamster origin, are infected with polyomavirus, the early stages of infection proceed normally, resulting in the synthesis of ST, MT and LT; however, viral DNA does not replicate and the late region genes are not expressed. Rather, cellular DNA synthesis is stimulated and the infected cells are driven through at least one to several rounds of division. During this period, actin cables are disassembled and the cells acquire a phenotype associated with transformed cells. After a couple of days, most of the cells in the culture resume a normal appearance and viral nucleic acid and proteins can no longer be detected. This phenomenon is termed abortive transformation.

During such infections a small percentage of the infected cells permanently acquire the transformed phenotype, including the ability to: (1) form foci on a monolayer of normal cells; (2) proliferate in low serum concentrations; (3) grow independent of anchorage to a solid substrate; and (4) form tumors in test animals. Such cells continuously express the

viral T antigens and contain viral DNA covalently integrated into the cellular chromosome. A number of genetic and biochemical experiments have led to the conclusion that the ST, MT and LT are responsible for initiating and maintaining the transformed phenotype. The TT has only been recently discovered and its role in transformation is unclear.

The rate-limiting step in neoplastic transformation appears to be the integration of viral DNA into the cellular chromosome. Polyomavirus has not evolved any specific mechanism for integration. In fact, integration is not a normal part of infection and appears to occur by cell-mediated nonhomologous recombination. Insertion occurs at random locations with respect to both the viral and cellular chromosomes. This appears to explain the phenomenon of abortive transformation. During the viral infection of nonpermissive cells, nearly all the cells are initially infected and thus express the viral T antigens. As a result the cells temporarily acquire the transformed phenotype. However, in most cells, as proliferation proceeds the viral genome is diluted out and thus nearly all progeny cells lose expression of the T antigens and revert to a normal appearance. In a minority of the infected cells, the viral DNA integrates in such a manner as to allow continuous expression of the T antigens. Since the viral genome, and thus T antigen expression, is passed on to all progeny cells, this leads to a stably transformed phenotype.

Much effort has been directed towards understanding the role that each of the viral tumor antigens plays in transformation and the molecular mechanisms by which they achieve their effects. ST increases the efficiency of transformation in certain cell types or when quiescent cells are infected. LT is sufficient to immortalize primary lines. While lines expressing LT exhibit a reduced requirement for serum, they do not form foci, grow independent of anchorage, or form tumors in animals. MT is the only viral gene product required to transform a number of established cell lines; however, both LT and MT are required to induce the fully transformed phenotype in primary cells. Established cell lines transformed by MT alone form foci, grow independent of anchorage and are tumorigenic. They do not show a reduced serum requirement.

Each of the viral T antigens are thought to act by targeting key cellular proteins that normally play a role in the regulation of proliferation. ST complexes with the cellular phosphatase pp2A and alters its activity. LT complexes the Rb protein and probably other targets. MT complexes with at least three nonreceptor tyrosine kinases, c-*src*, c-*fyn* and c-*yes*, phosphoinositide kinase, 14-3-3 proteins and phospholipase C-γ, all of which are components of cellular signal transduction pathways. Details concerning polyomavirus-induced tumorigenicity can be found in the accompanying article.

Genetics

The genetic analysis of polyomavirus began with the isolation of mutants that were temperature-sensitive for the ability to form plaques on a monolayer of permissive cells. In these experiments the permissive temperature was usually 32–35°C, while 39–40.5°C was nonpermissive.

These mutants fell into distinct phenotypic classes which corresponded to genetic complementation groups. Simultaneous studies with SV40 led to the identification of similar classes. The *ts-a* mutants fail to accumulate progeny viral DNA at the nonpermissive temperature. All of the *ts-a* mutants carry base-pair substitutions in sequences encoding the viral LT. All temperature-sensitive mutants mapping in the T antigen gene fall into a single complementation group and are defective for viral DNA replication at the nonpermissive temperature. The *ts-a* mutants also lose the ability to transform nonpermissive cells at the nonpermissive temperature. However, there is a class of mutants mapping to the T antigen gene that are temperature-sensitive for viral DNA replication while retaining the ability to transform at all temperatures.

The remaining classes of temperature-sensitive mutants carry lesions that affect the structure of the viral capsid proteins. One class, exemplified by *ts-10*, fail to accumulate the major viral capsid VP1 and to assemble mature virions. Another class, exemplified by *ts-3*, maps to the VP2/VP3 coding sequences, and seem to exhibit a defect in the uncoating step of infection.

Another class of polyomavirus mutants exhibit a host-range phenotype, in that they undergo a complete productive infection on murine cells transformed by polyomavirus, but are defective on normal murine cells. The lesions in these mutants map to sequences encoding both the LT and MT, but it is their effect on MT function that is thought to result in the defect. These mutants, exemplified by *NG18*, are also defective for viral transformation.

In recent years, a large collection of polyomavirus mutants have been generated by *in vitro* mutagenesis. Most of these mutations have been targeted to the regulatory region of the virus in order to better define *cis*-acting sequences important for the regulation of DNA replication and transcription, or to the viral early protein genes. Site-directed mutagenesis of the T antigen genes has also been used to establish structure–function relations in these proteins.

Relation to Other Members of the Polyomavirus Group

To date the complete nucleotide sequence of 12 members of the polyomavirus group have been completed and the partial sequences of two others are available. These studies demonstrate that all members of this group have essentially the same genome organization and use very similar strategies for controlling genome replication and expression.

There are important differences between various members of the polyomavirus group. First of all, murine polyomavirus and hamster polyomavirus both encode three early viral tumor antigens (LT, MT, ST), whereas other members of the group, such as SV40, encode only an LT and ST. SV40 LT possesses a C-terminal domain, termed the host-range domain, that appears to play a role in virus assembly. Viruses that encode an MT lack a host-range domain on the LT.

A second difference is that murine polyomavirus expresses each of the major capsid proteins, VP1, VP2 and VP3, off separate mRNAs. On the other hand, SV40 expresses only two late mRNAs. One encodes VP1, while the other encodes VP2 and VP3. Differential synthesis of VP2 and VP3 is controlled by the efficiency of translational initiation.

Summary

Murine polyomavirus has proved to be an excellent model for studying mechanisms of genome replication and expression in higher eukaryotes. While members of this group are not important pathogens, they have served as models for viral infection and spread in a natural population. But most of the effort has been put into learning how these small DNA viruses propagate. In addition, these viruses have served as invaluable models for discerning the mechanisms involved in neoplastic transformation.

See also: **Polymaviruses – murine (*Papovaviridae*): Molecular biology; Persistent viral infection; Transformation: Animal viruses.**

Further Reading

Fields BN and Knipe DM (1990) *Virology* 2nd edn. New York: Raven Press.

Salzman NP (ed.) (1986) *The Papovaviridae*. Vol. 1: *The Polyomaviruses*. New York: Plenum Press.

Molecular Biology

Walter Eckhart, The Salk Institute, San Diego, California, USA

Properties of the Virion

The polyoma virion is spherical in shape, with icosahedral symmetry and a diameter of about 45 nm. It contains 360 copies of a major protein, VP1, and 30–60 copies of minor proteins, VP2 and VP3, encoded by the viral DNA. The VP1 proteins are assembled as 72 pentamers. Twelve of the pentamers are surrounded by five others, whereas 60 are surrounded by six others. The pentamers are roughly cylindrical, with a tapering cavity. Each pentamer has one copy of VP2 or VP3 associated with it in the region of the cavity.

Properties of the Genome

The polyoma genome is a covalently closed circle of double-stranded (ds) DNA about 5300 bp in size. The DNA in the virion is supercoiled. The viral DNA is associated with four cellular histones, H2A, H2B, H3 and H4, organized into nucleosomes and packaged as a 'minichromosome'.

The genome is divided into early and late regions. The early region, which is expressed before the onset of viral DNA replication, encodes three proteins called T antigens. The T antigens are designated as small (ST), middle (MT) and large (LT). The late region, which is expressed after viral DNA replication begins, encodes the virion proteins, VP1, VP2 and VP3. There is a single origin of DNA replication. The origin 'core' comprises about 66 bp. The early and late coding regions are located on either side of the origin of replication. There is a noncoding region of about 150 bp on the early side of the replication origin which contains early promoter sequences. A noncoding region of about 250 bp on the late side of the replication origin contains enhancer sequences, which are binding sites for cellular transcription factors, notably PEA-1/AP-1 and PEA-3/ets. A diagram of the coding regions of the polyoma genome is shown in **Fig. 1.**

Properties of the Viral Proteins

Polyoma LT consists of 782 amino acids. It contains two independent nuclear location signals, spanning amino acids 189–195 and 280–286, respectively. The protein is located in the nucleus, where it functions in viral DNA replication, binding to the origin of

Relation to Other Members of the Polyomavirus Group

To date the complete nucleotide sequence of 12 members of the polyomavirus group have been completed and the partial sequences of two others are available. These studies demonstrate that all members of this group have essentially the same genome organization and use very similar strategies for controlling genome replication and expression.

There are important differences between various members of the polyomavirus group. First of all, murine polyomavirus and hamster polyomavirus both encode three early viral tumor antigens (LT, MT, ST), whereas other members of the group, such as SV40, encode only an LT and ST. SV40 LT possesses a C-terminal domain, termed the host-range domain, that appears to play a role in virus assembly. Viruses that encode an MT lack a host-range domain on the LT.

A second difference is that murine polyomavirus expresses each of the major capsid proteins, VP1, VP2 and VP3, off separate mRNAs. On the other hand, SV40 expresses only two late mRNAs. One encodes VP1, while the other encodes VP2 and VP3. Differential synthesis of VP2 and VP3 is controlled by the efficiency of translational initiation.

Summary

Murine polyomavirus has proved to be an excellent model for studying mechanisms of genome replication and expression in higher eukaryotes. While members of this group are not important pathogens, they have served as models for viral infection and spread in a natural population. But most of the effort has been put into learning how these small DNA viruses propagate. In addition, these viruses have served as invaluable models for discerning the mechanisms involved in neoplastic transformation.

See also: **Polymaviruses – murine (*Papovaviridae*): Molecular biology; Persistent viral infection; Transformation: Animal viruses.**

Further Reading

Fields BN and Knipe DM (1990) *Virology* 2nd edn. New York: Raven Press.

Salzman NP (ed.) (1986) *The Papovaviridae*. Vol. 1: *The Polyomaviruses*. New York: Plenum Press.

Molecular Biology

Walter Eckhart, The Salk Institute, San Diego, California, USA

Properties of the Virion

The polyoma virion is spherical in shape, with icosahedral symmetry and a diameter of about 45 nm. It contains 360 copies of a major protein, VP1, and 30–60 copies of minor proteins, VP2 and VP3, encoded by the viral DNA. The VP1 proteins are assembled as 72 pentamers. Twelve of the pentamers are surrounded by five others, whereas 60 are surrounded by six others. The pentamers are roughly cylindrical, with a tapering cavity. Each pentamer has one copy of VP2 or VP3 associated with it in the region of the cavity.

Properties of the Genome

The polyoma genome is a covalently closed circle of double-stranded (ds) DNA about 5300 bp in size. The DNA in the virion is supercoiled. The viral DNA is associated with four cellular histones, H2A, H2B, H3 and H4, organized into nucleosomes and packaged as a 'minichromosome'.

The genome is divided into early and late regions. The early region, which is expressed before the onset of viral DNA replication, encodes three proteins called T antigens. The T antigens are designated as small (ST), middle (MT) and large (LT). The late region, which is expressed after viral DNA replication begins, encodes the virion proteins, VP1, VP2 and VP3. There is a single origin of DNA replication. The origin 'core' comprises about 66 bp. The early and late coding regions are located on either side of the origin of replication. There is a noncoding region of about 150 bp on the early side of the replication origin which contains early promoter sequences. A noncoding region of about 250 bp on the late side of the replication origin contains enhancer sequences, which are binding sites for cellular transcription factors, notably PEA-1/AP-1 and PEA-3/ets. A diagram of the coding regions of the polyoma genome is shown in **Fig. 1.**

Properties of the Viral Proteins

Polyoma LT consists of 782 amino acids. It contains two independent nuclear location signals, spanning amino acids 189–195 and 280–286, respectively. The protein is located in the nucleus, where it functions in viral DNA replication, binding to the origin of

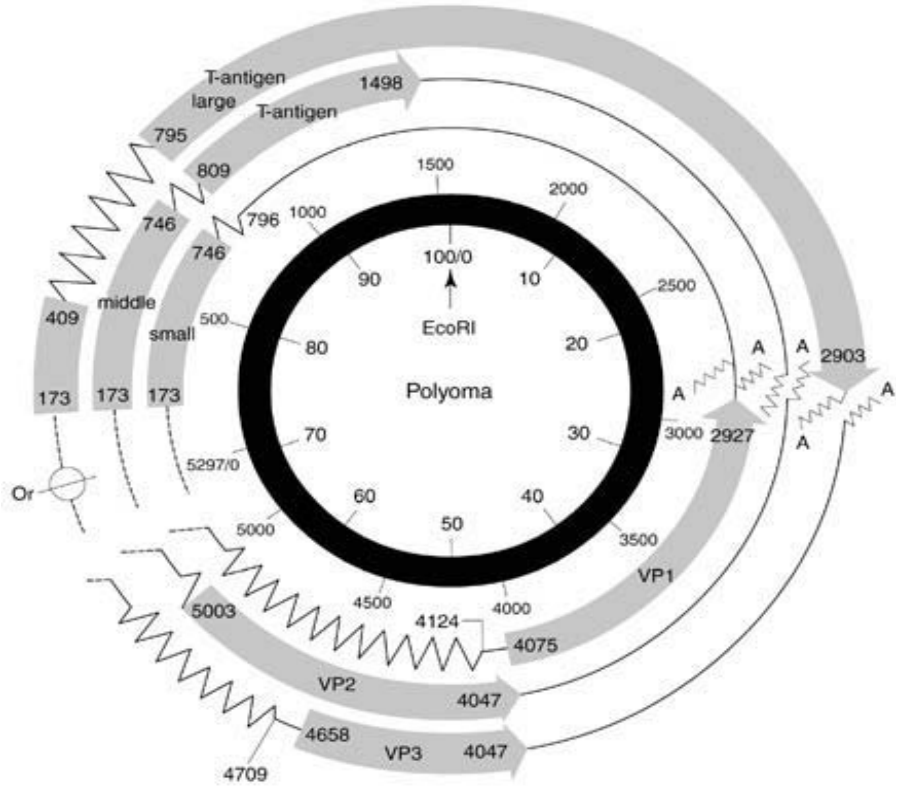


Figure 1 Coding regions of the polyoma genome. The coding regions for the early viral proteins (T antigens) and the late viral proteins (VP1, VP2 and VP3) are shown. (Adapted from Eckhart W (1990) In: Fields BN and Knipe DM, *Fields Virology*, 2nd edn. New York, Raven Press.)

replication and unwinding DNA in the region of the origin. Several biochemical activities are associated with LT, including ATPase and helicase activities. The protein helps to 'immortalize' primary rodent cells in culture, and cooperates with certain activated oncogenes to produce neoplastic cell transformation. Polyoma LT forms a complex with the retinoblastoma susceptibility protein, Rb, blocking the function of Rb in cell cycle progression. Mutants of polyoma LT that fail to bind Rb grow poorly in cells expressing functional Rb, suggesting that viral replication is coupled to induction of progression through the cell cycle. Unlike the simian virus 40 (SV40) LT, polyoma LT does not form a complex with the tumor suppressor protein, p53. However, polyoma LT can overcome p53-induced growth arrest through interaction with Rb.

The amino-terminal region common to all three T antigens contains a sequence similar to the domains of DnaJ proteins that activate molecular chaperones of the DnaK family. LT binds a cellular DnaK, heat shock protein 70. Mutation of the LT J domain abolishes the ability of LT to block the function of Rb, suggesting a role for J domains in regulation of Rb family members.

Polyoma MT contains 421 amino acids. MT is associated with the plasma membrane of the cell through a sequence of 22 uncharged and hydrophobic amino acids, bordered by positively charged amino acids, near the C terminus, which comprise a transmembrane domain. When expressed in established mouse or rat cell lines, MT induces neoplastic cell transformation. The transformed cells form tumors in animals.

The MT protein forms complexes with a variety of growth regulatory proteins in the cell. MT binds and activates three members of the src family of protein tyrosine kinases, $pp60^{c\text{-}src}$, $pp62^{c\text{-}yes}$, and $pp59^{c\text{-}fyn}$. Activation of protein tyrosine kinase activity occurs through decreased phosphorylation of a regulatory tyrosine in the C-terminal region of the $pp60^{c\text{-}src}$ associated with MT.

Activated $pp60^{c\text{-}src}$ phosphorylates tyrosines in MT itself, which serve as binding sites for proteins containing Src homology 2 (SH2) domains or phosphotyrosine-binding (PTB) domains. The SH2

domain of the 85 kDa regulatory subunit of phosphatidylinositol 3′ kinase (PI3K) binds phosphorylated tyrosine 315 of MT. The SH2 domain of phospholipase C-gamma 1 (PLC-γ1) binds phosphorylated tyrosine 322 of MT. As a consequence of binding the enzymatic activities of PI3K and PLC-γ1 are stimulated. Binding of PI3K by MT leads to enhanced glucose transport and activation of pp70 S6 kinase.

The PTB domain of the adaptor protein, Shc, binds phosphorylated tyrosine250 of MT. Shc itself then becomes phosphorylated on tyrosine, providing a binding site for the SH2 domain of the adaptor molecule, Grb2, which in turn brings the guanine nucleotide exchange factor, mSos into the complex. The presence of mSos in a membrane site activates p21*ras* and MAP kinase pathways, however PI3K association with MT is also required for maximum activation. One of the consequences of p21*ras* and MAP kinase activation is an increase in the transcriptional activating activity of the transcription factor, AP-1. The increase is caused by increased phosphorylation of regulatory serine residues in the N-terminal transactivating domain of c-Jun, which is a component of AP-1.

Mutation of tyrosine250, 315 or 322 decreases or abolishes the transforming activity of MT, assayed by anchorage-independent growth of transformed cells. There are variable and less pronounced effects on the frequency, time of appearance and spectrum of tumors induced in animals by the mutant viruses. Mutation of both tyrosine250 and 315 has more severe effects than mutation of either alone. Mutation of either tyrosine250 or tyrosine315 causes increased sensitivity to apoptosis induced by serum starvation, suggesting that imbalanced signal transduction driven by MT may potentiate apoptosis.

The N-terminal region of MT (amino acids 1–185) binds the regulatory and catalytic subunits (A and C) of protein phosphatase 2A (PP2A). Binding of PP2A appears to be required for subsequent binding of $pp60^{c\text{-}src}$ by MT, as mutations in MT that disrupt PP2A binding also abolish binding of $pp60^{c\text{-}src}$, and equimolar amounts of PP2A are present in the MT–$pp60^{c\text{-}src}$ complex. Amino acids 185–210 of MT are additionally required for the interaction with $pp60^{c\text{-}src}$. The phosphatase activity of PP2A may be required to facilitate the interaction of MT with $pp60^{c\text{-}src}$. The majority of MT in the cell is associated with PP2A, whereas only a small fraction (5–10%) of MT is associated with $pp60^{c\text{-}src}$.

MT also associates with 14.3.3 proteins, which have been implicated in signaling and cell cycle regulation. Serine257, which is the major site of serine phosphorylation in MT, is required for association with 14.3.3 proteins. Mutation of serine257 does not affect transformation or tumor formation by MT.

Table 1 Cellular proteins complexed with MT

Protein	*MT binding site/region*
PP2A	AA 1–185
$pp60^{c\text{-}src}$	AA 1–210
Shc	Tyrosine 250
PI3K	Tyrosine 315
PLC-γ1	Tyrosine 322
14.3.3	Serine 257

Table 1 summarizes the cellular proteins found in complexes with MT.

ST contains 195 amino acids. It is partitioned between the nucleus and cytoplasm. The protein is not absolutely required for viral replication, but facilitates the accumulation of viral DNA under certain conditions. Virus mutants lacking ST replicate poorly and fail to form tumors in newborn mice. Like MT, ST forms a complex with PP2A.

The major virion protein, VP1, contains 384 amino acids. It is responsible for the binding of polyoma virions to cellular receptors. Mutations in VP1 affect the plaque morphology, virion stability, hemagglutination properties and pathogenicity of polyoma strains. The minor virion proteins, VP2 and VP3, contain 319 and 204 amino acids, respectively. VP1 binds DNA, whereas VP2 and VP3 do not. The first five amino acids of VP1 are essential for DNA binding. Nuclear location signals are found in the N-terminal region of VP1, amino acids 1–11, and in the C-terminal region of VP2 and VP3, amino acids 307–318. Although the coding regions of the nuclear location signals of VP1 and VP2/3 overlap, as described below, the amino acid sequences are different because the proteins are translated in different reading frames.

Physical Properties

Polyoma infectivity survives months at 4°C and weeks at 37°C. Infectivity is inactivated at 70°C, but is not inactivated by heating to 60°C for 30 min. The virus is resistant to ether, 2% (v/v) phenol and 50% (v/v) ethanol. Virus preparations contain several kinds of particles: infectious viruses, containing intact viral DNA, noninfectious viruses, containing deleted or rearranged viral DNA molecules, empty particles, lacking DNA, and pseudovirions, containing cellular DNA. The density of DNA-containing viral particles is 1.34 g ml^{-1}. The virus agglutinates erythrocytes of a number of species. The ratio of physical particles to infectious units under conditions of standard plaque assays is 100:1–1000:1.

Replication: Strategy of Replication of Nucleic Acid

Polyoma produces a lytic infection in mouse cells, characterized by viral replication, production of infectious progeny and lysis of the infected cell. In hamster or rat cells, the infection is generally abortive; the virus replicates poorly and many infected cells survive. In lytically infected cells, viral DNA replication begins 12–18 h after infection and continues for about 24–48 h until the cell dies.

Polyoma DNA replication requires the origin core and an element from the enhancer region. Replication begins at the origin core and proceeds bidirectionally, terminating about 180° away on the circular viral DNA. An initiation complex containing LT binds to repeated pentanucleotide sequences in the origin core of a circular covalently closed viral DNA molecule and unwinds a region of dsDNA. This creates a replication 'bubble' of diverging replication forks. Nascent chains are initiated by DNA primase–DNA polymerase α. The nascent chains begin with an RNA primer, six to nine ribonucleotides long, which is extended as DNA. At the replication fork, one strand is synthesized continuously in the direction of fork movement, while the other strand is synthesized discontinuously in segments about 135 nucleotides long in the opposite direction. As replication proceeds, the torsional strain created by the unwinding of the parental strands of DNA is released by a topoisomerase activity. Bidirectional replication proceeds until the replication forks meet, at which point the daughter molecules separate.

Binding of cellular transcription factors to the enhancer region is necessary for efficient viral DNA replication. Binding is required on the molecule being replicated (i.e. in *cis* configuration), suggesting that the transcription factors may interact with LT, or with components of the replication complex. The transcription factor AP-1, which binds in the enhancer region, enhances DNA unwinding by LT *in vitro*.

Polyoma LT can be divided into N-terminal and C-terminal domains with independent functions in cellular and viral DNA replication. The N-terminal region, amino acids, 1–260, is sufficient to induce cellular DNA synthesis, but fails to replicate viral DNA, whereas the C-terminal region, amino acids 264–782, is sufficient to replicate viral DNA in actively growing cells, but fails to induce cellular DNA synthesis in serum-starved cells.

Characterization of Transcription

The early and late regions of the polyoma genome are transcribed from opposite strands of the DNA. Transcription begins at sites on either side of the origin of replication and proceeds in opposite directions. Transcription is carried out by the cellular RNA polymerase II. Transcription of early mRNA does not require viral proteins. The early promoter resembles other eucaryotic promoters in having a TATA box about 30 bp upstream of the early mRNA initiation site. The major 5′ termini of polyoma early mRNAs are located about 20 bp upstream of the initiation codon for the early proteins. Three kinds of early mRNAs are produced by alternative splicing. Each of the mRNAs encodes one of the T antigens.

Transcription of early mRNA is regulated by sequences in the enhancer region, and by LT. Mutations in binding sites for cellular transcription factors in the enhancer region reduce transcription of early mRNA.

Late transcription occurs primarily after the onset of viral DNA synthesis. Polyoma late mRNAs are heterogeneous in size. The nuclei of infected cells contain large transcripts that are tandem repeats of the entire viral genome. These transcripts are processed by splicing to produce mature polyadenylated mRNAs with unique coding regions attached to a common untranslated leader sequence. The 5′ termini of the late mRNAs are heterogeneous. The leader sequence is tandemly repeated, presumably because of successive leader to leader splicing which occurs during the processing of the large primary transcripts. Alternative splicing produces individual mRNAs encoding each of the virion proteins.

During the late phase of infection the amount of early RNA detectable by hybridization decreases. This effect is caused by the presence of antisense transcripts present in the late RNA (the early region is transcribed in opposite orientation in the late RNA). The antisense transcripts hybridize with early RNA in the nucleus. The double-stranded molecules are susceptible to modification by dsRNA-specific adenosine deaminase, which modifies adenosine to inosine, rendering the early RNA undetectable by hybridization. The modified RNAs accumulate in the nucleus, and thus are inert for protein synthesis. This process suggests a mechanism by which naturally occurring antisense transcripts downregulate early gene expression during the late phase of infection.

Characterization of Translation

The coding regions of the T antigens overlap, producing proteins with common N-terminal regions and unique C-terminal regions, determined by alternative splicing of the early mRNAs and translation in different reading frames. The three T antigens share

79 amino acids at their N-termini. ST and MT share an additional 112 amino acids which are not present in LT. The unique C-terminal regions of ST, MT and LT are four, 230 and 703 amino acids, respectively. The T antigens are synthesized in the cytoplasm. After synthesis they are partitioned to different cellular compartments: LT to the nucleus, MT to the plasma membrane and ST to the nucleus and cytoplasm.

The virion proteins, VP1, VP2 and VP3, are synthesized in the cytoplasm, but are transported to the nucleus, where assembly of infectious virions occurs. The coding regions of VP2 and VP3 overlap. The proteins have different N-terminal initiation codons, determined by alternative splicing of the late mRNAs, and a common C-terminal termination codon, so that the sequence of VP3 is entirely contained in VP2. The C-terminal coding region of VP2/3 overlaps the N-terminal coding region of VP1 for 29 nucleotides, but the amino acid sequences of the proteins are different because they are translated in different reading frames.

Post-translational Processing

Polyoma LT is phosphorylated on serine and threonine residues. Threonine278, which is phosphorylated by S- and G2-specific cyclin/cyclin-dependent kinase (cdk) complexes, is required for viral DNA replication. Mutation of threonine278 to alanine results in defects in origin binding and unwinding by LT. The phosphorylation of threonine278 by cyclin/cdk complexes suggests a reason why polyoma replication requires actively growing cells. Serines267, 271 and 274, which are also sites of phosphorylation, are not required for replication of viral DNA by the C-terminal domain of LT, but may regulate the relationship of the N- and C-terminal domains.

The MT protein is phosphorylated on serine 257, and on tyrosines250, 315 and 322, as described above. Serine257 phosphorylation regulates binding of 14.3.3 proteins by MT, whereas tyrosine phosphorylation provides binding sites for Shc, PI3K and PLC-γ1.

The virion proteins of polyoma are also modified by phosphorylation. About 15% of the VP1 molecules in virions are phosphorylated. VP2 and VP3 are phosphorylated to a lesser extent. The major phosphorylated amino acid of VP1 is threonine; serine is a minor component. The major sites of threonine phosphorylation are threonine63 and threonine156. VP1 is phosphorylated *in vitro* on serine66 by casein kinase II. Serine66 also appears to be a site of *in vivo* phosphorylation. Phosphorylation of VP1 takes place in the cytoplasm of the cell, possibly during translation. Phosphorylation of VP1 plays a role in virus assembly, because mutation of threonine156 to alanine causes defects in assembly. VP1 is also modified by acetylation, methylation, tyrosine sulfuration and proline hydroxylation. The role of these modifications is unknown.

The N-terminal glycine residue of VP2 is acylated with myristic acid. This modification takes place cotranslationally. Mutation of the glycine residue, preventing myristoylation, results in decreased infectivity.

Assembly Site, Uptake, Release and Cytopathology

Polyoma virions are assembled in the nucleus of the infected cell. VP2 binds to VP1, and VP2 can transport VP1 lacking nuclear location signals into the nucleus, suggesting that complexes of VP1 and VP2 may form in the cytoplasm before moving to the nucleus. Binding of calcium by VP1 is required for efficient virus assembly.

Infectious virus particles adsorb to susceptible cells through the interaction of VP1 with cellular receptors. Infection can be blocked by treatment of cells with sialidase, suggesting that *N*-acetylneuraminic acid (sialic acid) on the cell surface is required for polyoma infectivity. Polyoma attaches to the surface of susceptible cells by stereospecific recognition of oligosaccharides terminating in (α 2,3)-linked sialic acid. The structure of VP1 complexed with 3′-sialyl lactose shows van der Waals' contacts between the side chain of valine296 of VP1 and the sialic acid ring. Alanine at position 296 reduces the contacts and decreases the affinity of VP1 for the receptor fragment. Viruses with alanine at position 296 are more virulent than viruses with valine, suggesting that decreased binding of polyoma to its receptors promotes spread of the virus and increases pathogenicity. After adsorption, the virus is internalized and transported to the nucleus where replication occurs. Progeny virus particles are passively released when the infected cell dies. Each infected cell produces 10 000–100 000 virions (100–1000 PFU).

See also: **JC and BK viruses (*Papovaviridae*); Retroviral Oncogenes; Polyomaviruses – murine (*Papovaviridae*): General features; Simian virus 40 (*Papovaviridae*); Transformation: Animal viruses.**

Further Reading

Fields BN, Knipe DM, Howley PM *et al.* (eds) (1996) *Fields Virology*, 3rd edn. Philadelphia: Lippincott-Raven.

POMOVIRUSES

L. Torrance, Scottish Crop Research Institute, Invergowrie, Dundee, UK

History

Fungus-transmitted, tubular rod-shaped viruses were classified as furoviruses in 1987 based on the properties of particle shape, transmission by zoosporic Plasmodiophorid 'fungi' and the possession of a divided genome. However, as more nucleotide sequences became available, it was apparent that the *Furovirus* genus contained species with different genome organizations and sequence relatedness. The classification was revised in 1998 and three new genera (*Pomo-*, *Peclu-* and *Benyvirus*) were established in addition to the genus *Furovirus* with no assignment to families.

Taxonomy and Classification

Pomovirus is a siglum derived from *po*tato *mo*p-top virus (PMTV), the type species. There are four member species in the genus *Pomovirus:* PMTV, beet soil-borne virus (BSBV), beet virus Q (BVQ) and broad bean necrosis virus (BBNV). Pomoviruses have genomes comprising three species of linear positive-sense single-stranded RNA of c. 5.8–6.5 kb, 2.9–3.5 kb and 2.5–3 kb, respectively. The RNA is not polyadenylated, and the genome encodes a triple block of proteins thought to be involved in virus movement.

Physical Properties of Particles

Pomovirus particles have helical symmetry, and are 18–20 nm in diameter comprising multiple copies of the major coat protein (c. 19–20 kDa). The stop codon of the coat protein gene can be suppressed to produce a larger readthrough protein. The readthrough domain of PMTV is expressed as a fusion with the coat protein and remains attached to one extremity of some virus particles. Virus particles are fragile and preparations contain large numbers of short fragments; the protein helix is sometimes uncoiled at one end in particles of PMTV. The particle size distribution is reported to be 100–150 nm and 250–310 nm. Measurements of PMTV particles gave predominant lengths of 125 nm, 137 nm and 283 nm. Virus particles sediment as three components and sedimentation coefficients ($s_{20,w}$) of PMTV are reported to be 126, 171 and 236S.

Genome Properties

The complete sequence has been determined for the genomes of BSBV (**Fig. 1**) and BVQ, and for RNA 2 and 3 of PMTV. Partial sequence information is available for PMTV RNA 1 and the three RNA species of BBNV.

RNA 1 contains a single large open reading frame (ORF) that is interrupted by a UAA codon followed in phase by an additional coding region. The ORF encodes putative proteins of c. 145–149 kDa and c. 204–207 kDa (readthrough protein). The smaller protein contains methyltransferase and helicase motifs, and the readthrough protein also contains a RNA-dependent RNA polymerase (RdRp) motif suggesting that these proteins are involved in virus replication.

RNA 2 (RNA 3 of PMTV) encodes the coat protein gene which is terminated by a UAG stop codon and followed in phase by an additional coding region of variable size. Suppression of the UAG stop codon produces a readthrough protein of 54–104 kDa. The molecular mass of the readthrough protein of a glasshouse-maintained isolate of PMTV (PMTV-T) is 67 kDa, but is c. 82 kDa in several field isolates. The shorter size of PMTV-T is caused by internal sequence deletion in the 3′ half of the gene.

RNA 3 (RNA 2 of PMTV) contains three or four ORFs. The first three ORFs partially overlap and

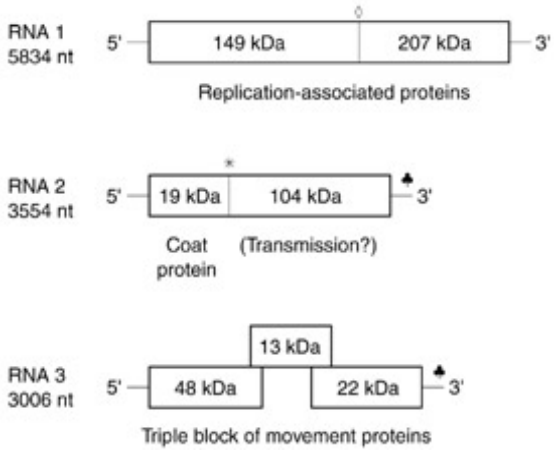


Figure 1 Diagram of BSBV genome organization showing the molecular mass of predicted protein products and their putative functions. * represents an UAG stop codon; ◇ represents a UAA stop codon; ♣ represents a tRNA-like structure.

share some characteristics with the triple gene block (TGB) found in the genomes of other rod-shaped viruses such as barley stripe mosaic hordeivirus and beet necrotic yellow vein benyvirus (BNYVV). By analogy they are thought to encode proteins that are involved in virus movement. The pomovirus-encoded proteins share the greatest sequence identity with each other in the second TGB protein (68–75%), and share 53–58% and 35–41% identity in the first and third TGB proteins, respectively. Also, the dNTP binding site motif in the first TGB protein is conserved. Pomovirus TGBs share some sequence identity with the respective TGB proteins of furo-, peclu-, beny- and hordeiviruses, but fewer identical amino acids when compared with those of other TGB containing viruses. PMTV (RNA 2) and BBNV (RNA 3) contain a fourth ORF which in PMTV encodes a putative 8 kDa cysteine-rich protein and in BBNV a putative 6 kDa protein. The RNA 3 of BVQ is shorter than the equivalent RNA in the other pomoviruses mainly because of the shorter lengths of its 5′ and 3′ untranslated regions. The three prime ends of the BSBV and BVQ RNAs and of PMTV RNA 2 can be folded into tRNA-like structures which contain an anticodon for valine.

Serological Relationships

The viruses are serologically distinct. PMTV particles contain an immunodominant epitope at the extreme N-terminus of the coat protein which is exposed along the entire length of the particles and reacts with monoclonal antibody SCR 69 (**Fig. 2**). The coat proteins of PMTV, BSBV and BVQ contain a conserved sequence of amino acids (SALNVAHQL) which are not exposed in the assembled particles but which react with a monoclonal antibody SCR 70 (raised to PMTV) in western immunoblotting tests. Distant serological relationships have been reported between BSBV and BVQ, and between soil-borne wheat mosaic furovirus and tobamoviruses with PMTV or BBNV.

Host Range and Geographical Distribution

The host range of pomoviruses is narrow, and restricted to dicotyledonous plants principally in the families Aizoaceae, Solanaceae, Chenopodiaceae and Leguminosae (only BBNV). The principal hosts of PMTV, BSBV and BBNV are potato, sugar beet and broad bean, respectively. PMTV occurs in Europe, South and North America and Asia in regions that have a cool, wet potato growing season. BSBV is widespread in sugar beet growing areas in Europe and North America. BVQ has been reported to occur only in sugar beet crops in Europe and BBNV has been reported only from Japan.

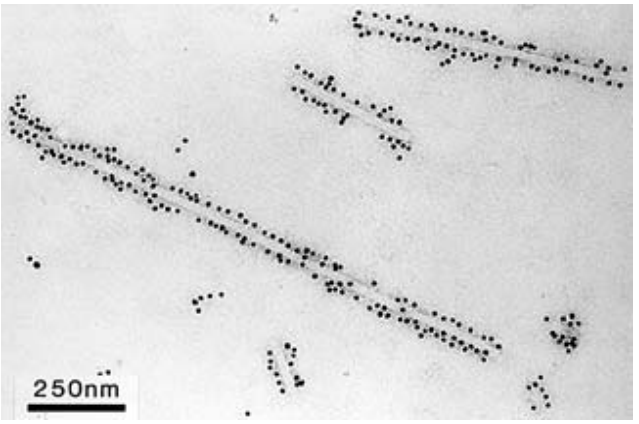


Figure 2 Electron micrograph of PMTV particles labelled with monoclonal antibody SCR 69/gold conjugate.

Cytopathology

Virus-induced inclusions are seen in thin sections of virus-infected plants. Sheaves of PMTV particles have been found in thin sections of all cell types including vascular tissues. In addition, microtubules have been seen in the cytoplasm, apoplast and vacuole of most cell types, sometimes associated with membranes of the endoplasmic reticulum and tonoplast. Enlarged endoplasmic reticulum and distorted membranes are seen in thin sections of plants infected with BSBV and BVQ.

Transmission

Pomovirus vectors are zoosporic protozoans in the family *Plasmodiophoridae*. They are obligate parasites with a complex life cycle, producing resting spores that survive in soil for long periods and biflagellate zoospores that swim in soil water and infect plant roots. The viruses are acquired during infection of root cells by the vector, and virus particles are thought to be carried within the zoospores and resting spores. Soil remains infective after air-drying. PMTV is transmitted by the potato powdery scab pathogen *Spongospora subterranea* f. sp. *subterranea*. *Polymyxa betae* is thought to be the vector of BSBV and BVQ. The viruses can also be transmitted by mechanical inoculation.

By analogy with BNYVV, the readthrough domain of the coat protein may play a role in vector transmission of virus. A correlation has been found between the deletion of amino acids in the C-terminal

portion of the readthrough domain and lack of transmission of PMTV by *S. subterranea*.

Ecology and Control

BBNV causes spots, streaks and veinal necrosis on the leaves of broad bean and pea and severely affected plants are stunted and defoliated. BSBV is widespread in sugar beets, it has been found in mixed infections with BNYVV, but no distinct symptoms have been attributed to infection by BSBV alone.

PMTV induces internal brown lines (spraing) and raised surface lines on potato tubers of sensitive cultivars infected directly from infested soil (**Fig. 3**). The spraing symptoms can be confused with those induced by the nematode-transmitted tobacco rattle tobravirus. Potato plants grown from infected tubers display yellow chevrons or markings on leaves or the mop-top dwarfing symptom caused by shortened internodes. PMTV decreases the quality of tubers produced for processing and consumption, and decreases the yield of tubers produced from plants of sensitive cultivars. Not every stem on an infected plant displays symptoms, and PMTV is erratically distributed in plants. The virus infects only a small proportion of plants grown from infected tubers, and it can be eliminated from stocks of cultivars that display obvious symptoms by removing infected plants. However, this method of control is limited to sensitive cultivars, and it carries the risk of establishment of PMTV at new sites. Haulm and tuber symptoms vary greatly with potato cultivar and environmental conditions especially temperature.

Resting spores of viruliferous vectors can be spread to new sites on contaminated farm vehicles or equipment, by planting contaminated tubers, by wind-blown surface soil or contaminated drainage water. Once the soil is infested by the viruliferous vector it is very difficult to control virus incidence. The Plasmodiophorid resting spores are resistant to chemical and environmental stresses and can remain viable in soil for many years, constituting an important reservoir of infection. The viruses are transmitted by the motile zoospores that swim in soil water, and their spread is favored by wet conditions. Soil fumigants such as methyl bromide are effective in controlling the Plasmodiophorids but it is environmentally damaging and its use is being restricted. Other chemical methods can provide partial control, e.g. decreasing soil pH to 5.0, by the application of sulphur, inhibits the spread of *S. subterranea* zoospores but the resting spores are not affected. At present there are no effective and environmentally safe chemical control methods. The best prospect for control of these soil-borne viruses is the development of virus-resistant cultivars. There are no sources of natural resistance in potato to PMTV but plants transformed with the PMTV coat protein gene have shown high levels of resistance to virus infection by manual or fungal inoculation. This form of resistance may provide effective control if incorporated into potatoes.

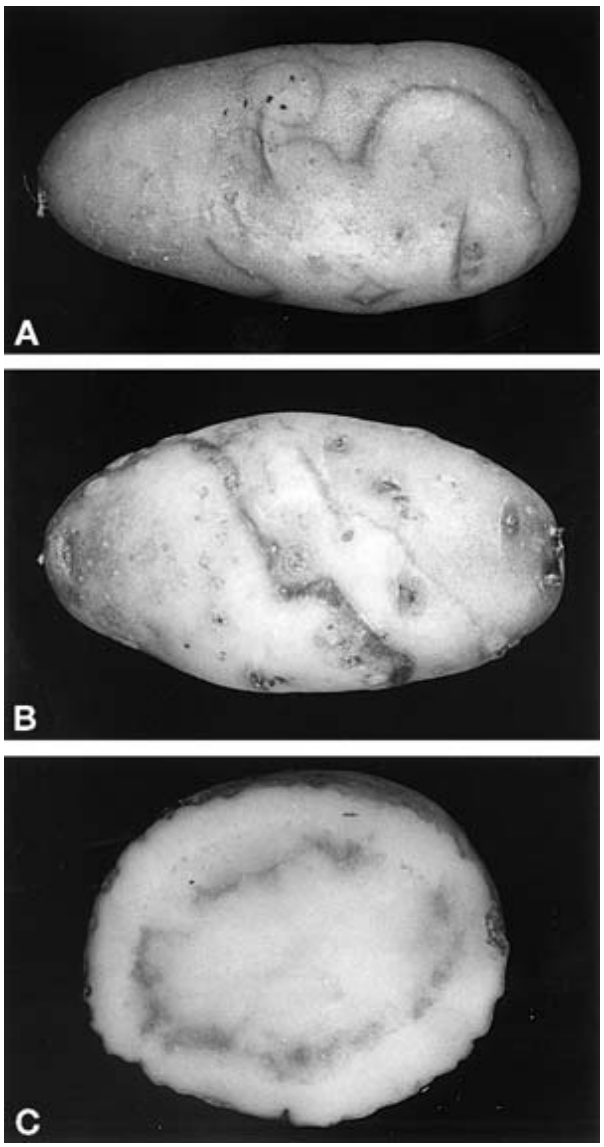


Figure 3 PMTV symptoms on potato tubers. Surface lines on **A** cv. Pentland Marble and **B** cv. Arran Pilot; **C** internal spraing on cv. Arran Pilot.

See also: **Benyviruses; Furoviruses; Pecluviruses; Tobamoviruses.**

Further Reading

Arif M, Torrance L and Reavy B (1995) Acquisition and transmission of potato mop-top furovirus by a culture of

Spongospora subterranea f. sp. *subterranea* derived from single cystosorus. *Ann. Appl. Biol.* 126: 493.

Jones RAC (1988) Epidemiology and control of potato mop-top virus. In: Cooper JI and Asher MJC (eds). *Applied Biology II. Viruses with Fungal Vectors*, p. 225. Wellesbourne, UK: Association of Applied Biologists.

Kashiwazaki S, Scott KP, Reavy B and Harrison BD (1995) Sequence analysis and gene content of potato mop-top virus RNA 3: further evidence of heterogeneity in the genome organization of furoviruses. *Virology* 206: 701.

Koenig R and Loss S (1997) Beet soil-borne virus RNA1: genetic analysis enabled by a starting sequence generated with primers to highly conserved helicase-encoding domains. *J. Gen. Virol.* 78: 3161.

Koenig R, Pleij CWA, Beier C and Commandeur U (1998) Genome properties of beet virus Q, a new furo-like virus from sugar beet, determined from unpurified virus. *J. Gen. Virol.* 79: 2027–2036.

Reavy B, Arif M, Kashiwazaki S, Webster KD and Barker H (1995) Coat protein-mediated immunity to potato mop-top virus in *Nicotiana benthamiana* is effective against fungal inoculation. *Mol. Plant–Microbe Interact.* 8: 286.

Reavy B, Arif M, Cowan GH and Torrance L (1998) Association of sequences in the coat protein/read-through domain of potato mop-top virus with transmission by *Spongospora subterranea*. *J. Gen. Virol.* 79: 2343–2347.

POTEXVIRUSES

Mounir G AbouHaidar and **Duncan Gellatly**, University of Toronto, Toronto, Ontario, Canada

General Features

Potexviruses consist of a genus (no family) of about 30 definite and possible member viruses, with the prototype member being potato virus X (PVX). The potexviruses share common properties with the carlavirus genus of plant viruses. The two genera are now being considered for merger into a single family. All potexviruses are morphologically similar, flexuous, filamentous viruses with virions ranging in size from 470 to 580 nm in length and about 14 nm in width. The central canal of the particle is about 3 nm in diameter. Infectious potexvirus particles typically consist of 1000–1500 subunits of a single species of capsid protein (CP) wrapped around one single-stranded, messenger-sense genomic RNA (gRNA) that ranges in size from 5845 to 7057 nucleotides (nt) (**Table 1**). These viruses have a broad geographic range reflecting the distribution of their hosts. Although any one potexvirus member has a limited number of hosts, many dicotyledonous and monocotyledonous plant species are susceptible to infection when the group is considered together. Unlike many other plant viruses that are transmitted by insect vectors, potexviruses are apparently spread via a mechanical mode of transmission. Seed transmission has been reported in a few cases, but it is not confirmed. Systemically infected plants usually display chlorotic mosaic or mottle symptoms. Yield losses due to potexvirus infection of crops can be as high as 30%.

Virion Structure and Assembly

Potexvirus particles are highly hydrated particles that have molecular weights ranging in the order of 35×10^6 kDa and sediment from 100 to 130 S. The single species of CP, which ranges in size from 21 to 29 kDa, coats the genomic RNA in a helical fashion giving rise to a flexible rod. Optical and x-ray diffraction studies of virus particles have shown that there are $8\frac{3}{4}$ subunits per turn of the helix, which repeats one every four turns and exhibits a pitch of 3.3–3.6 nm, depending on the degree of hydration of the particle. The RNA is localized to a position about 3.5 nm radius (in contrast to tobacco mosaic virus (TMV)-RNA which is located at about 4 nm radius). The tendency for viral RNA to be encapsidated at a low radius may afford the RNA better protection and allow for bending (flexibility) of the particle. There are approximately five nucleotides of the RNA which interact with the protein subunit. A considerable degree of variability (40–65%) in the amino acid sequences of the CPs of potexviruses, much of which is localized to the amino terminus (located on the outside of the virus particle), results in low serological cross-reactivity between different potexvirus members. The gRNA comprises 6.5–7.5% of the viral mass and ranges from 5845 to 7057 nt in size.

Self-assembly of purified CP monomers and gRNA to produce reconstituted virion particles resistant to ribonuclease A can occur *in vitro* in low ionic-strength buffers (e.g. pH 8.0). The assembly process

Spongospora subterranea f. sp. *subterranea* derived from single cystosorus. *Ann. Appl. Biol.* 126: 493.

Jones RAC (1988) Epidemiology and control of potato mop-top virus. In: Cooper JI and Asher MJC (eds). *Applied Biology II. Viruses with Fungal Vectors*, p. 225. Wellesbourne, UK: Association of Applied Biologists.

Kashiwazaki S, Scott KP, Reavy B and Harrison BD (1995) Sequence analysis and gene content of potato mop-top virus RNA 3: further evidence of heterogeneity in the genome organization of furoviruses. *Virology* 206: 701.

Koenig R and Loss S (1997) Beet soil-borne virus RNA1: genetic analysis enabled by a starting sequence generated with primers to highly conserved helicase-encoding domains. *J. Gen. Virol.* 78: 3161.

Koenig R, Pleij CWA, Beier C and Commandeur U (1998) Genome properties of beet virus Q, a new furo-like virus from sugar beet, determined from unpurified virus. *J. Gen. Virol.* 79: 2027–2036.

Reavy B, Arif M, Kashiwazaki S, Webster KD and Barker H (1995) Coat protein-mediated immunity to potato mop-top virus in *Nicotiana benthamiana* is effective against fungal inoculation. *Mol. Plant–Microbe Interact.* 8: 286.

Reavy B, Arif M, Cowan GH and Torrance L (1998) Association of sequences in the coat protein/read-through domain of potato mop-top virus with transmission by *Spongospora subterranea*. *J. Gen. Virol.* 79: 2343–2347.

POTEXVIRUSES

Mounir G AbouHaidar and **Duncan Gellatly**, University of Toronto, Toronto, Ontario, Canada

General Features

Potexviruses consist of a genus (no family) of about 30 definite and possible member viruses, with the prototype member being potato virus X (PVX). The potexviruses share common properties with the carlavirus genus of plant viruses. The two genera are now being considered for merger into a single family. All potexviruses are morphologically similar, flexuous, filamentous viruses with virions ranging in size from 470 to 580 nm in length and about 14 nm in width. The central canal of the particle is about 3 nm in diameter. Infectious potexvirus particles typically consist of 1000–1500 subunits of a single species of capsid protein (CP) wrapped around one single-stranded, messenger-sense genomic RNA (gRNA) that ranges in size from 5845 to 7057 nucleotides (nt) (**Table 1**). These viruses have a broad geographic range reflecting the distribution of their hosts. Although any one potexvirus member has a limited number of hosts, many dicotyledonous and monocotyledonous plant species are susceptible to infection when the group is considered together. Unlike many other plant viruses that are transmitted by insect vectors, potexviruses are apparently spread via a mechanical mode of transmission. Seed transmission has been reported in a few cases, but it is not confirmed. Systemically infected plants usually display chlorotic mosaic or mottle symptoms. Yield losses due to potexvirus infection of crops can be as high as 30%.

Virion Structure and Assembly

Potexvirus particles are highly hydrated particles that have molecular weights ranging in the order of 35×10^6 kDa and sediment from 100 to 130 S. The single species of CP, which ranges in size from 21 to 29 kDa, coats the genomic RNA in a helical fashion giving rise to a flexible rod. Optical and x-ray diffraction studies of virus particles have shown that there are $8\frac{3}{4}$ subunits per turn of the helix, which repeats one every four turns and exhibits a pitch of 3.3–3.6 nm, depending on the degree of hydration of the particle. The RNA is localized to a position about 3.5 nm radius (in contrast to tobacco mosaic virus (TMV)-RNA which is located at about 4 nm radius). The tendency for viral RNA to be encapsidated at a low radius may afford the RNA better protection and allow for bending (flexibility) of the particle. There are approximately five nucleotides of the RNA which interact with the protein subunit. A considerable degree of variability (40–65%) in the amino acid sequences of the CPs of potexviruses, much of which is localized to the amino terminus (located on the outside of the virus particle), results in low serological cross-reactivity between different potexvirus members. The gRNA comprises 6.5–7.5% of the viral mass and ranges from 5845 to 7057 nt in size.

Self-assembly of purified CP monomers and gRNA to produce reconstituted virion particles resistant to ribonuclease A can occur *in vitro* in low ionic-strength buffers (e.g. pH 8.0). The assembly process

Table 1 Propagation host species, complete and partial nucleotide sequences of some potexviruses

Potexviruses	*Propagation species*	*gRNA*[a] *(nucleotides)*	*Capsid protein*[b] *(kDa)*
Bamboo mosaic (BaMV)	*Hordeum vulgare*	6366	25
Cactus X (CVX)	*Chenopodium quinoa*		
Cassava common mosaic (CCMV)	*Euphorbia prunifolia*	6376	24.3
Clover yellow mosaic (CYMV)	*Vicia faba*	7015	23.5
Cymbidium mosaic (CybMV)	*Datura stramonium*	6227	23.5
Dioscorea latent (DLV)	*Nicotiana megalosiphon*		
Foxtail mosaic (FMV)	*H. vulgare*		
Hippeastrum latent (HsLV)	*Hippeastrum hybridum*		
Hydrangea ringspot (HRSV)	*Hydrangea macrophylla*		
Lily virus X (LVX)	*Lilium* hybrid		21.6
Nandina mosaic (NdMV)	*Nicotiana benthamiana*		
Narcissus mosaic (NMV)	*Narcissus tazetta*	6955	26.1
Nerine virus X (NVX)	*Nerine samiensis*		
Papaya mosaic (PMV)	*Carica papaya*	6656	23
Pepino mosaic (PpMV)	*Nicotiana glutinosa*		
Plantago asiatica mosaic (P1AMV)	*Plantago asiatica*	6128	22
Potato aucuba mosaic (PAMV)	*Nicotiana tabacum* cv. *Xanthi* nc	7057	27
Potato X (PVX) (strain X 3)	*N. tabacum*	6435	25.1
Strawberry mild yellow edge-associated (SMYEAV)	*Rubus rosifolius*	5966	25.7
Viola mottle (VMV)	*C. quinoa*		
White clover mosaic (WC1MV)	*Phaseolus vulgaris*	5846	22.5
Wineberry latent (WbLV)	*C. quinoa*		
Zygocactus X (zvx)	*C. quinoa*		

[a] Genomic RNA (gRNA) size is determined from nucleotide sequence of cDNA of viral RNA.
[b] Molecular mass is calculated from the nucleotide sequence of cloned viral cDNA.

occurs in two stages: initiation and elongation. The initiation step is rapid (less than 20 s) and independent of temperature. Initiation occurs at a specific sequence (packaging signal, 38 to 47 nt in size) at the 5′ end of the gRNA. In papaya mosaic virus (PMV), this sequence contains several repeats (of the type GCAAA) which are postulated to interact with $8\frac{3}{4}$ protein subunits of the 14S double disk (or helix) to specifically initiate assembly. Particles approximately 50 nm in length are formed. Synthetic poly(A) and poly(C) can also be specifically recognized and packaged at pH 8.0 by PMV protein. The elongation phase is a slower, temperature-driven process in which small polymers of CP are added, with growth toward the 3′ end. Elongation, unlike the initiation process, seems to be non-specific. Any RNA can be packaged by PMV protein provided that the initiation sequence (packaging signal) is present at the 5′ end. At lower pH values, the specificity of PMV protein for its RNA is lost. Any viral and non-viral RNAs may be packaged by the PMV CP but no complete particles are formed because initiation simultaneously starts at several sites of the RNA, resulting in multi-initiated 'kinky' particles. In the absence of RNA, CP monomers will assemble to form helical or stacked disk structures at low pH. Assembly of potexviruses is unlike that of tobamoviruses because it is unidirectional (starts at the 5′ end of RNA) and no site of internal initiation is present.

Genome Organization

The monopartite genome of potexviruses consists of a messenger-sense, single-stranded RNA that is 5′ capped (m^7GpppG) and 3′ polyadenylated. An A-C-rich 5′ noncoding region, from 80 to 107 nt in length, occurs upstream of five open reading frames (ORFs) which are well conserved in sequence and position among different potexviruses. The largest ORF, ORF 1, codes for a protein that has two motifs of amino acid sequences that are present in the conserved domains of NTP-binding helicases and RNA-dependent RNA polymerases. Mutation of PVX ORF 1 in one of the conserved glycine-lysine-serine (GKS) sequence, a motif that is present among the replicating enzymes of many RNA viruses, eliminates PVX replication. The product of ORF 1 thus appears to represent a component of a replicase complex that directs, together with host proteins, and possibly other viral proteins, the synthesis of viral RNA

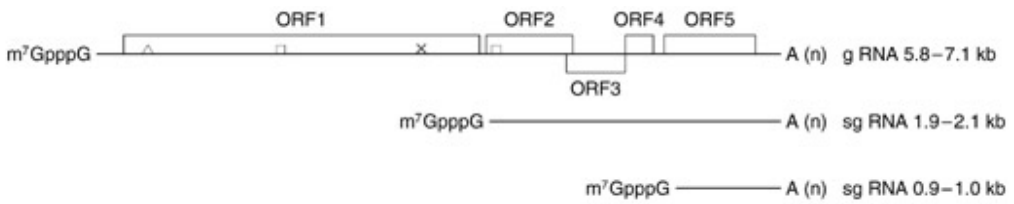


Figure 1 Organization of a potexvirus genome (gRNA). The diagram represents genomic organization of PVX, the type member of potexviruses. Five open reading frames (ORF) encoding the viral proteins with the following molecular weights: 166 kDa (ORF 1/putative RNA polymerase), 25 kDa (ORF 2), 12 kDa (ORF 3), 8 kDa (ORF 4) (ORF 2–4/triple gene block involved in viral transport) and 25 kDa (ORF 5/capsid protein). The sizes of the viral proteins vary among the other potexviruses of which the genomes have been sequenced. The sizes are: 147–191 kDa, 24–26 kDa, 11–14 kDa, 6–11 kDa and 21–26 kDa for ORF 1 to ORF 5, respectively. The two most abundant subgenomic RNAs (sgRNA) are also depicted. m^7GpppG represents the cap structure. $A_{(n)}$ represents the poly(A) tail. Triangle depicts the methyltransferase motif. Squares represent the NTPase/helicase motif (GSK). × depicts the GDD polymerase motif.

(Fig. 1). ORFs 2, 3 and 4 slightly overlap, and are commonly referred to as the 'triple gene block'. Products of the triple gene block function in cell-to-cell movement, being required for infectivity in a host plant but not in protoplasts. The amino acid sequences of ORF 2 from various potexviruses indicate an ATPase–helicase function, and the ORF 2 product of PVX has been shown to bind nucleic acids *in vitro*. Hydrophobic sequences in the products of ORFs 3 and 4 suggest an association with host cell membranes. Immunological localization has demonstrated the product of PVX ORF 4 to be associated with the cell wall. There are some apparent exceptions to the organization of ORFs 2–4. The genome of lily virus X (LVX) lacks the initiation codon for ORF 4, strawberry mild yellow edge-associated potexvirus (SMYEAV) lacks one for ORF 2. However, the rest of the coding sequences of these ORFs are present and are similar to other potexviruses. The exact role(s) of these ORFs in the potexvirus life cycle is not fully determined at present. ORF 5 codes for the CP. In two potexviruses, clover yellow mosaic (CYMV) and foxtail mosaic virus (FoMV), ORF 5 is contained within a larger ORF that appears to encode a 'read-through' protein. Various potexviruses also contain other small putative ORFs that are embedded in, and often out of frame from, the five major ORFs. Other putative ORFs found on the negative (complement) strand have also been described. The *in vivo* significance of such ORFs remains speculative. The 3′ noncoding region ranges from 43 to 138 nt in length and precedes a poly(A) tail that varies in length. A U-rich 8-nt sequence within the 3′ noncoding region of PVX is required for virus replication by binding two host proteins (28 and 32 kDa in tobacco) of unknown function. A consensus polyadenylation sequence (5′-AAUAAA-3′) occurs at different distances from the 3′ end of the gRNA.

During potexvirus replication, capped and poly(A)-tailed subgenomic RNAs (sgRNAs) are produced. The two most abundant sgRNAs are 1.9–2.1 kilobase (kb) and 0.9–1.0 kb in size. These have 5′ ends which correspond to internal regions of the gRNA, and are coterminal with the gRNA at their 3′ ends. The formation of sgRNAs was previously thought to alleviate the problem of translation of polycistronic mRNAs by eukaryotic ribosomes, which follow the Kozak scanning model. However, recent work has demonstrated that the PVX CP gene can be expressed in transgenic plants from an internal ribosome binding site. Consequently, the formation of sgRNAs in this group of viruses (and probably in other viruses) is merely for amplification (and not for initiation of translation) purposes. A 1.2-kb sgRNA in CYMV has been isolated, consisting of 757 and 415 nt segments from the 5′ and 3′ ends, respectively, joined so that the N-terminus of ORF 1 and C-terminus of ORF 5 are fused in frame. This sgRNA is clearly defective and requires the full-length genome as a helper for replication. Furthermore, this CYMV 1.2-kb sgRNA appears to be the only defective-interfering (DI)-like RNA seen among potexviruses.

Replication

The essential potexvirus replication strategy is believed to involve an RNA intermediate, in which the messenger-sense gRNA is initially copied to produce a full-length antisense complement. This antisense complement is then used as a template for the synthesis of full-length messenger-sense gRNA. Detection of double-stranded gRNA in infected tissue supports this model. The full-length antisense complement also serves as a template for the synthesis of sgRNAs. These sgRNAs are 3′ coterminal with the gRNA, and function as mRNAs for viral products coded for in the 3′-half of the gRNA. It is believed the sgRNAs are produced from the full-length antisense

complement via internal initiation. Membrane-containing extracts isolated from PVX-infected tobacco plants have been shown to support synthesis of all viral RNA types, both single and double stranded. Several conserved sequence motifs, identified at the 5′ and 3′ ends of the gRNA as well as regions immediately 5′ to the initiation sites of sgRNAs synthesis, may act as promoter elements for a replicase complex. The putative promoter sequence at the 5′ end of all potexvirus gRNAs is 5′-GAAAACAAAAC-3′ and at the 3′ end is 5′-ACUUAA-3′. PVX mutants in the 5′ noncoding region have demonstrated that sequence elements in this region also play a crucial role in the synthesis of both gRNA and sgRNA. The presence of a conserved octanucleotide sequence, and the spacing between this sequence and the 5′ start site of sgRNA synthesis initiation, has been shown to be essential for accumulation of the two major sgRNAs in PVX replication. The RNA-dependent RNA polymerase (RdRp) of PVX (ORF 1) contains a glycine-lysine-serine (GKS), a glycine-aspartate-aspartate (GDD) and a methyltransferase (for capping) motif which are also found in many RdRps of RNA viruses. The GKS motif is thought to be required for the binding of nucleoside triphosphates. Mutation of GKS to GNS or GES rendered that virus unable to replicate in plants or protoplasts. Substitution of glycine to alanine had only a minor effect on the replication of PVX. However, mutation of any of the three amino acids GDD rendered the viral genome noninfectious.

The function of the triple gene block products appears to be that of cell-to-cell movement, as mutations in ORFs 2, 3 or 4 prevent the development of systemic infection in plants but not of viral replication in protoplasts. Systemic spread of a transport-defective PVX ORF 2 mutant was restored by transformation of host cells with transport proteins from either a tobamovirus, dianthovirus, or bromovirus. Mutations to the conserved elements of ORF 2 (ATPase-helicase domain) and ORF 3 (hydrophobic domain) also inhibited viral spread in intact hosts. In PVX, the ORF 2 product has been localized to complex lamellar structures and inclusion bodies in the cytoplasm and possibly the nucleus, whereas in bamboo mosaic potexvirus, the ORF 2 product is found in electron-dense crystalline bodies in both the cytoplasm and nucleus. From studying size-exclusion limits of plasmodesmata, the 25-kDa protein (ORF 2) of PVX was shown to be required for full plasmodesmatal modification. A nucleotide triphosphate/helicase consensus sequence (GSGKS/T), similar to that found in ORF 1, has also been found in the ORF 2 of some potexviruses. The ORF 4 product of PVX has been localized to the cell wall. An intact product of ORF 5, the CP, is essential for systemic infection with PVX as well as for replication of PVX gRNA and packaging of this RNA to form complete virus particles.

The messenger-sense gRNA of potexviruses can be translated directly after uncoating in the plant cell. *In vitro* translation of gRNAs isolated from virions typically generates products that correspond to the putative replicase protein encoded by ORF 1 at the 5′ end of the gRNA. Only small and variable amounts of CP, encoded by ORF 5 at the 3′ end, are produced. Production of any CP is likely a result of internal initiation of translation. Efficient *in vitro* synthesis of CP can be achieved by using the smaller (0.9–1.0 kb) of the two major sgRNAs as template. *In vitro* translation of the larger (1.9–2.1 kb) major sgRNA yields product corresponding to ORF 2. The mechanism of expression of the products of ORFs 3 and 4 is less clear, apparently involving internal initiation or the presence of less abundant sgRNAs.

The 5′ cap structure, present on all potexvirus gRNAs and sgRNAs, is essential for infectivity. For instance, infectious *in vitro* RNA transcripts of white clover mosaic potexvirus (WCMV) are only 4% as infectious as capped viral gRNA. Similarly, the length of the 3′ poly(A) tail is critical; shortening the tail length from 71 to 24 nt of *in vitro* RNA transcripts of papaya mosaic virus reduced infectivity from 43% to 16% of native viral gRNA. Mutations to the polyadenylation signal of WCMV reduced both transcript infectivity and the average length of the poly(A) tail in progeny virus.

Fusion of a 27-kDa reporter gene (*Aequorea victoria* green fluorescent protein) to the amino terminus of PVX ORF 5 (CP) resulted in assembly of intact virions that were over twice the diameter of wild-type PVX virions. However, free CP was also required. Efficient functioning of the reporter-gene product was observable, and the assembled virus was capable of both local and systemic movement. The further potential of the use of potexviruses as vectors for the production of high levels of foreign proteins in plants remains to be seen.

Pathology

Symptoms on systemically infected hosts range from undetectable to moderate, depending on the individual potexvirus. Generally, systemic symptoms include chlorotic mottle or mosaic patterns, with stunting also being common. Potexviruses are capable of infecting most host tissues. Both primary and systemic cytopathology can occur in a single host. *Gomphrena globosa* is the most common local lesion host among members of the potexvirus group.

Inclusion bodies, composed of potexvirus particles

packed in parallel arrays, often form in the cytoplasm and sometimes in the nucleus of infected cells. These aggregates often vary in size and have irregular shapes, but can form spindle-shaped structures in some potexviruses. Another type of inclusion, laminated inclusion components (LIC), are thin proteinaceous sheets associated with bead-like structures. LICs display no viral antigens. A third type, the amorphous inclusions, occur in the cytoplasm and vacuoles. Amorphous inclusions contain viral antigens.

Co-infecting a single host with a potexvirus and another virus (e.g. a potyvirus) can have a synergistic effect on symptoms. For instance, mixed infections with PVX and potato virus Y (PVY) produces a dramatic increase in the severity of PVX symptoms, pathogenicity and accumulation, compared to that observed by infection with PVX alone. Other potyviruses such as tobacco vein mottling virus (TVMV), tobacco etch virus (TEV) and pepper mottle virus are also capable of the synergistic effect on PVX replication and symptom severity. The replication of the entire potyviral genome is not increased nor required for PVX/potyviral synergism. The synergistic response is mediated by expression of the 5′-proximal third of the genomic potyviral RNA (i.e. protease-1, helper component protease (HC-Pro) and protein-3 gene). The exact molecular basis for PVX/PVY synergism is not presently well understood.

Host Resistance

The product of the N gene in tobacco confers resistance to PVX. Similarly, three genes in potato have been identified which are associated with potexvirus resistance: Nx and Nb are involved in hypersensitivity, and Rx with immunity. Rx1 has been mapped to the distal end of potato chromosome XII. PVX is recognized to comprise five subgroups (i.e. I, II, III, IV and HB) based on the ability to infect hosts of different resistance genotypes. For instance, group III is the largest PVX subgroup, which systemically infects Nb hosts. Within a group, sequence conservation and host susceptibility are difficult to correlate. In PVX, the CP has been shown to be a strain-specific elicitor of Rx-mediated resistance in potato. Amino acids 121 and 127 may interact with cellular components involved in spread of the virus and with products of Rx resistance gene. The PVX CP also harbors at least two types of virulence determinants, X and B, which differ by only 14 amino acids. Type X CPs are avirulent on potato cultivars carrying the Nx resistance gene, whereas type B CPs are capable of overcoming Nx resistance. Resistance 'breaking' strains of PVX have emerged by minor sequence changes in both the CP and other regions of the viral genome. Expression of the PVX CP or mutant movement proteins in transgenic plants can result in a delay in the onset of symptoms upon subsequent challenge with PVX virus or gRNA. It has been shown recently that a replicating viral RNA (i.e. PVX) is a potent trigger of 'gene silencing', a phenomenon characterized by a genetic control mechanism implicated in virus resistance. Gene silencing is associated with the induced natural defense response of plants against viruses. The signal may move ahead of the inducing virus and result in delaying the spread of the infectious front. The exact molecular mechanisms involved in this phenomenon are currently not well understood.

This work is dedicated to the memory of Duncan Gellatly (1969–1998).

See also: **Carlaviruses; Plant resistance to viruses: Natural resistance, Engineered resistance; Synergism: Plant viruses; Virus structure: Atomic structure, Principles of virus structure.**

Further Reading

AbouHaidar MG and Erickson JW (1985) Structure and *in vitro* assembly of papaya mosaic virus. In: Davies JW (ed.) *Molecular Plant Virology*, Vol. I. Boca Raton, Florida: CRC Press.

Commonwealth Mycological Institute/Association of Applied Biologists (CMI/AAB) Description of Plant Viruses, No. 200, August 1978 (Potexvirus Group) and No. 354, December 1989 (potato virus X).

Francki RIB, Milne RG and Hatta T (1985) Potexvirus Group. In: *Atlas of Plant Viruses*, Vol. II. Boca Raton, Florida: CRC Press.

White KA, Rouleau M, Bancroft JB and Mackie GA (1995) Potexviruses. In: *Encyclopedia of Virology* 1st edn, p. 1142. London: Academic Press.

POTYVIRUSES (*POTYVIRIDAE*)

Juan José López-Moya and **Juan Antonio García**, Centro Nacional de Biotecnologia, CSIC, Campus UAM, Cantoblanco, Madrid, Spain

History, Taxonomy and Classification

The *Potyvirus* genus (type species potato virus Y) of the *Potyviridae* family currently contains the largest group of plant viruses – up to 30% of the total number of plant viruses. The family *Potyviridae* includes five other genera: *Rymovirus, Macluravirus, Ipomovirus, Bymovirus* and *Tritimovirus*. Historically, several viruses later classified in the family *Potyviridae* were known for a long time; some were reported as early as the beginning of the twentieth century, some during the 1930s and the number has increased further from the 1960s to the present day. Viruses of the *Potyviridae* family are among the most damaging plant viruses and a considerable literature has been devoted to them. Since the mid-1980s, complete genome sequences of potyviruses have become available, and genetic studies were made possible by the production of full-length directly infectious cDNA clones, or from infectious transcripts. This technique has also become available for a bymovirus.

The taxonomic standards currently in use for classification into the family *Potyviridae* include properties of the virus particles (long flexuous, as shown in **Fig. 1A**), their genome structure (positive sense single-stranded (ss) RNA, with a 5′ terminal protein and a 3′ poly(A) tail), their expression strategy (polyprotein containing several gene products, separable by proteolysis) and their cytopathological manifestations (particularly the formation of pinwheel- or scroll-shaped cylindrical cytoplasmic inclusions in infected plant cells, as shown in **Fig. 1B**).

Potyviruses can be differentiated from other genera in the family because they are aphid-transmissible and possess a monopartite genome. Bymoviruses are transmitted by fungi and have a genome composed of two molecules of ssRNA which are encapsidated separately. Rymoviruses and Tritimoviruses have monopartite genomes, and their natural vectors are eriophyd mites. Ipomoviruses and Macluraviruses also have monopartite genomes, and they are whitefly and aphid transmitted, respectively.

Although an extensive review of taxonomic issues exceeds the purpose of this entry, recent molecular information has essentially confirmed the classification shown in **Table 1**. Relationships between members of the family should be further examined when more complete sequences become available.

Geographic Distribution, Host Range, Epidemiology and Propagation

Members of the *Potyviridae* family are distributed throughout the world. Some of them have restricted natural and experimental host ranges; others may infect a considerable number of plant species distributed in many families. In general, there are members that are able to infect the most economically important crops, including grain, legumes, forage, vegetables, fruits and ornamentals.

Severity of outbreaks is commonly related to the abundance of initial foci of infection, vector populations and other factors. Vector organisms are mainly involved in the propagation of viruses within a localized region, although they might also have a role in initiation of epidemics at long distance. Human intervention is responsible in many cases for the spread of diseases in larger areas, and the introduction of new diseases. Seed transmission is also important in some potyviruses, and weeds can act as reservoirs of viruses. A typical example of well-documented spread of a potyvirus over a territory and over time is the progressive emergence of plum pox virus (PPV) in European countries during the twentieth century, and its recent spread to other continents.

Properties of Virions

Virion particles of potyviruses are long and flexuous (**Fig. 1A**), 680–900 nm long and 11–15 nm wide. They comprise protein (95%) and RNA (5%). Viruses belonging to other genera in the family possess particles with similar properties. Ipomovirus particles are around 900 nm long, while macluravirus ones are shorter (around 650 nm). The two bymovirus particles are about 250–300 and 500–600 nm in length.

Around 2000 subunits of a single protein (CP) compose the capsid of potyviruses. The molecular weight of each subunit ranges between 30 and 47 kDa, and they are disposed helicoidally around the nucleic acid. Variations in CP size are due mainly to

Table 1 Classification of members of the family *Potyviridae* into genera, with indication of the number of members, transmission vectors and number of genomic RNAs

	Members[a]			
Genus	*Definite*	*Possible*	*Transmission*	*Genome*
Potyvirus	75	93	Aphids	Monopartite
Bymovirus	5	—	Fungi	Bipartite
Rymovirus	3	2	Mites	Monopartite
Tritimovirus	2	—	Mites	Monopartite
Ipomovirus	1	1	Whiteflies	Monopartite
Macluravirus[a]	2	—	Aphids	Monopartite

[a] Number of members according to the International Committee on Taxonomy of Viruses.

differences in the length of the variable N-terminal region, while the internal core (of about 220 amino acids) is more conserved, as expected for its involvement in particle architecture. Although the structure of the potyvirus virions has not yet been determined, Shukla and others have established some structural characteristics (**Fig. 2**). It is known that both N- and C-termini of CP are surface exposed on the particle, the N-terminus being the immunodominant region. In addition, superficially located residues in this region play a role during aphid transmission and are involved in other important functions such as long distance movement in the host plant. A nonspecific nucleic acid binding-domain has been characterized recently in the CP of barley yellow mosaic bymovirus (BaYMV).

Serologic Relationships

Relationships among members of the *Potyviridae* family, based on serology, have been problematic because of their extreme complexity. Virus particles are strongly immunogenic as a general rule, but for many years antisera had limited application in taxonomy. Nonstructural proteins had even more restricted use in serological identification.

The unexpected serological crossreactivities between viruses, and inconsistent relationships derived from virion-based serology, may be due to the fact that group-specific epitopes are mainly located in the conserved internal core region of CP, while the virus-specific ones map in the variable N-terminus, a region that is surface-exposed and prone to undergo degradation. Once the origin of the problem has been identified, solutions have been proposed to obtain virus-specific antibodies by targeting towards the N-terminal epitopes. In addition, the highly conserved CP core may be used to generate *Potyviridae*-specific antibody probes, useful in detecting numerous viruses.

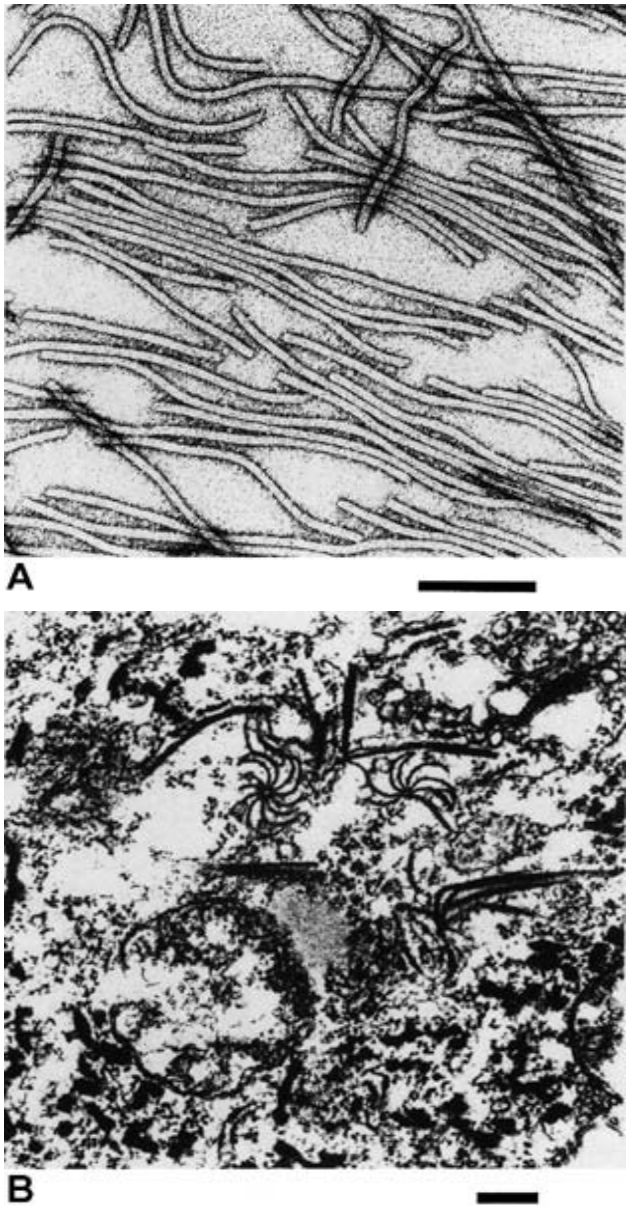


Figure 1 (**A**) Negative stain preparation of purified tobacco etch potyvirus particles. (**B**) Thin section showing the typical pinwheel shaped cytoplasmic inclusions present in cells of a *Nicotiana benthamiana* plant infected with plum pox potyvirus. Bar = 200 nm. (Courtesy of D. López-Abella, CIB, CSIC.)

Properties of the Genome

The nucleic acid of potyviruses is an ssRNA molecule of 9.4–10.3 kb, messenger (positive) sense, with a 5′ terminal protein (VPg), and polyadenylated at its 3′ end. It comprises a single open reading frame (ORF) coding for a long polyprotein (340–370 kDa) that

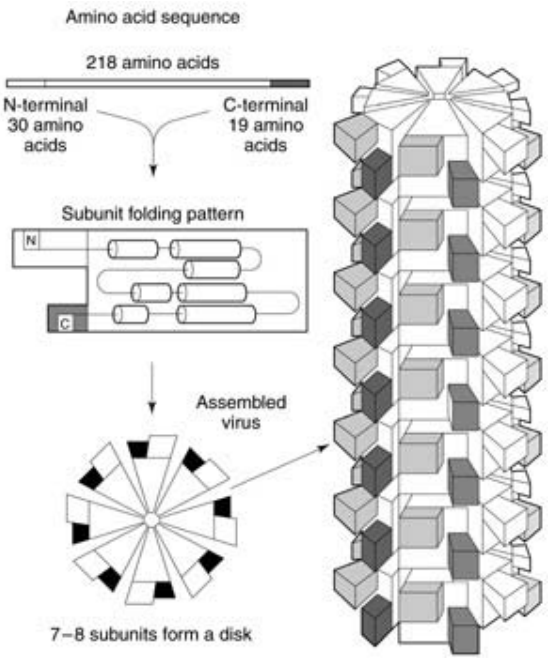


Figure 2 Structural model of potyvirus particles. The predicted folding of individual CP subunits is shown. CP cores unite together to build the virion, leaving both N- and C-termini superficially exposed. (Reproduced with permission from Shukla DD and Ward CW (1989) *Adv. Virus Res.* 36: 273.)

generates mature products (**Fig. 3**) after initiating an autoproteolysis processing cascade. Genomes of rymo- and ipomoviruses are similar in size and organization. In contrast, bymoviruses have their genome divided into two RNAs: the long RNA1 is analogous to the 3′ three-quarters portion of potyvirus genome, and the short RNA2 includes sequences corresponding to others in the 5′ portion of potyviruses, not having in the rest of the molecule clearly matching counterparts in the potyvirus genome. The majority of molecular data available have been obtained with potyviruses. Unless otherwise noted, the following sections describe characteristics of potyviruses.

Three potyvirus-encoded proteinases are involved in the proteolytic cleavage of gene products. The processing events have been studied in depth *in vitro*, and the present figure includes probable cotranslational and autocatalytic cleavages, followed by *trans* cleavages. Processing is dependent on the sequences surrounding each particular cleavage site. A review of this process can be found in the article by Riechmann and coworkers cited in the further reading section.

A typical potyvirus genome starts at the 5′ end with a noncoding region (NCR), usually less than 200 bp long. Studies performed with PPV showed that most of this region is dispensable for infectivity, although it contributes to viral competitiveness and pathogenesis. Although there is some controversy about the existence of internal ribosome entry sites in the 5′ NCR of potyviruses, recent results seem to indicate that translation takes place by a cap-independent leaky scanning mechanism. The 5′ NCR of poty-

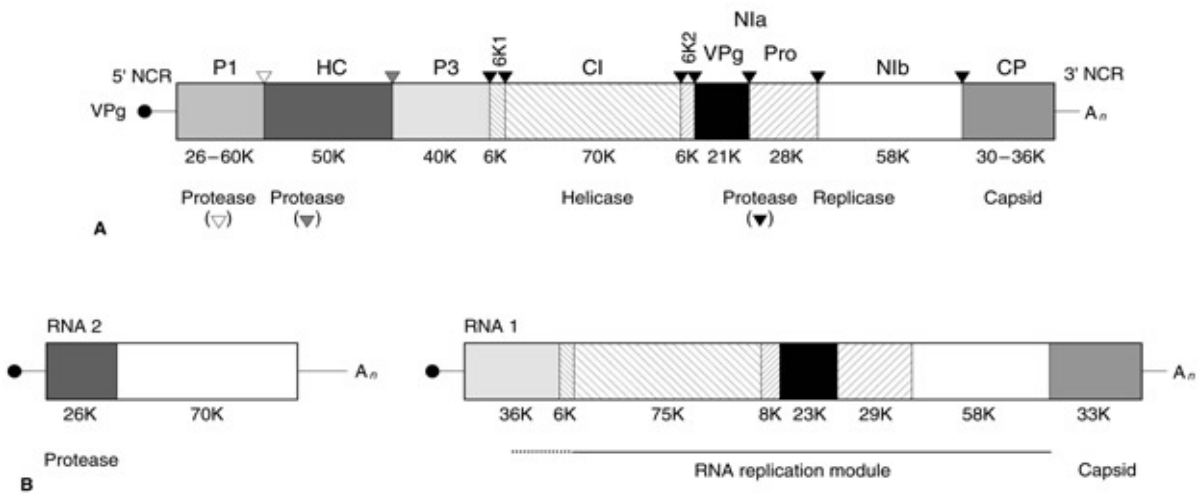


Figure 3 Genomic maps of (**A**) potyviruses and (**B**) bymoviruses. The ssRNA genomes are shown with boxes representing ORFs flanked by NTRs as lines. VPgs are represented by solid circles at each 5′ end, and poly(A) tails are located at each 3′ end. Names of gene products are indicated, as well as some of their known functions and their approximate size K (kDa). The three protease-specific cleavage sites (rendering functional proteins or regulating their activity) are also indicated by triangles above the potyvirus polyprotein. Genomic structures of rymo-, ipomo-, tritimo- and macluraviruses are similar to that of potyviruses.

viruses has been used as an enhancer of translation in transgenic plants.

Another NCR of about 200 bp is located at the 3′ end of the genome, before a variable-length poly(A) tail. Putative RNA structures in this region confer pathogenic properties, as shown in tobacco vein mottling virus (TVMV). In addition, work performed with tobacco etch virus (TEV) has demonstrated the existence of a *cis*-acting element necessary for infectivity near the 3′ end of the genomic RNA, including sequences contained in the last cistron of the ORF.

Properties and Functions of Gene Products

The polyprotein coded in the genome of a potyvirus comprises the following gene products. The first N-terminal protein, named P1, is a protease (similar to chymotrypsin-like serine proteinases) responsible for cleavage at its C-terminus. This region accounts for the largest sequence differences among potyviruses, being extremely variable in size (26–60 kDa) and sequence. Its functions have not yet been established. In TEV, it acts in *trans* as an accessory factor for genome amplification, and experiments suggest that it must be separated from the next gene product to restore debilitated infectivity caused by inactivation of its proteolytic activity. It also has RNA-binding activity.

After P1, the next gene product is the helper component, HC (about 50 kDa), a protein implicated in aphid transmission. Its N-terminal region, which includes a cysteine-rich portion with a putative zinc-finger-type structure, seems to be essential for transmission but is also needed for efficient RNA replication. The carboxy half of the protein is a papain-like cysteine protease, which serves to cleave at its own C-terminus. It has been reported that, like P1, HC is able to interact with RNA *in vitro*. Other functions have been assigned to this protein, such as a factor for long-distance transport and genome amplification. HC also forms amorphous-type inclusions in the cytoplasm of several potyviruses.

All other gene products are excised from the polyprotein by the action of the NIa protease (see below). The third gene product in the ORF is known as P3 (about 40 kDa), and there is little information regarding its role during infection. Immunocytological studies have found P3 associated with cylindrical inclusions in TVMV, and nuclear inclusions in TEV. A small protein $6K_1$ is located next. However in potyviruses such as TEV cleavage between P3 and $6K_1$ has not been observed *in vitro*, and in PPV excision seems not to be required for virus viability. Immunological data suggest that both P3 and $P3{+}6K_1$ products are present in infected cells. The region contains hydrophobic domains which might result in association to membranes. It has been hypothesized that the functional product of this genome region is $P3{+}6K_1$, whose activity would be modulated by cleaving off the $6K_1$ fragment.

The next protein, CI, is the largest potyvirus gene product in size (around 70 kDa). It forms very distinctive cylindrical inclusions in the cytoplasm of infected cells, with a high taxonomic value because they are unique to members of the *Potyviridae* family (**Fig. 1B**). CI includes domains peculiar to RNA helicases, and this activity has been proved experimentally. Two domains have been shown to bind RNA. Functionally, CI is believed to act during unwinding of RNA chains in replication. In addition, it has been found associated to the plasma membrane and plasmodesmata in early stages of infection and it is required for cell-to-cell spread.

A second small peptide, $6K_2$, is involved in virus replication, and its role seems to be related to its membrane-anchoring properties, maintaining the replication complex in place. Separation of $6K_2$ from NIa by autoproteolysis regulates localization of the latter in the nucleus.

The next protein, NIa, of about 49 kDa, may undergo a further cleavage in an internal suboptimal processing site, giving origin to NIaVPg (21 kDa) and NIaPro (27 kDa). VPg is found covalently attached to the 5′ end of genomic RNAs, and it may act as a protein primer during initiation of replication. However, parental VPg is not needed for infectivity (RNA synthesized *in vitro* without VPg is infectious). Recently, a role for VPg during long-distance movement has been identified. In addition to forming VPg, NIa is the protease responsible for the majority of proteolytic events leading to the appearance of final gene products, acting in *cis* and *trans* at processing sites mainly defined by characteristic heptapeptides. Although NIa is structurally related to serine proteases, it has a cysteine at the active site. Moreover, the protease domain of NIa resembles P1 and HC in having nonspecific RNA-binding activity. The complete NIa forms inclusion bodies in the nucleus, where it is translocated by a bipartite signal sequence.

NIb (58 kDa), the second component of nuclear inclusions, is located next. It is the RNA-dependent RNA polymerase (RdRp). NIb contains domains distinctive of polymerases, including a highly conserved GDD motif. Recruitment for the replication complex is postulated to occur via its interaction with NIa. As well as NIa, it is directed to the nucleus by signals in its sequence.

The last product in the ORF is CP. Many roles have been assigned to this protein. First, it forms the virus

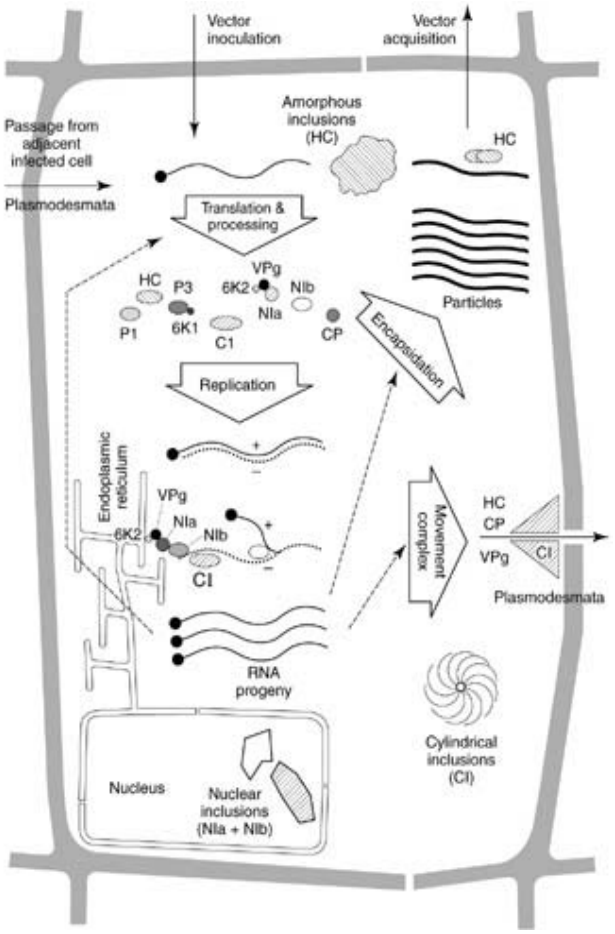


Figure 4 Different events during potyvirus infection of a plant cell. The eventual accumulation of viral proteins as inclusions in different compartments is also indicated.

particles, acting as protection for the genomic RNA. It is implicated in aphid transmission by interaction with HC (see Transmission). CP also participates in cell-to-cell and long-distance movement.

Gene products of viruses belonging to other genera in the family are believed to have equivalent functions. In the case of bymovirus RNA2, the large gene product has no sequence similarity with any potyvirus protein, and it has been postulated to play a role in fungus transmission.

Replication

Potyviruses replicate in the cytoplasm of infected cells. A scheme, based on works done by many researchers, is given in **Fig. 4**. After entering into a cell and undergoing desencapsidation, the genomic RNA must first be translated to give origin to the replication complex, which is composed of several viral products and probably host factors. The replication complex uses the genomic RNA (+ sense) as a template to generate a complementary chain (− sense), which serves as a template for the synthesis of numerous genomic RNAs. Translation of these genomic molecules results in accumulation of viral proteins, including the structural CP which encapsidates RNA to generate viral progeny particles. Movement to adjacent cells in the plant occurs via passage of the RNA through the plasmodesmata, in a form not totally identified, with the involvement of many products such as HC, CI, VPg and CP, and in the newly infected cell the replication cycle is repeated. Proteins involved could vary depending on cell type. Movement has been thoroughly studied by J. C. Carrington.

As a result of the potyvirus expression strategy using a polyprotein, the generation of many copies of CP needed to encapsidate progeny genomes implies the production of equimolar amounts of all other gene products. Some of them accumulate in inclusion bodies in different cellular compartments, while others might be secreted, degraded or remain in a nonnoticeable pattern. It is interesting that, whereas in all potyviral infections cylindrical inclusions (made by CI) are formed in the cytoplasm of infected cells, amorphous or nuclear inclusions (made by HC or NIa and NIb, respectively) are formed by some, but not all, potyviruses.

Potyvirus RNA replication takes place in membranous structures, probably derived from endoplasmic reticulum. NIb forms the core of the viral RNA replicase. NIb protein from TVMV shows *in vitro* RdRp activity, as recently proved. All NIb functions essential for RNA amplification may be provided *in trans*, as free protein supplied in transgenic plants complement TEV deletion mutants. Other proteins probably involved in viral RNA replication are the RNA helicase CI, the putative replication primer VPg, and the membrane anchoring factor $6K_2$. On the other hand, NIa interacts with NIb and RNA, serving perhaps to recruit products during the process. Other factors such as P3 might be involved as found by mutagenesis studies. P1 is also believed to play a role in replication, although it is the only nonessential potyvirus protein. Host factors also might be involved. In this regard, turnip mosaic virus (TuMV) VPg is able to interact with the translational eukaryotic initiation factor (iso) 4E, which may be involved in virus replication or/and translation.

A novel approach for the study of potyvirus replication and its effects on host cells has been developed by Maule. Using pea seed-borne mosaic virus (PSbMV) synchronous infection of cotyledon tissue and *in situ* hybridization, early events during infection have been studied. An infectious front can be identified, which involves active synthesis of minus-strain

RNA and subsequent later accumulation of genomic forms. Along with the presence of the viral RNAs in newly infected cells, a shutoff mechanism of translation of many host proteins was observed, as well as upregulation of other products (interestingly heat shock-related proteins or ubiquitin). These findings have been extended to other viruses and tissues, suggesting common molecular processes which might be significant in pathogenesis.

Evolution

Members of the family *Potyviridae* were considered within the Picorna-like supergroup of viruses, due to a fairly conserved set of proteins with replication-related functions present in analogous placement in the genome.

Genera in the family show a close relationship, indicated by gene product order in the polyprotein, strategy of expression and protein homologies. Each genus within the family seemed to be adapted to specific vector organisms. Cassette evolution has been proposed as the means used by viruses to incorporate new functions in their genomes, and this must have been the case for members of the family *Potyviridae*.

One intriguing issue is the shocking number of aphid-transmitted potyviruses. A combination of a very efficient transmission system and adaptation to new hosts must have contributed to this large expansion. In particular, vector transmission acting as bottlenecks might lead to broad speciation in the highly variable quasispecies structure of RNA genomes. Indications of recombination events, partial duplications, point mutations which confer different host responses, as well as other facts, might help to explain the extraordinary variability observed among potyviruses.

Transmission

As mentioned above, viruses in the *Potyviridae* family have different vector organisms. Bymoviruses are believed to be present and travel in the zoospores of their fungus vector. Rymoviruses and tritimoviruses are transmitted by eriophyd mites through a little known mechanism. Similarly, relationships between ipomoviruses and their whitefly vectors have not been clearly established. In contrast, the process of aphid transmission of potyviruses has been intensely studied in several aspects, specially in Pirone's laboratory. Transmission occurs in a nonpersistent manner, with the involvement of two viral proteins, CP and HC. There are nontransmissible variants of potyviruses exhibiting defects in either of these proteins, and by mutagenesis analysis alterations responsible for these phenotypic effects have been identified in TVMV and other potyviruses. The integrity of a highly conserved DAG motif near the N-terminus of CP has proved to be essential for transmission. In HC, mutations in several domains, mainly located in its N-terminal portion, result in failure of transmission.

The hypothesis currently suggested for the mechanism of aphid transmission involves a function for HC serving as a bridge to retain virus particles to internal structures in the aphid stylets. Using radioactively-labeled virions, it has been found that they are retained mainly in the distal part of the stylets. An *in vitro* interaction between HC and CP has been shown recently to correlate with transmissibility of CP variants of TVMV, and the domain of CP involved in the interaction includes the DAG motif. Recent results show that a PTK domain identified in the HC of zuchini yellow mosaic virus (ZYMV) is likely to be related to this binding to CP. Presumably other motifs in the HC might be involved in the interaction with the vector. In addition, specificity for each virus–vector combination is probably determined by compatibility between HC and the aphid.

Pathogenicity

The information about how potyviruses cause diseases in their host plants is limited. Recent research has begun to identify sequences and products in the genome of potyviruses that are directly implicated in the production of symptoms. As mentioned above, symptom alteration determinants have been identified in the 3′ NCR of TVMV, and sequences in the 5′ NCR of PPV, not essential for infectivity, contribute to pathogenesis. Chimeras between variants of viruses differing in their pathogenic responses have been generated, and thus used to identify the differences accounting for their phenotypic behavior. Single products are not always responsible for the effects. For instance, two separate domains have been identified in TEV implicated in the specific wilting response of Tabasco pepper.

VPg seems to be an important determinant of pathogenicity in potyviruses. Thus, sequence variations in the VPg are involved in resistance-breaking of TVMV and TEV in tobacco, and VPg determines pathotype-specificity of PSbMV in pea.

Sequences of the P1/HC region are involved in a synergistic effect in the case of mixed infections with other viruses. It has been proposed that HC is a broad-range pathogenicity enhancer that seems to transactivate viral replication by interfering with a plant defense response.

For bymoviruses, determinants for pathogenicity

and symptomatology have been found in the RNA1 of barley mild mosaic virus (BaMMV).

Control

Strategies considered for potyvirus control are diverse, from culture practices to the use of genetic resistance. Insecticide treatment of vectors has limited use because of the transmission type. The knowledge currently available about potyvirus replication, movement and transmission will eventually allow design strategies directed at interfering with these key roles during the virus life cycle.

Presently, a considerable effort (with not always equally gratifying results) has been placed in the exploitation of pathogen-derived resistance. Transgenic plants have been generated incorporating viral sequences. Since the early reports of CP-mediated resistance to plant viruses, this approach has been tried with several potyviruses. Results show that expression of one potyvirus CP in transgenic plants might confer resistance to other unrelated potyviruses. Nonstructural genes of viruses have been tested as sources of resistance, with promising results. Replicase sequences, for instance, create several resistant phenotypes, considered to be RNA-mediated. Elucidation of the mechanisms involved in resistance has been initiated after works done by Dougherty with potyvirus-derived transgenic lines, which pointed out the relationship of resistance and the cellular phenomenon known as cosuppression or gene silencing.

Future Perspectives

Analysis of potyvirus molecular biology using full-length cDNA clones has been extraordinarily successful in many respects, and it is likely to continue to provide interesting data. In particular, new control strategies might be developed after much better understanding of the potyvirus life cycle is achieved. Virus–host relationships involved in virus multiplication and pathogenesis, as well as in virus resistance, must be further characterized. Transgenic plants will provide information at this level, and even extend beyond, to be used as tools in plant molecular biology studies.

In addition, biotechnological uses of potyviruses has begun. The junction of P1–HC has been manipulated as a potential insertion site for foreign genes, allowing applications of potyviruses as expression vectors in plants, and it is likely that this or similar strategies will be further exploited.

See also: **Comoviruses (*Comoviridae*); Nepoviruses (*Comoviridae*); Plant virus disease – economic aspects; Pathogenesis: Plant viruses.**

Further Reading

Carrington JC, Kasschau KD, Mahajan SK and Schaad MC (1996) Cell-to-cell and long-distance transport of viruses in plants. *Plant Cell* 8: 1669.

Dougherty WG and Dawn Parks T (1995) Transgenes and gene suppression: telling us something new? *Curr. Opin. Cell Biol.* 7: 399.

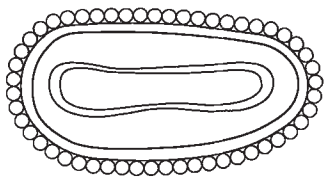
Maia IG, Haenni A-L and Bernardi F (1996) Potyviral HC-Pro: a multifunctional protein. *J. Gen. Virol.* 77: 1335.

Riechmann JL, Lain S and Garcia JA (1992) Highlights and prospects of potyvirus molecular biology. *J. Gen. Virol.* 73: 1.

Shukla DD, Ward CW and Brunt AA (1994) *The Potyviridae*. CAB International, Oxon, UK.

Wang D and Maule AJ (1995) Inhibition of host gene expression associated with plant virus replication. *Science* 267: 229.

POXVIRUSES (*POXVIRIDAE*)



Contents

Capripoxviruses

R Paul Kitching, World Reference Laboratory for Foot and Mouth Disease, Pirbright Laboratory, BBSRC Institute for Animal Health, Pirbright, UK

History

Sheeppox and goatpox are malignant pox diseases of sheep and goats easily recognizable by their characteristic clinical signs, and described in the earliest texts on animal diseases. Lumpy skin disease (Neethling) of cattle (LSD), however, was first described in 1929 in Northern Rhodesia (Zambia), having apparently been absent from domestic cattle until that time. From Zambia LSD spread south to Botswana and Zimbabwe and by 1944 the disease appeared in South Africa, where it caused a major epizootic, affecting over 6 million cattle. In 1957 LSD was first diagnosed in Kenya, and was thought at the time to be associated with the introduction of a flock of sheep affected with sheeppox on to the farm. Since then LSD has been present in most of the countries of sub-Saharan Africa, often associated with large epizootics followed by periods in which the disease is only rarely reported. In 1988 LSD caused a major outbreak in Egypt and in 1989 spread from Egypt to a village in Israel. This was the first time that a diagnosis of LSD outside of Africa had been supported by laboratory confirmation.

Taxonomy and Classification

The viruses that cause sheeppox, goatpox and LSD are all members of the genus *Capripoxvirus,* in the *Chordopoxvirinae* subfamily of the *Poxviridae* family, and have morphological, physical and chemical properties similar to vaccinia virus. Originally the viruses were classified according to the species from which they were isolated, but comparisons of their genomes indicate that the distinction between them is not so clear, and that recombination events occur naturally between isolates from different species. This is reflected in the ability of some strains to cause disease in both sheep and goats and in experimental results which show that all the sheep isolates examined could infect goats, and that goat isolates could infect sheep. The epidemiological relationship between sheeppox and goatpox isolates and cattle isolates is less clear, apparent in differences in the geographical distribution of sheeppox and goatpox and LSD (see later). However, some isolates recovered from sheep and goats in Kenya have genome characteristics very similar to cattle isolates. It has been proposed that confusion can be reduced by referring to the malignant pox diseases of sheep, goats and cattle, including Indian goat dermatitis and Kenyan sheep and goatpox, as capripox. It is envisaged that when sufficient isolates have been examined biochemically, no clear distinction will be possible between sheep, goat and cattle isolates, but a spectrum will emerge in which some strains have clear host preferences while others will be less defined and will naturally infect the host with which they come into contact.

Geographical and Seasonal Distribution

Capripox of sheep and goats is enzootic in Africa north of the equator, the Middle East and Turkey, Iran, Afghanistan, Pakistan, India, Nepal and parts of the People's Republic of China, and in 1986, Bangladesh. Sheeppox was eradicated from Britain in 1866, and from France, Spain and Portugal in 1967, 1968 and 1969 respectively. Sporadic outbreaks still occur in Europe, for instance in Italy in 1983 and Greece and Bulgaria both in 1995 and 1996, and Greece in 1997 and 1998.

LSD is enzootic in the sub-Saharan countries of Africa and is possibly still present in Egypt. The single outbreak in Israel was eradicated by slaughter of affected and in-contact cattle.

There is no clear seasonality to outbreaks of capripox in sheep and goats. In enzootic areas lambs and kids are protected against infection with capripoxvirus for a variable time dependent on the immunity of the mother. However, the spread of LSD is related to the density of biting flies and consequently major enzootics have been associated with humid weather when fly activity is greatest.

Host Range and Virus Propagation

Amongst domestic species, capripoxvirus is restricted to cattle, sheep and goats. Experimentally it is possible to infect cattle, sheep or goats with isolates derived

from any of these three species, although clinically the reaction following inoculation may be indiscernible. Viral genome analysis using restriction endonucleases has identified fragment size characteristics by which it is possible to classify strains into cattle, sheep or goat isolates. However, the identification of strains that have intermediate characteristics between typical sheep and goat isolates does suggest the movement of strains between these species. Analysis of some Kenyan isolates derived from sheep and goats shows very close homology with cattle LSD isolates.

The involvement of the African buffalo (*Syncerus caffer*) in the maintenance of LSD has not been clearly established. Some surveys have shown the presence of capripoxvirus antibody in buffalo, while others have failed to show its presence. Buffalo clinically affected with LSD have not been described. Experimental infection of giraffe (*Giraffe calemopardelis*), impala (*Aepyceros melampus*) and gazelle (*Gazella thomsonii*) has resulted in the development of clinical disease.

Bos indicus cattle are generally less susceptible to LSD and develop milder clinical disease than *B. taurus*, of which the fine-skin Channel Island breeds are particularly susceptible. Similarly, breeds of sheep and goats indigenous to capripoxvirus enzootic areas appear less susceptible to severe clinical capripox than do imported European or Australian breeds.

Capripoxvirus will grow on the majority of primary and secondary cells and cell lines of ruminant origin. Primary lamb testes cells are considered the most sensitive system for isolation and growth of capripoxvirus. The virus produces a characteristic cytopathic effect (cpe) on these cells which can take up to 14 days for field isolates, but can be as short as 3 days for well-adapted strains.

Isolates of capripoxvirus derived from cattle have been adapted to grow on the chorioallantoic membrane of embryonated hens' eggs, although attempts to grow isolates from sheep and goats in eggs have been unsuccessful. Vaccine strains of capripoxvirus have been adapted to grow on Vero cells. Capripoxvirus will not grow in any laboratory animals.

Genetics

Less is known concerning the specific genetics of capripoxvirus than is known about the orthopoxvirus genome. Studies on field isolates taken from cattle suggest that the virus is very stable, as *Hind* III restriction endonuclease digest patterns of isolates from the 1959 Kenya outbreak of LSD are identical to those obtained from 1986 LSD isolates. However, recombination has been shown to occur between cattle and goat isolates and this could be the natural method by which the virus evolves. By analogy with the orthopoxviruses, it is also likely that sequences are deleted or repeated within the genome in the normal replicative cycle.

Comparisons between the genomes of the different poxvirus genera show a relatively low level of nucleotide sequence homology. In common with other poxviruses, capripoxviruses contain a gene coding for thymidine kinase. Genes which code for attachment, fusion, interferon γ receptor and chemokine receptor proteins have also been identified, and sequenced, and shown to be highly conserved between isolates. Within the capripoxvirus genus, nucleotide divergence values suggest that the typical sheep and cattle isolates are more closely related to each other than to goat isolates. There is, however, less divergence between capripoxvirus genomes than seen in orthopoxvirus genomes, particularly in the near-terminal regions, which in the orthopoxvirus is considered to account for the considerably larger host range of this genus.

The usual technique for developing capripox vaccines has been to serially passage virulent isolates in tissue culture. This has been shown to reduce the virulence of the strain, although the mechanism by which it occurs is not known.

Evolution

The capripoxviruses have evolved into specific cattle, sheep and goat lines, but, as has been described above, intermediate strains exist, particularly those with cattle and goat genome characteristics. In Kenya there is evidence of movement of strains between all three species, but the absence of sheeppox or goatpox in LSD enzootic areas in Southern Africa, and the absence of LSD outside of Africa, would suggest that host-specific strains are being maintained and presumably are continuing to evolve.

Serologic Relationships and Variability

Polyclonal sera fail to distinguish in the virus neutralization test between any of the isolates of capripoxvirus so far examined. Sheep, goats or cattle that have been infected with any of the isolates are totally resistant to challenge with any of the other isolates. On this basis it has been possible to use the same vaccine strain to protect all three species. No monoclonal antibodies are yet available against capripoxvirus, but it can be expected that differences will emerge between strains using these reagents.

Capripoxviruses share a precipitating antigen with parapoxviruses, but no crossimmunity has been

shown between these two genera, or between capripoxvirus and any other pox virus genera.

Epidemiology

In sheeppox and goatpox enzootic areas the distribution of disease is frequently a reflection of the traditional form of husbandry. For instance, in the Yemen Arab Republic, the sheep and goat flocks kept on the grassland of the central plateau and better irrigated regions of the coastal plain move about in search of food, frequently mixing with flocks from neighboring villages at water holes, and in this situation disease is restricted to the young stock. Animals over 1 year of age have a solid immunity. The animals belonging to villages in the more mountainous regions and the arid areas of the coastal plain are isolated by terrain or semidesert from mixing with animals from other villages. It is not known what is the critical number of animals required to maintain capripoxvirus within a single population but it is over a thousand adult animals, which is the approximate village sheep and goat populations. In these villages disease is usually only seen following the introduction of new animals, typically from market, and generally affects animals of all age groups. The disease spreads through the village, usually within 3–6 months, and then disappears in the absence of more susceptible animals. Occasionally, even within areas of high sheep and goat density, it is possible to encounter animals that have been kept totally isolated in the confines of a domestic residence, and these may remain susceptible to infection until adult.

In Sudan, large numbers of sheep and goats are trekked from the west to the large collecting yards and markets of Omdurman, outside Khartoum. Here also it is possible to see capripox infection in adult animals. Many of the flocks originate in villages which, like in the Yemen, are isolated from their neighbors. Capripoxvirus does not persist in these villages, and as a result the animals acquire no resistance, and are fully susceptible when they first encounter disease on the long journey across Sudan. Animals being exported from countries that are free of capripoxvirus may suffer a similar fate when they arrive in a capripoxvirus enzootic area, as often seen in Australian or New Zealand sheep imported into the Middle East.

In a study of 49 outbreaks of capripox in the Yemen, only eight were reported to affect both sheep and goats, the remaining 41 causing clinical disease in either sheep or goats. It is possible that both sheep and goats could have been involved in more than the eight outbreaks, but that the disease was inapparent in one species; whether, therefore, the species in which the disease was inapparent could transmit virus and become a vector for disease has not been determined. In Kenya, capripox is frequently encountered in both sheep and goats within the same flock, and there is the possibility that the same strain of capripoxvirus could also cause LSD in cattle.

The epidemiology of sheeppox, goatpox and LSD is similar; the severity of outbreaks depends on the size of the susceptible population, the virulence of the strain of capripoxvirus, the breed affected (indigenous animals tending to be less susceptible to clinical disease than imported), and, with LSD, the presence of suitable insect vectors. Morbidity rates vary from 2 to 80%, and mortality rates may exceed 90%, particularly if the infection is in association with other disease or bad management.

Transmission and Tissue Tropism

Under natural conditions capripoxvirus is not transmitted very readily between animals, although there are circumstances when transmission appears very rapid; for example, in association with factors that damage the mucosae, such as peste des petits ruminants infection or feeding on abrasive forage. Animals are most infectious soon after the appearance of papules and during the 10 day period before the development of significant levels of protective antibody. High titres of virus are present in the papules, and those papules on the mucous membranes quickly ulcerate and release virus in nasal, oral and lachrymal secretions, and into milk, urine and semen. Viremia may last up to 10 days, or in fatal cases until death. Those animals that die of acute infection before the development of clinical signs and those that develop only very mild signs or single lesions rarely transmit infection, while those that develop generalized lesions produce considerable virus and are highly infectious. Aerosol infection over a few meters only, as with other poxvirus infections, is probably the usual form of transmission. Contact transmission of LSD virus under experimental conditions in the absence of insect vectors has only rarely been reported. Biting flies are significant in the mechanical transmission of LSD, and *Stomoxys calcitrans* and *Biomyia fasciata* have been implicated. There are probably a number of insects capable of mechanically transmitting LSD virus, but insects such as mosquitoes, which preferentially feed on hyperaemic sites such as papules and if interrupted inoculate a new host intravenously, are considered the most likely to be involved in outbreaks characterized by large numbers of affected animals with generalized infections. Experimentally, *S. calci-*

trans has also been shown to be capable of transmitting sheeppox and goatpox.

During the recovery phase following infection, the papules on the skin become scabs. It is relatively easy to demonstrate virions in the scab, but difficult to isolate virus on tissue culture, probably because of the complexing of antibody and virus within the scab. Capripoxvirus is reported to remain viable in wool for 2 months and in contaminated premises for 6 months, and is reported to remain infectious in skin lesions of cattle for 4 months. The true epidemiological significance of the virus within the scab, and ultimately the environment, is not clear. It has been suggested that the protein material that envelops the virus within the type A intracytoplasmic inclusion bodies of infected cells protects the virus in the environment.

There is no evidence for the existence of animals persistently infected with capripoxvirus. Transplacental transmission of capripoxvirus may be possible in association with simultaneous pestivirus infection, as may occur with pestivirus-contaminated capripox vaccine.

Capripoxvirus can be isolated from the leukocytes during viremia, and has been isolated from lesions in the liver, urinary tract, testes, digestive tract and lungs; however, the cells of the skin and skin glands and the internal and external mucous membranes appear to be the major sites of virus replication.

Pathogenicity

There is considerable variation in the pathogenicity of strains of capripoxvirus. Nothing is known concerning the genes responsible in the capripoxvirus genome for virulence or host restriction.

Clinical Features of Infection

The incubation period of capripox infection, from contact with virus to the onset of pyrexia, is approximately 12 days, although it frequently appears longer as transmission is often not immediate between infected and susceptible animals. Following experimental inoculation of virus the incubation period is approximately 7 days, and this is similar to that shown experimentally using biting flies to transmit virus.

The clinical signs of malignant disease are similar in sheep, goats and cattle. Twenty-four hours after the development of pyrexia of between 40 and 41°C, macules (2–3 cm diameter areas of congested skin) can be seen on the white skin of sheep and goats, particularly under the tail. Macules are not seen on the thicker skin of cattle, and are frequently missed on

Figure 1 Sheep pox showing rhinitis and conjunctivitis.

skin of pigmented sheep and goats. After a further 24 h the macules swell to become hard papules of between 0.5 and 2 cm diameter, although they may be larger in cattle. In the generalized form of capripox, papules cover the body, being concentrated particularly on the head and neck, axilla, groin and perineum, and external mucous membranes of the eyes, prepuce, vulva, anus and nose. In cattle these papules may exude serum, and there may be considerable edema of the brisket, ventral abdomen and limbs. The papules on the mucous membranes quickly ulcerate, and the secretions of rhinitis and conjunctivitis become mucopurulent (**Fig. 1**). Keratitis may be associated with the conjunctivitis.

All the superficial lymph nodes, particularly the prescapular, are enlarged. Breathing may become labored as the enlarged retropharyngeal lymph nodes put pressure on the trachea. Mastitis may result from secondary infection of the lesions on the udder.

The papules do not become vesicles and then pustules, typical of orthopoxvirus infections. Instead they become necrotic, and if the animal survives the acute stage of the disease, change to scabs over a 5–10

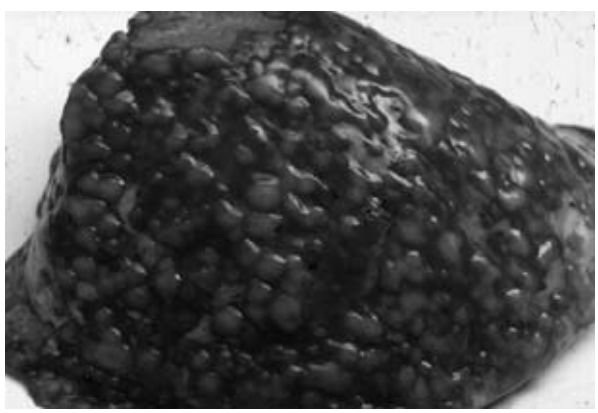


Figure 2 Sheep pox showing severe lung lesions.

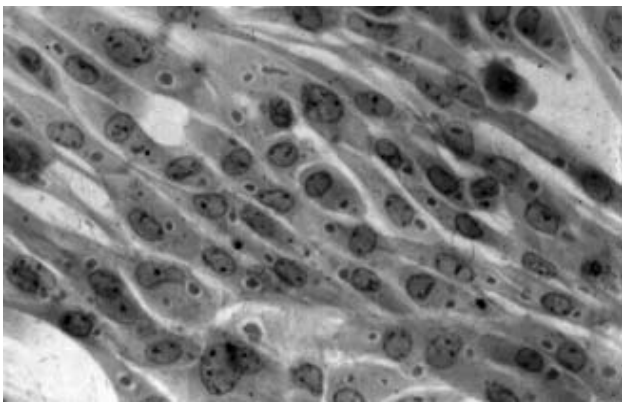


Figure 3 Capripoxvirus growing in lamb testis cells showing many intracytoplasmic inclusion bodies. ×400.

day period from the first appearance of papules. The scabs can persist for up to a month in sheep and goats, whereas in cattle the necrotic papules which penetrate the thickness of the skin may remain as 'sitfasts' for up to a year.

Severe disease is accompanied by significant loss of condition, agalactia, possibly secondary abortion and pneumonia. Eating, drinking and walking may become painful, and death from dehydration is not uncommon. Secondary myiasis is also a major problem in tropical areas.

Pathology and Histopathology

The lesions of capripox are not restricted to the skin, but also may affect any of the internal organs, in particular the gastrointestinal tract from the mouth and tongue to the anus, and the respiratory tract. In generalized infections papules are prominent in the abomasal mucosa, trachea and lungs. Those in the lungs are approximately 2 cm in diameter, and papules may coalesce to form areas of gray consolidation (**Fig. 2**).

In affected skin there is an initial epithelial hyperplasia followed by coagulation necrosis as thrombi develop in the blood vessels supplying the papules. Histiocytes accumulate in the areas of the papules, and the chromatin of the nuclei of infected cells marginates. The cells appear stellate as their boundaries become poorly defined, and many undergo hydropic degeneration with the formation of microvesicles. Intracytoplasmic inclusion bodies are present in infected cells of the dermis and also in the columnar epithelial cells of the trachea where frequently gross lesions may not be apparent. These are initially type B inclusions at the sites of virus replication (**Fig. 3**), but later in infection they are replaced by type A inclusions (see earlier). The maximum titer of virus is obtained from papules approximately 6 days after their first appearance.

Immune Response

Capripoxvirus, like orthopoxvirus, is released from an infected cell within an envelope derived from modified cellular membrane. The enveloped form of the virus is more infectious than the nonenveloped form, which can be obtained experimentally by freeze–thawing infected tissue culture. By analogy with orthopoxvirus, antigens on the envelope and on the tubular elements of the virion surface may stimulate protective antibodies. Animals immune to nonenveloped virus are still fully susceptible to the enveloped form. Passively transferred antibody, either colostral or experimentally inoculated, will protect susceptible animals against generalized infection; however, in the vaccinated or recovered animal there is no direct correlation between serum levels of neutralizing antibody and immunity to clinical disease. Antibody may limit the spread of capripoxvirus within the body, but it is the cell-mediated immune response which eliminates infection. In sheep, major histocompatibility complex (MHC)-restricted cytotoxic T lymphocytes are required in the protective immune response to orthopoxvirus infection, and therefore probably also capripoxvirus infection.

Immune animals challenged with capripoxvirus by intradermal inoculation develop a delayed-type hypersensitivity reaction at the challenge site. This may not be apparent in animals with high levels of circulating antibody. It has been suggested that the very severe local response shown by some cattle at the site of vaccination against LSD may be a hypersensitivity reaction due to previous contact with the antigens of parapoxvirus.

There is total crossimmunity between all strains of capripoxvirus, whether derived from cattle, sheep or goats.

Prevention and Control

In temperate climates capripox can be effectively controlled by slaughter of affected animals, and movement control of all susceptible animals within a 10 km radius for 6 months. In tropical climates, particularly in humid conditions when insect activity is high, movement restrictions are not sufficient and vaccination of all susceptible animals should be considered. In outbreaks of LSD it is not considered necessary to vaccinate sheep and goats, although theoretically cattle strains of virus could infect them. Similarly, in outbreaks of capripox in sheep and goats, cattle are not normally vaccinated.

Countries in which capripoxvirus is absent can maintain freedom by preventing the importation of animals from infected areas. There is always a possibility that skins from infected animals could introduce infection into a new area, although there have been no proven examples of this. The insect transmission of capripoxvirus into Israel from Egypt over a distance of between 70 and 300 km would indicate that it is impossible for countries neighboring enzootic areas totally to secure their borders.

In enzootic areas annual vaccination of susceptible animals with a live vaccine will control the disease. Calves, kids and lambs up to 6 months of age may be protected by maternal antibody, but this would only occur if the mother had recently been severely affected with capripox. Although maternal antibody will inactivate the vaccine, it is advisable to vaccinate all stock over 10 days of age. No successful dead vaccines have been developed for immunization against capripoxvirus infection, other than those that give only very short-term immunity.

Future Perspectives

Capripox of sheep and goats is present in most of Africa and Asia, whereas LSD is restricted to Africa. There is no good explanation as to why LSD has not spread into the Middle East and India, carried by the considerable trade in live cattle. Unless there is a reservoir host in Africa which is required for the maintenance of the cattle-adapted capripoxvirus, it can be anticipated that LSD will spread out of Africa, with major economic consequences.

While considerable attention has been given to vaccinia virus as a vector of other viral genes for development as a recombinant vaccine, little attention has been given to capripoxvirus as a potential vector vaccine. Although its use would be restricted to the not inconsiderable capripoxvirus enzootic area, it would have the advantage of not being infectious to humans, and being a useful vaccine in its own right.

See also: **Cowpox virus (*Poxviridae*); Fowlpox virus (*Poxviridae*); Immune response: Cell mediated immune response, General features; Mousepox and rabbitpox viruses (*Poxviridae*); Yabapox and Tanapox viruses (*Poxviridae*); Vaccinia virus (*Poxviridae*); Vectors: Animal viruses, Plant viruses.**

Further Reading

Black DN, Hammond JM and Kitching RP (1986) Genomic relationship between capripoxviruses. *Virus Res.* 5: 277.

Carn VM and Kitching RP (1995) An investigation of possible routes of transmission of lumpy skin disease (Neethling). *Epidemiol. Infect.* 114: 219.

Gershon PD, Ansell DM and Black DN (1989) A comparison of the genome organization of capripoxviruses with that of the orthopoxviruses. *J. Virol.* 63: 4703.

Gershon PD, Kitching RP, Hammond JM and Black DN (1989) Poxvirus genetic recombination during natural virus transmission. *J. Gen. Virol.* 70: 485.

Kitching RP, Hammond JM and Taylor WP (1987) A single vaccine for the control of capripox infection in sheep and goats. *Res. Vet. Sci.* 42: 53.

Kitching RP, Bhat PP and Black DN (1989) The characterization of African strains of capripoxvirus. *Epidemiol. Infect.* 102: 335.

Leporipoxviruses and Suipoxviruses

Grant McFadden, The John P. Robarts Research Institute, University of Western Ontario, London, Ontario, Canada

History

Poxviruses of leporids and swine cause a broad range of symptoms varying from mild lesions of the skin right up to lethal systemic diseases (**Table 1**). The agent of myxomatosis, a virulent disease of domestic rabbits described originally by G. Sanarelli in 1896, was in fact the first viral pathogen discovered for a laboratory animal. The close similarity of myxoma virus (MYX) with other members of the poxvirus family, such as variola and fowlpox, was first recognized by Aragão in 1927. MYX is notable because, although it causes rather benign lesions in the native *Sylvilagus* rabbit (the brush rabbit in North America and the tropical forest rabbit in South America), when introduced to the European (*Oryctolagus*) rabbit it causes an invasive disease syndrome with up to 100% mortality. MYX was the first viral agent ever introduced into the wild for the purpose of eradicating a vertebrate pest, namely the feral European rabbit population in Australia in 1950 and, two years later, in Europe. The resulting genetic selection of virus isolates with lesser pathogenicity and upsurgence of rabbits with greater resistance to the viral disease was studied intensively by Frank Fenner and his colleagues as a model system to investigate the ecological consequences of virus/host evolution in an outbred population.

Also of interest to the history of animal virology is the fact that the first DNA virus associated with

Countries in which capripoxvirus is absent can maintain freedom by preventing the importation of animals from infected areas. There is always a possibility that skins from infected animals could introduce infection into a new area, although there have been no proven examples of this. The insect transmission of capripoxvirus into Israel from Egypt over a distance of between 70 and 300 km would indicate that it is impossible for countries neighboring enzootic areas totally to secure their borders.

In enzootic areas annual vaccination of susceptible animals with a live vaccine will control the disease. Calves, kids and lambs up to 6 months of age may be protected by maternal antibody, but this would only occur if the mother had recently been severely affected with capripox. Although maternal antibody will inactivate the vaccine, it is advisable to vaccinate all stock over 10 days of age. No successful dead vaccines have been developed for immunization against capripoxvirus infection, other than those that give only very short-term immunity.

Future Perspectives

Capripox of sheep and goats is present in most of Africa and Asia, whereas LSD is restricted to Africa. There is no good explanation as to why LSD has not spread into the Middle East and India, carried by the considerable trade in live cattle. Unless there is a reservoir host in Africa which is required for the maintenance of the cattle-adapted capripoxvirus, it can be anticipated that LSD will spread out of Africa, with major economic consequences.

While considerable attention has been given to vaccinia virus as a vector of other viral genes for development as a recombinant vaccine, little attention has been given to capripoxvirus as a potential vector vaccine. Although its use would be restricted to the not inconsiderable capripoxvirus enzootic area, it would have the advantage of not being infectious to humans, and being a useful vaccine in its own right.

See also: **Cowpox virus (*Poxviridae*); Fowlpox virus (*Poxviridae*); Immune response: Cell mediated immune response, General features; Mousepox and rabbitpox viruses (*Poxviridae*); Yabapox and Tanapox viruses (*Poxviridae*); Vaccinia virus (*Poxviridae*); Vectors: Animal viruses, Plant viruses.**

Further Reading

Black DN, Hammond JM and Kitching RP (1986) Genomic relationship between capripoxviruses. *Virus Res.* 5: 277.

Carn VM and Kitching RP (1995) An investigation of possible routes of transmission of lumpy skin disease (Neethling). *Epidemiol. Infect.* 114: 219.

Gershon PD, Ansell DM and Black DN (1989) A comparison of the genome organization of capripoxviruses with that of the orthopoxviruses. *J. Virol.* 63: 4703.

Gershon PD, Kitching RP, Hammond JM and Black DN (1989) Poxvirus genetic recombination during natural virus transmission. *J. Gen. Virol.* 70: 485.

Kitching RP, Hammond JM and Taylor WP (1987) A single vaccine for the control of capripox infection in sheep and goats. *Res. Vet. Sci.* 42: 53.

Kitching RP, Bhat PP and Black DN (1989) The characterization of African strains of capripoxvirus. *Epidemiol. Infect.* 102: 335.

Leporipoxviruses and Suipoxviruses

Grant McFadden, The John P. Robarts Research Institute, University of Western Ontario, London, Ontario, Canada

History

Poxviruses of leporids and swine cause a broad range of symptoms varying from mild lesions of the skin right up to lethal systemic diseases (**Table 1**). The agent of myxomatosis, a virulent disease of domestic rabbits described originally by G. Sanarelli in 1896, was in fact the first viral pathogen discovered for a laboratory animal. The close similarity of myxoma virus (MYX) with other members of the poxvirus family, such as variola and fowlpox, was first recognized by Aragão in 1927. MYX is notable because, although it causes rather benign lesions in the native *Sylvilagus* rabbit (the brush rabbit in North America and the tropical forest rabbit in South America), when introduced to the European (*Oryctolagus*) rabbit it causes an invasive disease syndrome with up to 100% mortality. MYX was the first viral agent ever introduced into the wild for the purpose of eradicating a vertebrate pest, namely the feral European rabbit population in Australia in 1950 and, two years later, in Europe. The resulting genetic selection of virus isolates with lesser pathogenicity and upsurgence of rabbits with greater resistance to the viral disease was studied intensively by Frank Fenner and his colleagues as a model system to investigate the ecological consequences of virus/host evolution in an outbred population.

Also of interest to the history of animal virology is the fact that the first DNA virus associated with

Table 1 Members of the Leporipoxvirus and Suipoxvirus Genera

Member	*Abbreviation*	*Natural host*	*Major arthropod vector*	*Natural host disease*	*Disease in domesticated European rabbit* (Oryctolagus cuniculus)
Leporipoxvirus					
Myxoma	MYX	California brush rabbit[a] S. American tapeti[b] (*Sylvilagus* sp.)	Mosquito, flea	Localized benign fibroma	Systemic lethal myxomatosis
Rabbit fibroma (Shope fibroma)	SFV	N. American cottontail rabbit (*Sylvilagus floridans*)	Mosquito, flea	Localized benign fibroma	Localized benign fibroma
Malignant rabbit fibroma[c]	MRV	Lab. rabbit[d] (*Oryctolagus cuniculus*)	—	Not observed in wild	Systemic lethal syndrome similar to myxomatosis
Squirrel fibroma	SqFV	Gray squirrel (*Sciurus* sp.)	Probably mosquito	Localized or multiple fibromas	Occasional nodular dermal lesions
Hare fibroma	HFV	Wild hares (*Lepus* sp.)	Probably mosquito	Localized benign fibroma	Localized benign fibroma
Suipoxvirus					
Swinepox	SPV	Domestic pigs (*Suidae* sp.)	Hog lice	Localized cutaneous lesions	Intradermal lesions but no serial propagation

[a]Also called Marshall–Regnery myxoma.
[b]Also called Aragão's (or Brazilian) myxoma.
[c]Laboratory recombinant between MYX and SFV.
[d]MRV has been propagated only by serial inoculation of lab. rabbits and in cultured cells.

transmissible tumors was Shope fibroma virus (SFV), described in 1932 by Richard Shope as an infectious agent of fibroma-like hyperplasia in cottontail rabbits (*Sylvilagus floridanus*) in eastern USA. It is likely that the agent of 'hare sarcoma', described first in Germany in 1909, was also a poxvirus, now called hare fibroma virus (HFV). HFV remains the only leporipoxvirus to have arisen outside the Americas but its biology closely resembles that of SFV.

Very little is known about the remaining leporipoxviruses. Subcutaneous fibromatosis in gray squirrels of eastern USA and western gray squirrels in California, caused by poxviruses now collectively called squirrel fibroma virus (SqFV), have been observed since 1936, but their rigorous classification within the MYX–SFV family was not made until 1951 by L. Kilham. Similarly, HFV, described first in 1959 in the European hare (*Lepus europaeus*), was also shown to be a closely related poxvirus in 1961. In 1983, an outbreak of a disease resembling myxomatosis in laboratory rabbits in San Diego was caused by a novel leporipoxvirus later shown to be a genetic recombinant between SFV and a still-undefined strain of MYX. This virus, called malignant rabbit fibroma virus (MRV), has never been observed in wild rabbit populations but is of interest as an experimental model for poxvirus-induced immunosuppression and tumorigenesis. In most respects, MRV can be considered to be a substrain of MYX.

Based on landmark experiments with pneumococcus in the 1920s, the very first example of what was believed to be genetic interaction between viruses was reported in 1936 with the discovery that heat-inactivated myxoma could be reactivated with live SFV ('Berry-Dedrick' transformation), but later work showed this to be a genome rescue phenomenon rather than true recombination.

The only known member of the *Suipoxvirus* genus, swinepox virus (SPV), has been observed sporadically in pig populations throughout the world, but is not considered a serious pathogen because infected animals usually have only moderate symptoms and recover completely.

Taxonomy and Classification

The *Leporipoxvirus* and *Suipoxvirus* genera are in the subfamily *Chordopoxvirinae* of the family *Poxviridae*. The name lepori- comes from Latin *lepus* or *leporis* ('hare') and sui- from Latin *sus* ('swine'), to denote the relatively restricted host range of these viruses. All the viruses in the genus *Leporipoxvirus* can be shown to be closely related to each other by serology, immunodiffusion and fluorescent antibody tests although antigenic differences can be detected in strains of MYX. SPV (genus *Suipoxvirus*) is antigenically unique and is not known to have any closely related members. In terms of broad features, all are typical poxviruses, with characteristic brick-shaped virions containing a double-stranded (ds) DNA genome with covalently closed hairpin termini and terminal inverted repeat (TIR) sequences. Like other poxviruses, viral macromolecular synthesis takes place exclusively in the cytoplasm of infected cells.

Properties of the Virion

Like all members of the poxvirus family, the virions have a characteristic brick-shaped morphology with dimensions of approximately 250–300 nm × 250 nm × 200 nm. The leporipoxviruses are uniquely sensitive to ether and chloroform but otherwise the virions are very stable at ambient temperatures and in skin lesions. In all other respects, such as chemical composition and physical properties, the virus particles are very similar to those of vaccinia.

Properties of the Viral DNA and Protein

Detailed information about the viral DNA is available only for SFV, MYX and SPV. The leporipoxviruses have dsDNA genomes of 160–163 kb, with hairpin termini and TIR sequences of 10–13 kb. SPV DNA is somewhat larger (175 kb) and the TIR is only 4–5 kb but otherwise the genome has similar characteristics. It is believed that each virus encodes in excess of 200 genes. Restriction cleavage maps have been deduced for these viral genomes and generally the profiles are unique for each genus member but relatively well conserved amongst substrains and variants. Viral DNAs of leporipoxviruses crosshybridize at moderate stringencies only with other members of the genus, and SPV DNA is unique and is not known to crosshybridize with any other poxvirus DNA. The MRV DNA genome is 95% identical to MYX, except that it encodes five genes derived from SFV plus three SFV/MYX fusion genes.

The GC content of the leporipoxviruses (40% for SFV) is higher than that of the orthopoxviruses (35% for vaccinia) but there is evidence that many of the viral genes important for replication, gene expression and viral assembly are conserved between the genera. These essential genes for viral replication are clustered near the central regions of the viral genome. In contrast, viral genes mapping near the termini show considerable variability, and are believed to encode many of the specific determinants of pathogenesis that dictate host range and disease characteristics.

The protein complexities of these viruses as determined by one-dimensional gel electrophoresis

are comparable to that of vaccinia virus, although the profiles are unique for each member. In the cases where specific genes involved in viral propagation have been sequenced, such as thymidine kinase and topoisomerase, the proteins have been shown to be highly homologous to their counterparts from other poxviruses. Leporipoxviral proteins involved in virulence and pathogenesis, such as growth factors and serine proteinase inhibitors, tend to diverge more extensively from homologues in other poxviruses.

DNA Replication, Transcription and Translation

All of the major features of macromolecular synthesis by these viruses are very analogous to those deduced for the prototype poxvirus, vaccinia. DNA synthesis is restricted to cytoplasmic sites, although replication tends to be initiated somewhat more slowly than for vaccinia. The virus-encoded transcriptional apparatus is well conserved between the poxvirus genera, and many of the important regulatory signals that are utilized by vaccinia, such as promoters and transcription termination sequences, are also utilized with comparable efficiency in the leporipoxviruses. Thus, viral genes from one genus can be introduced to another by recombination or by DNA transfection technologies to generate chimeric virus constructs that maintain the correct regulation of the new genetic information. As in the case of vaccinia, transcriptional units can be of different kinetic classes (early/intermediate/late) and there is no splicing of viral mRNA.

The leporipoxviruses replicate in cytoplasmic factories that appear by microscopic analysis as eosinophilic B-type inclusion bodies. These factories, also called virosomes, can also be visualized by Feulgen, Giemsa or fluorescent antibody staining. SPV produces nuclear inclusions and vacuolations in addition to cytoplasmic bodies but these nuclear alterations are not believed to be sites of viral replication.

Molecular Mechanisms of Pathogenesis

Since these viruses are of only minor veterinary importance, recent research has focused on the elucidation of the determinants for viral virulence, particularly with respect to the cellular hyperplasia associated with viral replication in affected tissue and the mechanism(s) underlying the immune dysfunction caused by MYX in *Oryctolagus* rabbits. To date, at least two classes of leporipoxvirus gene products have been directly implicated in viral pathogenesis.

1. 'Virokines' are secreted virus-encoded proteins that are targeted to host-specific pathways outside the infected cell. For example, SFV and MYX encode growth factors related to epidermal growth factor and transforming growth factor alpha that participate in stimulating fibroblastic proliferation at primary and secondary tumors.
2. 'Viroceptors' are viral proteins that mimic cellular receptors and function by sequestering important host cytokines that normally participate in the antiviral immune response. Leporipoxviral-encoded receptor-like molecules have been discovered for tumor necrosis factor (TNF) and interferon γ (IFN-γ), and may exist for other antiviral lymphokines as well. Swinepox virus encodes a novel homologue of cellular chemokine receptors, and the leporipoxviruses express secreted chemokine binding proteins that are important for virus pathogenesis.

Interference with antigen presentation by MYX is believed to play a role in circumventing T-cell recognition during early stages of virus infection. One MYX gene product responsible for evading immune clearance, designated Serp1, is an extracellular inhibitor of cellular serine proteinases but its precise target remains to be identified.

Geographic and Seasonal Distribution

All three major species of *Sylvilagus* rabbits in the Americas have endemic fibroma-like poxviruses, and myxomatosis is now established in wild *Oryctolagus* rabbit populations of South America, Europe and Australia. SqFV and HFV have been reported to date only in North America and Europe, respectively. The leporipoxviruses in the wild undergo seasonal fluctuations that correlate well with increased populations of arthropod vectors in summer and autumn, most prominently mosquitoes. An exception to this is found in Britain, where the major vector of MYX is the flea, which is not as seasonally variable.

In the case of SPV, outbreaks are not tied to seasonal cycles but are generally associated with the degree of hog lice infestation.

Host Range and Virus Propagation

These viruses demonstrate a very restricted host range in terms of ability to cause disease, although viral replication can also occur in cultured cells from some nonsusceptible hosts as well. In some cases viral replication in tissue culture monolayers or chicken chorioallantoic membranes produces 'foci' in which infected cells manifest minimal cytopathic effects, thus permitting macroscopic cell aggregations to

develop. The extent of cytopathology is markedly influenced by both the cell type and the virus strain and in some instances the infected cells may detach from the monolayer to produce visible plaques. When viral replication is relatively slow and the toxicity to the target cell sufficiently moderate, a chronically infected carrier culture can be established in which progeny virus production persists for extended passages. Although poxviruses cannot permanently transform primary cells into an immortalized state, cells persistently infected with the fibroma-inducing leporipoxviruses assume many of the phenotypic characteristics associated with the transformed phenotype, such as novel morphology, growth in reduced serum and ability to form colonies in soft agar. It is likely that some of these phenotypic characteristics are facilitated by secreted poxviral proteins which mimic cellular mitogens, such as epidermal growth factor, and trigger neighboring cells into excessive proliferation.

In the cases of the benign leporipoxviruses and SPV, replication is restricted to dermal and subcutaneous sites, with occasional involvement of draining lymph nodes. However, MYX and MRV are unique in that they also replicate efficiently in lymphoid cells, such as macrophages, B cells and T cells. MYX, like HIV-1, replicates in either resting or stimulated T cells, and can be readily isolated from splenocyte cultures. The molecular basis for the uniquely permissive nature of MYX replication in lymphocytes is unknown, but is unquestionably an important factor in the extreme virulence of myxomatosis. Several MYX genes have been identified (M-T2, M-T5 and M11L) which function as host range determinants in infected lymphocytes by blocking the apoptosis response to infection.

Evolution and Genetic Variability

The deliberate release of MYX into rabbit populations of Australia, France and Britain in the early 1950s provided a unique opportunity to study the natural selection pressures exerted on a particularly virulent virus/host interaction. There is an extensive literature on the ecological consequences of this eradication program and the rapid evolution of myxomatosis in the wild is well documented. Although the original South American MYX virus strain that was introduced left very few survivors in selected populations, within a few years attenuated viral strains with reduced virulence took over and more resistant rabbits became predominant.

In terms of the categories of viral virulence, some strains of MYX are classified as highly virulent (e.g. Moses and Lausanne), and attenuated variants exist down to relatively nonpathogenic (e.g. neuromyxoma and the Nottingham strains). Little is known about the extent of genetic variation in other leporipoxviruses, although different isolates of SFV show marked variations in tumorigenicity. Generally, leporids which recover from infection with one member either become resistant or undergo partial protection from infection by another member.

SPV shares some antigenic crossreactivity with vaccinia, but neutralizing antibody does not confer crossprotection for secondary infections by members of different genera.

Transmission and Tissue Tropism

The principal mode of transmission is by biting arthropod vectors, and the major inoculation route is dermal. Since these viruses do not replicate in the vector, the transmission is purely mechanical and hence virus spread can be readily accomplished by alternative routes as well. Thus, mosquitoes, fleas, blackflies, ticks, lice, mites, and even thistles and the claws of predatory birds, have all been implicated in leporipoxvirus transmission. The efficiency of transmission by arthropods is quite variable, and is related to viral titers in skin lesions as well as the size of the vector populations. There are no known respiratory or oral routes of infection with members of either genus, but in some infections, such as MYX in domestic rabbits, the disease can be transmitted by direct contact with ocular discharges or open cutaneous lesions.

The sui- and leporipoxviruses in their native hosts are specific for the epidermis or subdermis and usually do not progress to secondary sites, although draining lymph nodes can be affected. However, in the case of MYX infection of the domestic rabbit the virus can propagate efficiently in lymphocytes and migrates via infected leukocytes through lymphatic channels to establish secondary sites of infection.

Pathogenicity

The leporipoxviruses are restricted to rabbits, squirrels and hares and swinepox is found only in domestic pigs. For SFV infection of *Sylvilagus* rabbits, tumors can last for many months before regressing, whereas in *Oryctolagus* rabbits recovery is usually complete within a few weeks. Only MYX manifests dramatic alterations in pathogenicity when the European rabbit is infected. For all of these viruses the immune status of the host rabbit plays a critical role; for example, in adult rabbits SFV rarely causes disease symptoms except for the primary fibroblastic lesion but in newborn or immunocompromised animals the infec-

tion can lead to invasive tumors and much higher titers of infectious virus in infected tissues. Agents such as cortisone, x-rays or immunosuppressants can dramatically increase SFV tumor development, and chemical promoters like 3,4-benzopyrene or methylcholanthrene can predispose progression to invasive fibromatosis or even metastatic fibrosarcoma.

The ability to evade the host immune response, replicate in lymphocytes and spread efficiently to secondary sites is a unique property of MYX in *Oryctolagus* rabbits. The myxomatosis syndrome can be associated with multiple external signs (e.g. South American MYX) or may have relatively fewer gross symptoms (e.g. California MYX) and mortalities can range up to 100%. Supervening Gram-negative bacterial infections in the respiratory tract and conjunctiva are often observed concomitantly with MYX, particularly by the adventitious pathogens *Pasteurella multocida* and *Bordetella bronchoseptica,* and contribute to the lethality of the disease.

SPV is only mildly pathogenic in pigs although it can cause a minor level of mortality, usually associated with milk feeding reduction in younger animals.

Clinical Features of Infection

The cutaneous tumors induced by the different leporipoxviruses in their natural hosts are clinically very similar to each other. The fibromas are rarely associated with any other symptoms, such as fever or appetite loss, and invariably regress as long as the animal is not otherwise immunocompromised. In the case of MYX in *Oryctolagus* rabbits, however, the symptoms rapidly become severe as the tumors fail to regress and the concomitant immunosuppression contributes to the lethal myxomatosis syndrome. The clinical features of myxomatosis are influenced by the genetic background of both the virus strain and the rabbit host. In the preacute form of the disease caused by California MYX the rabbits succumb in less than a week, and often have only minor external symptoms, such as inflammation and edema of the eyelids. Skin hemorrhages can be observed in some cases and convulsions often precede death. In the acute form caused by South American strains of MYX, the rabbits survive 1–2 weeks and develop more distinctive symptoms. The primary tumor can be either flat and diffuse or protuberant, and secondary site tumors around the nose, eyes and ears become prominent by 6–7 days, at which time purulent exudates from the nose and eyes frequently develop. The cutaneous tumors often become necrotic and a generalized immune dysfunction exacerbates the progressive secondary bacterial infestation of the respiratory tract. In the case of the more attenuated MYX isolates, such as neuromyxoma, the disease course is less severe and may be associated with little or no mortality.

The disease course of SPV in pigs is rather different, and resembles vaccinia in humans. Inoculation results in localized dermal papules, which progress on to vesicles and pustules, after which the lesions crust and scab over. The only clinical symptom is occasional minor fever and the animals recover completely within three weeks.

Pathology and Histopathology

The primary tumors caused by leporipoxviruses in *Sylvilagus* rabbits, squirrels and hares all closely resemble proliferant fibromas. Following inoculation, an acute inflammatory reaction occurs with infiltration of polymorphonuclear and mononuclear cells and proliferation of fibroblast-like cells of uncertain origin. The 'tumor' consists of pleomorphic cells imbedded in a matrix of intercellular fibrils of collagen. Unlike the transformed cells induced by other DNA tumor viruses, cells from poxviral tumors are not immortalized and cannot be propagated independently. Instead they appear to require secreted virus-encoded proteins in order to sustain the hyperproliferative state. Inclusion bodies characteristic of poxviral replication can be observed in the cytoplasms of epithelial and some fibroma cells. As the tumor develops, mononuclear leukocyte cuffing of adjacent vessels is observed and at the base of the tumor there is accumulation of lymphocytes, plasma cells, macrophages and neutrophils. The ratio between influx of inflammatory cells and fibroblast proliferation is variable but generally there is little or no necrosis. The speed with which immune cells clear the viral infection and reverse the hyperproliferation can range from 1–2 weeks up to 6 months, depending on both the virus and the host.

The principal difference between the benign fibroma syndrome described above and the devastating disease caused by MYX in *Oryctolagus* rabbits is that the latter viruses efficiently propagate in host lymphocytes and are able to circumvent the cell-mediated immune response to the viral infection. The subcutaneous tumors consist of proliferating undifferentiated mesenchymal cells which become large and stellate with prominent nuclei ('myxoma' cells). In surrounding tissue there can be extensive proliferation of endothelial cells of the local capillaries and venules, often to the point where complete occlusion leads to extensive necrosis of the infected site. The overlying epithelial cells can show hyperplasia or degeneration, depending on the virus strain, and

poxviral inclusion bodies are frequently observed in the prickle-cell layer. In some MYX strains primary and secondary skin tumors can undergo extensive hemorrhage and internal lesions may be found in the stomach, intestines and heart. The virus readily migrates to secondary sites within infected immune cells and concomitant cellular proliferation can be detected in the reticulum cells of lymph nodes and spleen, as well as the conjunctival and pulmonary alveolar epithelium. The nasal mucosa and conjunctiva overlying secondary tumors undergo squamous metaplasia such that the epithelia become nonciliated and nonkeratizing. Disruption of the ciliary architecture may be one of the factors which facilitate the extensive Gram-negative bacterial infections of the eyes, nose and respiratory tract. Varying degrees of inflammatory cell infiltration by polymorphonuclear heterophils occur soon after infection but there is only a limited effective cellular immune response. The lymph nodes and spleen show evidence of aberrant T-cell activation and hyperplasia and infectious virus can be isolated from all lymphoid organs except the thymus. Death is believed to be caused by a combination of tissue damage from the increasing tumor burden, generalized immunosuppression and debilitating bacterial colonization of the respiratory tract.

Little is known about SPV pathogenesis but gross features closely resemble those of the noninvasive orthopoxviruses in their native hosts.

Immune Response

The benign fibromas caused by SFV/SqFV/HFV regress, albeit slowly, due to a combined cellular and humoral immune response. These viruses are excellent antigens and neutralizing antibody produced during recovery will also crossreact with other members of the genus. All of the leporipoxviruses are strongly cell-associated and cell-mediated immunity is probably the single most important mechanism of viral clearance. Other immune mechanisms are also activated, including interferon production, antibody-mediated cell lysis, sensitized macrophages and natural killer (NK) cells. Neutralizing antibody can last for many months after viral clearance and immunity is usually crossprotective to the other leporipoxviruses.

In the case of MYX in *Oryctolagus* rabbits the picture is very different. Although circulating antibody can be detected against virions, as determined by neutralization or agglutination, and against soluble antigens, as determined by complement fixation and precipitin tests, the antibody provides little protection against the disease progression. Instead, cellular immunity is severely compromised, and by day 6–7 lymphocytes (especially splenocytes) are demonstrably dysfunctional in their response to mitogens and lose the ability to secrete critical cytokines such as interleukin-2. Unlike the case of SFV, there is a notable absence of virus-specific T-cells in either the spleen or draining lymph nodes. Immune dysfunction is common in viruses that replicate in lymphocytes, but the precise levels at which MYX intervenes in cellular immunity remain to be clarified. There is some evidence that these viruses interfere with the function of cell surface MHC class I molecules, which could prevent proper viral antigen presentation and hence interfere with immune recognition of infected cells. Also, several virus-specific gene products have been shown to be secreted homologues of the cellular receptors for TNF and IFN-γ that are believed to bind and sequester these extracellular ligands in the vicinity of virus-infected cells and thus short-circuit immune pathways dependent on TNF and IFN-γ.

SPV-infected pigs generally recover from the infection and become immune to secondary challenge. There are few data on the nature of this immunity, but it bears close resemblance to that of vaccinia immunization in humans.

Prevention and Control

Since these viruses are spread principally by biting arthropods, vector control is the single most effective method of disease prevention. The viruses are susceptible to standard anti-poxvirus chemical agents, such as phosphonoacetic acid, arabinosyl cytosine and rifampicin, but these are of limited utility in infected animals. Immunization against myxomatosis can be accomplished with live SFV or attenuated strains of MYX.

Future Perspectives

Now that DNA sequencing studies have been initiated for many different poxviruses, it is likely that more viral genes which determine the clinical characteristics of their diseases will be discovered. Studies on viral gene products which stimulate fibroblastic and endothelial cells to proliferate will likely provide information on how mitogenesis is regulated by surface receptors on these target cells. The ability of MYX to replicate in lymphocytes offers an important system in which to elucidate the mechanisms of cellular tropism by which these viruses suppress the innate apoptosis response to virus infection. Furthermore, the analysis of virus-induced immunosuppression should shed light on the various immune strategies used by the host to combat viral infections

in general. Finally, the restricted host ranges of the lepori- and suipoxviruses suggests the potential for the genetic manipulation of these viruses such that heterologous foreign antigen genes can be expressed for the purpose of developing novel vaccines against important pathogens of domestic leporids and swine.

See also: **Cowpox virus (*Poxviridae*); Fowlpox virus (*Poxviridae*); History of virology: General; Immune response: Cell mediated immune response, General features; Molluscum contagiosum virus (*Poxviridae*); Mousepox and rabbitpox viruses (*Poxviridae*); Pathogenesis: Animal viruses; Smallpox and monkeypox viruses (*Poxviridae*); Vaccinia virus (*Poxviridae*); Yabapox and Tanapox viruses (*Poxviridae*).**

Further Reading

DiGiacomo RF and Maré CJ (1994) Viral diseases. In: Manning P, Ringler DH and Newcomer CE (eds) *The Biology of the Laboratory Rabbit,* 2nd edn, p. 171. San Diego: Academic Press.

Fenner F and Ratcliffe FN (1965) *Myxomatosis*. Cambridge, UK: Cambridge University Press.

McFadden G and Graham K (1994) Modulation of cytokine networks by poxviruses: the myxoma virus model. *Semin. Virol.* 5: 421.

McFadden G (ed.) (1995) *Viroceptors, Virokines and Related Immune Modulators Encoded by DNA Viruses*. Austin: R. G. Landes.

Robinson AJ, Jackson R, Kerr P *et al.* (1997) Progress towards using recombinant myxoma virus as a vector for fertility control in rabbits. *Reprod. Fertil. Dev.* 9: 77.

Tripathy DN, Hanson LE and Crandall RA (1981) Poxviruses of veterinary importance: diagnosis of infections. In: Kurstak E and Kurstak C (eds) *Comparative Diagnosis of Viral Diseases III. Vertebrate Animal and Related Viruses. Part A – DNA Viruses*. p. 268. New York, London, Toronto, Sydney, San Francisco: Academic Press.

PRIONS

PrP

Contents

Human and Animal

Stanley B Prusiner, Departments of Neurology and Biochemistry and Biophysics, University of California, San Francisco, California, USA

Introduction

Prions are novel transmissible pathogens causing a group of invariably fatal neurodegenerative diseases that present as genetic, infectious or sporadic disorders, all of which involve modification of the prion protein (PrP). This unprecedented spectrum of disease presentations demanded a new mechanism; prions provide a conceptual framework within which this remarkably diverse spectrum can be accommodated.

Prion diseases of humans are referred to as Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker disease (GSS), fatal familial insomnia (FFI), fatal sporadic insomnia (FSI) and kuru (**Table 1**). In animals the prion diseases are called scrapie of sheep and goats, bovine spongiform encephalopathy (BSE), chronic wasting disease (CWD) of mule deer and elk, feline spongiform encephalopathy (FSE) and transmissible mink encephalopathy (TME).

Because prions and the mechanism of disease pathogenesis are without precedent, classification of the prion diseases has been quite varied. For many years, the human prion diseases were classified as neurodegenerative disorders of unknown etiology based upon pathologic changes being confined to the central nervous system (CNS). With the transmission of kuru and CJD to apes, investigators began to view these diseases as CNS infectious illnesses caused by slow viruses. Even though the familial nature of a subset of CJD cases was well described, the significance of this observation became more obscure with the transmission of CJD to animals. Eventually, the meaning of heritable CJD became clear with the discovery of mutations in the PrP gene of these patients.

in general. Finally, the restricted host ranges of the lepori- and suipoxviruses suggests the potential for the genetic manipulation of these viruses such that heterologous foreign antigen genes can be expressed for the purpose of developing novel vaccines against important pathogens of domestic leporids and swine.

See also: **Cowpox virus (*Poxviridae*); Fowlpox virus (*Poxviridae*); History of virology: General; Immune response: Cell mediated immune response, General features; Molluscum contagiosum virus (*Poxviridae*); Mousepox and rabbitpox viruses (*Poxviridae*); Pathogenesis: Animal viruses; Smallpox and monkeypox viruses (*Poxviridae*); Vaccinia virus (*Poxviridae*); Yabapox and Tanapox viruses (*Poxviridae*).**

Further Reading

DiGiacomo RF and Maré CJ (1994) Viral diseases. In: Manning P, Ringler DH and Newcomer CE (eds) *The Biology of the Laboratory Rabbit,* 2nd edn, p. 171. San Diego: Academic Press.

Fenner F and Ratcliffe FN (1965) *Myxomatosis*. Cambridge, UK: Cambridge University Press.

McFadden G and Graham K (1994) Modulation of cytokine networks by poxviruses: the myxoma virus model. *Semin. Virol.* 5: 421.

McFadden G (ed.) (1995) *Viroceptors, Virokines and Related Immune Modulators Encoded by DNA Viruses*. Austin: R. G. Landes.

Robinson AJ, Jackson R, Kerr P *et al.* (1997) Progress towards using recombinant myxoma virus as a vector for fertility control in rabbits. *Reprod. Fertil. Dev.* 9: 77.

Tripathy DN, Hanson LE and Crandall RA (1981) Poxviruses of veterinary importance: diagnosis of infections. In: Kurstak E and Kurstak C (eds) *Comparative Diagnosis of Viral Diseases III. Vertebrate Animal and Related Viruses. Part A – DNA Viruses*. p. 268. New York, London, Toronto, Sydney, San Francisco: Academic Press.

PRIONS

PrP

Contents

Human and Animal

Stanley B Prusiner, Departments of Neurology and Biochemistry and Biophysics, University of California, San Francisco, California, USA

Introduction

Prions are novel transmissible pathogens causing a group of invariably fatal neurodegenerative diseases that present as genetic, infectious or sporadic disorders, all of which involve modification of the prion protein (PrP). This unprecedented spectrum of disease presentations demanded a new mechanism; prions provide a conceptual framework within which this remarkably diverse spectrum can be accommodated.

Prion diseases of humans are referred to as Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker disease (GSS), fatal familial insomnia (FFI), fatal sporadic insomnia (FSI) and kuru (**Table 1**). In animals the prion diseases are called scrapie of sheep and goats, bovine spongiform encephalopathy (BSE), chronic wasting disease (CWD) of mule deer and elk, feline spongiform encephalopathy (FSE) and transmissible mink encephalopathy (TME).

Because prions and the mechanism of disease pathogenesis are without precedent, classification of the prion diseases has been quite varied. For many years, the human prion diseases were classified as neurodegenerative disorders of unknown etiology based upon pathologic changes being confined to the central nervous system (CNS). With the transmission of kuru and CJD to apes, investigators began to view these diseases as CNS infectious illnesses caused by slow viruses. Even though the familial nature of a subset of CJD cases was well described, the significance of this observation became more obscure with the transmission of CJD to animals. Eventually, the meaning of heritable CJD became clear with the discovery of mutations in the PrP gene of these patients.

Table 1 The prion diseases

Disease	*Host*	*Mechanism of pathogenesis*
A. Kuru	Fore people	Infection through ritualistic cannibalism
iCJD	Humans	Infection from prion-contaminated hGH, dura mater grafts, etc.
vCJD	Humans	Infection from bovine prions?
fCJD	Humans	Germline mutations in PrP gene
GSS	Humans	Germline mutations in PrP gene
FFI	Humans	Germline mutation in PrP gene (D178N, M129)
sCJD	Humans	Somatic mutation or spontaneous conversion of PrP^{C} into PrP^{Sc}?
FSI	Humans	Somatic mutation or spontaneous conversion of PrP^{C} into PrP^{Sc}?
B. Scrapie	Sheep	Infection in genetically susceptible sheep
BSE	Cattle	Infection with prion-contaminated MBM
TME	Mink	Infection with prions from sheep or cattle
CWD	Mule deer, elk	Unknown
FSE	Cats	Infection with prion-contaminated beef
Exotic ungulate encephalopathy	Greater kudu, nyala, oryx	Infection with prion-contaminated MBM

BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt–Jakob disease; sCJD, sporadic CJD; fCJD, familial CJD; iCJD, iatrogenic CJD; vCJD, (new) variant CJD; CWD, chronic wasting disease; FFI, fatal familial insomnia; FSE, feline spongiform encephalopathy; FSI, fatal sporadic insomnia; GSS, Gerstmann–Sträussler–Scheinker disease; hGH, human growth hormone; MBM, meat and bone meal; TME, transmissible mink encephalopathy.

The Prion Concept

Prions are unprecedented infectious pathogens that are devoid of nucleic acid and seem to be composed exclusively of a modified isoform of PrP designated PrP^{Sc}. The normal, cellular PrP, denoted PrP^{C}, is converted into PrP^{Sc} through a process whereby a portion of its α-helical and coil structure is refolded into a β sheet. This structural transition is accompanied by profound changes in the physicochemical properties of the PrP. The amino acid sequence of PrP^{Sc} corresponds to that encoded by the PrP gene of the mammalian host in which it last replicated. In contrast to pathogens with a nucleic acid genome that encode strain-specific properties in genes, prions encipher these properties in the tertiary structure of PrP^{Sc}. Transgenetic studies argue that PrP^{Sc} acts as a template upon which PrP^{C} is refolded into a nascent PrP^{Sc} molecule through a process facilitated by another protein.

Perhaps the best current working definition of a prion is a proteinaceous infectious particle that lacks nucleic acid. Because prions appear to be composed entirely of a protein that adopts an abnormal conformation, it is not unreasonable to think of prions as infectious proteins. Although PrP^{Sc} is the only *known* component of the infectious prion particles, these unique pathogens share several phenotypic traits with other infectious entities such as viruses.

In a broader view, prions are elements that impart and propagate variability through multiple conformers of a normal cellular protein. The species of a particular prion is encoded by the sequence of the chromosomal PrP gene of the mammal in which it last replicated. In contrast to pathogens with a nucleic acid genome that encode strain-specific properties in genes, prions seem to encipher these properties in the tertiary structure of PrP^{Sc}.

The discovery that mutations of the PrP gene caused dominantly inherited prion diseases in humans linked the genetic and infectious forms of prion diseases and presented another hurdle for investigators who continued to argue that prion diseases are caused by viruses. More than 20 mutations of the PrP gene are now known to cause the inherited human prion diseases and significant genetic linkage has been established for five of these mutations. The prion concept readily explains how a disease can manifest as a heritable as well as an infectious illness. Moreover, the hallmark common to all of the prion diseases, whether sporadic, dominantly inherited or acquired by infection, is that they involve the aberrant metabolism of the prion protein.

Prion Protein Isoforms

PrP^{C} and PrP^{Sc} have the same covalent structure and each consists of 209 amino acids in Syrian hamsters (**Fig. 1**). The N-terminal sequencing, the deduced

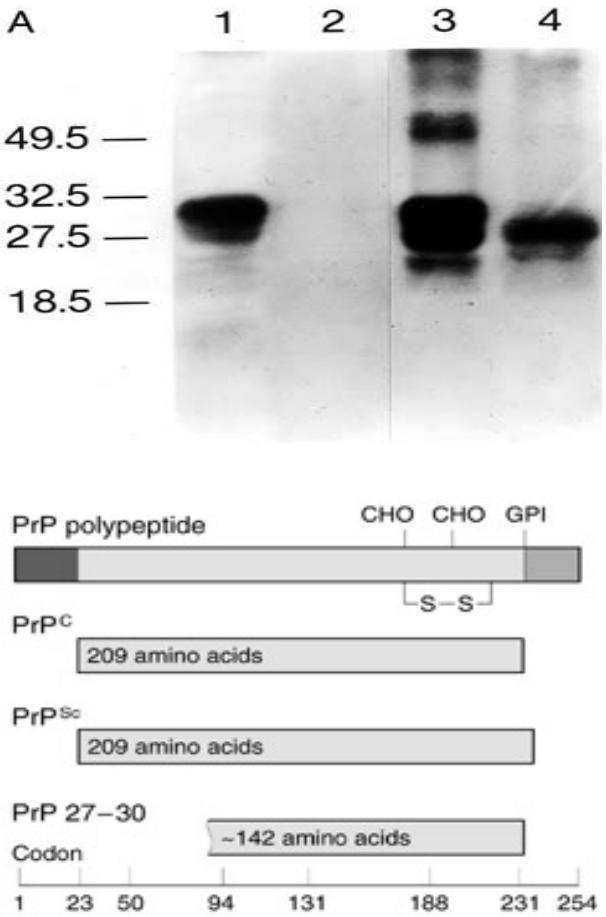


Figure 1 Prion protein isoforms. (**A**) Western immunoblot of brain homogenates from uninfected (lanes 1 and 2) and prion-infected (lanes 3 and 4) Syrian hamsters. Samples in lanes 2 and 4 were digested with 50 μg ml^{-1} of proteinase K for 30 min at 37°C. PrP^C in lanes 2 and 4 was completely hydrolyzed under these conditions, whereas approximately 67 amino acids were digested from the N-terminus of PrP^{Sc} to generate PrP 27–30. After polyacrylamide gel electrophoresis (PAGE) and electrotransfer, the blot was developed with anti-PrP R073 polyclonal rabbit antiserum. Molecular size markers are in kilodaltons (kDa). (**B**) Bar diagram of SHaPrP which consists of 254 amino acids. After processing of the N- and C-termini, both PrP^C and PrP^{Sc} consist of 209 residues. After limited proteolysis, the N-terminus of PrP^{Sc} is truncated to form PrP 27–30 which is composed of approximately 142 amino acids. (Reprinted with permission from *Molecular Neurology*, pp 175–204. Copyright 1998 Scientific American, Inc.)

amino acid sequences from PrP cDNA and immunoblotting studies argue that PrP 27–30 is a truncated protein of about 142 residues that is derived from PrP^{Sc} by limited proteolysis of the N-terminus.

In general, ~10^5 PrP^{Sc} molecules correspond to one ID_{50} unit of prions, using the most sensitive bioassay. PrP^{Sc} is probably best defined as the abnormal isoform of the prion protein that stimulates conversion of PrP^C into nascent PrP^{Sc}, accumulates and causes disease. Although resistance to limited proteolysis has proved to be a convenient tool for detecting PrP^{Sc}, not all PrP^{Sc} molecules possess protease resistance.

The prion diseases are caused by the accumulation of PrP^{Sc}. In accord with the autosomal dominant inheritance of familial prion diseases caused by mutations of the PrP gene, PrP^{Sc} represents a gain of dysfunction.

Human Prion Diseases

Most humans afflicted with prion disease present with rapidly progressive dementia, but some manifest cerebellar ataxia. Although the brains of patients appear grossly normal upon postmortem examination, they usually show spongiform degeneration and astrocytic gliosis under the light microscope (**Fig. 2**). In all cases of GSS and variant (v) CJD, PrP amyloid plaques are found. Before PrP immunostaining was available, histochemical staining was used to examine brains from kuru patients where ~70% of cases were thought to have amyloid plaques. The presence or absence of PrP amyloid plaques in sporadic and inherited CJD is quite variable.

Human prion disease should be considered in any patient who develops a progressive subacute or chronic decline in cognitive or motor function. Typically, adults between 40 and 80 years of age are affected. The young age of more than 30 people who have died of vCJD in Britain and France has raised the possibility that these individuals were infected with bovine prions that contaminated beef products. Over 100 young adults have also been diagnosed with iatrogenic CJD between 4 and 30 years after receiving human growth hormone (hGH) or gonadotropin derived from cadaveric pituitaries. The longest incubation periods (20–30 years) are similar to those associated with more recent cases of kuru.

Sporadic CJD

Sporadic forms of prion disease comprise most cases of CJD and possibly a few cases of GSS (**Table 1A**). In these patients, mutations of the PrP gene are not found. How prions causing disease arise in patients with sporadic forms is unknown; hypotheses include horizontal transmission of prions from humans or animals, somatic mutation of the PrP gene, and spontaneous conversion of PrP^C into PrP^{Sc}. Since numerous attempts to establish an infectious link between sporadic CJD and a pre-existing prion disease in animals or humans have been unrewarding,

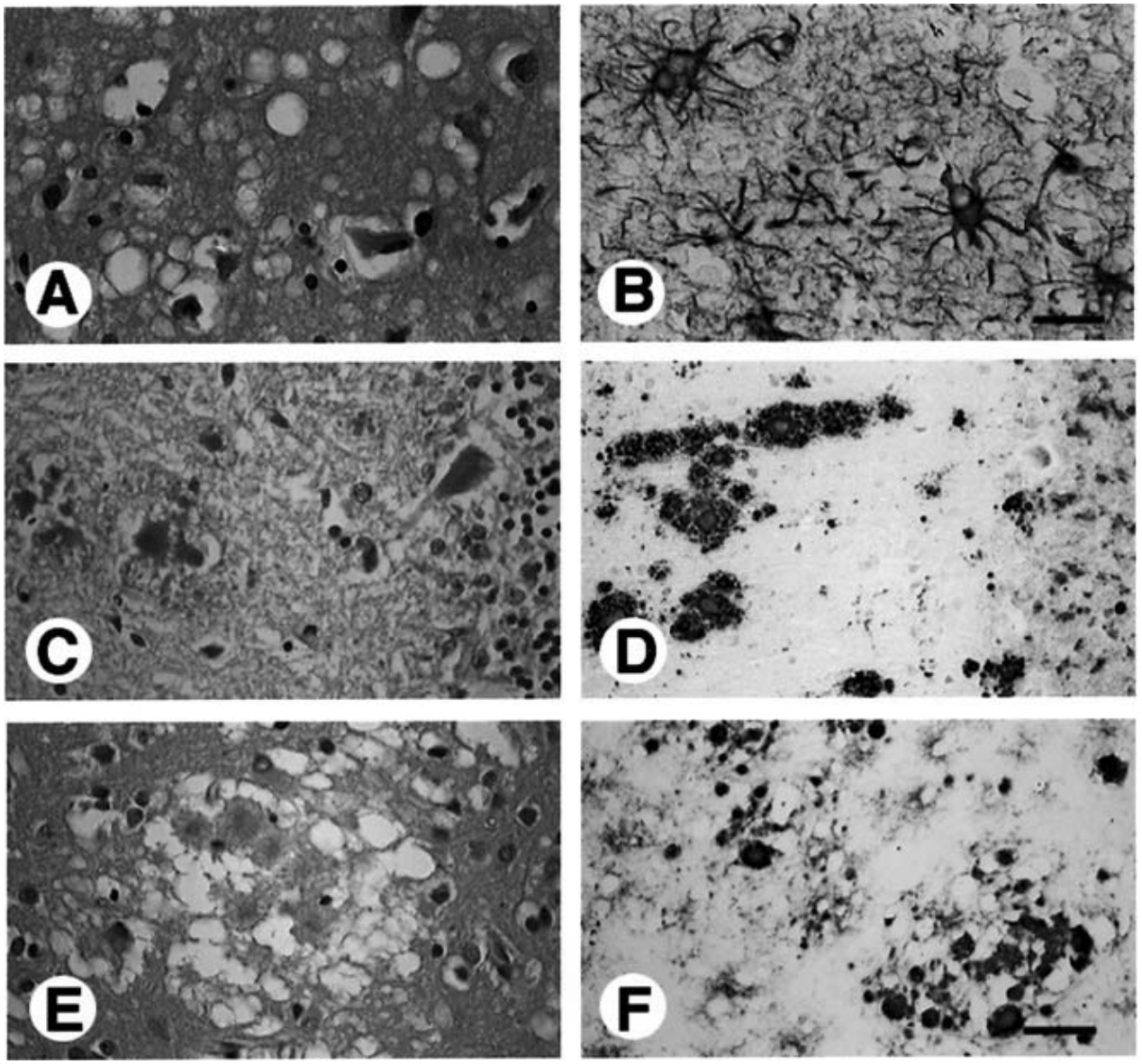


Figure 2 Neuropathology of human prion diseases. Sporadic CJD is characterized by vacuolation of the neuropil of the gray matter, by exuberant reactive astrocytic gliosis, the intensity of which is proportional to the degree of nerve cell loss, and, rarely, by PrP amyloid plaque formation (not shown). The neuropathology of familial CJD is similar. GSS(P102L), as well as other inherited forms of GSS (not shown), is characterized by numerous deposits of PrP amyloid throughout the CNS. New variant CJD (vCJD) has clinical and epidemiological features that suggest it was acquired by infection with prions. The neuropathological features of vCJD are unique among CJD cases because of the abundance of PrP amyloid plaques that are often surrounded by a halo of intense vacuolation. (**A**) Sporadic CJD, cerebral cortex stained with hematoxylin and eosin showing widespread spongiform degeneration. (**B**) Sporadic CJD, cerebral cortex immunostained with anti-GFAP antibodies demonstrating the widespread reactive gliosis. (**C**) GSS, cerebellum with most of the GSS-plaques in the molecular layer (left 80% of micrograph) and many but not all are periodic acid Schiff (PAS) reaction positive. Granule cells and a single Purkinje cell are seen in the right 20% of the panel. (**D**) GSS, cerebellum at the same location as panel C with PrP immunohistochemistry after the hydrolytic autoclaving reveals more PrP plaques than seen with the PAS reaction. (**E**) Variant CJD, cerebral cortex stained with hematoxylin and eosin shows that the plaque deposits are uniquely located within vacuoles. With this histology, these amyloid deposits have been referred to as 'florid plaques'. (**F**) Variant CJD, cerebral cortex stained with PrP immunohistochemistry after hydrolytic autoclaving reveals numerous PrP plaques often occurring in clusters as well as minute PrP deposits surrounding many cortical neurons and their proximal processes. Bar in B = 50 μm and applies also to panels A, C, and D. Bar in F = 100 μm and applies also to panel D. **(For color references see Color Plate 27.)**

it seems unlikely that transmission features in the pathogenesis of sporadic prion disease.

Inherited prion diseases

To date, 20 different mutations in the human PrP gene resulting in nonconservative substitutions have been found that segregate with the inherited prion diseases (**Fig. 3**). Familial (f) CJD cases suggested that genetic factors might influence pathogenesis, but this was difficult to reconcile with the transmissibility of fCJD and GSS. The discovery of genetic linkage between the PrP gene and scrapie incubation times in mice raised the possibility that mutation might feature in the hereditary human prion diseases. The P102L mutation was the first PrP mutation to be genetically linked to CNS dysfunction in GSS (**Fig. 3**) and has since been found in many GSS families throughout the world. Indeed, a mutation in the protein coding region of the PrP gene has been found in all reported kindred with familial human prion disease; besides the P102L mutation, genetic linkage has been established for four other mutations.

Tg mouse studies confirmed that mutations of the PrP gene can cause neurodegeneration. The P102L mutation of GSS was introduced into the MoPrP transgene, and five lines of Tg(MoPrP-P101L) mice expressing high levels of mutant PrP developed spontaneous CNS degeneration consisting of widespread vacuolation of the neuropil, astrocytic gliosis and numerous PrP amyloid plaques similar to those seen in the brains of humans who die from GSS(P102L). Brain extracts prepared from spontaneously ill Tg(MoPrP-P101L) mice transmitted CNS degeneration to Tg196 mice but contained no protease-resistant PrP. The Tg196 mice do not develop spontaneous disease, but express low levels of the mutant transgene MoPrP-P101L and are deficient for mouse PrP ($Prnp^{0/0}$). These studies, combined with the transmission of prions from patients who died of GSS to apes and monkeys or to Tg(MHu2M-P101L) mice, demonstrate that prions are generated *de novo* by mutations in PrP. Additionally, brain extracts from patients with some other inherited prion diseases, fCJD(E200K) or FFI, transmit disease to Tg(MHu2M) mice.

Infectious prion diseases

The infectious prion diseases include kuru of the Fore people in New Guinea, where prions were transmitted by ritualistic cannibalism. With the cessation of cannibalism at the urging of missionaries, kuru began to decline long before it was known to be transmissible. Sources of prions causing infectious CJD on several different continents include improperly sterilized depth electrodes, transplanted corneas, hGH and gonadotropin derived from cadaveric pituitaries, and dura mater grafts. As noted above, many young adults have developed CJD after treatment with cadaveric hGH. Dura mater grafts implanted during neurosurgical procedures seem to have caused more than 60 cases of CJD; these incubation periods range from 1 to more than 14 years.

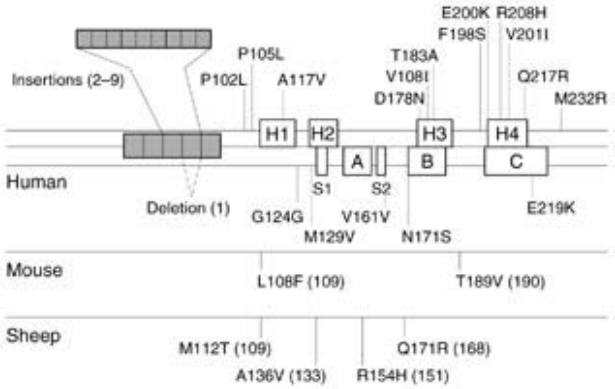


Figure 3 Mutations and polymorphisms of the prion protein gene. Mutations causing inherited human prion disease and polymorphisms in human, mouse and sheep. Above the line of the human sequence are mutations that cause prion disease. Below the lines are polymorphisms, some but not all of which are known to influence the onset as well as the phenotype of disease. Data were compiled by Paul Bamborough and Fred E Cohen. (Reprinted with permission from *Science* 278: 245–251. Copyright 1997 American Association for the Advancement of Science.)

Studies of the prion diseases have taken on new significance with the recent reports of ~30 cases of an atypical vCJD in teenagers and young adults. To date, all of these cases have been reported in Great Britain, with the exception of one case from France. It now seems possible that bovine prions passed to humans through the consumption of tainted beef products. How many cases of vCJD caused by bovine prions will occur in the years ahead is unknown. Until more time passes, we shall be unable to assess the magnitude of this problem. These tragic cases have generated a continuing discourse concerning mad cows, prions and the safety of human and animal food supplies throughout the world. Untangling politics and economics from the science of prions seems to have been difficult in disputes between Great Britain and other European countries over the safety of beef and lamb products.

Strains of Prions

The existence of prion strains raises the question of how heritable biological information can be enci-

Table 2 Distinct prion strains generated in humans with inherited prion diseases and transmitted to transgenic mice

Inoculum	*Host species*	*Host PrP genotype*	*Incubation time (days ± SEM) (n/n_o)*	*PrP^{Sc} (kDa)*
None	Human	FFI(D178N, M129)		19
FFI	Mouse	Tg(MHu2M)	206 ± 7 (7/7)	19
FFI → Tg(MHu2M)	Mouse	Tg(MHu2M)	136 ± 1 (6/6)	19
None	Human	fCJD(E200K)		21
fCJD	Mouse	Tg(MHu2M)	170 ± 2 (10/10)	21
fCJD → Tg(MHu2M)	Mouse	Tg(MHu2M)	167 ± 3 (15/15)	21

phered in any molecule other than nucleic acid. Strains or varieties of prions have been defined by incubation times and the distribution of neuronal vacuolation. Subsequently, the patterns of PrP^{Sc} deposition were found to correlate with vacuolation profiles and these patterns were also used to characterize strains of prions.

The typing of prion strains in C57BI, VM and F1(C57BI × VM) inbred mice began with isolates from sheep with scrapie. The prototypic strains, called Me7 and 22A, gave incubation times of ~150 and ~400 days in C57BI mice, respectively. The PrPs of C57BI and I/Ln (and later VM) mice differ at two residues and control incubation times (**Fig. 3**).

Until recently, support for the hypothesis that the tertiary structure of PrP^{Sc} enciphers strain-specific information was minimal, except for the DY strain isolated from mink with transmissible encephalopathy. PrP^{Sc} in DY prions showed diminished resistance to proteinase K digestion as well as an anomalous site of cleavage. The DY strain presented a puzzling anomaly as other prion strains exhibiting similar incubation times did not show this altered susceptibility to proteinase K digestion of PrP^{Sc}. Also notable was the generation of new strains during passage of prions through animals with different PrP genes.

PrP^{Sc} conformation enciphers variation in prions

Persuasive evidence that strain-specific information is enciphered in the tertiary structure of PrP^{Sc} comes from transmission of two different inherited human prion diseases to mice expressing a chimeric MHu2M PrP transgene. In FFI, the protease-resistant fragment of PrP^{Sc} after deglycosylation has an M_r of 19 kDa, whereas in fCJD(E200K) and most sporadic prion diseases it is 21 kDa. This difference in molecular size was shown to be due to different sites of proteolytic cleavage at the NH_2-termini of the two human PrP^{Sc} molecules, reflecting different tertiary structures. These distinct conformations were not unexpected because the amino acid sequences of the PrPs differ.

Extracts from the brains of FFI patients transmitted disease into mice expressing a chimeric MHu2M PrP gene about 200 days after inoculation and induced formation of the 19 kDa PrP^{Sc}, whereas fCJD(E200K) and sporadic (s) CJD produced the 21 kDa PrP^{Sc} in mice expressing the same transgene. On second passage, Tg(MHu2M) mice inoculated with FFI prions showed an incubation time of ~130 days and a 19 kDa PrP^{Sc}, while those inoculated with fCJD(E200K) prions exhibited an incubation time of ~170 days and a 21 kDa PrP^{Sc} (**Table 2**). The experimental data demonstrate that MHu2MPrP^{Sc} can exist in two different conformations based on the sizes of the protease-resistant fragments, yet the amino acid sequence of MHu2MPrP^{Sc} is invariant.

The results of our studies argue that PrP^{Sc} acts as a template for the conversion of PrP^{C} into nascent PrP^{Sc}. Imparting the size of the protease-resistant fragment of PrP^{Sc} through conformational templating provides a mechanism for both the generation and propagation of prion strains.

Interestingly, the protease-resistant fragment of PrP^{Sc} after deglycosylation with an M_r of 19 kDa has been found in a patient who died after developing a clinical disease similar to FFI. Since both PrP alleles encoded the wt sequence and a Met at position 129, we labeled this case fatal sporadic insomnia (FSI). At autopsy, the spongiform degeneration, reactive astrogliosis, and PrP^{Sc} deposition were confined to the thalamus. These findings argue that the clinicopathologic phenotype is determined by the conformation of PrP^{Sc}, in accord with the results of the transmission of human prions from patients with FFI to Tg mice.

Interplay between the species and strains of prions

Studies on the role of the primary and tertiary structures of PrP in the transmission of disease have given new insights into the pathogenesis of the prion diseases. The amino acid sequence of PrP encodes the species of the prion, and the prion derives its PrP^{Sc}

sequence from the last mammal in which it was passaged. While the primary structure of PrP is likely to be the most important, or even sole, determinant of the tertiary structure of PrP^C, existing PrP^{Sc} seems to function as a template in determining the tertiary structure of nascent PrP^{Sc} molecules as they are formed from PrP^C. In turn, prion diversity appears to be enciphered in the conformation of PrP^{Sc}, and prion strains may represent different conformers of PrP^{Sc}.

Evidence for different conformations of PrP^{Sc} in eight prion strains

Using a highly sensitive conformation-dependent immunoassay for measurement of PrP^{Sc} in tissue homogenates, eight different prion strains passaged in Syrian hamsters were examined. Brains from Syrian hamsters were collected when the animals displayed signs of neurologic dysfunction; the incubation times for the prion strains varied from 70 to 320 days. Most of the PrP in the brains of Syrian hamsters with signs of neurologic disease was PrP^{Sc} as defined by the β-sheet conformation. The level of PrP^{Sc} in the brains of these clinically ill animals exceeded that of PrP^C by 3- to 10-fold. The highest levels of PrP^{Sc} were found in the brains of Syrian hamsters infected with the Me7-H strain; in contrast, the lowest levels were found in the brains of Syrian hamsters inoculated with the SHa(Me7) strain. Interestingly, the Me7-H and SHa(Me7) strains were both derived from Me7 passaged in mice, but they accumulated PrP^{Sc} to quite different levels.

Using this conformation-dependent immunoassay, each strain was found to initiate formation of PrP^{Sc} molecules with a distinct conformation. When the incubation times of the eight strains were plotted as a function of the concentration of either PrP^{Sc} or PrP 27–30, no relationship could be discerned.

To assess the fraction of PrP^{Sc} that is sensitive to proteolysis during limited digestion with proteinase K, we subtracted the protease resistant PrP 27–30 fraction from the total PrP^{Sc} for each of the eight prion strains. It was asked whether the proteinase K-sensitive fraction of PrP^{Sc} ($[PrP^{Sc}] - [PrP\ 27–30]$) might reflect those PrP^{Sc} molecules that are most readily cleared by cellular proteases. The clearance of PrP^{Sc} is of considerable interest with respect to control of the length of the incubation time and other phenotypic features of prion strains. When the $[PrP^{Sc}] - [PrP\ 27–30]$ fraction was plotted as a function of the incubation time, a linear relationship was found with an excellent correlation coefficient ($r = 0.94$) (**Fig. 4**).

The above results demonstrate that eight different strains possess at least eight different conformations. Additional data argue that each strain is composed of a spectrum of conformations as revealed by limited protease digestion and GdnHCl denaturation studies. These findings contrast with the until recently held notion that the primary structure of a protein determines a single tertiary structure.

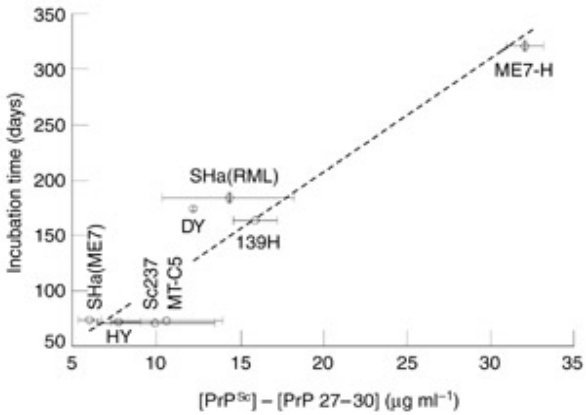


Figure 4 Eight prion strains distinguished by the conformation-dependent immunoassay. Brain homogenates of Syrian hamsters (LVG/LAK) inoculated with different scrapie strains were either undigested or digested with 50 μg ml^{-1} of proteinase K for 2 h at 37°C prior to the conformation-dependent immunoassay. The immunoassay was used to measure the concentration of PrP^{Sc} and PrP 27–30 for each strain. Incubation time plotted as a function of the concentration of the proteinase K-sensitive fraction of PrP^{Sc} ($[PrP^{Sc}] - [PrP\ 27–30]$). (Reprinted with permission from *Nature Medicine*, in press. Copyright 1998 Nature America, Inc.)

How many formations can PrP^{Sc} adopt? The conformation-dependent immunoassay described here provides a rapid tool capable of discriminating the secondary and tertiary structures of a substantial number of PrP^{Sc} molecules.

As noted above for studies of strains passaged from humans with fCJD(E200K) and FFI, PrP^{Sc} must act as a template in the replication of nascent PrP^{Sc} molecules. Also as discussed above, it seems likely that the binding of PrP^C or a metastable intermediate PrP* to protein X is the initial step in PrP^{Sc} formation and that this is the rate-limiting step in prion replication. PrP^{Sc} interacts with PrP^C but not protein X in the PrP^C–protein X complex. When PrP^C or PrP* is converted into a nascent PrP^{Sc} molecule, protein X is released.

It also follows from these observations that the different incubation times of various prion strains should arise predominantly from distinct rates of PrP^{Sc} clearance rather than from different rates of PrP^{Sc} formation. Thus, prion strains that are readily cleared should have prolonged incubation times,

while those that are poorly cleared should display abbreviated incubation periods. This hypothesis was investigated by relying upon the difference in brain PrP^{Sc} concentrations before and after proteinase K treatment as a surrogate for *in vivo* clearance of each prion strain. When clearance, as approximated by $[PrP^{Sc}] - [PrP\ 27–30]$, was plotted as a function of the incubation time for eight strains, a linear relationship was found (**Fig. 4**). It is important to recognize that proteinase K sensitivity is an imperfect model for *in vivo* clearance and that only one strain with a long incubation time exceeding 300 days has been studied.

It has been suggested that Asn-linked carbohydrates (CHOs) specify prion strains, but this proposal is difficult to reconcile with the addition of high mannose oligosaccharides to Asn-linked consensus sites on PrP in the endoplasmic reticulum and subsequent remodeling of the sugar chains in the Golgi. Modification of the complex CHOs attached to PrP^{C} is clearly completed prior to the PrP^{C} trafficking to the cell surface, which indicates that the Asn-linked CHOs of PrP^{Sc} do not instruct the addition of such complex-type sugars to PrP^{C}.

Mutagenesis of the complex-type sugar attachment sites seemed to increase PrP^{Sc} formation in cultured cells, but resulted in prolonged incubation times in Tg mice and differences in the patterns of PrP^{C} distribution and PrP^{Sc} deposition in mice expressing mutant PrPs. These studies suggest that Asn-linked glycosylation might alter the stability of PrP, and in particular PrP^{Sc}, which results in various patterns of PrP^{Sc} deposition. Thus, different clearance rates of PrP^{Sc} may be important in determining not only strain-specific neuropathology but also the length of the incubation time.

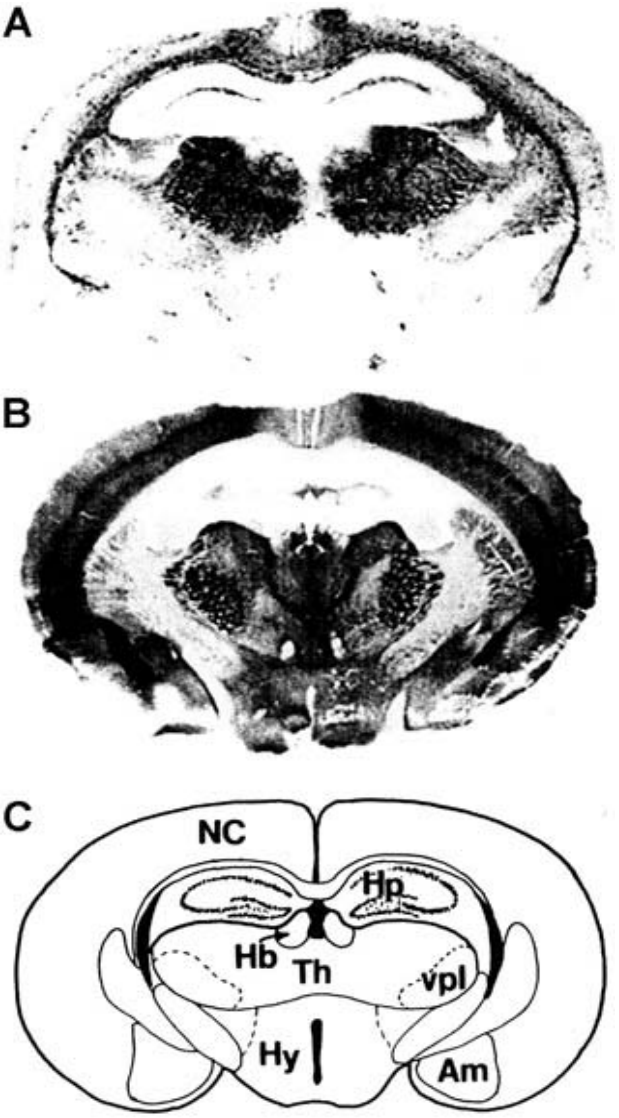


Figure 5 Regional distribution of PrP^{Sc} deposition in $Tg(MHu2M)Prnp^{0/0}$ mice inoculated with prions from humans who died of inherited prion diseases. Histoblot of PrP^{Sc} deposition in a coronal section of a $Tg(MHu2M)Prnp^{0/0}$ mouse through the hippocampus and thalamus. (**A**) The Tg mouse was inoculated with brain extract prepared from a patient who died of FFI. (**B**) The Tg mouse was inoculated with extract from a patient with fCJD(E200K). Cryostat sections were mounted on nitrocellulose and treated with proteinase K to eliminate PrP^{C}. To enhance the antigenicity of PrP^{Sc}, the histoblots were exposed to 3-guanidinium isothiocyanate before immunostaining using α-PrP 3F4 mAb. (**C**) Labeled diagram of a coronal section of the hippocampus/thalamus region. NC, neocortex; Hp, hippocampus; Hb, habenula; Th, thalamus; vpl, ventral posterior lateral thalamic nucleus; Hy, hypothalamus; Am, amygdala. (Reprinted with permission from *Cell* 93: 337–348. Copyright 1998 Cell Press.)

Mechanism of selective neuronal targeting?

In addition to incubation times, neuropathologic profiles of spongiform change have been used to characterize prion strains. However, recent studies with PrP transgenes argue that such profiles are not an intrinsic feature of strains. The mechanism by which prion strains modify the pattern of spongiform degeneration was perplexing, as earlier investigations had shown that PrP^{Sc} deposition precedes neuronal vacuolation and reactive gliosis. When FFI prions were inoculated into Tg(MHu2M) mice, PrP^{Sc} was confined largely to the thalamus (**Fig. 5A**), as is the case for FFI in humans. In contrast, fCJD(E200K) prions inoculated into Tg(MHu2M) mice produced widespread deposition of PrP^{Sc} throughout the cortical mantel and many of the deep structures of the CNS (**Fig. 5B**), as is seen in fCJD(E200K) of humans. To examine whether the diverse patterns of PrP^{Sc} deposition are influenced by Asn-linked glycosylation of PrP^{C}, we constructed Tg mice expressing PrPs mutated at one or both of the Asn-linked

glycosylation consensus sites. These mutations resulted in aberrant neuroanatomic topologies of PrP^C within the CNS, whereas pathologic point mutations adjacent to the consensus sites did not alter the distribution of PrP^C. Tg mice with mutation of the second PrP glycosylation site exhibited prion incubation times of >500 days and unusual patterns of PrP^{Sc} deposition. As noted above, glycosylation can modify the conformation of PrP and affect either the turnover of PrP^C or the clearance of PrP^{Sc}. Regional differences in the rate of deposition or clearance would result in specific patterns of PrP^{Sc} accumulation.

Prion Diseases of Animals

The prion diseases of animals include scrapie of sheep and goats, bovine spongiform encephalopathy, transmissible mink encephalopathy, chronic wasting disease of mule deer and elk, feline spongiform encephalopathy and exotic ungulate encephalopathy (**Table 1B**).

Sheep and cattle PrP gene polymorphisms

Parry argued that host genes were responsible for the development of scrapie in sheep. He was convinced that natural scrapie is a genetic disease that could be eradicated by proper breeding protocols. He considered its transmission by inoculation of importance primarily for laboratory studies and communicable infection of little consequence in nature. Other investigators viewed natural scrapie as an infectious disease and argued that host genetics only modulates susceptibility to an endemic infectious agent.

In sheep, polymorphisms at codons 136, 154 and 171 of the PrP gene that produce amino acid substitutions have been studied with respect to the occurrence of scrapie (**Fig. 3**). Studies of natural scrapie in the USA have shown that ~85% of the afflicted sheep are of the Suffolk breed. Only those Suffolk sheep homozygous for Gln (Q) at codon 171 developed scrapie, although healthy controls with QQ, QR and RR genotypes were also found. These results argue that susceptibility in Suffolk sheep is governed by the PrP codon 171 polymorphism. In Cheviot sheep, the PrP codon 171 polymorphism has a profound influence on susceptibility to scrapie, as in the Suffolk breed, and codon 136 seems to play a less pronounced role.

In contrast to sheep, different breeds of cattle have no specific PrP polymorphisms. The only polymorphism recorded in cattle is a variation in the number of octarepeats: most cattle, like humans, have five octarepeats but some have six; however, the presence of six octarepeats does not seem to be overrepresented in BSE.

Bovine spongiform encephalopathy

Prion strains and the species barrier are of paramount importance in understanding the BSE epidemic in Britain, in which it is estimated that almost one million cattle were infected with prions. The mean incubation time for BSE is about 5 years. Therefore, most cattle did not manifest disease because they were slaughtered between 2 and 3 years of age. Nevertheless, more than 170 000 cattle, primarily dairy cows, have died of BSE over the past decade. BSE is a massive common source epidemic caused by meat and bone meal (MBM) fed primarily to dairy cows. The MBM was prepared from the offal of sheep, cattle, pigs and chickens as a high-protein nutritional supplement. In the late 1970s, the hydrocarbon-solvent extraction method used in the rendering of offal began to be abandoned, resulting in MBM with a much higher fat content. It is now thought that this change in the rendering process allowed scrapie prions from sheep to survive rendering and to be passed into cattle. Alternatively, bovine prions were present at low levels prior to modification of the rendering process and, with the processing change, survived in sufficient numbers to initiate the BSE epidemic when inoculated back into cattle orally through MBM. Against the latter hypothesis is the widespread geographical distribution throughout England of the initial 17 cases of BSE, which occurred almost simultaneously. Furthermore, there is no evidence of a pre-existing prion disease of cattle, either in Great Britain or elsewhere.

Origin of BSE prions?

The origin of the bovine prions causing BSE cannot be determined by examining the amino acid sequence of PrP^{Sc} in cattle with BSE, as the PrP^{Sc} in these animals has the bovine sequence, whether the initial prions in MBM came from cattle or sheep. The bovine PrP sequence differs from that of sheep at seven or eight positions. In contrast to the many PrP polymorphisms found in sheep, only one PrP polymorphism has been found in cattle. Though most bovine PrP alleles encode five octarepeats, some encode six. PrP alleles encoding six octarepeats do not seem to be overrepresented in BSE, as noted above (**Fig. 3**).

Brain extracts from BSE cattle cause disease in cattle, sheep, mice, pigs and mink after intracerebral inoculation, but prions in brain extracts from sheep with scrapie fed to cattle produced illness substantially different from BSE. However, no exhaustive effort has been made to test different strains of sheep prions or to examine the disease following bovine to bovine passage. The annual incidence of sheep with scrapie in Great Britain over the past two decades has

remained relatively low. In July 1988, the practice of feeding MBM to sheep and cattle was banned. Recent statistical analysis argues that the epidemic is now disappearing as a result of this ruminant feed ban, reminiscent of the disappearance of kuru in the Fore people of New Guinea.

Monitoring cattle for BSE prions

Although many plans have been offered for the culling of older cattle in order to minimize the spread of BSE, it seems more important to monitor the frequency of prion disease in cattle as they are slaughtered for human consumption. No reliable, specific test for prion disease in live animals is available, but immunoassays for PrP^{Sc} in the brainstems of cattle might provide a reasonable approach to establishing the incidence of subclinical BSE in cattle entering the human food chain. Determining how early in the incubation period PrP^{Sc} can be detected by immunological methods is now possible because a reliable bioassay has been created by expressing the BoPrP gene in Tg mice. Prior to development of $Tg(BoPrP)Prnp^{0/0}$ mice, non-Tg mice inoculated intracerebrally with BSE brain extracts required more than 300 days to develop disease. Depending on the titer of the inoculum, the structures of PrP^{C} and PrP^{Sc} and the structure of protein X, the number of inoculated animals developing disease can vary over a wide range. Some investigators have stated that transmission of BSE to mice is quite variable, with incubation periods exceeding 1 year, while others report low prion titers in BSE brain homogenates compared to rodent brain scrapie.

Have bovine prions been transmitted to humans?

In 1994, the first cases of CJD in teenagers and young adults that were eventually labelled new variant (v) CJD occurred in Great Britain. In addition to the young age of these cases, the brains of these patients showed numerous PrP amyloid plaques surrounded by a halo of intense spongiform degeneration. These unusual neuropathologic changes have not been seen in CJD cases in the USA, Australia or Japan. Both macaque monkeys and marmosets developed neurologic disease several years after inoculation with bovine prions, but only the macaques exhibited numerous PrP plaques similar to those found in vCJD.

The restricted geographical occurrence and chronology of vCJD have raised the possibility that BSE prions have been transmitted to humans. That only ~30 vCJD cases have been recorded and the incidence has remained relatively constant make establishing the origin of vCJD difficult. No set of dietary habits distinguishes vCJD patients from apparently healthy people. Moreover, there is no explanation for the predilection of vCJD for teenagers and young adults. Why have older individuals not developed vCJD-based neuropathologic criteria? It is noteworthy that epidemiological studies over the past three decades have failed to find evidence for transmission of sheep prions to humans. Attempts to predict the future number of cases of vCJD, assuming exposure to bovine prions prior to the offal ban, have been uninformative because so few cases of vCJD have occurred. Are we at the beginning of a human prion disease epidemic in Britain like those seen for BSE and kuru, or will the number of vCJD cases remain small, as seen with iatrogenic (i) CJD caused by cadaveric hGH?

Strain of BSE prions

Was a particular conformation of bovine PrP^{Sc} selected for heat resistance during the rendering process, and then reselected multiple times as cattle infected by ingesting prion-contaminated MBM were slaughtered and their offal rendered into more MBM? Recent studies of PrP^{Sc} from brains of patients who died of vCJD show a pattern of PrP glycoforms different from those found for sCJD or iCJD. But the utility of measuring PrP glycoforms is questionable in trying to relate BSE to vCJD because PrP^{Sc} is formed after the protein is glycosylated and enzymatic deglycosylation of PrP^{Sc} requires denaturation. Alternatively, it may be possible to establish a relationship between the conformations of PrP^{Sc} from cattle with BSE and those from humans with vCJD by using Tg mice, as was done for strains generated in the brains of patients with FFI or fCJD. A relationship between vCJD and BSE has been suggested, based on the finding of similar incubation times in non-Tg RIII mice of ~310 days after inoculation with human or bovine prions.

Summation

Although prions were originally defined in the context of an infectious mammalian pathogen, it is now becoming widely accepted that prions are elements that impart and propagate variability through multiple conformers of a normal cellular protein. Such a mechanism must surely not be restricted to a single class of transmissible pathogens. Indeed, proteins that act like prions have recently been reported in fungi. It is likely that the original definition will need to be extended to encompass other situations where a similar mechanism of information transfer occurs.

The study of prions has taken several unexpected directions over the past three decades; a novel and

fascinating story of prion biology is emerging. Investigations of prions have elucidated a previously unknown mechanism of disease in humans and animals. While learning the details of the structures of PrPs and deciphering the mechanism of PrP^{C} transformation into PrP^{Sc} will be important, the fundamental principles of prion biology have become reasonably clear. Though some investigators prefer to view the composition of the infectious prion particle as unresolved, such a perspective denies an enlarging body of data, none of which refutes the prion concept. Moreover, the discovery of prion-like phenomena mediated by proteins unrelated to PrP in yeast and other fungi serves not only to strengthen the prion concept but also to widen it.

The discovery that prion diseases in humans are uniquely both genetic and infectious greatly strengthened and extended the prion concept. To date, 20 different mutations in the human PrP gene, all resulting in nonconservative substitutions, have been found either to be linked genetically to or to segregate with the inherited prion diseases (**Fig. 3**). Yet the transmissible prion particle is composed largely, if not exclusively, of an abnormal isoform of the prion protein designated PrP^{Sc}.

The wealth of data establishing the essential role of PrP in the transmission of prions and the pathogenesis of prion diseases has provoked consideration of how many biological processes are controlled by changes in protein conformation. The extreme radiation-resistance of the scrapie infectivity suggested that the pathogen causing this disease and related illnesses would be different from viruses, viroids and bacteria. Indeed, an unprecedented mechanism of disease has been revealed where an aberrant conformational change in a protein is propagated. The future of this emerging area of biology should prove even more interesting and productive as many new discoveries emerge.

See also: **Prions: Kuru.**

Acknowledgements

I thank Drs Fred Cohen, Stephen DeArmond, Jiri Safar and Michael Scott for helpful discussions. This research was supported by grants from the National Institute of Aging and the National Institute of Neurologic Diseases and Stroke of the National Institutes of Health, International Human Frontiers of Science Program and American Health Assistance Foundation, as well as by gifts from the Sherman Fairchild Foundation, Keck Foundation, G Harold and Leila Y Mathers Foundation, Bernard Osher Foundation, John D French Foundation and Centeon.

Further Reading

Alper T, Cramp WA, Haig DA and Clarke MC (1967) Does the agent of scrapie replicate without nucleic acid? *Nature* 214: 764.

DeArmond SJ and Prusiner SB (1997) Prion diseases. In: Lartos P and Graham D (eds) *Greenfield's Neuropathology* 6th edn, p. 235. London: Edward Arnold.

Gajdusek DC (1977) Unconventional viruses and the origin and disappearance of kuru. *Science* 197: 943.

Prusiner SB (1998) Prions. *Proc. Natl. Acad. Sci* 95: 13363.

Prusiner SB, Scott MR, DeArmond SJ and Cohen FE (1998) Prion protein biology. *Cell* 93: 337.

Safar J, Wille H, Itri V *et al* (1998) Eight prion strains have PrP^{Sc} molecules with different conformations. *Nat. Med.* 4: 1157.

Kuru

Carlo Masullo, Institute of Neurology, Catholic University School of Medicine, Rome, Italy

History

Kuru was the first naturally occurring subacute spongiform encephalopathy of humans shown to be caused by an unconventional transmissible agent. Kuru means shivering or trembling in the Fore language. It was first described by Gajdusek and Zigas in 1957 in the Fore cultural and linguistic group of isolated regions of Papua New Guinea. It was found to affect all ages, being common in male and female children and in adult females, but rare in adult males. Kuru is characterized by cerebellar ataxia and a shivering-like tremor that produces complete motor incoordination. The disease is inevitably fatal and death usually occurs in less than 1 year.

Classification

Kuru belongs to the group of subacute spongiform encephalopathies or prion diseases of humans which also include Creutzfeldt–Jakob disease (CJD), Gerstmann–Straussler syndrome (GSS) and fatal familial insomnia (FFI); related animal diseases are scrapie of sheep and goats, chronic wasting disease (CWD) of mule and deer, transmissible mink encephalopathy (TME) and bovine spongiform encephalopathy (BSE).

Geographic and Seasonal Distribution

Kuru has been found in the Eastern Highlands

fascinating story of prion biology is emerging. Investigations of prions have elucidated a previously unknown mechanism of disease in humans and animals. While learning the details of the structures of PrPs and deciphering the mechanism of PrP^{C} transformation into PrP^{Sc} will be important, the fundamental principles of prion biology have become reasonably clear. Though some investigators prefer to view the composition of the infectious prion particle as unresolved, such a perspective denies an enlarging body of data, none of which refutes the prion concept. Moreover, the discovery of prion-like phenomena mediated by proteins unrelated to PrP in yeast and other fungi serves not only to strengthen the prion concept but also to widen it.

The discovery that prion diseases in humans are uniquely both genetic and infectious greatly strengthened and extended the prion concept. To date, 20 different mutations in the human PrP gene, all resulting in nonconservative substitutions, have been found either to be linked genetically to or to segregate with the inherited prion diseases (**Fig. 3**). Yet the transmissible prion particle is composed largely, if not exclusively, of an abnormal isoform of the prion protein designated PrP^{Sc}.

The wealth of data establishing the essential role of PrP in the transmission of prions and the pathogenesis of prion diseases has provoked consideration of how many biological processes are controlled by changes in protein conformation. The extreme radiation-resistance of the scrapie infectivity suggested that the pathogen causing this disease and related illnesses would be different from viruses, viroids and bacteria. Indeed, an unprecedented mechanism of disease has been revealed where an aberrant conformational change in a protein is propagated. The future of this emerging area of biology should prove even more interesting and productive as many new discoveries emerge.

See also: **Prions: Kuru.**

Acknowledgements

I thank Drs Fred Cohen, Stephen DeArmond, Jiri Safar and Michael Scott for helpful discussions. This research was supported by grants from the National Institute of Aging and the National Institute of Neurologic Diseases and Stroke of the National Institutes of Health, International Human Frontiers of Science Program and American Health Assistance Foundation, as well as by gifts from the Sherman Fairchild Foundation, Keck Foundation, G Harold and Leila Y Mathers Foundation, Bernard Osher Foundation, John D French Foundation and Centeon.

Further Reading

Alper T, Cramp WA, Haig DA and Clarke MC (1967) Does the agent of scrapie replicate without nucleic acid? *Nature* 214: 764.

DeArmond SJ and Prusiner SB (1997) Prion diseases. In: Lartos P and Graham D (eds) *Greenfield's Neuropathology* 6th edn, p. 235. London: Edward Arnold.

Gajdusek DC (1977) Unconventional viruses and the origin and disappearance of kuru. *Science* 197: 943.

Prusiner SB (1998) Prions. *Proc. Natl. Acad. Sci* 95: 13363.

Prusiner SB, Scott MR, DeArmond SJ and Cohen FE (1998) Prion protein biology. *Cell* 93: 337.

Safar J, Wille H, Itri V *et al* (1998) Eight prion strains have PrP^{Sc} molecules with different conformations. *Nat. Med.* 4: 1157.

Kuru

Carlo Masullo, Institute of Neurology, Catholic University School of Medicine, Rome, Italy

History

Kuru was the first naturally occurring subacute spongiform encephalopathy of humans shown to be caused by an unconventional transmissible agent. Kuru means shivering or trembling in the Fore language. It was first described by Gajdusek and Zigas in 1957 in the Fore cultural and linguistic group of isolated regions of Papua New Guinea. It was found to affect all ages, being common in male and female children and in adult females, but rare in adult males. Kuru is characterized by cerebellar ataxia and a shivering-like tremor that produces complete motor incoordination. The disease is inevitably fatal and death usually occurs in less than 1 year.

Classification

Kuru belongs to the group of subacute spongiform encephalopathies or prion diseases of humans which also include Creutzfeldt–Jakob disease (CJD), Gerstmann–Straussler syndrome (GSS) and fatal familial insomnia (FFI); related animal diseases are scrapie of sheep and goats, chronic wasting disease (CWD) of mule and deer, transmissible mink encephalopathy (TME) and bovine spongiform encephalopathy (BSE).

Geographic and Seasonal Distribution

Kuru has been found in the Eastern Highlands

Province of Papua New Guinea, which has a population of about 35 000 people living in 160 villages. These villages are located from 1000 to 2500 meters above sea level. No cases of Kuru have been found outside this geographical region. No evidence has emerged from the clinico-epidemiological studies on different seasonal trends of the disease.

Host Range and Virus Propagation

Kuru is naturally occurring only in humans and its clinical course is inevitably fatal.

Experimental transmission of Kuru has been obtained in nonhuman primates and in some small rodents, such as minks and ferrets, through intracerebral inoculation of brain material of patients who died from Kuru. Fourteen strains of Kuru have been isolated and 11 of these human strains have been inoculated into small laboratory rodents, i.e. hamsters, mice and guinea pigs. Only three of these 11 strains produced clinical disease and neuropathological lesions in an occasional mouse on primary passage, and none of these have been successfully serially transmitted from mouse to mouse. Hamsters and guinea pigs failed to develop clinical disease or evidence of brain pathology during several years of follow-up after the experimental inoculation.

A similar pattern of strain adaptation occurred in attempting the transmission of strains of Kuru adapted into nonhuman primates to small rodents. In fact, several strains of chimpanzee, capuchin, spider, rhesus and squirrel monkey-adapted Kuru virus failed to transmit the disease or induce subclinical neuropathological lesions in small rodents for their entire lifespan. On the contrary, two primate-adapted strains of Kuru have caused disease in one of several minks and one of several ferrets, respectively. Serial passage of the Kuru virus in mink has not been successful, while primary passage into ferrets required an incubation of 31 months; serial passage of this same strain in ferrets has been associated with incubation periods of 59 and 70.5 months, respectively, in the two animals which developed the disease on the first ferret-to-ferret passage, and 9 months in the one ferret which developed the disease on the second ferret-to-ferret passage. This variable behavior of a given strain of Kuru has been observed with the passage of the virus between species and, to a lesser degree, within the same species of experimental host. The time sequence of disease progression also mimics that in humans, ranging from several months to over a year until death. A single strain of Kuru virus may cause severe neuronal spongiosis in different brain areas such as the cerebral cortex in chimpanzees without any major neuropathological involvement of the brainstem or spinal cord. On the contrary, this same virus strain may produce extensive brainstem and spinal cord lesions in the squirrel monkey.

Kuru is a naturally occurring disease and its mode of dissemination and maintenance is not completely clarified. A possible explanation is the contamination of close kinsmen by opening of the skull of dead victims in a rite of cannibalism, during which all girls, women and children of the Kuru victim's family were certainly exposed to the virus. The disease is gradually disappearing with the cessation of ritualistic cannibalism.

There are several hundred Kuru orphans born since 1957 to mothers dying of Kuru and none has yet developed the disease. Thus, the many children with Kuru seen in the 1950s were not infected prenatally, perinatally or neonatally by their mothers. Kuru has progressively disappeared, first among children and thereafter among adolescents.

Genetics

Experimental studies have shown that there are several strains of the Kuru agent. In fact, at least 14 strains of Kuru have been isolated by direct inoculation of human brain into nonhuman primates.

Recent studies on the search for possible mutations of the PRNP gene found no mutations occurred in some Kuru cases. The polymorphism at the codon 129 of the PRNP (prion protein) gene has been determined in a group of 38 Kuru patients and the genotypes were M/V in 50%, M/M in 30% and V/V in 20% of the cases; in a control group of the same ethnic background, the genotypes were M/V in 48%, M/M in 30% and V/V in 22%. Therefore, no differences between Kuru patients and their controls have been found, irrespective of the codon 129 genotype.

Evolution

The origin of Kuru is not clear; it is possible that it resulted from a sporadic case of CJD, which, in that particular environment of New Guinea, produced a unique epidemic. Serial passages of brain in successive cannibalistic rituals might have changed the clinical picture of the disease. A spontaneous case of CJD in a 26-year-old native Chimbu New Guinean from the Central Highlands has been reported – the clinical diagnosis was proved only by the neuropathological study of a brain biopsy specimen; this case might represent the basis for spread of the agent, with modifications of the virulence related to serial passages through cannibalistic procedures.

Serologic Relationships and Variability

Transmission studies in experimental animals have shown that several strains of the Kuru agent do exist. Owing to the still unknown nature of the agent causing the disease and the lack of any immune-mediated response in the course of Kuru, no antigenic variations are known.

Epidemiology

The natural incubation period of Kuru can be as long as 25–30 years and it is at times identical in two or more individuals infected at the same time, even over this span of years; the natural incubation period does not seem to be determined by age at exposure.

In the Fore group, Kuru had a yearly incidence rate and prevalence ratio of 1% of the population. As mentioned earlier, there was a marked excess of deaths of adult females over males with a male:female ratio of 1:3 for the whole South Fore group. More than 2500 patients died of Kuru in the 17 year period of surveillance by year since its discovery in 1957 through 1975.

In the last 20 years, there has been a constant and progressive decline of the incidence of Kuru among Fore people. The disease has progressively disappeared from the younger age-groups up to the adult ones. No new Kuru patients have been recorded as born after 1959, and prospective evaluations estimate that Kuru will disappear by the end of the twentieth century.

Transmission and Tissue Tropism

The natural (human-to-human) mode of transmission is not totally understood, but ritual cannibalism has been the probable mode of propagation and maintenance of the disease. This is because the brain tissue, with which the officiating women contaminated both themselves and all their infants and toddlers, contained over ten infectious doses per gram, with self-inoculation occurring through the eyes, nose and skin as well as by mouth.

Experimental transmission of Kuru was first demonstrated in a chimpanzee by intracerebral inoculation of brain homogenate of a Kuru patient; since then, experimental transmission studies of Kuru were conducted in several animal species (other nonhuman primates and various small laboratory rodents).

Studies in cell cultures derived from tissues of patients and animals with Kuru have been performed, inoculating nonhuman primates by the intracerebral route. Primary cell lines that maintain the infectious Kuru agent did not largerly differ in appearance from neuronal cultures derived from brains of non-Kuru cases. Some of these cell cultures became infected with Kuru and the persistence of Kuru infection was demonstrated for 170 days in human cultures and for 215 days in animal cultures. It has never been clearly proved that the Kuru agent replicates *in vitro*.

Kuru has a selective neurotropism; in fact, histological lesions are found only in the central nervous system, involving neurons as well as astrocytes. In about 10% of Kuru cases there is an accumulation and deposition of typical amyloid plaques. Lesions have never been described outside nervous tissue. It is likely that, as in other subacute spongiform encephalopathies of humans and animals, the etiological agent penetrates the lymphoreticular system, reaching the midthoracic level of the spinal cord and then spreading towards the central nervous system, resulting in the clinical appearance of the disease.

Pathogenicity

The etiological agent of Kuru has not yet been isolated; the agent produces a disease which is always fatal for the host and it is regularly detected in the brain tissue where the infectivity titer reaches 10^8 infectious doses per gram. In extraneural tissues, such as liver and spleen, Kuru agent has been found only rarely at death and with lower titers. Infectivity has never been detected in blood, urine, leukocytes, cerebrospinal fluid, placenta or embryonal membranes originating from patients with Kuru.

A pathological amyloidotic protein, PrPres (resistant), has been systematically found in the brains of patients dead from Kuru. PrPres is a distinctive pathological hallmark of Kuru and other spongiform encephalopathies of animals and humans; however, whether this pathological protein represents an important component of the infectious agent or is a by-product of the infection is still a matter of controversy.

Clinical Features of Infection

There is considerable consistency in the clinical characteristics of Kuru, suggesting genetic stability of the agent. In fact, Kuru is constantly characterized by cerebellar ataxia and a shivering-like tremor and its clinical course is remarkably uniform, with cerebellar symptomatology progressing to total inability and death, usually within 3–9 months. Generally, patients complain of joint pain, with the subsequent appearance of walking difficulties prior to clinical onset. During the clinical course of the

disease, some patients develop neurological signs of cognitive impairment, mainly related to damage to the frontal lobes, but most Kuru patients show signs of dementia only in the late stages of the disease. During the progression of the disease, truncal ataxia and shivering tremor become so severe that patients are not able to walk without assistance. At least a third of Kuru patients show clinical signs of basal ganglia impairment, with rigidity and cogwheeling.

Pathology and Histopathology

Macroscopically, the cerebral hemispheres appear normal but the cerebellum regularly shows atrophy, often especially severe in the phylogenetically old paleocerebellum, i.e. the vermis and flocculonodular lobe. Microscopically, the lesions are almost homogenous and no large variations are noted that could be related to differences in age, sex or duration of the disease. The most regular and striking degeneration always occurs in the cerebellum, where there is a loss of Purkinje and granule cells. At the level of the molecular layer, masses of microglial cells are usually found. A diffuse and severe proliferation of astrocytes is observed throughout all layers of the cerebellar cortex. Amyloid plaques, a distinctive neuropathological feature of Kuru, are abundant in more than 80% of cases and are seen within the granular and molecular layers of the cerebellum. These plaques are morphologically characterized by a multicentric core with radiating filamentous deposits. The core consists of granular or homogenous substances of various sizes which are densely stained with periodic acid–Schiff reaction, birefringent with Congo red staining and fluorescent with thioflavin staining.

Immunohistochemical staining with antibodies specifically raised against the pathological protein PrPres shows an intense positive reaction in the core and the periphery of these multicentric amyloid 'Kuru' plaques. Immunocytochemistry of some Kuru brains has been recently carried out in the light of potential similarities with the amyloid plaques deposited in the brain of cases of the new variant CJD (nvCJD), recently reported in young British patients. The conclusion of these studies using antiprion protein immunocytochemistry shows that the pathology, including immunomorphology of PrP deposition, is within the lesion spectrum of CJD, although plaques are unusually prominent and the topography of PrP deposition parallels that of spongiform change and/or astrogliosis, while it seems that Kuru does not share the neuropathological hallmarks of nvCJD. The cerebellar white matter shows no signs of a primary demyelination. Spongiform changes associated with proliferation of reactive astrocytosis and some degree of neuronal loss are diffusely observed throughout the cerebral cortex. In the striatum, many large neurons show a severe and multilocular vacuolation. Other changes are variably distributed in the rest of the basal ganglia and are most frequently observed in the anterior nuclear group of the thalamus.

Immune Response

There is no recognizable inflammatory/immune-mediated response during the clinical course of Kuru, as is the case in all the other subacute spongiform encephalopathies.

Prevention and Control

The most likely mode of natural propagation and maintenance of the infection appears to be contamination during ritualistic cannibalistic ceremonies; this has been confirmed by the dramatic decrease of the number of new cases of Kuru since the cessation of cannibalistic rituals in the Fore region. This decline in Kuru incidence occurred during the period of transformation from a Stone Age cultural enviroment, in which the endocannibalistic ritual consumption of dead relatives was regularly practiced, to a modern society organized with a cash economy. The decline in the incidence of Kuru victims has therefore followed the cessation of cannibalism, and the disease has progressively disappeared from the youngest age group (4–9 years) up to the adult age groups.

The number of Kuru cases has steadily declined and each year the youngest patients have been older than those in the previous year. Now, only two or three cases are occurring annually, none younger than 40 years of age. According to prospective estimates it will probably take only a few years before the appearance of the last Kuru case.

See also: **Prions: Human and Animal.**

Further Reading

Gajdusek DC (1977) Unconventional viruses and the origin and disappearance of Kuru. *Science* 197: 943.

Gajdusek DC (1996) Infectious amyloids: spongiform subacute encephalopathies as transmissible cerebral amyloidoses. In: Fields BN, Knipe DM, Howley PM *et al.* (eds) *Fields Virology*, 3rd edn. p. 2851. Philadelphia: Lippincott-Raven.

Gajdusek DC (1996) Kuru in childhood: implications for the problem of whether bovine spongiform encephalopathy affects humans. In: Court L and Dodet B (eds) *Transmissible Subacute Spongiform Encephalopathies: Prion Diseases*, p. 15. Paris: Elsevier.

Gajdusek DC and Zigas V (1957) Degenerative disease of the central nervous system in New Guinea: the endemic occurrence of 'kuru' in the native population. *N. Engl. J. Med.* 257: 974.

Klatzo I, Gajdusek DC and Zigas V (1959) Pathology of Kuru. *Lab. Invest.* 8: 799.

Yeast and Fungi

Marie-Lise Maddelein, Herman Edskes, Kim Taylor and **Reed B Wickner**, Laboratory of Biochemistry and Genetics, National Institute of Diabetes Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland, USA

History

The notion that a protein could be infectious without a nucleic acid as part of the infectious material arose from studies of scrapie of sheep, kuru and Creutzfeldt–Jakob disease (CJD) of man, and the equivalent diseases of other mammals. These diseases are called transmissible spongiform encephalopathies because the diseased brain has a spongy appearance due to loss of neurons and accumulation of vesicles in others. The transmissibility requires injection or feeding of the diseased tissues.

Origin of the infectious protein concept

Gajdusek showed in the 1960s that the inherited and spontaneous forms of CJD were infectious for monkeys, just like the infectious form. He believed that CJD (and scrapie) was due to a slow virus. Studies by Tikva Alper showed that the scrapie agent was remarkably UV-resistant, compared with viruses and bacteriophage. She proposed that scrapie may be infectious without a nucleic acid. Griffith proposed that an infectious protein might be an altered form of a cellular protein that had acquired the ability to change the normal form of the protein into the altered form by forming an oligomer with it. This 1967 proposal is precisely what the transmissible spongiform encephalopathies are widely believed to be today.

In 1975, Dickinson identified a critical gene involved in scrapie of mice that he named *Sinc*, for *s*crapie *inc*ubation period. Prusiner purified the scrapie agent and found that it contained a protease-resistant protein (named PrP). Weissmann and Chesebro cloned the PrP genes of hamster and mouse, respectively, and found they were normal cellular genes. In fact, the PrP gene was the same as Dickinson's *Sinc* gene. Weissmann showed that PrP was necessary for the propagation of scrapie, as mice deleted for the gene were immune to the disease. Prusiner showed that mice transgenic for the hamster gene had now become sensitive to the hamster scrapie agent. Although these studies have not yet proven that scrapie is due to an infectious protein, the concept was widely circulated in this connection.

The definition of 'prion'

The term 'prion' was coined by Prusiner in 1982 to mean simply the scrapie agent. In view of its unusual properties, scrapie was considered to be a new type of entity. It was suggested that if scrapie were an infectious protein, without a nucleic acid, it could be transmitted via 'reverse translation' or 'protein-dependent protein synthesis', but infectious protein was not part of the definition of the word prion. Indeed, many current authors use 'prion' as a synonym for 'scrapie agent'.

We have perhaps confused matters by using the word 'prion' to mean 'infectious protein' and have treated the question of whether there are such things as a separate issue. We continue that usage here.

Expected properties of a yeast or fungal infectious protein

If there were an infectious protein of yeast, it would be expected, like yeast viruses, to spread to other cells via the cell fusion that accompanies mating. Thus, it would appear as a nonchromosomal genetic element.

We proposed three genetic criteria that should distinguish an infectious protein (a prion) from the majority of non-chromosomal genetic elements, which are plasmids or viruses (**Fig. 1**).

1. If one can cure a prion, it should be possible for it to arise again in the cured strain (reversible curability).
2. Overproduction of the normal form of the protein should increase the frequency with which the prion form arises.
3. Recessive mutations of the chromosomal gene for the protein should produce the same phenotype as the presence of the prion, because both result in absence of the normal form of the protein. Deletion of the chromosomal gene should also result in loss of the prion.

Note that scrapie is not known to satisfy any of these criteria.

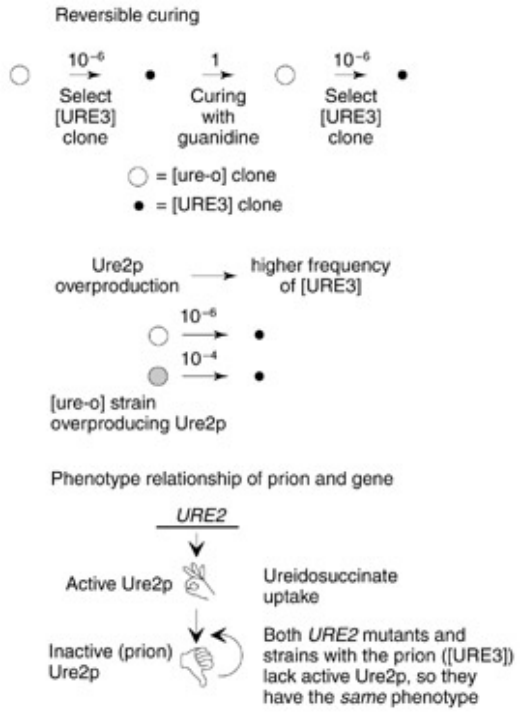


Figure 1 Genetic properties expected of a prion. We proposed that a prion should be reversibly curable, that overexpression of the normal form should increase the *de novo* appearance of the prion form and that the presence of the prion and the absence of the normal form should have similar phenotypes (if the prion phenotype comes from absence of the normal form). From Wickner RB (1994) with permission.

[URE3], an Infectious Form of Ure2p, a Regulator of Nitrogen Metabolism

[URE3] satisfies the three genetic criteria for a prion

[URE3] is a nonchromosomal genetic element identified by Lacroute in 1971 in his search for yeast mutants able to take up ureidosuccinate in the presence of a good nitrogen source, such as ammonia. Ureidosuccinate is an intermediate in uracil biosynthesis, the product of aspartate transcarbamylase (**Fig. 2**). It is taken up by Dal5p, the permease for allantoate, which is structurally similar to ureidosuccinate. Allantoate is a poor, but usable nitrogen source for yeast, but genes needed for allantoate utilization (such as *DAL5*) are repressed when a good nitrogen source is available. This 'nitrogen catabolite repression' is mediated in part by Ure2p, which blocks the positive transcription regulator, Gln3p, when a good nitrogen source is at hand. Gln3p is necessary for transcription of *DAL5* and other nitrogen-regulated genes.

Lacroute found that both the chromosomal *ure2* mutants and strains with the nonmendelian element [URE3] could take up ureidosuccinate. But Aigle and Lacroute found that *ure2* mutants could not propagate the [URE3] genetic element. This is criterion (3) above. [URE3] may be cured by growing cells in the presence of low concentrations of guanidine, but we found that from the cured strains can again be isolated strains that have spontaneously developed [URE3]. This is reversible curability (criterion (1) above). Finally, overproduction of Ure2p results in a 20–200-fold increase of the frequency with which [URE3] arises (criterion (2)).

Further evidence that [URE3] is a prion form of Ure2p

Not only can overproduction of Ure2p induce appearance of [URE3] in a normal strain, but it can do the same thing in a strain which was initially deleted for *URE2*. This argues against [URE3] being the altered form of a virus or plasmid present in the normal strains, as the putative normal replicon should depend on the same chromosomal genes (i.e. *URE2*).

If [URE3] is an infectious protein form of Ure2p, it should be possible to show some difference in Ure2p in [URE3] strains compared with normal strains. Indeed, Ure2p is more protease resistant in [URE3] strains, than in either the parent strain or in a cured strain.

One type of nonnucleic acid 'genetic element' is a sort of regulatory circuit that is self-maintaining. Since Ure2p is a transcription regulator, it is critical to show that this is not the case here. Several lines of evidence show that the regulatory capacity of Ure2p and its ability to undergo or induce the [URE3] change are distinct. Moreover, the [URE3] genetic element can be equally well propagated under conditions of nitrogen derepression or repression.

Molecular biologic proof that Ure2p is infectious

We have shown that it is actually the overproduction of the Ure2 protein, not the *URE2* mRNA or the *URE2* gene in high copy, that induces the appearance of [URE3]. This may be viewed as the molecular biological equivalent of purifying Ure2p from [URE3] strains and showing that it is infectious.

Mechanism of [URE3] generation and propagation

The Ure2 protein may be divided into an N-terminal 65-residue prion domain and a C-terminal nitrogen regulation domain. The prion domain is sufficient when overproduced to induce appearance of [URE3] at a frequency over 1000 times the spontaneous rate. It is also necessary for a Ure2p molecule to have a

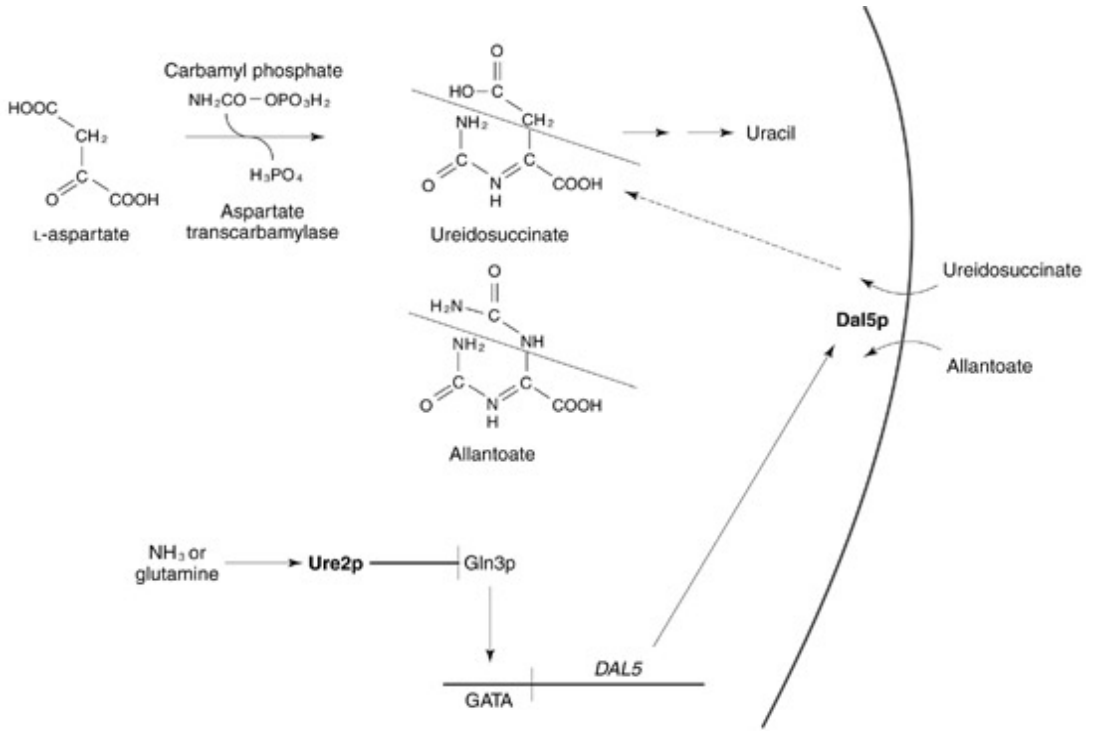


Figure 2 Nitrogen regulation affects uptake of ureidosuccinate through Ure2p. In the presence of a good nitrogen source (ammonia or glutamine) the Ure2p protein prevents the Gln3p protein from activating transcription of genes whose products are involved in utilization of poor nitrogen sources. Dal5p is such a protein, a transporter for allantoate, a poor nitrogen source structurally similar to ureidosuccinate. Ureidosuccinate is an intermediate in uracil biosynthesis.

covalently attached prion domain in order for it to be subject to the prion change as transmitted from another molecule (**Fig. 3**). Indeed, the N-terminal prion domain can transmit [URE3] in the complete absence of the C-terminal part of the Ure2 molecule. Likewise, the C-terminal part of the molecule can regulate nitrogen without the N-terminal domain.

Although the N-terminal prion domain and the C-terminal nitrogen regulatory domain of Ure2p can each act in the absence of the other, there are several lines of evidence that they have specific functional interaction: (1) the C-terminal domain must be overproduced to regulate nitrogen as well as the full-length protein, suggesting that the N-terminal domain somehow helps in this activity; (2) deletions in the C-terminal domain *increase* the frequency with which the molecule converts to the prion form, suggesting that the C-terminal domain stabilizes the N-terminal domain in the normal form; and (3) attaching the prion domain to β-galactosidase does not make that enzyme subject to inactivation by the introduction of [URE3].

The precise mechanism by which the prion domain inactivates the C-terminal nitrogen regulation domain is not yet known. It is possible that this involves a specific interaction, or that the aggregation of Ure2p in [URE3] strains prevents Ure2p from entering the nucleus, where it acts to modulate Gln3p activity.

[PSI], an Infectious Form of Sup35p, a Translation Release Factor

[PSI] as a infectious form of Sup35p

[PSI] was discovered in 1965 by Cox as a nonchromosomal genetic element that increased the strength of chromosomal suppressor mutations (suppressor tRNAs). Weak suppressors became strong and strong suppressors became lethal. Even [PSI] by itself had some weak suppressor activity. [PSI] could affect any nonsense mutation.

[PSI] can be cured by growth of strains in high osmotic strength medium, or by guanidine. But from the cured strains could again be isolated strains that had developed [PSI]. This is reversible curability. *Sup35* mutants, like [PSI], have 'omnipotent suppressor' activity, working on UAA, UGA and UAG codons. Moreover, [PSI] fails to propagate in certain *sup35* mutant strains. This is the relation expected if [PSI] is a prion form of Sup35p (**Fig. 3**). Finally, overproduction of Sup35p results in a 100-fold increase of the frequency with which [PSI] appears.

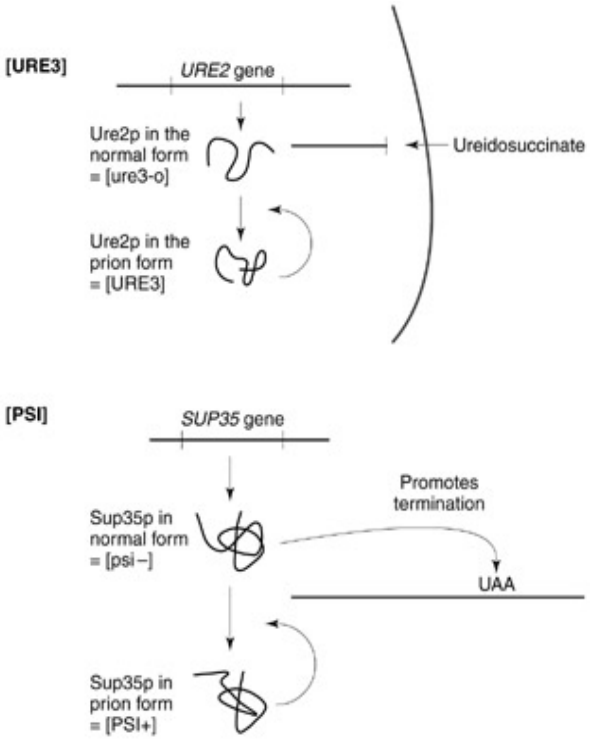


Figure 3 [URE3] and [PSI] as prion forms of Ure2p and Sup35p. If an altered protein can convert the normal form into the same altered form, this altered form can be a prion if it has a means to pass from cell to cell, or individual to individual. We proposed that [URE3] and [PSI] are altered forms of Ure2p and Sup35p, respectively.

Thus, [PSI] has the three genetic properties expected if it is an infectious protein form of Sup35p. Sup35p is a subunit of the translation release factor. It is to be expected that a change of Sup35p that partially inactivates it (or completely inactivates some proportion of the Sup35p molecules) would make translation termination less active and less able to compete with a suppressor tRNA.

Support for [PSI] being a prion

In normal strains, Sup35p is a soluble protein, complexed with the other subunit of the translation release factor, Sup45p, as a heterodimer. In extracts of [PSI] strains, most of Sup35p is present as an aggregate. The Sup35p in extracts of [PSI] strains is resistant to digestion with protease, perhaps because it is in this aggregated form.

Prion domain of [PSI]

Ter-Avanesyan and coworkers examined various deletion mutants of Sup35p and showed that the C-terminal domain is essential for cell growth, presumably the translation termination domain. They found that the N-terminal 114 residues were dispensable for cell growth, but were necessary for the propagation of [PSI]. This region has a series of repeats of an eight amino acid motif and is rich in glutamine and glycine residues. PrP also has an octapeptide repeat near its N-terminus, but transgenic mouse studies indicate that this repeat is not necessary for the propagation of scrapie.

Hsp104 is necessary for [PSI] propagation *and* can cure [PSI]

Chernoff and coworkers isolated a high-copy plasmid that cured [PSI] and found that it encoded Hsp104, a heat shock protein known to disaggregate proteins which had been denatured by heat. Overproduction of Hsp104 by other means also results in loss of [PSI]. Surprisingly, underproduction of Hsp104, for example, by deletion of the gene, also results in loss of [PSI].

The fact that a protein renaturing factor can eliminate [PSI] supports the notion that [PSI] is a conformationally altered form of a normal protein. At the same time, this important result suggests an avenue to approach the treatment of CJD and other transmissible spongiform encephalopathies. That [PSI] propagation requires Hsp104 could suggest that Hsp104 is actually involved in the conversion of Sup35p from the normal to the altered form (along with the altered form of Sup35p itself). Another possibility is that in the absence of Hsp104 there is essentially a single aggregate of Sup35p in [PSI] strains, and that when the cell divides, only one of the daughter cells will receive this aggregate (and thus receive [PSI]). Perhaps normal levels of Hsp104 breaks the aggregates into several peices, at least one of which goes to each daughter cell, insuring the stable propagation of [PSI].

In vitro propagation of [PSI]

Recently, Paushkin *et al* have reported an *in vitro* system in which soluble Sup35p, isolated from a wild-type strain, is converted to the aggregated form by addition of highly purified Sup35p aggregates from a [PSI] strain. A small aliquot of the product can prime the aggregation reaction of a new batch of soluble Sup35p. This reaction can apparently be continued as long as there is a supply of fresh normal Sup35p. The reaction requires the same N-terminal domain of Sup35p that has been shown to be critical for propagation of [PSI]. This is a remarkable confirmation of the prion model for [PSI].

[Het-s] as a Prion Form of the *het-s* Protein of *Podospora*

Heterokaryon incompatibility in fungi

A colony of the filamentous fungus, *Podospora*, is not really a collection of cells. Rather, the cells of the colony are interconnected, separated by incomplete cell walls and septae. Thus, each colony is a single multinuclear cell, a syncytium. When two colonies of a single fungal strain grow toward each other, the cellular processes (called hyphae) of the two colonies fuse, forming what is, in effect, one colony. The nuclei migrate between what was the two colonies so that a 'heterokaryon' is formed – a combination of the two colonies in which each 'cell' may have a mixture of the two kinds of nuclei, one kind from one parent colony, and one from the other. When these colonies fuse, a virus or plasmid present in one colony will spread throughout the other as well. Many fungal viruses and plasmids are quite debilitating to their hosts. For this reason, it is believed, most fungi have a system that limits with what strains it will carry out this 'hyphal fusion' (or heterokaryon formation). This system limiting heterokaryon formation is called 'heterokaryon incompatibility'.

Genetic control of heterokaryon incompatibility

Sexual mating, leading to meiosis, is a process designed to create diversity by shuffling the genetic cards. Thus, sexual mating requires *differences* between the individuals at one or more loci (the mating type loci). Heterokaryon formation, as described above, is designed to allow cooperation (e.g. sharing of nutrients) between genetically identical individuals, and so heterokaryon formation only occurs between strains identical at each of several genes, called *het* genes in the case of *Podospora*.

One of the *Podospora het* genes is called *het-s*, and it can have two alleles, *het-S* and *het-s*. Two *het-s* or two *het-S* strains readily fuse and form healthy heterokaryons with each other. But when a *het-s* strain meets a *het-S* strain, hyphal fusion is followed quickly by death of fused hyphae and the formation of a barrier to further fusion between the two colonies.

Genetic properties of *het-s* suggest a prion

The *het-s* strains can have either of two phenotypes, one showing the incompatibility described above, and another 'neutral' phenotype in which cells form heterokaryons equally well with *het-s* and *het-S* cells. This difference is controlled by a nonmendelian genetic element, called [Het-s], whose presence leads to the incompatibility phenotype, and whose absence results in the neutral phenotype (**Fig. 4**). [Het-s] can be cured, but then spontaneously reappears at a low frequency ('reversible curability'). Begueret and coworkers have recently found that overproduction of the *het-s* protein results in an increase of the frequency with which [Het-s] appears. Deletion of the *het-s* gene results in inability to propagate [Het-s], but the deleted strains show the same neutral phenotype as is found in the absence of [Het-s], so this does not specifically point to [Het-s] being a prion (although it is not against this conclusion). Finally, the *het-s* protein shows increased resistance to protease digestion in extracts of [Het-s] strains compared to strains lacking [Het-s].

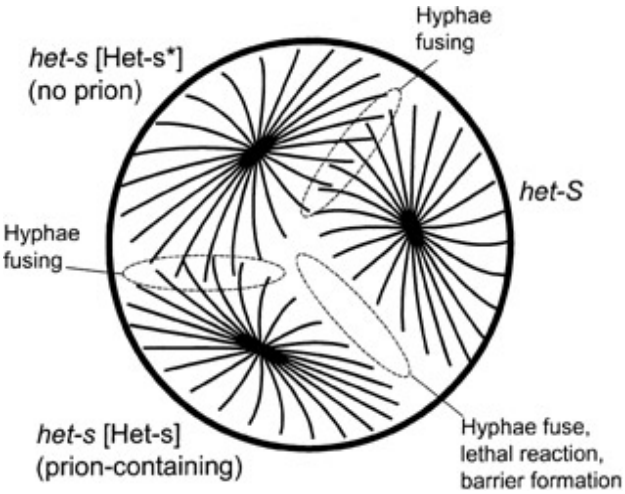


Figure 4 Heterokaryon incompatibility in *Podospora* and the [Het-s] prion. A *het-s* strain shows heterokaryon incompatibility with a *het-S* strain only if the former carries a nonmendelian genetic element, called [Het-s]. Begueret and coworkers have presented evidence that [Het-s] is a prion form of the *het-s* protein.

These results all point to [Het-s] being a prion form of the *het-s* protein. This is the first case in which a prion appears to be facilitating a normal cellular function. Heterokaryon incompatibility is a phenomenon found in most filamentous fungi, and in the many other cases examined genetically, there is no evidence of any unusual genetic features suggestive of prions.

Features of the *het-s* protein

The *het-s* and *het-S* alleles differ by only 14 amino acids in a 289 amino acid open reading frame. A single difference at residue 33 is sufficient to convert *het-s* to *het-S* behavior. These proteins have no similarity to any other proteins in the databases, including the other putative prion proteins, PrP, Ure2P and Sup35p.

Table 1 Comparison of the putative prion systems

Prion	*Protein*	*Phenotype of prion*	*Function of normal protein*	*Structure of protein*
Scrapie	PrP	Ataxia, dementia, death	Unknown	Normal – α helix; prion – β sheet
[URE3]	Ure2p	Nitrogen derepression	Nitrogen repression	Asparagine-rich prion domain
[PSI]	Sup35p	Translation readthrough	Translation termination	Glutamine and asparagine rich
[Het-s]	*het-s* protein	Heterokaryon incompatibility	None known	??

Comparison of the Putative Prion Systems (Table 1)

Although the evidence certainly suggests that the scrapie agent is an altered form of PrP, there is other data that calls this into question. Although PrP can be made in large amounts in bacterial or yeast cells, sometimes as protease-resistant material with high β-sheet content, it has never proven infectious. Moreover, there are several reports of dissociation between the finding of abnormal PrP and appearance of infectivity. Finally, the genetic criteria proposed for fungal prions have not yet been satisfied for PrP and scrapie.

The yeast and fungal systems, because of the ease with which they may be studied genetically, have already produced some important results:

- The fungal systems have provided evidence that there can be such a thing as a prion – evidence of a type not yet available for scrapie and PrP.
- These systems show that an inherited trait can be a prion: a prion can be a 'gene'.
- The role of chaperones in prion propagation has long been speculated, but is first shown for [PSI]. It is expected that these systems will soon lead to the definition of other factors affecting prion generation and propagation.
- [Het-s] shows that a normal cellular function can be determined by a prion.
- The definition of prion domains in Ure2p and Sup35p is beginning to contribute to our understanding of how a prion arises and is propagated.
- The *in vitro* system for [PSI] is the best available for any prion to date.

We expect that many more prions will be discovered, some causing pathologic conditions of animals, plants or microorganisms, while others mediate normal cellular functions. The genetic approaches to identifying prions that have proven useful in yeast and filamentous fungi will probably continue to be valuable in identification of other prions.

See also: **Prions: Human, Human and Animal; Yeast RNA viruses (*Totiviridae*); Viroids.**

Further Reading

Chernoff YO, Lindquist SL, Ono BI, Inge-Vechtomov SG and Liebman SW (1995) Role of the chaperone protein Hsp104 in propagation of the yeast prion-like factor [psi$^+$]. *Science* 268: 880.

Coustou V, Deleu C, Saupe S and Begueret J (1997) The protein product of the *het-s* heterokaryon incompatibility gene of the fungus *Podospora anserina* behaves as a prion analog. *Proc. Natl Acad. Sci. USA* 94: 9773–9778.

Cox BS (1993) Psi phenomena in yeast. In: Hall MN and Linder P (eds) *The Early Days of Yeast Genetics*, p. 219. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Masison DC and Wickner RB (1995) Prion-inducing domain of yeast Ure2p and protease resistance of Ure2p in prion-containing cells. *Science* 270: 93–95.

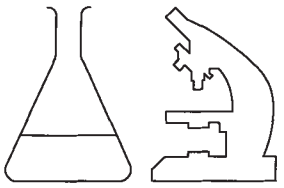
Paushkin SV, Kushnirov VV, Smirnov VN and Ter-Avanesyan MD (1997) *In vitro* propagation of the prion-like state of yeast Sup35 protein. *Science* 277: 381.

Wickner RB (1994) Evidence for a prion analog in *S. cerevisiae:* the [URE3] non-Mendelian genetic element as an altered *URE2* protein. *Science* 264: 566.

Wickner RB (1996) Prions and RNA viruses of *Saccharomyces cerevisiae. Annu. Rev. Genet.* 30: 109.

Wickner RB (1997) *Prion Diseases of Mammals and Yeast: Molecular Mechanisms and Genetic Features.* Austin, TX: Landes.

PROPAGATION OF VIRUSES



Contents

Animal

Steffen Faisst, Novo Nordisk AIS, Soeborg, Denmark

Introduction

Viruses exist in two functionally distinct forms. The viral particle, referred to as virion, represents the static extracellular form without any metabolic activity, serving as a vehicle for the viral genetic material. The second, the dynamic form, consists of the viral genetic material itself once it is uncoated in a host cell. This active form of a virus uses the host cell biosynthesis machinery and energy supplies to multiply and to generate progeny virions. Hence, virus multiplication exploits host cells in a parasitic way. This results in the disturbance of normal cellular functions in many virus-infected cells, one of the basic mechanisms of viral diseases.

Virus propagation under defined laboratory conditions provides an important experimental tool in basic research in virology, allowing studies of virus multiplication, virus–host cell interactions and viral pathogenesis. Furthermore, virus propagation provides the basis for diagnoses of viral disease and vaccine production, as well as the generation of recombinant viruses for potential use in gene therapy.

Here, the mechanisms of intracellular virus multiplication are summarized, and techniques used for virus propagation, purification and titration are described.

The Virus Multiplication Cycle

To multiply, a virus has to enter a living cell. Thereafter, the viral genome is released from the capsid, and interacts with the host cell in order to replicate and to produce viral proteins. New capsids are assembled, and the newly synthesized genomes are packaged into these capsids either concomitant with or after their assembly. This results in progeny virions, which are released from the cell in order to transfer the viral genome to new host cells.

The initial step of virus–cell contact, referred to as adsorption, is mediated by the binding of a viral protein, located at the virion surface, to a receptor at the cell surface. Cellular receptors of many viruses have been identified. Most viruses adsorb to cell surface proteins that have specific metabolic functions, and that are expressed only in a subset of differentiated cells. Rabies virus, for example, binds to the acetylcholine receptor, and accordingly adsorbs to nerve cells. Human immunodeficiency virus adsorbs to CD4 molecules of T lymphocytes and macrophages, but needs co-receptors for viral entry into these cells. These co-receptors have been identified recently, and belong to the family of chemokine receptors. It should also be noted that carbohydrates have been shown to act as virus receptors. For example, polyomavirus, Sendai virus and vaccinia virus adsorb to sialyloligosaccharides of glycoproteins and glycolipids, which can be found on the surface of many cell types.

After adsorption, viruses have to cross the plasma membrane in order to enter a cell. The mechanism of penetration differs from virus to virus, depending on the respective virion structure. Adsorption to cell receptors brings viruses into intimate contact with the plasma membrane. Subsequently, viruses with a membranous envelope may directly inject the viral capsid into the cytoplasm by membrane fusion. However, the virus–receptor complex can also be internalized by coated pit-mediated endocytosis and delivered to endosomes. Subsequent fusion of viral and endosome membranes leads to the release of the viral capsid into the cytoplasm.

Viruses lacking an envelope also enter cells by coated pit-mediated endocytosis, but entry of the capsid into the cytoplasm obviously cannot occur via membrane fusion. For the non-enveloped adenovirus, evidence has been presented that capsid proteins induce the lysis of endosomes, thus releasing the capsid into the cytoplasm.

Once within the cytoplasm, the viral genome has to be liberated from the capsid and transported to the appropriate intracellular site for transcription or replication. This event is referred to as uncoating.

For RNA viruses, cellular factors like proteases are thought to dismantle the virus capsids immediately after entry, releasing the viral RNA into the cyto-

plasm, where it is replicated and translated. Also poxviruses, which contain a double-stranded DNA genome, replicate in the cytoplasm, using a virus-encoded DNA polymerase. Only retroviruses transcribe their RNA genome in DNA, which subsequently translocate to the nucleus and integrate into the cellular genome. The integrated retroviral genome serves as a template to synthesize new RNA genomes.

For nuclear-replicating DNA viruses, the capsid is supposed to be translocated along the cytoskeleton to the nuclear pores. At the nuclear pores, the viral DNA is released into the nucleus, where it is replicated.

When the viral genome is delivered to its appropriate intracellular site, it has to interact with the host cell biosynthesis machinery in order to get amplified and transcribed into mRNAs allowing the synthesis of viral (capsid and noncapsid) proteins. Thereafter, cellular and viral factors mediate the assembly of the capsids, and the packaging of the viral genome into these capsids. These steps are referred to as capsid maturation. The assembly of capsid proteins of nonenveloped viruses occurs either in the nucleus (e.g. parvoviruses, adenoviruses and papovaviruses) or in the cytoplasm (e.g. reoviruses). Most of the nonenveloped viruses rely on host cell lysis for their egress.

The assembly of the capsids of enveloped viruses also takes place either in the cytoplasm (most RNA viruses, poxviruses), or in the nucleus (herpesviruses), but the last step in virion assembly is linked to the release of the virions. Most RNA viruses egress by budding through the plasma membrane. Herpesviruses have been shown to leave the nucleus by budding through the inner nuclear membrane. They are then released from the cells by transport through the endoplasmic reticulum. Poxviruses may be released from the cells either by budding through the Golgi apparatus, or by cell disruption, resulting in enveloped and nonenveloped particles, respectively. Both enveloped and nonenveloped particles are infectious. Once progeny virus has been released, new cells can become infected, and further multiplication cycles are initiated.

Cytopathic Effect

Different types of interaction between viruses and host cells can be observed. Many viruses kill or morphologically modify their host cells when they multiply. This is called the cytopathic effect (CPE), and the respective virus is said to be cytopathogenic.

Generally, cytopathogenic viruses code for proteins that shut off synthesis of cellular macromolecules, or that are cytotoxic. Furthermore, the capsid proteins of nonenveloped viruses seem to be implicated in cell lysis, on which these viruses may rely for their egress. Enveloped viruses may additionally insert viral proteins into the cell membrane, which also may impair the viability of the target cells. Cell death within a few days is the result of productive infection with many types of viruses, such as togaviruses, picornaviruses or autonomous parvoviruses. Infection with poxviruses, reoviruses or adenoviruses also leads to cell death, but less rapidly.

Another type of CPE is the induction of cell fusion by viruses such as paramyxoviruses, human immunodeficiency viruses and herpesviruses. Induction of cell fusion is also due to the insertion of viral proteins into the host cell membrane, and results in the formation of syncytia (giant cells with up to several hundred nuclei).

As a result of mild CPEs, a balance between cell growth and virus production can sometimes be observed (e.g. after paramyxovirus infections). Such 'carrier cultures' may be seen as the cell culture counterparts of chronic infections in animals. Several RNA viruses that are not cytopathogenic (like arenaviruses and most retroviruses) may also provoke such steady-state infections.

Host Range, Permissiveness and Susceptibility

Any cell that can be infected by a virus is said to be susceptible. However, infection of a susceptible cell does not necessarily result in a productive infection. Productive infections occur only in cells able to support a complete viral multiplication cycle. Such cells are said to be permissive. In terms of cell cultures, the spectrum of permissive cells makes up the host range of a virus. However, the host range may also define the animal species that support a productive infection.

The host range of viruses may be wide, but it may also be very limited. For example, the host range of the parvovirus H-1 comprises humans, monkey, hamster and rat as animal species, and most cell types (fibroblasts, keratinocytes, lymphocytes) of human, monkey, hamster or rat origin. On the other hand, the host range of B19, another parvovirus, is restricted to humans and human erythroid precursor cells.

Infections of susceptible, but nonpermissive cells do not result in virus production. Three distinct types of such nonproductive infections have been described, and are referred to as abortive, latent and restrictive infections, respectively.

An abortive infection occurs in susceptible cells, which sustain some, but not all steps of the viral multiplication cycle. As stated earlier, after successful

entry of a virus into a cell, the viral genome has to become uncoated, amplified and expressed. Newly synthesized genomes have to be packaged into progeny particles that need to be released from infected cells.

Virus multiplication can be blocked at all stages of this cycle in susceptible, but nonpermissive cells. For example, the polyoma virus multiplication cycle is blocked at the stage of DNA replication and transcription in embryonal carcinoma cells lacking specific DNA-binding proteins. Similarly, the multiplication of the parvoviruses H-1 and MVM is blocked at the level of DNA and RNA synthesis in some cells that are refractory to virus propagation. Furthermore, some diploid cell strains infected with parvovirus H-1 are proficient in capsid assembly, but no DNA is packaged, and no particles are released from the cells. Cellular functions implicated in these last stages of infection have not yet been identified.

It should also be stated that infection of a permissive cell with a defective virus, lacking one or several essential viral functions, results in an abortive infection. During abortive infections, the virus may exert different cytopathic effects, depending on the viral functions that the host cells allow to be expressed. Hence, an abortive infection may result in cell death, if the virus was able to express its cytotoxic proteins, but may also be inapparent.

A latent infection consists of the persistence of viral genomes in infected cells for many cell generations. The viral genomes may persist in an integrated or in an episomal state or both. For example, retroviruses have been found as 'endogenous viruses' in human cells, and have been calculated to persist in these cells since approximately 40 million years. Such latent infections do not lead to host cell killing, but may nevertheless influence biologic characteristics of the cells. As an extreme example, persistence of tumor viruses is associated with cellular transformation leading to increased growth rates, abnormal cell morphology, altered cell metabolism and chromosomal aberrations.

Most viruses establishing latent infections can be rescued from their host cells, provided they have not become defective. Latent infections that can become productive are called persistent infections. To convert a latent into a productive infection, specific treatments have to be applied. For example, superinfection with adenovirus results in a productive infection of persistent adeno-associated viruses, and treatment with phorbol esters activates the multiplication cycle of persistent human immunodeficiency virus type 1 in the human macrophage cell line U1.

Finally, a rare type of nonproductive infection is the restrictive infection, that is observed in cell cultures where only a small subset of the cell population is permissive, or where the cells are only transiently permissive.

Virus Propagation in Whole Organisms

In initial studies in virology, the experimental tools for virus propagation and purification were whole laboratory animals or embryonated hen's eggs. Laboratory animals can be infected using the natural route of virus entry. To achieve this, the virus must be brought into contact with either the skin (e.g. papillomaviruses), the digestive tract (e.g. enteroviruses), the respiratory tract (e.g. orthomyxoviruses) or the conjunctiva (e.g. herpes simplex virus). Viral replication may be confined to the site of entry, or progeny virions may spread through the body (generally via the blood or lymphatic stream) with subsequent targeting to specific organs. Laboratory animals also can be infected by injecting the virus directly into specific organs (e.g. the brain for rabies virus). Several days or weeks after infection, the animals are killed, and cell-free extracts from the organs sustaining virus multiplication are used as a source of virus.

Furthermore, fertilized hen eggs can be infected after several days of incubation (depending on the stage of embryonic development required). Most viruses can be grown in the embryonic membranes of fertilized eggs, i.e. the yolk sac (e.g. herpesviruses), the chorion (e.g. poxviruses), the allantois (e.g. influenza virus) and the amnion (e.g. mumps virus). Although laboratory animals or embryonated eggs are still the most appropriate propagation systems for some viruses (animals for arboviruses, coxsackieviruses and rabies virus; eggs for orthomyxoviruses), they tend to be replaced by cell cultures, which are much more convenient to handle. For present-day virology, the use of animals is restricted mostly to research on viral pathogenesis and for production of vaccines.

Virus Propagation in Cell Culture

Multiplicity of infection, defective-interfering particles

The term 'cell culture' refers to *in vitro* cultures derived from dispersed cells taken from original tissues by enzymatical, mechanical or chemical disaggregation. Cultured cells may serve as hosts for the propagation of a number of viruses, provided these cells express all the factors allowing a complete viral replication cycle.

The first cells to be cultured were primary cells freshly isolated from animal tissues. The growth of

these cultures is restricted to five to ten cell generations at most, which limits their value for routine virus propagation. Such primary cultures contain a variety of cell types providing a broad viral spectrum. Thus, they represent a system in which as yet uncharacterized viruses can be propagated and isolated with a high probability of success, and are therefore valuable in virus diagnosis.

Cell lines derived from cancer tissues, capable of indefinite growth in culture, and strains of diploid cells prepared from human or animal tissues (in particular from embryos), capable of growing in culture up to 100 cell generations, contain cells of a specific type and allow the propagation of viruses under defined conditions. The choice of the most suitable cell line to propagate a virus will depend mainly on the host range and tissue specificity of the virus investigated. Continuous cell lines are useful for the propagation of viruses such as adenoviruses and rhinoviruses. Insect viruses (e.g. baculoviruses) can be propagated *in vitro* in immortal insect cells (e.g. SF cells, derived from *Spodoptera frugiperda* pupal ovarian tissue). Other viruses, like herpesviruses or enteroviruses, may preferentially be propagated in diploid cell strains of finite life.

However, attempts to propagate a series of viruses in cultured cells failed. This is mainly due to differences in cell behavior between cultured cells and their counterparts *in vivo*. Many of these differences stem from the dissociation of cells from a three-dimensional geometry and the lack of several systemic compounds involved in homeostatic regulation *in vivo*. As a consequence, cellular metabolism may not be truly representative of the tissue from which the cells were derived (see also below, 'Virus Propagation in Three-Dimensional Cell Culture Systems'). Hepadnaviruses, for example, require liver-specific receptor(s) to enter their host cells. In cell culture, only primary liver cells fulfill this requirement. Continuous hepatoma cell lines, however, do not express these receptor(s) in culture and therefore prohibit hepadnavirus propagation *in vitro*. Most data on the hepadnavirus replication cycle have therefore been obtained by genetic approaches using transfection of terminally redundant hepadnavirus DNA constructs, directing the synthesis of the RNA pregenome, into cultured hepatoma cells. This method allows only a single round of virus production, and precludes the study of early steps of the viral replication cycle such as the uncoating of the viral genome and its conversion into covalently closed circular DNA. Recently, receptor(s) required for internalization of hepadnavirus particles have been identified and cloned. The availability of cloned receptor genes now will allow cultured hepatoma cells to be genetically engineered in order to induce constitutive expression of these molecule(s) on their surface. Such cells subsequently may represent an efficient *in vitro* system for hepadnavirus propagation.

To obtain the best yield of progeny virus, it is important to determine the optimal conditions for the initial infection of the culture. One important parameter is the ratio of the number of cells to the number of infectious particles inoculated. This ratio is called multiplicity of infection (moi). A multiplicity of infection of two (moi = 2) defines an infection with an average of two infectious virus particles per exposed cell.

Most, if not all, viruses generate defective genomes, and, consequently, defective particles during their multiplication. This defectiveness is due to deletions in the viral genome, which render the virus incapable of expressing all the functions needed for a productive infection. These particles can arise because their defectiveness is complemented by genes of co-infecting 'wild-type' viruses. Therefore, the formation of defective particles is favored by infections at high moi. Adenovirus or parvovirus preparations may contain up to 1000 defective particles per infectious virion. In order to generate as few defective particles as possible, the standard protocol for the propagation of many viruses involves an initial infection at moi of 1×10^{-3} (i.e. one infectious virion per 1000 cells).

Infections are usually performed under cell culture conditions (37°C, pH 7.4, 5% CO_2) for 30 min to 1 h. The volume of the solution in which cells and viruses are brought together should be as small as possible in order to enhance the probability of host cell–virus contact. The presence of cations facilitates the adsorption process of most viruses. After infection, cell cultures are maintained at 37°C, except in the case of infections with viruses such as rhinoviruses or coronaviruses, which multiply best at 33°C, the temperature encountered in the nasal mucosa. After one or several multiplication cycles, progeny virus can be purified from the infected cultures.

Virus propagation in three-dimensional cell culture systems

As mentioned above for hepadnaviruses, papillomaviruses also cannot be propagated in conventional cell cultures. *In vivo,* papillomaviruses infect basal epithelial cells and establish their genomes as extrachromosomal elements. Following cell division, infected daughter cells migrate from the basal region and begin the process of differentiation. Upon terminal differentiation, papillomavirus genome amplification, late gene expression and virion assembly

are induced. Hence, attempts to propagate papillomaviruses in conventional cell cultures, which do not allow the cells to terminally differentiate, have not been successful, but were facilitated by the utilization of three-dimensional (3D) cell cultures (also called organotypic cultures or raft cultures). In organotypic cultures, cells of different lineages are recombined in a 3D matrix in spatial relationships and in experimentally determined ratios to recreate a component of the organ under study. Organotypic cultures of epithelial cells, for example, recreate important features, both morphological and physiological, of epithelial differentiation *in vitro* by raising the cells to an air–liquid interface, and even allow the achievement of terminal differentiation after treatment with 12-*O*-tetradecanoyl phorbol-13-acetate (TPA). When epithelial cells containing extrachromosomal papillomavirus genomes were grown in raft cultures, differentiation-dependent viral late gene expression, genome amplification and virion biosynthesis were observed.

Virus Purification

Viruses are purified from tissue (culture) homogenates, using various fractionation procedures. Different methods can be used depending on the physicochemical properties of the virions to be purified. Most virions are very sensitive to inactivation by heat, acid, alkali and lipid solvents. Accordingly, in most purification protocols, the virus is maintained at neutral pH and 4°C.

Generally, the supernatant of infected cell cultures provides a relatively clean virus suspension, but in some instances, viruses must be released by breaking up the cells by sonication, homogenization, or repeated freeze–thaw cycles. Thereafter, viruses can be concentrated and partially purified from cellular debris by adsorbing viral particles to erythrocytes, DEAE cellulose, aluminum hydroxide or ion-exchange resins. Subsequently, virions are eluted with buffers of specific pH and ionic strength. Viruses may also be precipitated with ammonium sulfate.

These partially purified viruses can be further separated from contaminants by physical methods, in particular by centrifugation. Differential centrifugation, which consists of multiple centrifugation cycles at increasing speed, is used to pellet first the contaminants and then the virions. Centrifugation through a cushion of a dense sucrose solution or through a preformed sucrose gradient (rate zonal centrifugation) separates viruses from contaminants based on their size and shape, i.e. their sedimentation coefficient. Equilibrium centrifugation in cesium chloride or potassium tartrate solutions is used to purify viruses as a function of their buoyant density.

Since virus purification aims at the elimination not only of cellular debris but also of defective viral material, equilibrium centrifugation may be the method of choice. This method allows the separation of infectious virions, defective particles, and empty capsids, which generally band at distinct densities (for example, infectious, defective and empty particles of the parvovirus H-1 have a density of 1.41, 1.38 and 1.32 g ml^{-1}, respectively). Before equilibrium centrifugation, it may be useful to treat the virus suspension with DNase, since contaminating cellular or viral DNA stick to some viruses and therefore would be copurified.

Virus preparations can be concentrated by ultracentrifugation, freeze-drying or dialysis against hydrophilic agents such as polyethylene glycol. Generally, concentration will be the last step of a purification protocol, but may be useful before centrifugation steps. Most viruses are heat labile. Therefore, virus preparations should be stored as cold as possible. As a rule, the half-life of many (enveloped) viruses will be years at −196°C, months at −70°C, days at 4°C and only hours at 20°C or minutes at 37°C.

Virus Titration

There is a variety of ways to determine the amount of virus in a preparation. Two types of methods should be distinguished: (1) infectivity assays that measure the amount of infectious virions in a preparation; and (2) particle assays that determine the number of all virus particles.

For lytic viruses, the most commonly used infectivity assay is the plaque assay. Monolayers of highly permissive cells are infected with serial dilutions of the virus suspension to be tested. After infection, the cells are overlaid with a semisolid agarose gel containing the culture medium, in order to restrict spread of progeny virus to cells next to the initially infected cells. Each infectious particle will give rise to a focus of infected cells, which can be seen as an area of CPE. The number of areas of CPE (plaques) within a cell monolayer can be easily counted after staining of the cell monolayer with a vital dye such as neutral red. Living cells stain red, and the areas of CPE appear as clear plaques against a red background (**Fig. 1**). From the number of plaques detected by this method, the number of infectious particles in a virus suspension can be calculated, and is expressed in terms of plaque-forming units (PFU) per milliliter.

To determine the titer of noncytopathogenic viruses, *in situ* hybridization assays can be used. Prerequisites for this test are the identification of permissive cells allowing the amplification of the viral

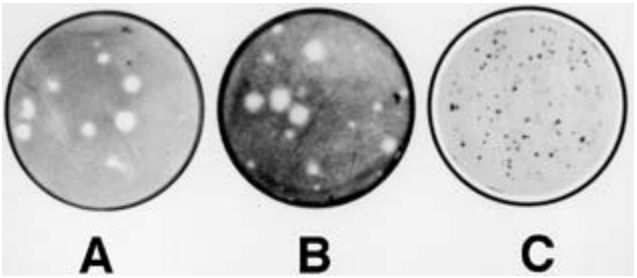


Figure 1 Virus titration. Plaque assay of the papovavirus simian virus type 40 (SV40) yieding clear-cut plaques (**A**) and of the parvovirus minute virus of mice (MVM) yielding more diffuse plaques (**B**). Areas of virus-induced cytopathic effect appear as clear plaques within the cell monolayer which has been stained with the vital dye Neutral Red. (**C**) An *in situ*-hybridization assay of an MVM-infected cell monolayer in which single cells containing amplified viral DNA appear as black dots. From the number of plaques (**A, B**) and dots (**C**), respectively, the total number of infectious particles in the virus suspension used for infection can be calculated. For more details see text.

DNA by factors of more than 200, and the availability of corresponding viral DNA that can be used as a probe. After infection of the cells, at the time when DNA amplification is maximal but before release of progeny virus, the monolayer is transferred to a nitrocellulose membrane. Cells attached to the membrane are subsequently lysed by alkali treatment, and the DNA (cellular and viral) is fixed to the membrane. A single cell that was infected and the viral DNA amplified, can be detected by autoradiography after hybridization of the filter-bound DNA with the corresponding radioactively labeled viral DNA (**Fig. 1**). Viral titers determined by this method give the number of viral particles within a virus suspension which can infect and replicate in a host cell, irrespective of whether this infection is productive or not.

Particle assays are much faster and simpler to handle. Two methods are widely used: the hemagglutination assay and particle counting by electron microscopy. Hemagglutination assays are based on the capacity of many viruses to adsorb to erythrocytes. Serial dilutions of a virus suspension are incubated with red blood cells. The viruses can bridge red blood cells, preventing them from precipitating. The virus dilution no longer capable of agglutinating erythrocytes gives a measure of the particle content of the suspension, and is expressed in terms of hemagglutinating units (HAU). This test is not very sensitive and reflects the presence of all the viral particles capable of binding to red blood cells, including defective particles and empty capsids.

Virus particles can also be counted directly by electron microscopy after negative staining. A known volume of virus suspension is deposited on a formvar-coated or carbon-coated copper grid, water and salts are removed, and viruses are subsequently negatively stained and counted. Since some virus may be lost during the washing and staining processes, it is convenient to add a marker to the virus suspension, e.g. in the form of a known concentration of latex particles. The number of latex particles counted enables the determination of particle loss during preparation and therefore allows the original virus particle concentration to be determined.

See also: **Cell structure and function in virus infections; Defective interfering viruses; Diagnostic techniques: Isolation and identification by culture and microscopy; Replication of viruses; Vectors: Animal viruses, Plant viruses; Viral receptors; Virus–host cell interactions.**

Further Reading

Freshney RI (1987) *Culture of Animal Cells. A Manual of Basic Technique*, 2nd edn. New York: Wiley–Liss.

Bacteria

Gail E Christie, Department of Microbiology and Immunology, Virginia Commonwealth University, Richmond, Virginia, USA

Introduction

Bacterial viruses, or bacteriophages, consist of a genome, either DNA or RNA, encapsidated in a protein coat. Occasionally this nucleoprotein capsid may also be surrounded by a membrane, derived from that of the host cell. The virus particle, or virion, serves to protect the nucleic acid and deliver it into a susceptible bacterial host. Expression of the viral genome results in infection of the host cell and production of new progeny phages. Bacteriophage multiplication depends upon the macromolecular synthetic machinery and energy generating systems of the infected bacterial cell. There are different outcomes of bacteriophage infection, depending on the nature of the interactions of a particular type of phage with the bacterial host. Virulent phages are those which invariably follow a lytic cycle of multiplication, resulting in the destruction of the infected cell and release of progeny phages. Temperate phages can undergo a similar lytic cycle, but also have an alternative mode of propagation in which their

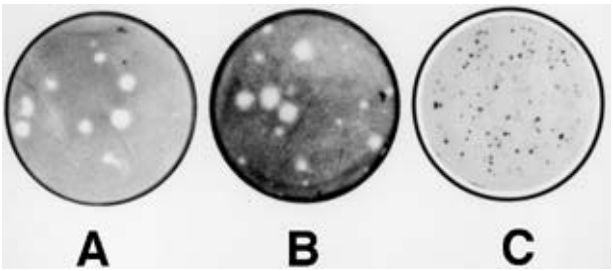


Figure 1 Virus titration. Plaque assay of the papovavirus simian virus type 40 (SV40) yieding clear-cut plaques (**A**) and of the parvovirus minute virus of mice (MVM) yielding more diffuse plaques (**B**). Areas of virus-induced cytopathic effect appear as clear plaques within the cell monolayer which has been stained with the vital dye Neutral Red. (**C**) An *in situ*-hybridization assay of an MVM-infected cell monolayer in which single cells containing amplified viral DNA appear as black dots. From the number of plaques (**A, B**) and dots (**C**), respectively, the total number of infectious particles in the virus suspension used for infection can be calculated. For more details see text.

DNA by factors of more than 200, and the availability of corresponding viral DNA that can be used as a probe. After infection of the cells, at the time when DNA amplification is maximal but before release of progeny virus, the monolayer is transferred to a nitrocellulose membrane. Cells attached to the membrane are subsequently lysed by alkali treatment, and the DNA (cellular and viral) is fixed to the membrane. A single cell that was infected and the viral DNA amplified, can be detected by autoradiography after hybridization of the filter-bound DNA with the corresponding radioactively labeled viral DNA (**Fig. 1**). Viral titers determined by this method give the number of viral particles within a virus suspension which can infect and replicate in a host cell, irrespective of whether this infection is productive or not.

Particle assays are much faster and simpler to handle. Two methods are widely used: the hemagglutination assay and particle counting by electron microscopy. Hemagglutination assays are based on the capacity of many viruses to adsorb to erythrocytes. Serial dilutions of a virus suspension are incubated with red blood cells. The viruses can bridge red blood cells, preventing them from precipitating. The virus dilution no longer capable of agglutinating erythrocytes gives a measure of the particle content of the suspension, and is expressed in terms of hemagglutinating units (HAU). This test is not very sensitive and reflects the presence of all the viral particles capable of binding to red blood cells, including defective particles and empty capsids.

Virus particles can also be counted directly by electron microscopy after negative staining. A known volume of virus suspension is deposited on a formvar-coated or carbon-coated copper grid, water and salts are removed, and viruses are subsequently negatively stained and counted. Since some virus may be lost during the washing and staining processes, it is convenient to add a marker to the virus suspension, e.g. in the form of a known concentration of latex particles. The number of latex particles counted enables the determination of particle loss during preparation and therefore allows the original virus particle concentration to be determined.

See also: **Cell structure and function in virus infections; Defective interfering viruses; Diagnostic techniques: Isolation and identification by culture and microscopy; Replication of viruses; Vectors: Animal viruses, Plant viruses; Viral receptors; Virus–host cell interactions.**

Further Reading

Freshney RI (1987) *Culture of Animal Cells. A Manual of Basic Technique,* 2nd edn. New York: Wiley–Liss.

Bacteria

Gail E Christie, Department of Microbiology and Immunology, Virginia Commonwealth University, Richmond, Virginia, USA

Introduction

Bacterial viruses, or bacteriophages, consist of a genome, either DNA or RNA, encapsidated in a protein coat. Occasionally this nucleoprotein capsid may also be surrounded by a membrane, derived from that of the host cell. The virus particle, or virion, serves to protect the nucleic acid and deliver it into a susceptible bacterial host. Expression of the viral genome results in infection of the host cell and production of new progeny phages. Bacteriophage multiplication depends upon the macromolecular synthetic machinery and energy generating systems of the infected bacterial cell. There are different outcomes of bacteriophage infection, depending on the nature of the interactions of a particular type of phage with the bacterial host. Virulent phages are those which invariably follow a lytic cycle of multiplication, resulting in the destruction of the infected cell and release of progeny phages. Temperate phages can undergo a similar lytic cycle, but also have an alternative mode of propagation in which their

genome can be maintained stably for many generations within the bacterial host. Filamentous phages and some mycoplasma viruses establish an ongoing infection within a bacterial cell, leading to the continuous production and release of progeny phage without killing the host.

Bacteriophages are generally grown under environmental conditions that are optimal for the host bacterium. Susceptibility of a bacterial strain to infection by a particular phage is determined primarily by the presence of the appropriate receptors on the bacterial cell surface. Other factors that can play a role in host range include restriction/modification systems, immunity or resistance encoded by resident prophages or plasmids, and the availability of specific bacterial enzymes required by the phage for replication or gene expression. In the case of satellite phages, lytic growth requires the presence of a suitable helper phage, usually present as a prophage in the bacterial host.

This entry summarizes mechanisms involved in the different modes of bacteriophage propagation and provides an overview of basic methods used in the quantitative study of bacteriophage infection and in preparation of bacteriophage stocks from infected cells.

Virus Multiplication Cycle

Adsorption and infection

Viral infection is initiated by the adsorption of the infecting particle to a bacterial cell. This attachment involves highly specific interactions between specialized phage structures and receptors on the bacterial cell surface. Most bacteriophages are tailed, and use tail fibers or tail spikes for adsorption. Other phages may use apical spikes or special attachment proteins; some lipid-containing phages adsorb by their envelopes. A variety of bacterial cell wall components are known to be exploited as bacteriophage receptors. Outer membrane proteins involved in uptake systems for other molecules may be used. For example, phage T1 binds to proteins involved in the transport of ferrichrome, while λ adsorption requires proteins involved in maltose transport. O-antigen side chains and residues in the lipopolysaccharide core of Gram-negative bacteria and complexes of peptidoglycan with carbohydrate or teichoic acid in Gram-positive bacteria have also been identified as receptors. Capsules or extracellular slime are used as the initial site of adsorption by certain phages, as are pili and flagella. Adsorption to the plasma membranes of mycoplasmas has also been documented. Cofactors may be required for adsorption. Ca^{2+} is the most frequently required cofactor, followed by Mg^{2+}. These divalent cations sometimes also play a role in stabilizing the phage particle. Other known cofactors include NaCl, in the case of some marine phages and cyanophages, and L-tryptophan, which is required by some T-even phages for proper unfolding of the tail fibers.

Following adsorption, the bacteriophage genome enters the cell and initiates infection. The protein coat generally remains outside the cell, although in the case of the fd-type filamentous phages and cystovirus φ6 the capsid has been shown to penetrate the cell wall but does not enter the cytoplasm. Occasionally, specific viral proteins are also injected; examples of this include the spike protein of φX174, N4 RNA polymerase, and the T4 *alt* gene product. Phages that adsorb to porins or other specific outer membrane receptors are thought to take advantage of the solute transport channels of these membrane complexes during DNA penetration. Electron microscopy of infected cells indicates that the tails or tail cores of some phages penetrate the cell wall and provide a channel for DNA entry. Structural components of some phage baseplates also possess lysozyme activity. Adhesion zones between the inner and outer membranes have been proposed as possible entry sites for some phages. Even in well-studied bacteriophages, however, the process of nucleic acid penetration is still poorly understood.

Lytic cycle

Expression of functions encoded by the bacteriophage genome results in the takeover of the host biosynthetic machinery for viral replication. Host DNA, RNA and protein synthesis are shut off to varying degrees by different phages. Phages with large genomes depend less on host functions than those with small genomes, and may encode redundant functions, particularly for nucleic acid metabolism. Phage gene expression is regulated largely at the transcriptional level, except in the case of the single-stranded (ss) RNA phages, in which gene expression is regulated by the secondary structure of the viral RNA. Transcription generally occurs in discrete temporal stages, reflecting the order in which the various phage functions are required during infection. Genes expressed early in infection encode proteins needed for phage replication and shut-off of host functions, while those expressed late are involved in phage assembly and lysis of the bacterial host. For some of the larger phages with more complicated regulatory circuitry, temporal expression is further subdivided into additional classes, while some small phages, such as φX174, express all genes more or less simultaneously. With few exceptions, early transcription is carried out by the host RNA polymerase while

later phases of transcription use phage-encoded RNA polymerases or phage-modified forms of the host transcriptional machinery. Phages N4 and PBS2, however, coinject phage-encoded RNA polymerases into the host cell that are used immediately for phage-specific transcription.

Phage nucleic acid replication depends on host functions, although certain bacteriophages encode their own polymerases or replicase subunits. Modes of bacteriophage genome replication vary widely, depending on the phage. Phages with single-stranded DNA or RNA genomes first synthesize a complementary strand. Replicating templates can be linear or circular, and replication may be uni- or bidirectional. The product of replication that is used as the packaging substrate may be a unit-length genome or a concatemer of multiple genomic copies.

Bacteriophage maturation is a highly regulated process in which the structural proteins (and in a few instances lipids) of the phage particle are assembled and the nucleic acid is packaged. The tailed phages assemble heads and tails in separate pathways; these components are then attached after encapsidation of the DNA. Entry of phage nucleic acid into preformed capsids or shells has also been shown to occur in most of the cubic phages. In the case of the ssRNA phages, however, coat protein aggregates around the viral RNA. Encapsidation is also quite different in the filamentous phages and plasmaviruses, as discussed below.

The lytic cycle, as its name implies, ends with the release of progeny phage by lysis of the infected cell. Tailed phages generally employ a two-component system involving a murein hydrolase that breaks down either glycosidic or peptide bonds in the peptidoglycan and a transmembrane holin that promotes access of the endolysin to the peptidoglycan across the cytoplasmic membrane. This strategy leads to total cellular destruction, accompanied by a characteristic loss of turbidity in a liquid culture of infected cells. The microvirus ϕX174 encodes a single lysis function that appears to form septal lesions in the cell wall which allow release of the cytoplasmic contents, leaving behind rod-shaped, nonrefractile ghosts. The RNA phages also encode a single lysis protein that is thought to be a transmembrane protein rather than a lysozyme, but the mechanism of action is not understood. For plasmaviruses and filamentous phages, infected cells are not killed and progeny phage are released by budding or extrusion, respectively.

Lysogenic cycle

The ability to undergo a stable association with a bacterial host, termed lysogeny, is a property exhibited by many phages. Bacteriophages capable of establishing lysogeny are called temperate. Most temperate phages are tailed phages with double-stranded (ds) DNA genomes, although at least one filamentous phage and one plasmavirus have also been reported to form lysogens. Following adsorption and entry of the phage nucleic acid, a temperate phage undergoes a choice between two alternate pathways. It can either enter the lytic cycle, in which the genome is replicated and progeny phage are produced, or the phage can establish a latent prophage state, in which the bacteriophage genome is replicated indefinitely along with the bacterial chromosome. Many temperate phages integrate their genomes into the bacterial chromosome during the establishment of lysogeny; for most of these phages the integration occurs at specific locations, called attachment sites. There are also examples of phages that replicate in the prophage state as either circular or linear plasmids; these include P1, satellite phage P4 and N15.

The prophage state is maintained by a phage-encoded immunity repressor that prevents expression of lytic phage functions. Synthesis of this repressor, which is usually a protein but can also be a regulatory RNA, confers upon the lysogenic bacterium immunity to superinfection by homologous phages. Prophages frequently encode additional functions that impart novel properties in addition to superinfection immunity to a lysogenic host, a phenomenon called lysogenic conversion. Examples of new properties that may be acquired by lysogenic bacteria include resistance to other unrelated phages, production of toxins, alterations in colony morphology, production of new surface antigens or receptors, and resistance to antibiotics. These properties persist as long as the cell retains the prophage.

Prophage loss occurs spontaneously at a low rate. If a prophage escapes repressor control, it can undergo vegetative replication and lyse the bacterial host. Cultures of lysogenic bacteria frequently contain a small number of free phages due to spontaneous release from a few cells in which lytic multiplication has occurred. Many prophages can also be induced, most commonly by agents that cause direct DNA damage or interfere with DNA replication and activate the RecA-dependent SOS pathway in the bacterial host.

Nonlytic multiplication

Filamentous phages of Gram-negative bacteria do not cause bacterial cell lysis. Phage replication is tightly controlled and the infected cells continue to grow and divide, albeit more slowly than usual. Assembly of filamentous phage particles, which contain a long

loop of circular single-stranded DNA surrounded by multiple copies of the major coat protein, occurs as the phage is secreted from the cell. Coat protein is inserted into the inner membrane of the infected cell via a signal sequence that is subsequently removed. Newly synthesized intracellular phage DNA, covered by a phage-encoded ssDNA binding protein, is drawn to the inner surface of the cytoplasmic membrane, where assembly begins. As the growing filament is extruded from the cell, the ssDNA binding protein is shed and replaced by a helical array of subunits of the major coat protein. The two ends of the filament are capped by structures formed by minor coat proteins that are present in 4–5 copies per phage particle. Bacteria infected by filamentous phages produce progeny phage on a continual basis, at a rate of several hundred phage per cell per generation.

Plasmaviruses are spherical enveloped viruses that infect mycoplasmas. Following infection, there is a latent period of up to 2 hours followed by a 'rise' period of 4–6 hours during which progeny viruses are released from an infected cell. Infected cells grow more slowly than uninfected cells, but the infectious cycle does not kill the bacteria. The plasmavirus DNA acquires an envelope by budding of the cell membrane. The plasmavirus genome can also integrate into the host chromosome during the rise period, leading to the establishment of lysogeny. A second group of mycoplasma viruses, the plectroviruses, also has a nonlytic infectious cycle. These viruses encapsidate their ssDNA genomes in a short rod-shaped particle. Virus assembly and release by extrusion through the cell membrane are coincident, similar to what is seen in the filamentous phages, but details of the process are not known. Progeny phage are produced continuously throughout the infectious cycle, which lasts for several hours.

Plating, Titration and Measurement of Growth Parameters

The plaque assay is a critical tool for phage isolation and quantitation. The number of infectious phage particles in a sample is measured in 'plaque-forming units' or pfu. Titration of a phage suspension is carried out by mixing serial dilutions of the sample with aliquots of sensitive bacteria. This mixture is plated in a soft agar overlay on a petri dish containing the appropriate nutrient agar and incubated, usually overnight. Individual infected cells release phages that infect the surrounding cells in the bacterial layer, and multiple rounds of infection continue until growth of the bacteria on the plate ceases due to nutrient depletion. At suitable phage dilutions, this will lead to the appearance of individual holes, or plaques, in an otherwise confluent bacterial lawn. Since each plaque is derived from a single viable phage, counting the number of plaques allows calculation of the number of plaque-forming units in the original suspension.

Plaque purification is used for the isolation of pure phage populations. Individual plaques can be picked (with a sterile toothpick or pasteur pipette), resuspended and replated prior to large-scale growth to ensure that the resulting phage preparation is genetically homogeneous.

A particular phage will exhibit a characteristic plaque morphology. Virulent phages produce clear plaques, and the plaques formed by phages with large genomes tend to be smaller than the plaques formed by phages with small genomes. Temperate phages give rise to turbid plaques, due to the growth of surviving lysogenic bacteria within the zone of lysis. Growth of lysogens is often heavier in the center of the plaque, with a surrounding clear margin. Plaques formed by filamentous phages and the mycoplasma viruses that have nonlytic infectious cycles are also turbid in appearance; these plaques are actually foci of infected cells that are growing more slowly than the surrounding bacteria and may disappear upon prolonged incubation of the plates. Plaques with a turbid appearance may also result from partially lysed cells or accumulation of plasma membranes, rather than growth of surviving bacteria. Phages infecting bacteria that produce slime or capsule often form plaques surrounded by halos due to the breakdown of capsule in the surrounding cells. Plating conditions can affect plaque size: a low moisture content in the agar or a dense bacterial inoculum will reduce the size of the plaques.

The parameters of latent period and burst size are often used to describe bacteriophage growth. The latent period is the time interval between the initial infection and the release of progeny phages. The burst size is the number of new phages released by a single infected cell. Both of these parameters can be measured by a one-step growth experiment, in which phage production by synchronously infected bacteria is monitored. A culture of exponentially growing bacteria is infected with phages, incubated for a short time to allow adsorption, and then diluted or treated with phage-specific antiserum to prevent further infection by unadsorbed phages. The diluted culture is then incubated further and samples are removed at various time intervals and assayed for plaque-forming units. The number of plaque-forming units will remain constant for a certain time interval; this corresponds to the latent period. There is then a period during which the phage titer rises rapidly, followed by a plateau when no further increase in

plaque-forming units occurs. The increase in titer corresponds to the burst, and the ratio of the final titer at the plateau to the initial titer of phage-infected bacteria is the average burst size. Those phages that are released from infected cells continuously by extrusion or budding will not yield a discrete burst, but the number of progeny viruses released from individual infected cells as a function of time has still been estimated using this method.

Preparation of High-Titer Stocks

To obtain a high yield of progeny phage, culture conditions that are optimal for the bacterial host should be used. Sensitive bacteria must be actively growing when infected, and at a sufficiently low density to allow multiple rounds of phage infection before the culture reaches stationary phase. Any necessary cofactors for adsorption or phage stabilization (e.g. Ca^{2+} or Mg^{2+} salts) must also be added. Bacteriophages can be propagated on solid media or in liquid culture, and there are commonly used techniques employing both approaches.

Propagation on solid media involves plating a mixture of bacterial cells, at a concentration that will result in a uniform lawn, in a soft agar overlay with just enough phages to give confluent or nearly confluent lysis after a suitable incubation time (generally overnight, although for phages of some slow-growing bacterial species it will take longer). Once lysis has occurred, the top agar is scraped off and suspended in a small volume of appropriate buffer. Alternatively, a small volume of buffer can be added to the plate, allowed to stand for a period of time to allow diffusion of the phage particles into the buffer, and then collected. The phage suspension must then be centrifuged at low speed to sediment agar and cell debris. This centrifugation is not generally sufficient to sterilize the phage stock. A common practice is the addition of a few drops of chloroform to the lysate to kill any remaining bacteria; while this works well for certain phages it is risky as a general practice because many phages are chloroform sensitive. Further purification of the phage suspension to ensure that it contains no viable bacteria can be accomplished by filtration through membrane filters with a pore size of 0.45 μm.

Propagation in liquid media is the method of choice for large-scale phage production. The optimum ratio of infecting phage to sensitive cells (multiplicity of infection) will vary, depending on the particular phage and host, but the bacterial culture should be young and actively growing. Following an adsorption step, the infected cells are allowed to grow for several cycles of infection under culture conditions that support good bacterial growth. Visible lysis of the culture may or may not occur, depending on the phage. Release of intracellular phages from unlysed cells can be accomplished in some cases by treatment with chloroform, trypsin or lysozyme. For phages that require divalent cations for adsorption, phage yield can be improved by adding EDTA or EGTA before harvesting to block adsorption to cell debris. If the phage requires Mg^{2+} for stabilization, EGTA is preferable since it will chelate Ca^{2+} but not Mg^{2+}. Cultures of lysogenic bacteria can be grown to mid-log phase and then induced; in this case nearly all cells are simultaneously infected by the induced prophages, and progeny are released after a single round of infection. Phages released by budding or extrusion are harvested from the culture medium after a suitable period of bacterial growth.

Concentration and Purification

Once most of the bacteria and debris have been removed from a lysate by low speed centrifugation, phages are generally further concentrated and purified. Direct ultracentrifugation can be used for small-scale phage preparation; the lysate is frequently treated with DNase and RNase prior to ultracentrifugation to degrade bacterial DNA and RNA released during lysis. Centrifugation at 50 000–60 000 *g* appears to be adequate for most phages. Precipitation is used to concentrate phages from large volumes of lysate. Polyethylene glycol 6000 (PEG) is an agent that appears to work for virtually all phages; ammonium acetate has also been used in some cases. For PEG precipitation, NaCl (up to 0.5 mol l^{-1}) and PEG (most commonly at a final concentration of about 10%) are dissolved in the lysate, and the PEG is then allowed to precipitate at 4°C, usually overnight. The PEG precipitate, which contains the phages as well as any remaining bacteria and bacterial debris present in the lysate, is collected by low speed centrifugation. Extraction of the PEG pellet with a small volume of buffer yields a concentrated phage suspension. Phages can be purified further by density gradient centrifugation. Equilibrium sedimentation in CsCl is commonly used. Lipid-containing phages are sensitive to CsCl, however, and are purified on sucrose gradients. After density gradient centrifugation, phages are dialyzed against a suitable storage buffer, often containing Mg^{2+} to help stabilize the phage particles. Lysates and purified phage stocks are usually stored at 4°C.

See also: **Lysogeny and prophage; Filamentous phages (*Inoviridae*); Cyanophages; Single-stranded RNA phages (*Leviviridae*); T4-like phages (*Myoviridae*); Coliphage lambda (*Siphoviridae*);**

Coliphage ϕX174 and related phages (*Microviridae*); P4 phage (*Satellites*); P2, 186 and related phages (*Myoviridae*); Mu-like phages (*Myoviridae*); Enterobacteria phage N4 (*Podoviridae*); Enterobacteria phage P1 (*Myoviridae*); Salmonella phage P22 (*Podoviridae*); Phage PRD1 (*Tectiviridae*); Phage ϕ6 (*Cystoviridae*); Bacillus phage ϕ29 (*Podoviridae*); *Bacillus subtilis* phages; SPO1 phage (*Myoviridae*); T1-like phages (*Siphoviridae*); T5-like phages (*Siphoviridae*); T7-like phages (*Podoviridae*).

Further Reading

Ackermann H-W and DuBow MS (1987) *Viruses of Prokaryotes*. Vol. 1: *General Properties of Bacteriophages*. Boca Raton: CRC Press.

Calendar R (ed.) (1988) *The Bacteriophages*. New York: Plenum Press.

Maniloff J (1988) Mycoplasma viruses. *Crit. Rev. Microbiol.* 15: 339.

Young R (1992) Bacteriophage lysis: mechanism and regulation. *Microbiol. Rev.* 56: 430.

Plant

William M Wintermantel, USDA-ARS, Salinas, California, USA

Choice of Propagative Host

One of the most important factors to consider when designing a method for virus propagation is the choice of plant material. Although many viruses are named for the plant from which they were isolated, often this is not the best host for virus propagation. When the virus is known, it is likely that literature is available to suggest suitable propagative hosts. If the virus has not been identified or is not well characterized, it will be necessary to passage the virus to a number of host plants to decide which will be suitable local lesion and propagative hosts. Local lesion hosts are particularly important when the virus may be a mixture of strains or even different viruses. Individual lesions are believed to represent infections originating from a single infectious unit. Consequently, strains can be separated from one another by establishing new infections in systemic hosts from individual lesions. Similarly, mixed viruses, and sometimes multiple strains of the same virus, can be separated from one another by inoculation to different hosts. Some standard hosts, such as *Nicotiana* spp., *Chenopodium* spp. and others, have proven useful for diagnosing and separating strains from a number of viruses.

In choosing a propagative host, the plant should be capable of being infected systemically by the virus, and should accumulate virus to high concentrations without becoming severely stunted. If possible choose a host with large, soft, spongy leaves, as such leaves produce more sap, resulting in greater virus yield. *Nicotiana* spp. are an excellent example, and work well for many viruses. Hosts which contain high levels of substances that interfere with virus purification and/or bind the virus should be avoided, particularly those with high levels of tannins, gums, waxes and phenolic compounds. Any host which contains excessive amounts of these substances can result in poor recovery of virus.

Once a host is chosen, some preliminary experiments should be conducted to identify the appropriate temperature and lighting conditions for virus propagation. A virus may prefer different conditions in different hosts, possibly reflecting the physiological variation among hosts. Virus strains may also exhibit variation dependent upon environmental conditions. Some cauliflower mosaic virus (CaMV) strains, for example, infect solanaceous hosts under conditions of low light intensity, but not high. Other strains are not affected by lighting conditions. Certain viruses, such as cucumber mosaic virus, exhibit a phenomenon known as cycling, in which virus concentration increases for several days, then declines fairly quickly. Cycling can be a host-specific phenomenon, occurring in some hosts, but not others. As a result, it is important to determine the amount of time necessary to achieve optimal virus concentration before virus titers begin to decline. Simple time-course experiments can be designed to address when and under what conditions optimal virus concentration can be achieved.

Virus Transmission

In order to propagate a plant virus effectively, it must first be passaged from the initially infected plant to the propagative host. This requires obtaining infectious virus from the original plant, and introducing it to the propagative host such that the virus is in the presence of all necessary components for infection.

Although mechanical transmission is not possible with some viruses, mechanical transmission is the simplest and most direct method of virus transmission available. A number of different methods can be used to transmit viruses mechanically, ranging from sap inoculation, to inoculation with purified virions, to inoculation with viral nucleic acid. Mechanically transmissible viruses which accumulate to reasonably

Coliphage ϕX174 and related phages (*Microviridae*); P4 phage (*Satellites*); P2, 186 and related phages (*Myoviridae*); Mu-like phages (*Myoviridae*); Enterobacteria phage N4 (*Podoviridae*); Enterobacteria phage P1 (*Myoviridae*); Salmonella phage P22 (*Podoviridae*); Phage PRD1 (*Tectiviridae*); Phage ϕ6 (*Cystoviridae*); Bacillus phage ϕ29 (*Podoviridae*); *Bacillus subtilis* phages; SPO1 phage (*Myoviridae*); T1-like phages (*Siphoviridae*); T5-like phages (*Siphoviridae*); T7-like phages (*Podoviridae*).

Further Reading

Ackermann H-W and DuBow MS (1987) *Viruses of Prokaryotes*. Vol. 1: *General Properties of Bacteriophages*. Boca Raton: CRC Press.

Calendar R (ed.) (1988) *The Bacteriophages*. New York: Plenum Press.

Maniloff J (1988) Mycoplasma viruses. *Crit. Rev. Microbiol.* 15: 339.

Young R (1992) Bacteriophage lysis: mechanism and regulation. *Microbiol. Rev.* 56: 430.

Plant

William M Wintermantel, USDA-ARS, Salinas, California, USA

Choice of Propagative Host

One of the most important factors to consider when designing a method for virus propagation is the choice of plant material. Although many viruses are named for the plant from which they were isolated, often this is not the best host for virus propagation. When the virus is known, it is likely that literature is available to suggest suitable propagative hosts. If the virus has not been identified or is not well characterized, it will be necessary to passage the virus to a number of host plants to decide which will be suitable local lesion and propagative hosts. Local lesion hosts are particularly important when the virus may be a mixture of strains or even different viruses. Individual lesions are believed to represent infections originating from a single infectious unit. Consequently, strains can be separated from one another by establishing new infections in systemic hosts from individual lesions. Similarly, mixed viruses, and sometimes multiple strains of the same virus, can be separated from one another by inoculation to different hosts. Some standard hosts, such as *Nicotiana* spp., *Chenopodium* spp. and others, have proven useful for diagnosing and separating strains from a number of viruses.

In choosing a propagative host, the plant should be capable of being infected systemically by the virus, and should accumulate virus to high concentrations without becoming severely stunted. If possible choose a host with large, soft, spongy leaves, as such leaves produce more sap, resulting in greater virus yield. *Nicotiana* spp. are an excellent example, and work well for many viruses. Hosts which contain high levels of substances that interfere with virus purification and/or bind the virus should be avoided, particularly those with high levels of tannins, gums, waxes and phenolic compounds. Any host which contains excessive amounts of these substances can result in poor recovery of virus.

Once a host is chosen, some preliminary experiments should be conducted to identify the appropriate temperature and lighting conditions for virus propagation. A virus may prefer different conditions in different hosts, possibly reflecting the physiological variation among hosts. Virus strains may also exhibit variation dependent upon environmental conditions. Some cauliflower mosaic virus (CaMV) strains, for example, infect solanaceous hosts under conditions of low light intensity, but not high. Other strains are not affected by lighting conditions. Certain viruses, such as cucumber mosaic virus, exhibit a phenomenon known as cycling, in which virus concentration increases for several days, then declines fairly quickly. Cycling can be a host-specific phenomenon, occurring in some hosts, but not others. As a result, it is important to determine the amount of time necessary to achieve optimal virus concentration before virus titers begin to decline. Simple time-course experiments can be designed to address when and under what conditions optimal virus concentration can be achieved.

Virus Transmission

In order to propagate a plant virus effectively, it must first be passaged from the initially infected plant to the propagative host. This requires obtaining infectious virus from the original plant, and introducing it to the propagative host such that the virus is in the presence of all necessary components for infection.

Although mechanical transmission is not possible with some viruses, mechanical transmission is the simplest and most direct method of virus transmission available. A number of different methods can be used to transmit viruses mechanically, ranging from sap inoculation, to inoculation with purified virions, to inoculation with viral nucleic acid. Mechanically transmissible viruses which accumulate to reasonably

high titers in the infected host can usually be prepared by grinding a small amount of infected tissue with a mortar and pestle in a suitable buffer. If the inoculation is being performed directly from triturated sap, the favored method is to dip an index finger directly in the inoculum, then gently spread the inoculum over the leaf surface, while supporting the leaf with the opposite hand. Pass over each portion of the leaf only once, otherwise excessive damage may result. Inoculation performed with gloved hands reduces the potential for contamination. Glove-free hands are more sensitive to the delicate nature of leaves, but this increases the potential for contamination. If gloves are not used, it is important to remember that hands must be thoroughly washed between inoculations to prevent cross-contamination with other inoculum. All equipment in contact with the inoculum must be free of contaminating virus. When many inoculations are to be performed, glass rods, cotton swabs or pestles also work well.

Effective inoculation usually requires that leaves be abraded by means of either carborundum dust or by Celite (diatomaceous earth) suspended in the inoculum. Carborundum is a fine metal powder that creates microwounds in leaves when rubbed on the leaf surface, allowing virus to enter through the wounds. Leaves are usually dusted with carborundum before inoculation, then washed briefly to prevent carborundum remaining on the leaves from producing scars or more wounds as the plant grows. Celite is also a fine powder, but is usually suspended with liquid inoculum and applied during inoculation to create wounding. Although Celite is more gentle on plant tissue than carborundum, it too should be washed from leaves after inoculation. Inoculation occurs almost immediately upon rubbing a leaf with a virus suspension; therefore, rinsing leaves with water soon after inoculation will not affect transmission. Although more difficult, some phloem-limited viruses can also be transmitted mechanically through stem slashing. Stem slash inoculation is performed by dipping a scalpel or razor blade in inoculum, then using the blade to make several consecutive horizontal cuts approximately halfway through one side of the stem of the plant to be inoculated. The procedure deposits virus directly in the phloem tissue of the inoculated plant. Care should be taken to avoid girdling the plant when this approach is implemented.

Some viruses are not mechanically transmissible, and must be passaged by insect, fungal or nematode vectors, or through direct vascular connections. Such connections can be established by grafting a portion of the source plant to the plant being infected. Depending on the plant, a number of grafting techniques are possible. The main advantage of grafting is to permit a direct vascular connection between the source plant and the plant being inoculated. Historically, some viruses have been transmitted through the use of the parasitic plant, dodder (*Cuscuta* spp.). Dodder establishes a vascular connection with plants, allowing virus to pass from one plant to another through its vasculature. If dodder is used, it must be from a source known to be free of contaminating viruses. Although virus transmission through dodder is no longer a common practice, dodder can facilitate movement of virus between hosts when other methods fail.

It is important to remember that any vector-transmitted virus should be passaged by the vector. Frequent mechanical transmission of vector-transmitted viruses often leads to loss of vector transmissibility. This does not mean such viruses should not be transmitted mechanically, rather that vector transmission should be performed at regular intervals to avoid selection against vector transmissibility during virus propagation. Some viruses, such as barley yellow dwarf virus which circulates in its aphid vector, require an intimate connection with the vector, as well as with plant hosts. If possible, it is best to routinely maintain and transmit all such viruses in the manner by which they are transmitted in nature.

Preserving Infectivity During Transmission and Isolation

Many mechanically transmitted viruses can be successfully transmitted by simply grinding infected tissue in 50–100 mmol l^{-1} sodium or potassium phosphate buffer with a pH between 7.0 and 8.0. This is a common buffer, used effectively for mechanical transmission of a large number of viruses. Experimentation has determined that the inclusion of phosphate in the inoculation buffer increases infectivity of the inoculum for many viruses. Although phosphate buffers of neutral pH are suitable for passaging many plant viruses, it is important to remember that each virus has unique properties, and the conditions which favor transmission of one virus may inhibit or decrease the efficiency of transmission of another. Some viruses are adversely affected by phosphate. It is sometimes possible to transmit these viruses through sap in phosphate buffer, but longer extraction procedures require alternative buffers such as sodium borate or sodium citrate. The pH of the buffer is also critical. If the pH of the buffer is not suitable, virions may swell and become unstable, or they may dissociate altogether, resulting in free nucleic acid and rapid degradation. A number of viruses require the addition of divalent cations in

specific concentrations, while still others require chelating agents, such as ethylenediaminetetra-acetic acid (EDTA), to bind such cations.

Most plant tissues contain phenolic compounds which are released upon tissue maceration and reduce infectivity. Many mechanically transmissible viruses can be prepared in buffer of the appropriate pH, and rubbed on leaves of a suitable propagative or local lesion host without substantial loss of infectivity. Other viruses are not so stable and require special care. Young leaves generally contain fewer phenolic compounds and are routinely used to avoid phenolics when these become a problem. To protect virions from degradation during processing, extraction should be performed at temperatures near 0°C, and reducing agents such as 2-mercaptoethanol, thioglycolic acid, diethyldithiocarbamate or sodium sulfite should be included in tissue grinding buffer to prevent enzymatic inactivation of inoculum during tissue homogenization. These measures are usually quite effective at preserving infectivity during virus purification. The addition of polyvinyl pyrrolidone (PVP) of varying molecular weights has also proven useful in maintaining the infectivity of some viruses. PVP complexes with tannins to prevent their interaction with the virus particle, and consequently enhances infectivity.

Virus Isolation from Propagative Hosts

Once a virus has reached optimal concentrations in the propagative host, as determined through experimentation, it can either be collected in tissue or isolated from the propagative host. In the case of the former, virus in infected leaf tissue can be collected and frozen. Some viruses are quite stable for many months when stored as frozen tissue. A more stable inoculum preservation method, however, is to freeze-dry the tissue in a lyophilizer. Leaf tissue can be cut into small pieces, frozen and dried until all moisture is removed. When the tissue is not fully dried, samples often lose infectivity more rapidly in storage. Once dried, the virus can be stored at 4°C, or −20°C. Many viruses are stable for several years when stored in this manner. Others are much less stable and may need to be stored as purified virions frozen in glycerol, or as frozen nucleic acid.

To obtain purified virions, tissue must be homogenized. This is usually performed in a blender because large volumes of tissue can be ground rapidly and effectively. Some researchers do not advocate the use of blenders for viruses which are composed of long rod-shaped particles, as these particles may be sheared by the blender. Long rods are protected well by grinding tissue in a mortar with liquid nitrogen, then adding buffer to the pulverized tissue. This procedure also works well for extraction of many phloem-limited viruses. Others claim, however, that blenders, particularly Virtis top-driven blenders, actually facilitate extraction of infectious long flexuous rods from phloem-limited viruses. As discussed above, the grinding buffer must not only allow the virus to be separated from host materials, but also preserve infectivity. Large-scale extraction of virus allows virions to be exposed to inactivating compounds for long periods of time, much longer than during a simple sap transmission. Consequently, all factors affecting infectivity must be considered in designing a suitable virus purification buffer.

Nonionic detergents are helpful in some extraction buffers. Certain viruses, such as CaMV, form inclusion bodies in the host, which contain large quantities of virus and would sediment at low speed if not disrupted. When a small amount of detergent is added to the extraction buffer, these inclusions dissociate more readily. Such detergents also facilitate removal of host membranes.

The best advice when developing a buffer for virus extraction is to begin with the simplest buffer possible. This could be a 50–100 mmol l^{-1} phosphate solution with a pH of 7.5, and a reducing agent. Add other components or change components as necessary, until a suitable amount of infectious virus is obtained. The suspension is then clarified with a low-speed centrifugation, and the supernatant is collected and filtered through cheesecloth or Miracloth™. If virus yields are low, precipitation with polyethylene glycol (PEG) can be used to concentrate the virus prior to high-speed centrifugation. With some viruses, PEG precipitation is performed as an alternative to high-speed centrifugation, as the latter can disrupt virions or create pellets which are difficult to resuspend. However, high-speed or ultracentrifugation remains the preferred method for isolating most plant viruses.

Ultracentrifugation of the supernatant at high speeds for an extended period of time removes virions from the supernatant, forming a tight pellet on the bottom of the tube. A cushion of concentrated sucrose in extraction buffer is often used during ultracentrifugation to prevent excessive debris from sedimenting with virions, resulting in a cleaner virus pellet. If pellets are difficult to resuspend, a small amount of a nonionic detergent included in the grinding buffer may ease resuspension. Following resuspension of the pellet, the concentrated virions can be inoculated to a host or purified further.

It is sometimes useful to have a highly purified source of inoculum, in which case the resuspended high-speed pellet or a PEG precipitated fraction can

be run on a sucrose density gradient. The viral band can be extracted from the gradient to obtain highly purified virions. Such purifications are not necessary for most virus transmissions, although they can be useful in determining the precise amount of inoculum administered to plants, as concentration can be determined on a spectrophotometer following gradient purification. In addition, gradient-purified virions can be mixed with glycerol and frozen for long-term storage. Infectivity of virions stored in this manner varies.

The most important aspect of virus propagation is to understand that each virus has unique characteristics and specific interactions with each host plant it infects. Although a great deal of literature is available on propagation and maintenance of known plant viruses, effective methods for propagation and maintenance of each newly discovered virus must be determined experimentally. Suggestions can be obtained from literature on similar viruses, but this cannot replace experimentation.

See also: **Propagation of viruses: Animal; Vectors: Plant viruses; Virus structure: Atomic structure, Principles of virus structure.**

Further Reading

Brunt AA, Crabtree K, Dallwitz MJ, Gibbs AJ and Watson L (1996) *Viruses of Plants: Descriptions and Lists from the VIDE Database*. Wallingford, UK: CAB International.

CMI/AAB Descriptions of Plant Viruses (1989) Wellesbourne, Warwick, UK: Association of Applied Biologists.

Hampton R, Ball E and De Boer S (1990) *Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens: A Laboratory Manual*. St Paul, MN: APS Press.

Hull R (1992) Viruses. In: Gurr SJ, McPherson MJ and Bowles DJ (eds) *Molecular Plant Pathology: A Practical Approach*, vol. 1, p. 1. Oxford: IRL Press.

Matthews REF (1991) Isolation. In: *Plant Virology* 3rd Edn, p. 54. San Diego: Academic Press.

PSEUDORABIES VIRUS (*HERPESVIRIDAE*)

Saul Kit, Novagene Inc., Houston, Texas, USA

History

Aujeszky's disease (AD), also known as infectious bulbar paralysis and as pseudorabies (PR) because of its clinical similarity to rabies, was one of the first virus diseases to be recognized. In 1902, Aujeszky recovered the causative agent from infected oxen, cats and dogs, and transmitted it to rabbits and guinea pigs, in which it produced the pruritis and central nervous system (CNS) signs characteristic of naturally occurring PR disease. AD was probably present in the United States as early as 1813, but it was not until 1931 that Richard Shope established that 'mad-itch' disease of Iowa cattle was serologically identical to AD, and that a mild and usually unrecognized disease of swine was produced by the mad-itch agent, that is, PR virus (PRV). Shope showed that PRV was infectious for adult swine and was transmitted through skin abrasions to cattle pastured with infected swine. In 1933, minced rabbit brain and testicles were first used by Traub to cultivate PRV *in vitro*. A year later, Sabin reported that the virus was immunologically related to herpes simplex virus (HSV-1) and herpes B. virus and PRV was classified into the herpesvirus group. In the 1940s and 1950s, established mouse cell cultures were used to serially propagate the virus. Virus isolations are now routinely made in sensitive rabbit cell cultures and propagated in a variety of vertebrate (porcine, bovine, monkey, chick, etc.) cell lines.

Taxonomy and Classification

PRV (swine herpesvirus-1) is a member of the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus*. PRV particles are about 150–180 nm in diameter and consist of an enveloped nucleocapsid (about 105–110 nm) that surrounds the genomic DNA. The DNA of PRV is a linear double-stranded, class 2 herpesvirus DNA molecule, approximately 90×10^6 Da (135 kbp) in size and encodes about 80 genes (**Tables 1** and **2**). Many of these genes are not required for replication in cultured cells. It is composed of two components, L and S. The S component consists of a short unique (U_S) sequence bracketed by large (about 10^7 Da) inverted repeats. (T_R/I_R). High-frequency inversion of the U compo-

be run on a sucrose density gradient. The viral band can be extracted from the gradient to obtain highly purified virions. Such purifications are not necessary for most virus transmissions, although they can be useful in determining the precise amount of inoculum administered to plants, as concentration can be determined on a spectrophotometer following gradient purification. In addition, gradient-purified virions can be mixed with glycerol and frozen for long-term storage. Infectivity of virions stored in this manner varies.

The most important aspect of virus propagation is to understand that each virus has unique characteristics and specific interactions with each host plant it infects. Although a great deal of literature is available on propagation and maintenance of known plant viruses, effective methods for propagation and maintenance of each newly discovered virus must be determined experimentally. Suggestions can be obtained from literature on similar viruses, but this cannot replace experimentation.

See also: **Propagation of viruses: Animal; Vectors: Plant viruses; Virus structure: Atomic structure, Principles of virus structure.**

Further Reading

Brunt AA, Crabtree K, Dallwitz MJ, Gibbs AJ and Watson L (1996) *Viruses of Plants: Descriptions and Lists from the VIDE Database*. Wallingford, UK: CAB International.

CMI/AAB Descriptions of Plant Viruses (1989) Wellesbourne, Warwick, UK: Association of Applied Biologists.

Hampton R, Ball E and De Boer S (1990) *Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens: A Laboratory Manual*. St Paul, MN: APS Press.

Hull R (1992) Viruses. In: Gurr SJ, McPherson MJ and Bowles DJ (eds) *Molecular Plant Pathology: A Practical Approach*, vol. 1, p. 1. Oxford: IRL Press.

Matthews REF (1991) Isolation. In: *Plant Virology* 3rd Edn, p. 54. San Diego: Academic Press.

PSEUDORABIES VIRUS (*HERPESVIRIDAE*)

Saul Kit, Novagene Inc., Houston, Texas, USA

History

Aujeszky's disease (AD), also known as infectious bulbar paralysis and as pseudorabies (PR) because of its clinical similarity to rabies, was one of the first virus diseases to be recognized. In 1902, Aujeszky recovered the causative agent from infected oxen, cats and dogs, and transmitted it to rabbits and guinea pigs, in which it produced the pruritis and central nervous system (CNS) signs characteristic of naturally occurring PR disease. AD was probably present in the United States as early as 1813, but it was not until 1931 that Richard Shope established that 'mad-itch' disease of Iowa cattle was serologically identical to AD, and that a mild and usually unrecognized disease of swine was produced by the mad-itch agent, that is, PR virus (PRV). Shope showed that PRV was infectious for adult swine and was transmitted through skin abrasions to cattle pastured with infected swine. In 1933, minced rabbit brain and testicles were first used by Traub to cultivate PRV *in vitro*. A year later, Sabin reported that the virus was immunologically related to herpes simplex virus (HSV-1) and herpes B. virus and PRV was classified into the herpesvirus group. In the 1940s and 1950s, established mouse cell cultures were used to serially propagate the virus. Virus isolations are now routinely made in sensitive rabbit cell cultures and propagated in a variety of vertebrate (porcine, bovine, monkey, chick, etc.) cell lines.

Taxonomy and Classification

PRV (swine herpesvirus-1) is a member of the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus*. PRV particles are about 150–180 nm in diameter and consist of an enveloped nucleocapsid (about 105–110 nm) that surrounds the genomic DNA. The DNA of PRV is a linear double-stranded, class 2 herpesvirus DNA molecule, approximately 90×10^6 Da (135 kbp) in size and encodes about 80 genes (**Tables 1** and **2**). Many of these genes are not required for replication in cultured cells. It is composed of two components, L and S. The S component consists of a short unique (U_S) sequence bracketed by large (about 10^7 Da) inverted repeats. (T_R/I_R). High-frequency inversion of the U compo-

Table 1 PRV genes: UL segment of PRV DNA[a]

BamH-I fragment	*Gene and orientation*	*Function*	*Essential in culture*	*Amino acids*	*Mol.wt (kDa)*
14′	Ori-L ??	Origin of replication			
5′	ORF-1 →	Membrane associated ?	Nonessential	207	22
5′	UL54 ←	Gene regulation ?		361	40
5′	UL53 ←	gK multiple membrane spanning	Nonessential (entry)	312	34
5′	UL52 ←	Helicase/primase		958	103
5′	UL51 →	Virion component	Nonessential	236	30
5′/1	UL50 ←	dUTPase	Nonessential	268	29
1	UL49.5 →	gN viral envelope (complex with gM)	Nonessential	98	14
1	UL49 →	Tegument?			
1	UL27 ←	gB membrane fusion in penetration and cell to cell spread	Essential	913	100
1	UL28 ←	ICP 18.5 concatemer DNA cleavage/encapsidation	Essential	723	79
1	UL29 ←	Major DNA binding protein			
1	UL30 →	DNA polymerase	Essential	1045	115
1	UL31 ←				
2	UL38 →	Capsid protein			
2	UL39 →	Large subunit RR reductase	Nonessential	835	91
2	UL40 →	Small subunit RR reductase	Nonessential	303	33
2	UL41 ←	Virion-associated host shut off	Nonessential	365	40
2	UL42 →	Polym. associated protein	Essential	384	40
2	UL43 →	Multiple membrane spanning protein	Nonessential	373	38
2/9	UL44 →	(gC) binding to host cell heparin sulfate, C3b receptor	Nonessential	479	51
9	UL26 ←	Serine protease	Nonessential	524	55
9	UL26.5 ←	Virion protein		278	28
9	UL25 ←	?		534	57
9/11	UL24 ←	Mutant gives small plaques	Nonessential	171	19
11	UL23 →	Thymidine kinase	Nonessential	320	36
11/16	UL22 →	(gH) penetration and cell to cell spread	Essential	686	72
15		Contains direct repeat elements			
4	ORI-L	Origin of replication			
4	UL21 ←	Cleavage/encapsidation	Nonessential	525	55
4	UL20 →	Membrane spanning protein, viral egress	Nonessential	161	17
4	UL19 →	Major capsid protein	Essential	1330	146
4	UL18 →	Capsid protein	Essential	295	32
4/3	UL15 ←	(Spliced)			
3	UL14 →				
3	UL13 →	Protein kinase/tegument	Nonessential		
3	UL12 →	Alkaline DNase	Nonessential	483	51
3	UL11 →	Membrane		63	7
3	UL10 ←	Membrane spanning protein (gM) – viral entry	Nonessential	393	45
3	UL9 →	Origin binding protein, helicase	Essential	843	91
3	UL8.5 →			470	51
3	UL8 →	Helicase/primase complex with UL5	Essential	683	71
3	UL7 ←	?		266	29
3	UL6 ←	Capsid associated protein mutants fail to encapsidate viral DNA	Essential	643	70
6	UL5 →	Helicase/primase	Essential	833	90
6	UL4 →	?	Nonessential	145	16
6	UL3.5 ←	Nonstructural protein, virus envelopment and maturation	Essential	224	24
6	UL3 ←	?		237	26
6	UL2 ←	Uracil-DNA glycosylase	Nonessential ?	316	33
6	UL1 ←	gL (complex with gH)	Essential	156	17
6/14/8′/8/5	LAT →	Long (spliced) latency-associated transcript			
14	EP0 ←	Promiscuous transactivator viral promoters	Essential	410	45

[a]Sequences of viral genes are available in Genebank and EMBL databases.

Table 2 PRV genes: IRS/TRS and US segments of PRV DNA

BamH-I fragment	*Genes and orientation*	*Function*	*Essential in culture*	*Amino acids*	*Mol. wt (kDa)*
8/5	TRS/IRS IE180 ←	Regulate positively early and late genes and negatively itself	Essential	1460	180
10/12	TRS/IRS RSp40 →	Gene regulation – homologous to HSV ICP22		364	40
10	US3 →	Protein kinase (serine/threonine)	Nonessential	390	43
7	US4 →	gG (gX) excreted sulfated protein	Nonessential	498	54
7	US6 →	gD (gp50) penetration into cell	Essential	402	45
7	US7 →	gI (gp63) complex with gE	Nonessential	350	36
7	US8 →	gE (gI) virus release and cell–cell spread	Nonessential	577	
7	US9 →	11 K tegument	Nonessential	106	11
7/12	US2 →	28 K	Nonessential	256	28

nent, but not the L component, is observed during virus growth, so that the S genome is found in two isomeric forms. The ends of the PRV genome are unique; no terminal redundancy is present. Antigenic differences have not been detected among strains of PRV, but strain differences demonstrable by restriction endonuclease fingerprinting are common and have been used for epidemiological studies and control programs. Deletion mutations in the U_S region of the PRV genome occur 'spontaneously' following passages in chick cells.

Molecular Biology

The PRV genome is, for the most part, colinear with the I_L arrangement of the HSV-1 genome. However, a peculiarity of the PRV genome is the presence of an inversion in the middle of the unique long (U_L) region (0.07 and 0.39 map units) compared to HSV-1 and other alpha herpesviruses. On one side, the inversion occurs between the *UL27* and *UL26* genes (**Tables 1** and **2**). It appears that PRV lacks a sequence homologous to the HSV-1 *UL45* gene at the *Bam*HI-9 junction downstream of *UL44* (gC). Due to the fact that in PRV the *UL26* and *UL44* genes are adjacent and their ends are conserved, the right border of the inversion must lie within their intergenic region. Most of the PRV genes are conserved relative to those of HSV-1, but there are notable differences. Some genes present in HSV-1 [*UL56, UL48 (VP16), UL45, US5 (gJ), US10, US11* and *US12*] have not been detected in PRV, whereas the PRV gene, *UL3.5,* has also been found in the varicella-zoster virus (VZV), bovine herpesvirus 1 (BHV1) and equine herpesvirus 1 (EHV1) alpha herpesvirus genomes, but not in that of HSV-1. Also, the PRV *US2* gene is not colinear with the *US2* gene of HSV-1.

PRV genes are expressed during lytic infection as three groups, immediate early (IE), early and late, in a coordinately regulated and sequentially ordered cascade. The IE genes are transcribed by the host cell RNA polymerase in the absence of *de novo* viral protein synthesis. Early genes are dependent upon IE proteins for their transactivation and can be transcribed in the absence of viral DNA synthesis. Late genes consist of two subclasses depending on their requirement for viral DNA synthesis. Inhibition of viral DNA synthesis moderately decreases the accumulation of quasi-late $\gamma 1$ transcripts but totally prevents formation of $\gamma 2$ late transcripts. $\gamma 1$ quasi-late genes reach maximum expression levels after DNA synthesis starts. Most late gene products are virion structural proteins.

The PRV genome contains only one IE gene, IE180, but since this IE gene is located in the T_R/I_R region of the PRV genome, there are two copies per genome. The IE gene is homologous to the HSV-1 $\alpha 4$ gene, and specifies an unspliced 5.1 kb transcript which is translated to a protein of about 180 kDa. The PRV IE gene is required for continuous transcription of early and late PRV genes and for shutting off the synthesis of its own RNA. The PRV IE gene contains five to six different consensus sequences in its promoter region and is capable of *trans*-activating a variety of viral and cellular class II and class III promoters by interacting with host transcription factors and specific promoter sites. Unlike PRV, HSV-1 encodes several IE genes. Homologous genes found in PRV and presumed to have regulatory gene functions include *EP0* and *RSp40,* homologues of the IE HSV-1 genes, *ICP0* and *ICP22,* respectively, but these PRV genes are early genes rather than IE genes. The PRV *UL54* gene encodes a protein homologous to the HSV-1 IE *ICP27* gene, but the mode of regulation of PRV *UL54* has not been elucidated.

A latency-associated transcription unit (LAT) overlaps the PRV IE gene. This transcription unit is at least 11.8 kb long, extends from the U_L region to

the T_R/I_R region of the PRV DNA (0.69–0.77 map units), and is transcriptionally active in the trigeminal ganglia of latently infected swine. The LAT transcription unit is spliced to a large latency associated transcript (LLT), an 8.5 kb poly(A^+) transcript, and to smaller 4.5–5.5 kb and 1.0–2.0 kb RNA species. The first and second exons of the LLT are antiparallel to and overlap the genes for EP0 in U_L and IE180 in the I_R. The roles of the LAT transcripts in the maintenance of or reactivation of PRV from the latent state have not as yet been delineated.

Early PRV genes encode proteins functioning in DNA metabolism and include the nonessential genes encoding thymidine kinase (*UL23*), ribonucleotide reductase (*UL39*, *UL40*), uracil–DNA glycosylase (*UL2*) and dUTPase (*UL50*). Some other PRV genes are essential for viral DNA replication and include *UL9* (origin binding protein), *UL30* (DNA polymerase) and the *UL5*, *UL8* and *UL52* helicase/primase complex, and probably *UL29* (major DNA binding protein).

Replication of PRV DNA occurs in two phases. In the first phase, the viral DNA enters the cell nucleus and circularizes by ligation of the ends of the linear DNA molecules. This process does not appear to require expression of virus functions. The first rounds of replication are associated with these circular structures. The main origin of replication is located in the BamHI-4 fragment in the middle of the U_L region. Replication probably proceeds by a rolling circle mechanism. Later in the replication process, concatamers in head-to-tail alignment are generated. Cleavage of concatameric DNA to generate mature, genome-sized linear molecules requires the expression of UL21 and UL28 and possibly other viral proteins and involves site-specific cleavages from signals present on both ends of the PRV genome. Both cleavage and packaging are intimately linked to capsid assembly. UL19 (major capsid protein), UL18 and UL38 have been identified as PRV capsid proteins. Genes encoding tegument and envelope proteins have also been identified (**Tables 1** and **2**). The molecular structure of latent PRV DNA in the trigeminal ganglia is uncertain, but appears to be concatameric or circular, and is presumably generated during an abortive DNA replication cycle.

Geographic Distribution

PR is enzootic in swine in most parts of the world, except Australia, Canada and Norway. PR has been a consistent source of significant losses in swine and cattle due to increased mortality, reproductive failures and lower growth performance. The cost of PR outbreaks has been estimated to be about $10 000 per year per outbreak, with a total annual cost in the US of from 30 to 72 million dollars. In most states in the US, PR diagnosis in a herd results in a quarantine of the swine with movement limited directly to a slaughter facility.

Host Range

Domestic and feral swine are the only identified reservoir hosts in which PR transmission is maintained. All other species are considered to be 'dead-end' hosts (i.e. the virus dies within the host without passing infection to other animals). PRV causes an acutely fatal infection in many nonporcine species, such as cattle, sheep, dogs, cats, raccoons, opossum, rats, mice and chicks. These species become infected after comingling with pigs or ingesting infected meat. The development of PR in carnivores may often be the first indication that the disease exists in swine on a farm. Reports of infection of humans have been rare and have not been substantiated.

Epidemiology

From 1902 to 1930 only single outbreaks of PRV were reported, predominantly in cattle and dogs. Pseudorabies in pigs was described in 1920 and the first enzootic in pigs appeared in 1931. In the following years, sporadic outbreaks occurred in several European countries, the USA, North Africa and Turkey. In areas where small pig farms predominated, PRV appeared suddenly and involved the majority of susceptible pigs. After that, there was no subsequent outbreak of disease on the premises for many years. The epidemiological pattern changed in the 1950s and 1960s with the development of large swine units. PRV became enzootic in the big farrowing and fattening farms in Eastern and Southeastern Europe and in the middle west of the USA. By the mid-1970s, PRV had spread to Central and Western Europe and Southeast Asia and had become a worldwide disease of great economic importance. Serologic surveys of swine sent to slaughter in the US revealed an increase in the PR-positive herds from 0.56% in 1974 to 8.78% in 1984. Until 1977, only 2–24 PR outbreaks in pigs were recorded in the then Federal Republic of Germany, but from 1977 onwards, their numbers increased, reaching 2000 recorded outbreaks in 1987. PRV was first isolated from New Zealand pigs on the North Island in 1976. PR was first reported in central Japan in 1981 and has been spreading rapidly throughout that country.

The increase and spread of PR disease may be, at least in part, attributed to the emergence of more virulent PRV strains or variants. A more significant

cause is the changed management within the swine industry. PR disease is most prevalent in areas that employ intensive swine housing practices and where swine are most concentrated. In Iowa in 1984/1985, only 2% of herds with fewer than 50 sows were seropositive for PRV, whereas the herd prevalence rate for herds with more than 100 sows was 55%. The incidence of PRV is highest in Europe in those areas with cold climates and intensive hog industries, such as Holland, Belgium, France and Hungary, and is less of a problem in countries with less intensive hog production.

A salient factor in the spread of PR disease is the capacity of PRV to establish latent infections in sensory ganglia of infected swine. A small percentage of these latently infected animals sporadically shed virus in saliva and nasal discharges, so that licking, biting and aerosols can cause transmission. Losses from overt disease in swine occur when nonimmune pregnant sows, or swine less than 3 months old born to nonimmune sows, are infected. When breeding sows have adequate antibody levels, overt clinical disease in their progeny may be greatly reduced, but the baby pigs may nevertheless become latently infected and later develop clinical signs of disease and/or transmit infectious virus. On farms where breeding, farrowing, nursery, growing and finishing operations are conducted separately, significant losses may occur when weaned swine from several sources are brought together into a growing–finishing facility.

Transmission and Pathogenesis

PR is usually transmitted by acutely infected pigs, which produce large amounts (10^4 PFU) of infectious PRV, or by latently infected animals, which sporadically shed small amounts of virus even though they may not show signs of disease. The primary source of virus spread is intimate nose-to-nose contact involving expelled body fluids of oronasal origin. Often, PRV is transmitted by pregnant sows to their highly susceptible newborn offspring. Latently infected pigs may be experimentally induced to excrete PRV by stress-related factors, simulated by the drug, dexamethasone. Factors influencing infection include the movement of swine (e.g. the introduction into a breeding herd of latently infected sows from outside the farm), swine and wildlife density, housing and ventilation design, access of other animals and wildlife to swine, dead pig disposal, and the source and handling of swine feed. Airborne transmission of droplets containing virus between farms can play a role in initiating disease outbreaks. The airborne transmission over a distance of 15–80 km of PRV originating in northern Germany caused the recurrent epizootics of 1984 to 1988 in both conventional and specific pathogen-free Danish herds. Contamination of feed, water, bedding and floors may likewise provide susceptible pigs with access to virus. After successful transmission, virus replication occurs in the tonsillar and pharyngeal tissues and in the olfactory epithelium. From these sites, the virus spreads to the medulla by way of the olfactory nerve system and the epineural lymph of the trigeminal and glossopharyngeal nerves, and on further replication reaches the brain. Multiplication of virus in the respiratory tract of swine facilitates its spread within the body, since macrophages and leukocytes become infected and may carry the virus to various body organs, particularly to the placenta in which it may multiply and subsequently invade the fetus. PRV can be isolated from buffy coat cells, providing further evidence of absorption on or within leukocytes. Feces and urine are not considered significant sources of infection. Most swine infected with virulent virus shed infectious virus for from 12 to 21 days. By contrast, most wildlife and domestic species excrete virus for only 1 to 2 days before death and in amounts insufficient for transmission of infection.

PRV, like other herpesviruses, is extremely labile, so that experimental virus stocks must be stored at −70°C. The virus can, however, survive in damp bedding for 140 days at 4°C and for 40 days at 37°C, for 5 weeks on shelled corn and for 3 weeks on moist meal. The virus has a very short survival time on clean concrete, green plants or well-cured hay. Heat, direct sunlight and dry conditions quickly inactivate PRV. PRV can survive in ground flesh for weeks. Dead baby pigs or infected placentas that are ingested by cats and dogs or wildlife, cause fatal infections. Dogs have become fatally infected with PRV after fighting with feral pigs. Cattle can become infected through contaminated feed or when secretions from infected pigs are introduced through existing bovine skin abrasions. Simultaneous wound contamination at the time of bite infliction also occurs, particularly in the rear limbs of cattle. To avoid fatal outbreaks of PR, it is essential that cattle on farms be isolated from swine. Iatrogenic infections, involving accidental injections of conventional, inadequately attenuated PRV vaccines into lambs and chicks, have caused fatal infections on several occasions.

Clinical Features of Infection

Generally, after infection of pigs, two different disease patterns may be seen depending on the age of the animal and the virulence of the virus strains. In piglets, the CNS is heavily affected whereas in older pigs, mostly respiratory symptoms are seen.

Young pigs infected with PRV exhibit a high morbidity, high mortality, fever, vomiting, diarrhea and CNS signs. The CNS signs include tremors, incoordination and pronounced muscle spasms, circling and intermittent convulsions accompanied by excess salivation. Death occurs by 8 days after infection. Maternal antibodies protect piglets from signs of disease, but not infection. Older (feeder) pigs show a high morbidity, variable mortality, fever, anorexia, constipation, vomiting, depression, growth arrest, listlessness, coughing and sneezing, labored breathing and CNS signs. In pregnant sows, mortality may be low, but respiratory and gastrointestinal signs are common, as are reproductive problems. Infection of sows before day 30 of gestation may result in death and resorption of the embryos. Infection after day 30 of pregnancy may terminate in abortion, or by the delivery of stillbirths and mummified fetuses. PR in cattle assumes a rapid and fatal course. Death often occurs by 2 days after the first signs of illness appear. The first sign is a decrease in milk yield followed by violent licking of parts of the body. As pruritis increases in intensity, the cow becomes frenzied, bites and gnaws at the skin and rubs its head and neck against hard objects. Rabbits, cats and dogs are among the most vulnerable of species. Subcutaneous injection of 1 PFU of PRV can be lethal to rabbits. In dogs, pruritis is accompanied by drooling of saliva and plaintive howling simulating true rabies. In cats, the disease may progress so rapidly that pruritis is not observed.

Pathology and Histopathology

Following primary oronasal infection of swine, virus replicates in the oropharynx. At 24 h after infection, viral antigens and DNA can be detected in cells of the nasal epithelium. After 48 h, areas of the epithelium become necrotic and the infection reaches the stroma killing many cells. Enveloped virus particles are detectable by electron microscopy in and around infected epithelial cells and fibroblasts. The stroma of the oronasal epithelium contains many blood vessels and neuronal axons of the trigeminal, olfactory and vegetative nerves, through which the virus can travel via the axoplasm to cranial nerve ganglia and the medulla and pons. Virus may establish latency in the nuclei of neurons, in which case production of infectious virus ceases, but the viral genome itself is maintained in a portion of the cells, or the virus may continue to spread within the CNS producing ganglioneuritis, meningoencephalitis, perivascular cuffing and focal gliosis associated with extensive necrosis of neuronal and glial cells. A common finding in severely infected swine is congestion of the nasal mucosa and pharynx, tonsillitis and rhinitis. Pulmonary congestion and edema are frequently observed in such cases. In the larger air passages, viral capsids and viruses may be seen in bronchial and bronchiolar epithelium, lymphocytes and alveolar macrophage. Swine that recover from PR may shed virus sporadically in their nasal secretions. Others from which virus cannot be isolated by conventional means may yield virus after cocultivation of tonsillar or trigeminal ganglia with susceptible cells. That recovered swine are latently infected may also be shown by demonstrating PRV DNA or RNA in trigeminal ganglia. *In situ* hybridization techniques with polymerase chain reaction-generated probes are particularly useful for this purpose.

Immune Response

One highly sulfated, secreted glycoprotein [gG (90–98 kDa)] of unknown function, eight Class I structural membrane glycoproteins [gB, gC, gD (gp50), gE, gI (gp63), gH, gL and gN], and five multiple membrane spanning Class III proteins [UL11, UL20, UL43, UL53 (gK) and UL10 (gM, 45 kDa)] are synthesized in PRV-infected cells (**Tables 1** and **2**). The gB glycoprotein is a complex of homodimeric molecules that are post-translationally cleaved by a cellular protease residing in the trans-Golgi into two subunits per monomer (72–78 kDa and 50–58 kDa) that remain linked via disulfide bond. gE (115–122 kDa) and gI (63 kDa) are noncovalently complexed with each other as are gH (95 kDa) and gL (20 kDa). Of the PRV glycoproteins, only four (gB, gH, gL and gD) are essential for virus replication. These glycoproteins are involved in virus penetration of host cells and cell-to-cell spread. The other glycoproteins are nonessential for PRV replication in cultured cells. Indeed, a quadruple deletion mutant lacking gE, gI, gG and gC is viable. The structural glycoproteins are found embedded in the nuclear and cellular membranes of infected cells as well as on the surface of mature enveloped viruses, and are major antigens that interact with the host immune system to elicit humoral and cell-mediated immune responses. Glycoprotein gC (92–98 kDa), although nonessential, has a primary role in virus attachment to host cells through a heparan sulfate proteoglycan receptor on the cell surface. However, in the absence of gC, PRV can attach to cells by an alternative pathway not mediated by the heparan sulfate receptor and virus infection, though retarded still occurs. Polylysine enhances the adsorption of gC-negative PRV mutants. In addition, PRV gC functions as a complement C3 receptor, thereby providing the virus with an immunoevasion mechanism that prevents complement-mediated cell lysis and virus

neutralization. Glycoproteins gE and gI influence the release of infectious virions from infected cells and promote cell-to-cell spread (as measured by plaque size). In animals, gE and gI are also important for efficient infection of the central nervous system and for neurovirulence. Glycoprotein gK is a virion structural component involved in virus release, but is not required for entry. gK mutants exhibit a dramatic reduction in the ability to form plaques by cell to cell spread on noncomplementing vero cells. A PRV mutant lacking gM displayed reduced infectivity and was delayed in penetration, indicating that the virion component, gM, though nonessential, has a modulatory role in the initiation of PRV infection.

PRV glycoproteins gG, gD, gI and gE map in the U_s region of the viral genome and are flanked on the left by a protein kinase gene and on the right by the genes encoding 11 and 28 kDa proteins. Glycoproteins gB, gC, gH, gL, gN, and gM map in the U_L region of the viral genome (**Tables 1** and **2**).

Until recently, it was accepted that most viruses have only one protein or glycoprotein that is important for induction of immune responses and protection in the host. However, it is now clear that several herpesvirus glycoproteins can induce protection. Convalescent sera from animals infected with PRV have antibodies to gD, gB and gC, which can neutralize viruses outside cells and destroy virus-infected cells by antibody-dependent cellular cytotoxicity or antibody-dependent, complement-mediated cytolysis. In pigs, monoclonal antibodies against gE, gB, gC and gD all neutralize virus, while passive immunization of mice or pigs with these monoclonal antibodies protects them from lethal virus challenge. The level of antibodies in pigs does not, however, correlate with protection against virulent virus infection, indicating that major histocompatibility complex-restricted (MHC) cytotoxic T lymphocytes and natural killer cells are also important. Unfortunately, data on these cellular immune elements are as yet limited. It is known that gE, gB and gC are important targets, but not the only targets, for cytotoxic lymphocytes. PRV downmodulates the expression of MHC Class I antigens in porcine and murine cells. Since MHC Class I antigens are critical for recognition of infected cells by virus-specific cytotoxic T lymphocytes (CTL), it is logical to suppose that suppressed Class I expression would reduce CTL activity and facilitate viral immunoevasion. The specific viral regulatory genes involved in downregulating the MHC Class I antigens have not been identified. The fact that the PRV envelope is composed of several glycoproteins is, in a sense, fortunate from the point of view of the host. Mutagenic alterations in a single viral glycoprotein do not abrogate the defense mechanisms of the host. Indeed, although almost all of the modified-live PRV vaccines harbor deletion mutations in viral glycoproteins, they are highly protective.

Prevention and Control of Pseudorabies

The strategy selected to control PR has depended on: (1) the type of operation carried out by the producer (farrow-to-finish, seedstock, feeder pig producer, feeder pig finisher); (2) financial considerations; (3) availability of suitable replacements; (4) the disease profile of the herd and; (5) PRV status in the area. Slaughter of an entire herd and repopulation is considered with single isolated outbreaks in non-infected districts, but may also be necessary when disease is detected in seed stock operations. Slaughter is performed on quarantined pigs after they reach market weight of 220–260 pounds. This is done to save the meat and market value of the animals. Hence, it may take 6 months to slaughter an entire herd. The slaughter strategy is expensive and disruptive, results in the loss of valuable blood lines, and is impractical if the source of infection cannot be identified or if the reinfection risk is high. Offspring segregation has been used to produce PRV-seronegative gilt replacements from positive sow herds. Serological testing and the culling of seropositive pigs has been recommended in low-prevalence areas when the proportion of seropositive individuals in the herd was about 20% or less and when there was no evidence that virus was circulating between the sows of the breeding herd and their offspring. Seropositive pigs were then replaced with seronegative gilts. Factors impeding control through test and slaughter are the expense of repeated serologic testing, large herd size and the density at which pigs are maintained, especially if they are housed in proximity to other infected farms. Test and slaughter programs in England and Denmark have greatly decreased PR outbreaks but have not completely eliminated them.

Vaccination has been used alone or together with other strategies for over 25 years to stop PR outbreaks, to reduce virus shedding by infected pigs, to minimize clinical disease and mortality, to reduce losses from secondary bacterial infections and to reduce the incidence of latent infections. Both killed and modified-live virus (MLV) vaccines have been used, but despite frequent vaccination with conventional vaccines, the incidence of PR outbreaks in areas of intense husbandry has continued to increase, principally because it has hitherto been impossible to identify pigs latently infected with virulent PRV in a vaccinated herd. This situation has now changed with the identification of several nonessential viral

genes that contribute to virulence. The thymidine kinase (*TK*) of PRV and other herpesviruses is one of the most important genes contributing to virus virulence. Therefore, irreversible deletion mutations in the *TK* gene have been engineered by recombinant DNA techniques to significantly attenuate the virus and greatly increase vaccine safety. All current genetically engineered PRV vaccines have deletion mutations in the *TK* gene. The elucidation of glycoprotein genes also led to an understanding of the action of conventional vaccines and the finding that some glycoprotein genes can be deleted. This motivated the development of differential diagnostic tests and provided the basis for PRV eradication programs.

Conventional MLV PRV vaccines, as illustrated by the Bucharest vaccine strains, were obtained by repeatedly passing PRV on the chick CAM and in cultured chick embryo fibroblast cells. gE deletion mutations were induced in most, but not all, of the Bucharest derived vaccine variants. It was shown, retrospectively, that the gE deletion mutations could account for much of the reduced neurovirulence of Bucharest vaccine strains. Other conventional PRV vaccines, such as the Bartha and NIA4 vaccines also harbored gE deletions, but in addition, the Bartha vaccine had a deletion in the *gI* gene and mutations in the *UL21* gene and in the promoter region of the glycoprotein *gC* gene. A partially attenuated, but gE-positive Bucharest variant was the first PRV strain used by the author to develop a genetically engineered TK-deleted PRV vaccine, but virulent Iowa, Aujeszky and NIA3 PRV strains have also been used by others as the starting material for TK-deleted PRV vaccines. In addition, deletion mutations have been engineered in the gC gene, the gE gene or the gG gene of TK-deleted vaccines to produce diagnostic markers by which vaccinated pigs could be distinguished serologically from pigs harboring virulent PRV.

Diagnosis

The clinical signs of PR are sufficiently characteristic to permit a preliminary diagnosis which may be verified by PRV isolation, the fluorescent antibody staining of frozen tissue sections, and serum neutralization (SN) tests. Screening ELISA and latex agglutination tests are, however, more rapid and more sensitive than SN tests. With the advent of gene-deleted marker vaccines, differential blocking ELISA diagnostic tests are now being used. Marker-specific differential diagnostic tests have been developed for each of the gC-, gE and gG deleted vaccines. When pigs are vaccinated with the OMNIMARK vaccine, a TK-deleted gC-deleted vaccine, for example, they develop antibodies to various PRV proteins, but not to gC. Wild-type PRV-infected pigs develop antibodies to most PRV proteins, including gC. Hence, a differential blocking ELISA test, which uses gC as the antigen to coat microtiter wells and a gC-specific monoclonal antibody conjugated to horseradish peroxidase to react with the antigen, distinguishes serologically between OMNIMARK-vaccinated and wild-type virus-infected pigs. Undiluted test sera are used with this gC-blocking ELISA test. The specificity of the test approaches 100% and the sensitivity is about the same as that of screening ELISA and latex agglutination tests. gC antibodies can be detected by about 7–10 days after pigs vaccinated with OMNIMARK become infected with wild-type virus and for a year or more after pigs are latently infected with wild-type PRV. SN antibody tests are often negative under these conditions. The wild-type virus-infected pigs identified by the differential diagnostic test can be removed during herd clean-up. Vaccinated swine that are negative in the differential tests are classified by the USDA as PRV-negative and their interstate and intrastate movement is permitted.

Future Perspectives

The availability of gene-deleted marker vaccines for PRV has stimulated new efforts for the control and eradication of PR disease. Such eradication and control programs are now in progress in the USA, Cuba, Mexico, Jamaica, Hungary, France, Germany, Bulgaria, the Czech and Slovakian republics, The Netherlands, Norway, Sweden, Rumania, the former USSR republics, Yugoslavia, Japan and Viet Nam. It has also been recognized that latent PRV infection is prevalent in free-roaming feral swine, which may come in contact with and transmit PR disease to domestic swine and other livestock. The US Animal Health Association has therefore recommended that serological surveillance of feral swine be expanded and that procedures for their vaccination (oral?) with marker PRV vaccines be developed.

Finally, it should be emphasized that attenuated, gene-deleted marker vaccines are promising candidates for live vaccine vectors, which can confer protection against both PR and other swine diseases. Model experiments in which foreign DNA sequences have been inserted at several sites in the PRV genome have recently been carried out. In one such experiment, a live, attenuated PRV expressing the envelope glycoprotein of hog cholera virus (classical swine fever) was prepared and its efficacy in protecting swine against both PR and hog cholera investigated, with promising results. Many more such PRV vector-based subunit vaccines may be anticipated.

See also: **Epidemiology of viral diseases; Herpes simplex viruses (*Herpesviridae*): General features, Molecular biology; Immune response: Cell mediated immune response, General features; Latency; Vaccines and immune response; Vectors: Animal viruses.**

Further Reading

Gustafson DP (1986) Pseudorabies. In: *Diseases of Swine*, 6th edn, p. 274. Ames, Iowa: Iowa State University Press.

Kit S and Kit M (1991) Genetically engineered herpesvirus vaccines: accomplishments in pigs and prospects in humans. In: Melnick JL (ed.) *Progress in Medical Virology*, vol. 38, p. 128. Basel: Karger.

Mettenleiter TC (1994) *Acta Vet. Hung.* 42: 153.

Mettenleiter TC, Kern H and Rauh I (1990) *Virology* 179: 498. van Oirschot JT (ed.) (1989) *Vaccination and Control of Aujeszky's Disease*. Dordrecht: Kluwer.

Wittman G and Rziha H-J (1989) Aujeszky's disease (pseudorabies) in pigs. In: Whittman G (ed.) *Herpesvirus Diseases of Cattle, Horses and Pigs*, p. 230. Boston, Dordrecht, London: Kluwer.

Q

QUASISPECIES

Esteban Domingo, Centro de Biología Molecular "Severo Ochoa" (CSIC-UAM), Universidad Autónoma de Madrid, Cantoblanco, Madrid, Spain

Origins of the Concept

Quasispecies describes a type of population structure in which collections of closely related genomes are subjected to a continuous process of genetic variation, competition and selection. Quasispecies has become very important in virology because it provides an interpretation for the extensive plasticity, both genetic and phenotypic (of biological features), displayed by many viruses, in particular RNA viruses. The quasispecies concept originated in theoretical studies on early informational macromolecules by Eigen and colleagues, and in experimental work on bacteriophage $Q\beta$ by Weissmann and colleagues. Eigen was the first to elaborate a quantitative treatment of the generation of error copies of simple replicating molecules (replicons) as those that probably populated the earth at the early stages of life. This treatment represented a link between principles of information theory and Darwinian evolutionary concepts, and was inspired to a great extent by experiments on evolution of simple RNA molecules replicating *in vitro* carried out by Spiegelman and his associates in the 1960s.

Eigen, Schuster and their colleagues defined the quasispecies as an organized, steady-state distribution of error copies of self-replicating macromolecules (nucleic acids). It represents a rated distribution of mutants (mutant spectrum) centered around one or several master sequences (**Table 1** and **Fig. 1**). The copying fidelity with which replication occurs determines the fraction of progeny molecules which will be identical to the parental replicon. This theoretical concept assumes a selection equilibrium in the distribution whose evolution can be described by a system of ordinary differential equations. This selection equilibrium is metastable in that it will collapse when an advantageous mutant appears in the distribution. The previous quasispecies will then be substituted by a new one, characterized by a new master sequence and a new mutant spectrum. The stability of a quasispecies depends on the complexity of the genetic information contained in the genome, the copying fidelity and the superiority of the master sequence. This is expressed mathematically in an error threshold relationship which limits the amount of genetic information that can be transmited when copying fidelity is limited (**Table 1**).

Quasispecies has a physical, a chemical and a biological definition. Physically a quasispecies distribution of genomes can be regarded as a cloud in sequence space. Sequence space refers to all possible sequences that theoretically could be occupied by a genome (**Table 2**). Viral quasispecies can be viewed as clouds that occupy tiny portions of such theoretical space. Occupation of sequence space by viruses is restricted by multiple genetic and functional constraints. The clouds have, at any given time, a defined population structure dependent on the distance of each point to the consensus sequence (that could be taken as the center of the distribution), and on the relative fitness values of the different mutant components. The distance or number of mutated positions between a given sequence and the best adapted one (the master sequence) is called the Hamming distance. This term is also used to indicate the number of mutations between two sequences.

Chemically, the definition of quasispecies is the one most familiar to virologists: a rated distribution of related, nonidentical genomes. Biologically, quasispecies are the target of selection. It is not easy to imagine an ensemble rather than an individual genome is the actual target of selection. However, individual RNA genome sequences may have a fleeting existence, and there are now a number of cases documenting that the evolutionary fate of an individual viral genome is strongly influenced by the mutant spectrum surrounding it.

Table 1 Important equations in quasispecies

• *Basic differential equation describing quasispecies evolution*

$$\frac{dx_i}{dt} = (A_i Q_i - D_i)x_i + \sum_{k \neq i} w_{ik} x_k + \phi_i$$

dx_i/dt = variation of concentration of mutant i as a function of time
x_i = concentration of mutant i
A_i = velocity constant for the replication of i
Q_i (quality factor) = probability that replication of i produces i
D_i = velocity of decomposition of i
W_{ik} x_k = velocity of formation of i as a result of erroneous replication of any other molecule k
ϕ_i = Constant that corrects for changes of concentration of i as a result of flux of molecules

• *Error threshold relationship*

$$\nu_{max} < \ln \sigma_o / (1 - \bar{q})$$

ν_{max} = the maximum length of the sequence (genetic complexity) that can be maintained during replication
σ_0 = selectivity or superiority of the master sequence relative to its mutant spectrum
$\bar{q}$ = average copying fidelity. The average error rate is $(1 - \bar{q})$. (Different positions of a replicating nucleic acid often show different copying fidelities. In this case $\bar{q}$ is the geometric mean of the copying fidelity value at each position.) The expected number of errors in a sequence of length ν replicating with a fidelity of $\bar{q}$ is $\nu\ (1 - \bar{q})$

The quasispecies theory was aimed at interpreting self-organization and evolutionary optimization of primitive genetic material. It was recognized that error rates lower than about 10^{-2} misincorporations per nucleotide and round of copying would be difficult to attain without participation of catalytic functions contained in some types of protein and RNA structures. This gave rise to the extended notion of catalytic hypercycle which combined the coding ability of nucleic acids (types of molecules well suited as a repository of genetic information) with the catalytic potential of proteins (more complex molecules that can combine multiple active surfaces to accelerate a variety of biochemical reactions).

Real Virus Quasispecies

The second origin of the quasispecies concept was experimental. It had long been suspected that RNA viruses were genetically unstable, as suggested by the abundance of mutants in viral stocks and by the difficulty of maintaining some mutants (conditional lethal, plaque-type) free of wild-type revertants. The first evidence that an RNA virus depicted features of quasispecies was obtained by Weissmann and associates in Zürich working with bacteriophage Qβ. Upon replication in its host *Escherichia coli*, a clone of bacteriophage Qβ generated error copies with high frequency. Quantification of the reversion of a site-specific mutant, and of its growth rate relative to the parental wild-type Qβ, allowed an estimate of the mutation rate for a specific purine transition: about 10^{-4} per round of copying, a value about 10^5-fold larger than that estimated for the mutation rate of DNA genomes. Furthermore, genomic RNA from many phage clones was analyzed by T1-oligonucleotide fingerprinting (rapid nucleotide sequencing techniques were not yet available). The results indicated that, assuming a random distribution of mutations among the genomes analyzed, each infectious RNA differed in 1–2 positions from the average or consensus sequence in the population. Interestingly, the T1-oligonucleotide map of the RNA from the uncloned phage population remained unchanged during 50 serial infections, a fact that must be kept in mind before considering stability of a consensus sequence as evidence against quasispecies. A highly dynamic mutant spectrum can nevertheless produce the same average sequence over many generations of viral replication. Weissmann and colleagues concluded that: 'The genome of Qβ phage cannot be described as a defined unique structure, but rather as a weighted average of a large number of different individual sequences.'

Subsequent studies with many human, animal and plant RNA viruses, or viruses which include an RNA

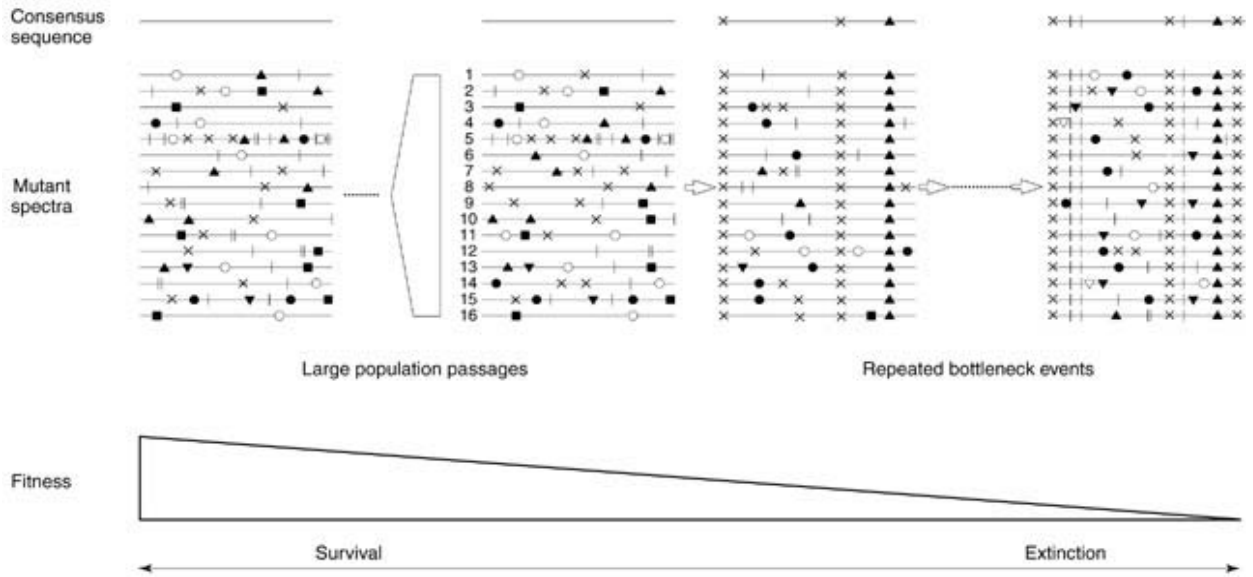


Figure 1 Representation of quasispecies and of possible outcomes of their evolution. Horizontal lines represent individual genomes, and symbols on the lines represent mutations. Large population passages (left) in a constant environment generally lead to fitness gains. The average sequence may or may not be modified as a result of replication. Repeated bottleneck events (right, small arrows) generally result in accumulation of mutations and average fitness losses that may drive viral populations near extinction. For a mathematical expression of quasispecies and definition of terms see **Tables 1** and **2**.

Table 2 Definition of some terms frequently used in the literature of quasispecies

- *Consensus or average sequence (of nucleotides or amino acids)* The sequence that results from taking for each position the most frequent residue (nucleotide or amino acid) found at the corresponding position in a set of aligned sequences. The consensus sequence may not exist physically as a component of the mutant distribution
- *Fitness* A parameter that describes the adaptation of an organism to its environment. For viruses, relative fitness values are quantitated as their ability to produce infectious progeny relative to a reference virus, in a defined environment
- *Master sequence* The dominant sequence in a quasispecies. It often shows the highest selection value among the mutant sequences present, but it may or may not coincide with the consensus or average sequence of the population
- *Mutant spectrum* The ensemble of mutant genomes that constitute a quasispecies
- *Mutation frequency* The proportion of mutants (average for an entire sequence or specific for a defined site) in a genome population
- *Mutation rate* The frequency of occurrence of a mutation event during genome replication
- *Population number* The number of individuals in a population. For viruses, it is the number of infectious genomes replicating in a given biological niche (cell, tissue, organ, organism). The number of genomes quantitated in an infected host is also termed the viral load
- *Rate of fixation of mutations* The frequency of mutations in the viral genome which become dominant per unit time in an infected host or among a set of infected hosts. This rate is generally not constant. The assumption of a molecular clock is not justified for RNA viruses
- *Sequence space* A theoretical representation of all possible variants of a genomic sequence. The sequence space is n^{ν} in which n is the number of symbols in an informational macromolecule and ν is the complexity (total number of residues) of the macromolecule. For a single-stranded RNA virus of 10 000 residues in length the sequence space is $4^{10\,000}$

as a replication intermediate, have documented that they all share high mutation rates or frequencies, and the dynamics of competition and selection predicted by the quasispecies model. The genetic plasticity of RNA replicons led Holland and colleagues to emphasize the multiple biological and evolutionary implications of such a highly dynamic RNA world parasitizing the relatively static DNA world of differentiated organisms.

A generalized quasispecies concept is now used by virologists to describe dynamic distributions of nonidentical but closely related mutant and recombi-

nant viral genomes subjected to a continuous process of genetic variation, competition and selection, and which act as a unit of selection.

Environmental Perturbations and Occupation of Sequence Space

Real virus quasispecies, such as those replicating in the course of acute or chronic infections of differentiated organisms, are permanently perturbed by a complicated array of environmental influences. One is compartmentalization of virus replication within infected organisms: viruses may replicate in disparate cell types, such as epithelial cells of the upper respiratory tract, lymphocytes or brain cells. Different sites within an infected organism will be subjected to different kinds of selective pressures derived either from variations in the internal environment (immune responses, metabolic or nutritional alterations, temperature, etc.) or from externally applied influences such as the presence of antiviral agents. Competition among components of the mutant spectrum will be established within each infected cell. Virus progeny from different cells may engage in subsequent rounds of competition (for entry into target cells, for effective replication, etc.). Such rating events may involve many genomes in the case of acute, systemic infections in which viruses may reach up to 10^{12} infectious units at a given time in the entire organism, and viral RNA loads composed of infectious and noninfectious genomes may reach exceedingly high levels. Evolving mutant distributions are easily shaped by selective forces and perturbed by random sampling events (**Fig. 1**), providing a genetic flexibility that could not be reached if mutations were rare events. Adaptability is facilitated by the limited genome size (generally between 3×10^3 and 3×10^4 nucleotides) and large population numbers. All possible single and double mutants, and decreasing proportions of triple, quadruple, etc. mutants are potentially present in many viral populations. In contrast, only a minute fraction of all possible single mutants is present at any given time in populations of cellular organisms. That is, the ability to explore their allotted share of sequence space is vastly superior for small, abundant replicons than for complex DNA genomes. Furthermore, one or a few nucleotide (or amino acid) replacements are often sufficient to trigger biologically relevant changes (alterations in cell tropism, virulence, escape from immune surveillance, resistance to antiviral inhibitors, etc.). Variant genomes with few mutations have a high probability of occurrence within the mutant spectra of evolving quasispecies. Of course, clouds of mutants are far from being symmetrical, as even single point mutants may have reduced fitness and may not be represented, or represented with low frequency, in the mutant spectra. Mathematical extensions of the initial theoretical quasispecies concept have been developed to cope with some of the perturbations undergone by real virus quasispecies; however, such treatments are complex and difficult to apply to experimental observations with viruses.

Multiple Biological Implications

Quasispecies represents a new description of the population structure of RNA viruses, and it has numerous implications for the biology of this important group of pathogens (**Table 3**). Particularly relevant to viral disease control is the pre-existence and selection of mutants completely or partially resistant to antiviral agents. Examples are influenza virus A mutants resistant to amantadine (1-aminoadamantane) and rimantadine (α-methy-1-adamantane methylamine), human rhinovirus mutants displaying low- or high-level resistance to disoxaril, 5-[7-[4-(4,5-dihydro-2-oxadozolyl)phenoxy]heptyl]-3-methyl-isoxazole, or other related compounds, and, more recently, human immunodeficiency virus mutants resistant to reverse transcriptase and protease inhibitors.

The frequency of antibody-resistant mutants of RNA viruses is generally high (10^{-4}–10^{-6} per infectious genome) both in cell culture and *in vivo*. The frequency of cytotoxic T lymphocyte-escape mutants is more difficult to determine but variations at specific T cell epitopes permitting virus escape *in vivo* have been documented in several systems, such as human hepatitis C virus, human immunodeficiency virus and lymphocytic choriomeningitis virus.

RNA viruses may use a number of alternative receptors or coreceptors for entry into cells, and shifts in receptor specificity may be triggered by amino acid replacements at surface residues on the viral capsid or viral envelope. Mutant viruses able to use either alternative receptors or altered forms of a standard receptor occur in nature and have also been selected in cell culture.

Virulent (disease-causing) variants may be generated in populations of avirulent viruses, or may be present as minority components of attenuated viral preparations. Beck, Levander and associates described that an avirulent coxsackievirus B3 became cardiopathic upon replication in selenium- or vitamin E-deficient mice, as a result of repeated selection of six specific mutations scattered along the viral genome. It is likely that such a phenotypic modification was facilitated by the impaired immune responses, and the larger ensuing viral loads, in infected hosts bearing

Table 3 Some implications of the quasispecies structure of viral populations

- It offers a general and highly effective adaptive strategy
- In particular, it explains the pre-existence and selection in viral populations of mutants with altered phenotypic properties:
  - resistant to antiviral agents
  - antibody and cytotoxic T lymphocyte escape
  - with altered cell receptor specificity
  - with different ability to induce interferon
  - with increased virulence
- It implies the existence of thresholds for phenotypic expression
- There is a possible association between the pathogenic potential of a virus and its mutant spectrum complexity

some nutritional deficiencies. Chumakov and colleagues quantitated the presence of virulent poliovirus in the mutant spectrum of attenuated poliovirus used for vaccine preparation. When the proportion of virulent poliovirus variants in the vaccine exceeded a certain value, the virulent phenotype was manifested and the vaccine failed the safety tests in monkeys. This, and additional results with other viral systems, have established that expression of a variant phenotype encoded in a subset of genomes from the mutant spectrum depends on their proportion in the mutant distribution. These results agree with theoretical predictions on the behaviour of viral quasispecies in that the fate of each individual component of the mutant spectrum is strongly dependent on the mutant cloud surrounding it.

Recent evidence suggests that the complexity of the mutant spectrum may influence the outcome of viral infections. The complexity of the coronavirus mouse hepatitis virus quasispecies may contribute to its pathogenic potential. Likewise, the nonresponse to treatment with interferon α in chronic hepatitis C virus infections may relate to the number of viral molecular species detected in the infected patients. Model experiments with the animal pathogen foot-and-mouth disease virus showed that indeed the repertoire of viral mutants that became dominant in an evolving quasispecies depended on the population size of the virus. Thus, it is not surprising that both the population size and the complexity of the mutant spectrum may be important determinants of the pathogenic potential of some viruses, although the number of well-documented cases is still limited.

Connections with Population Genetics and with Current Concepts of Complexity

The main departure of the quasispecies concept from previous models of population genetics is the emphasis on mutation (or, more generally, in genetic variation) to the point of invalidating the concept of the wild type as a defined genomic sequence. RNA viral genome sequences are statistically defined, but individually indeterminate. Recognition of such indetermination, together with the emphasis on continuous mutant generation, contributed to the success of quasispecies, rather than other quantitative models of population biology, as a descriptor of RNA viruses.

The rapid evolution of RNA viruses has been used to explore some principles and theories of population biology. A concept of increasing interest in virology is fitness (**Table 2**). This is a complex parameter which has been the object of considerable research and debate in biology. Fitness of a virus is measured as its relative ability to produce infectious progeny under a defined set of environmental conditions. Relative fitness values have been determined in tissue culture and in some cases in laboratory mice or other hosts. Cells or organisms are co-infected with the virus to be tested together with a genetically or phenotypically marked reference virus. The progeny virus is passaged for several transfers and the quantification of the proportion of the two viruses relative to the initial mixture yields a relative fitness value. Using this assay a number of observations on the dynamics of viral quasispecies have been made.

When large populations of RNA viruses are allowed to replicate in a defined environment, fitness gains are generally observed when measured in the same environment (**Fig. 1**). However, adaptation of virus to one environment (for example one cell type) may result in profound fitness losses in another environment. Two vesicular stomatitis virus clones with similar relative fitness competing in serial infections coexisted for many passages. However, in a rather unpredictable manner, one of the clones abruptly displaced the other and became dominant in the population. This observation agrees with the competitive exclusion principle of population genetics that states that unless there is a niche differentiation, one of two competing species will eventually outcompete the other. In such competitions both the

Table 4 Some practical implications of the quasispecies structure of RNA viruses

- Vaccines must be multivalent (multiple B and T epitopes)
- Antiviral agents must be used in combination (directed to independent targets; the number depends on viral population size and genome turnover)
- Completely new antiviral strategies should be explored (drugs capable of throwing viral replication into error catastrophe)
- The use of virulent RNA viruses as pest control agents should be avoided

winners and the losers gained fitness in such a manner that their relative position in the fitness landscape was similar at the end and at the start of the competition. This agrees with the Red Queen hypothesis of population biology, as, in the words of the Red Queen in Lewis Carroll's *Through the Looking Glass*, 'it takes all the running you can do, to keep in the same place'.

In contrast to large population passages, repeated bottleneck events such as those mediating serial plaque-to-plaque transfers, resulted in stochastic fitness losses (**Fig. 1**). This is known as the 'Muller's ratchet' effect, according to which asexual populations of organisms will tend to incorporate deleterious mutations in a rather irreversible fashion unless compensatory mechanisms such as recombination can restore the initial, better adapted, genomes. For well-adapted viral quasispecies, repeated plaque isolations will tend to deviate successive mutant distributions from the optimal one, resulting in average fitness losses (**Fig. 1**). The transmission population size needed to maintain viral fitness is dependent on the initial fitness of the viral clone tested. The higher the initial fitness the larger the transmission pool must be to avoid fitness losses. Studies with foot-and-mouth disease virus have documented that viral clones subjected to serial plaque-to-plaque transfers accumulate unusual mutations, providing insight into the types of low-frequency mutants that populate viral quasispecies.

RNA viruses in their evolutionary dynamics constitute attractive experimental systems of complex adaptive behaviour. They display a highly indeterminate fine structure as well as a rather unpredictable behavior. Indetermination arises from the stochastic nature of mutagenesis, in conflict with the need of the system to ensure genetic continuity. Adaptability stems from the variable degrees of success of subsets of genomes. The indetermination of mutagenesis, together with the directionality of selective forces, situate RNA viruses in a subtle border between reproducibility of some observations and unpredictability of others. Thus, RNA virus quasispecies may also become interesting model systems for studies on complexity.

Strategies for Disease Prevention and Control

The great adaptability of viral quasispecies creates difficulties for viral disease control, and may also contribute to the emergence of new viral pathogens. When quasispecies was recognized as the most adequate descriptor of pathogenic RNA viruses, the need to adjust antiviral strategies to the new findings became apparent to some scientists. These recommended strategies (**Table 4**) are not yet generally followed, probably because of an inherent tendency of thinking of RNA viruses still as genetically defined entities. If a single selective pressure is applied to limit virus replication, the highly dynamic mutant spectrum is likely to provide variants capable of overcoming the selective constraints. Research on entire new antiviral strategies, such as the possibility of displacement of virus replication into error catastrophe, seems justified in view of accumulating evidence that the copying fidelity properties of viral replicases can be modified by structural alterations of the enzymes.

See also: **Antivirals; Defective interfering viruses; Emerging and re-emerging virus diseases; Immune escape mechanisms; Persistent viral infection.**

Further Reading

Domingo E and Holland JJ (1997) RNA virus mutations and fitness for survival. *Annu. Rev. Microbiol.* 51: 151.

Domingo E, Sabo DL, Taniguchi T and Weissmann C (1978) Nucleotide sequence heterogeneity of an RNA phage population. *Cell* 13: 735.

Domingo E, Holland JJ, Biebricher C and Eigen M (1995) Quasispecies: the concept and the word. In: Gibbs AJ, Calisher CH and Garía-Arenal F *et al* (eds) *Molecular Basis of Virus Evolution*, p. 171. Cambridge: Cambridge University Press.

Eigen M and Schuster P (1979) *The Hypercycle – A Principle of Natural Self-Organization*. Berlin: Springer.

Holland JJ, Spindler K, Horodyski F *et al* (1982) Rapid evolution of RNA genomes. *Science* 214: 1577.

Nowak MA (1992) What is a quasispecies? *Trends* in *Ecol. Evol.* 7: 118.

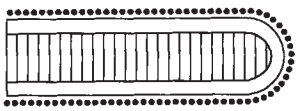
R

Rabbit Fibroma Virus *see* **Poxviruses**

Rabbit Hemorrhagic Disease Virus *see* **Caliciviruses**

Rabbitpox Virus *see* **Mousepox and Rabbitpox Viruses and Poxviruses**

RABIES VIRUS (*RHABDOVIRIDAE*)



George M Baer and **Noël Tordo**, Laboratorios Baer, Mexico City, Mexico

History

The dramatic clinical signs of rabies have been recorded from early times: hyperexcitability, increased tendency of animals to bite, jaw paralysis, changed facial expression, and transmission of the disease to other animals and, eventually, humans. One of the earliest references to the disease is from the third millenium BC, in the Eshnunna Code preceding the Code of Hammurabi:

> If a dog is mad and the authorities have brought it to the attention of the owner; if he does not keep it in and it bites a man and causes his death, then the owner shall pay 40 shekels of silver. If it bites a slave and causes his death he shall pay 15 shekels of silver.

Rabies in dogs and its transmission to man were well recognized by the time of Aristotle. Pliny and other writers referred to the influence of the dog-star Sirius, including the increased susceptibility of dogs to rabies during the so-called 'dog-days' of summer.

Outbreaks of rabies in dogs, foxes and wolves were reported from most European countries throughout the Middle Ages, predating the current European outbreak in foxes which began in the early 1940s. The first cases in the United States were reported in Virginia foxes in 1753. Bites by rabid wolves (animals often reported rabid in Iran, Afghanistan and the Soviet Union) still rank as the most dangerous source of the disease for humans. Mortality rates after wolf bites are well over 50%, although dogs still cause over 90% of human rabies deaths world-wide; in many developing countries the numbers of deaths still reach many thousands annually.

Rabies in vampire bats was first suggested by chronicles of Spanish conquistadores in the sixteenth and seventeenth centuries and confirmed in Brazil, Argentina and Trinidad in the early 1900s. Vampire rabies is still a major animal health problem in most Latin American countries, with hundreds of thousands of cattle deaths yearly. Insectivorous bats are a sporadic problem in North America, Latin America and Europe.

The classic studies of Pasteur beginning in 1881 showed that the central nervous system was the principal site of rabies virus replication, that the virus could be passaged in experimental animals, and that vaccines could be prepared from virus thus passaged. These studies led to his first preparing and then administering human vaccine in 1885. It is surprising that the same vaccine was not used for mass dog vaccination programs until 1921 when Umeno and Doi initiated urban control programs in Japan.

The first specific diagnostic tool, the 'Negri body', was discovered by Adelchi Negri in 1903. This rather insensitive tool was commonly used until the fluorescent antibody technique replaced it in the late 1950s.

Taxonomy and Classification

Rabies viruses belong to the family of RNA viruses *Rhabdoviridae*. These viruses have nonsegmented negative-strand RNA genomes enclosed in a lipid envelope derived from the host cell. The bullet-shaped virions are all composed of a nucleocapsid or ribonucleoprotein (RNP) core and an envelope in the form of a lipoprotein bilayer membrane closely surrounding the RNP core. The outer glycoprotein (G) coat is responsible for the induction of neutralizing (anti-G) antibodies. The virion RNP core is tightly wound into coils. Extending from the outer surface of the envelope is an array of spike-like G projections. The rabies-like viruses cross-react serologically with rabies nucleoprotein (N) antisera to varying degrees and include Lagos bat, Mokola, Duvenhage, Obodhiang and kotonkan viruses.

Geographic and Seasonal Distribution

Human rabies occurs world-wide, except in certain islands and peninsulae where the disease has never occurred (Australia and a number of small Caribbean islands) or where it has been eliminated (England, Ireland, Japan, Taiwan, Spain, Portugal). The infected countries can be divided into those that have controlled canine rabies with effective canine vaccination programs (but where wildlife rabies remains) such as all the western European countries, Canada, the US and some Latin American countries, and those countries where canine rabies is still endemic such as most of the African and Asian countries.

Genetics

Since 1981 the molecular genetics of rabies virus has followed two distinct strategies, work with viral messenger RNAs and work with the genome itself; the latter has permitted simultaneous analysis of the intergenic regions. Complete sequence is known for the PV and SAD strains of rabies and the rabies-related Mokola virus. The sequence data have helped to locate the glycosylation sites as well as the signal and transmembrane peptides on the G protein. The potential phosphorylation sites have also been characterized on the N and M1 (NS or phospho-protein). In addition, the important regions in eliciting humoral and T cell-mediated immunity were mapped along the G, M1 and N polypeptides.

Evolution

Conservation of the elements involved in transcription and replication

A comparative analysis of rabies virus strains, rabies-related viruses, rhabdoviruses in general, and paramyxoviruses indicate that strong selective pressure has stressed the following major elements controlling the gene expression: (1) the start and stop transcription signal bordering each cistron; (2) the promoters for RNA synthesis and encapsidation at the 3′ and 5′ genomic ends, respectively; (3) the RNA-dependent RNA polymerase (L protein), by far the most conserved polypeptide; it exhibits six highly conserved domains separated by variable areas, a distribution in agreement with the notion of independent functions (RNA synthesis, capping, polyadenylation and phosphorylation) concatenated within the polypeptide.

An intermediate place of unsegmented negative-strand RNA virus evolution

Proteins other than the L polymerase are poorly conserved. The G protein, although maintaining limited sequences around the main glycosylation site, has varied a great deal, as might be expected for the polypeptide that mediates the first contact with the host. The N protein has also retained small sequence stretches most likely involved in direct interaction with the genomic backbone. The M1 phosphoproteins and M2 matrix proteins are highly varied, as are the untranslated genomic areas.

The G–L intergene is of particular interest because of its large size and its inability to encode substantial peptides. In the same genomic location a fish rhabdovirus (infectious hematopoietic necrosis virus or IHNV) encodes an mRNA of similar size, as do the paramyxoviruses for the HN hemagglutinin protein. The rabies G–L intergene is presumed to be a remnant gene, baptized ψ for pseudogene. It places rabies virus in an intermediate position in the evolution of unsegmented negative-strand RNA viruses, between the rhabdoviruses with condensed genomes (vesicular stomatitis virus, for instance, shows the dinucleotide GA between the G and L), IHNV virus and the paramyxoviruses. The L protein homology studies independently confirm that rabies virus was closer to paramyxoviruses than to VSV.

The rabies ψ pseudogene: the best thermometer of evolution

Since it is a nonprotein coding region highly susceptible to mutation, changes in the rabies ψ pseudogene are more likely to represent the natural evolution of the virus outside any external selective pressure, and this site may therefore be a suitable target for

epidemiologic studies. By use of the polymerase chain reaction (PCR) technique directly from brain samples it was shown that:

- There is up to 18% divergence in the ψ pseudogene of different vaccine strains, most of which are derived from the original Pasteur strain.
- Wild isolates from a given geographic area are clearly related, with less than 2.5% divergence.
- Wildlife isolates differ by approximately 15% from vaccine strains (but complete cross-protection by those vaccines is still achieved).
- In West Africa (Ivory Coast, Cameroon, Niger and Morocco) where vaccine failures have often been noted, there is a greater (25–40%) divergence from the vaccine strains.
- European bat isolates tend to be completely different from vaccine strains at the ψ pseudogene. At the G and N gene, those known to be important in initiating the immune response, the divergence between isolates from European bats and isolates from vaccine strains is comparable to the difference vs Mokola virus (against which rabies vaccines are ineffective).

Serologic Relationships and Variability

The lyssavirus genus includes a wide variety of rabies viruses, both laboratory-adapted and naturally occurring 'street' viruses, almost all of which can be differentiated through the use of antinucleoprotein monoclonal antibodies. Monoclonal antibody analysis of various rabies variants indicates a remarkable stability in their pattern over many years, with a bat virus from all Mexican freetail bat, for instance, giving the same pattern in the early 1980s and the early 1990s. The analysis of variants also shows that the predominant virus circulating in a given epidemiologic area or 'niche' (such as raccoon rabies in eastern US) is also found in the animals that raccoons bite such as skunks and groundhogs; bat viruses tend to give a different pattern. The common rabies vaccines prepared from 'fixed' viruses such as LEP (low egg passage Flury), HEP (high egg passage Flury), ERA, PM and PV virus strains protect against all rabies 'street' viruses.

Within the lyssavirus genus there are also 'rabies-like' viruses, originally isolated in Africa and markedly different from the rabies viruses in their NP pattern. They include a virus isolated from a bat in Nigeria (Lagos bat), from a human bitten by a bat in South Africa (Duvenhage), or from shrews in Nigeria (Mokola). Common rabies vaccines do not protect against these virus strains.

Epidemiology

Rabies is endemic world-wide, either in dogs or in wild animals, except in those limited areas of the world (mostly islands) where rabies has never existed or where it has been eliminated. Most developing countries of Asia, Africa and Latin America have many cases of rabies in dogs which result in many human antirabies vaccinations and many human deaths. In most of these countries the approximate rate of human antirabies vaccination is 1:1000 population annually.

The picture is quite different in regions where rabies in dogs has been controlled and the disease is prevalent in a variety of wild animals, such as red foxes (western and eastern Europe and Ontario, Canada), arctic foxes (all circumpolar areas), skunks (midwest US), raccoons (eastern US), mongooses (Asia, South Africa, Cuba, Puerto Rico, the Dominican Republic, Haiti and Grenada), wolves (Iran, Afghanistan and the former USSR), vampire bats (northern Mexico to northern Argentina), insectivorous bats (Latin America, the US, Canada and, rarely, western Europe), and frugivorous bats (South America and the Caribbean). In those areas rabies is mostly transmitted within one species and rarely outside, with, for instance, rabies transmitted freely within skunk populations but only occasionally to other animals they bite, such as cows. Rabies rarely 'crosses over' and begins an outbreak in a species bitten by the original species involved.

But rabies in Latin America is almost always transmitted by one species of bat, the common vampire *Desmodus rotundus*, an animal that feeds solely on blood, mostly cattle blood. Every year there are hundreds of thousands of rabies deaths in cattle bitten by vampire bats; rare human outbreaks have also been reported, mostly in Trinidad, Brazil and Peru. Rabid insectivorous bats, on the other hand, rarely infect other species such as humans, cats and wild animals.

Transmission and Tissue Tropism

Rabies is almost always transmitted by saliva via bites or scratches. In rabid animals the submaxillary salivary glands (the most commonly involved extraneural organ) are infected about 75% of the time, with levels of virus often exceeding 10^8 infectious doses (mouse or tissue culture) per ml. After its introduction by a bite the virus stays at the local site for a variable incubation period, usually several weeks, then advances up the peripheral nerves to the central nervous system; it often reaches the brain and the salivary glands before changes in the behavior of the animal occur. There is, then, a dangerous

preclinical period of virus excretion in which the animal can infect other animals or humans without exhibiting any clinical abnormality such as jaw paralysis, excitation or changes in locomotion. During the terminal centrifugal spread of the disease (again by peripheral nerves) a variety of organs are commonly infected, including salivary glands, skin, lungs, kidneys and gonads.

Pathogenicity

Rabies viruses differ in their pathogenicity, but those differences have been difficult to measure. Certain laboratory adapted viruses (i.e. 'fixed' viruses) are highly invasive when injected either intracerebrally or intramuscularly. The invasiveness (as measured by the difference in mortality after intracerebral or intramuscular injection) of two 'street' isolates from one species in one geographic area can differ by as much as a thousandfold; it is not clear what factors this difference is due to, but it may include some factor in the saliva, the number of defective-interfering particles in the sample, and the particular characteristics of the virus. The pathogenicity also depends on the species of animal bitten, some species such as foxes being exquisitely susceptible to the virus, while others, such as the opossum, are much more resistant. Humans are quite resistant to rabies, with the expected mortality in persons bitten by rabid animals yet untreated being far below 50%; in observations made in the late 1800s, before rabies treatments were initiated, it was noted that 15% of persons died after severe and multiple hand bites by rabid dogs, while 85% survived without any treatment.

Clinical Features of Infection

Rabies in humans usually begins with mild and nonspecific symptoms which lead to an initial diagnosis of a common and minor bacterial or viral infection. A specific symptom often noted during the progression of the disease is pain or paresthesia at the bite site (usually the hand or foot). The acute neurological period begins with obvious nervous dysfunction, often including hyperactivity and, later, paralysis. Fever, nuchal rigidity, muscle fasciculation, convulsions, hyperventilation and excess salivation may be seen. The majority of agitated patients ('furious rabies') develop marked anxiety or agitation, sometimes accompanied by hydrophobia and aerophobia; during periods of agitation the patient's mental state fluctuates between periods of increasingly severe agitation and periods of normal behavior or depression. This acute period ends after 2–7 days. 'Paralytic rabies', with paralysis dominating the symptoms, is seen in about 20% of patients. Coma follows a transition period which begins with apneustic breathing; death is thought to be caused by respiratory arrest.

Pathology and Histopathology

There is little gross pathology in humans or animals that die of rabies; congestion of the meningeal vessels may occasionally be noted. The most common histologic change is perivascular infiltration, especially in the brainstem (the pons and medulla) as well as the spinal cord, basal ganglia and cerebral cortex. The neuronal degeneration and other inflammatory changes are variable. Negri bodies, specific (pathognomonic) cytoplasmic inclusions, are found in approximately 75% of rabid animals; these inclusions are most notable in the Purkinje cells of the cerebellum and in the hippocampal gyri (Ammon's horn). The absence of these inclusions does *not* rule out the diagnosis of rabies.

Geographic and Seasonal Distribution

The geographic distribution of the disease is summarized in the Epidemiology section. Rabies in dogs appears to have a somewhat seasonal character, with an increase in cases during the summer months; this was early attributed to the influence of the dog-star Sirius, but actually may be due to heat cycles in female dogs which lead to fighting among male dogs and an eventual increase in subsequent rabies cases. Rabies in terrestrial wild animals has not been reported to be seasonal; bat rabies, however, is distinctly seasonal in nontropical areas.

Host Range and Virus Propagation

Rabies virus infects a very broad array of animal species, perhaps the widest range of any animal virus. Commonly infected are dogs, wolves, foxes, jackals, skunks, raccoons, raccoon dogs, vampire bats, insectivorous bats and mongooses; the animals bitten by these species develop sporadic cases of rabies (these include cats, cows, horses, badgers and woodchucks) although the virus in those species rarely becomes enzootic. Humans are mostly infected after bites by rabid dogs.

Propagation is almost always by bite. Virus appears in the saliva of infected animals either when they are symptomatic, or for days or weeks before clinical signs appear. The presymptomatic excretion of virus in saliva is a very important epidemiologic characteristic for virus survival, and a crucial piece of information for physicians who judge whether an exposure has occurred in persons bitten by rabid (or possibly rabid) animals.

Experimental hosts include mice (the animal most commonly injected for confirmatory laboratory diag-

nosis), hamsters and rats (often used for pathogenesis studies), dogs (pathogenesis, vaccine efficacy), raccoons and skunks (oral vaccination). Suckling (1–3 day old mice) have been used for human and animal rabies vaccine production in many countries, mainly in Latin America, since their myelin content is much lower than that of adult animals, and virus titers in these young animals tend to be very high. Embryonating eggs have been used for propagation of attenuated vaccines (LEP and HEP). Street rabies viruses can be grown on mouse neuroblastoma cells with relative ease, and those cells are the most sensitive for confirming an initial diagnosis by fluorescent antibody. Tissue culture-adapted viruses grow to high titers on BHK cells. Other cells such as VERO, human diploid, chick and duck embryo cells are also commonly used for vaccine preparation.

Immune Response

Animals or humans vaccinated with classic rabies vaccines respond with a rise in neutralizing antibodies, the level reached generally being proportional to the potency of the vaccine. Neutralizing antibodies arise in direct response to the glycoprotein (G) gene; recombinant vaccines constructed with the cDNA of rabies virus glycoprotein also give rise to neutralizing (anti-G) antibodies. Animals with neutralizing antibodies, even at low levels, almost always survive subsequent challenge with 'street' rabies virus. Recently the protective role of the N gene has been recognized in experimental animals, although its significance in vaccinated animals (or humans) is not known. The cellular immune response after vaccination involves a wide array of cells. Serum neutralizing antibodies in individuals (humans or animals) that sicken with rabies are rarely noted before the eighth day of illness; antibodies appear in the cerebrospinal fluid 1 or 2 days later.

Prevention and Control of Rabies

Rabies may be controlled at three levels: human, domestic animal and wild animal. Until widespread canine vaccination programs were initiated in the late 1940s rabies was controlled only at the human level, with hundreds of thousands of persons vaccinated world-wide for exposure to rabid animals, mostly dogs. Rabies in dogs was controlled when potent animal vaccines became available (especially after the advent of proper vaccine potency tests) along with effective dog vaccination programs (those resulting in the immunization of at least 70% of community dogs) and stray dog control. This stopped dog-to-dog rabies transmission as well as serving as a barrier between infected wild animals and human populations. Recently an additional step has been taken involving the oral rabies vaccination of wild animals (foxes, raccoons) to eliminate rabies in those populations.

Future Perspectives

Many issues remain unsolved in rabies: what causes 15% of persons bitten in the hand by rabid dogs to die, while 85% survive without treatment? Where is the virus during the long incubation periods? What makes the virus break away at the end of that period? Why do some outbreaks in wild animals fade away after a few decades while others 'simmer' for much longer periods? Is there a better and less expensive treatment for exposed humans than that now administered (antiserum – or globulin – and vaccine)?

As the examination of molecular aspects of rabies permits the unravelling of the mysteries of pathogenesis we should see more investigation of antiviral substances and the development of more effective and less lengthy human treatments. Examination of viral isolates from various areas of the world will permit examination of the viral genomes of more and more rabies viruses, and clarify whether some are so far from the 'classic' strains as to require separate vaccines for the protection of humans and animals. The future should also bring a better understanding of just how effective vaccines can be delivered to unvaccinated animals such as wild animals, or, more important, community dogs in developing countries.

See also: **Epidemiology of viral diseases; Nervous system viruses; Rabies-like viruses (*Rhabdoviridae*); Vaccines and immune response; Vesicular stomatitus viruses (*Rhabdoviridae*); Zoonoses.**

Further Reading

Baer GM (ed.) (1991) *The Natural History of Rabies*, 2nd edn. Boca Raton, Florida: CRC Press.

Dietzschold B, Rupprecht CE, Zhen Fang Fu and Koprowski H (1996) Rhabdoviruses. In: Fields BN *et al.* (eds) *Fields Virology*, Third Edition, p. 1137. Philadelphia: Lippincott-Raven Publishers.

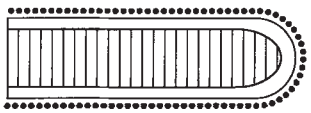
Dietzschold B *et al.* (1988) Antigenic diversity of the glycoprotein and nucleocapsid proteins of rabies and rabies-related viruses: implications for epidemiology and control of rabies. *Rev. Infect. Dis.* suppl. 4: 785.

Smith JS (1996) New aspects of rabies with emphasis on epidemiology, diagnosis, and prevention of the disease in the United States. *Clinical Microbiology* 9: 166–76.

Tordo N and Poch O (1988) Structure of rabies virus. In: Campbell JB and Charlton KM (eds) *Rabies. Developments in Veterinary Virology*, p. 25. Boston: Kluwer Academic.

[This article is reproduced from the 1st edn (1994).]

RABIES-LIKE VIRUSES (*RHABDOVIRIDAE*)



Robert E Shope, Center for Tropical Diseases, University of Texas Medical Branch, Galveston, Texas, USA

History

Rabies virus was thought to be a single serotype without relatives until 1969. That year workers at Yale University and the Centers for Disease Control in Atlanta, Georgia discovered that two viruses from Africa were bullet-shaped like rabies, and were related serologically to rabies virus. One of these, Lagos bat virus had been isolated in 1956 by L.R. Boulger and James Porterfield from brains of *Eidolon helvum* fruit bats captured on Lagos Island, Nigeria. The second, Mokola virus, was isolated by Graham Kemp in 1968 from the organs of shrews of the genus *Crocidura* captured in the Mokola District of Ibadan, Nigeria. Subsequently, it was discovered that the virus Obodhiang, isolated by Jack Schmidt in 1963 from *Mansonia* mosquitoes in the Sudan, and another virus isolated in 1967 by Graham Kemp and Vernon Lee from *Culicoides* midges in Ibadan, Nigeria and named kotonkan virus, were distant serologic relatives of rabies virus. The mosquito and midge viruses have not been recovered from naturally infected vertebrate animals and may be insect viruses.

The full significance of the finding in 1969 that viruses from Africa were related to rabies virus was not realized at the time. In 1971, however, Mokola virus was isolated from a fatal human case of central nervous system disease in Ibadan, Nigeria.

In 1970, a South African farmer was bitten on the lip by a bat. He later died of what was clinically thought to be rabies. The virus isolated from his brain was recognized as a new rabies-related virus and was named Duvenhage virus.

Lagos bat virus has since been isolated from a cat and a bat in South Africa, from a bat in Central African Republic, and from a rabid dog in Ethiopia, but has not been associated with human illness. Mokola virus has been subsequently isolated from apparently rabid dogs and a cat in Zimbabwe, and from a rabid dog in Ethiopia. Both Lagos bat and Mokola viruses have thus demonstrated their potential for rabies-like pathogenicity; Mokola virus was also isolated from shrews in Cameroon and from a rodent, *Lophuromys* in the Central African Republic. The most striking finding, however, was the discovery of viruses most closely related to Duvenhage virus in bats in Germany. In 1985, a bat biologist died of this virus (since named European bat lyssavirus) infection. It is now clear that close relatives of Duvenhage virus are widely distributed in bats throughout Europe, and represent a hazard to human health.

A lyssavirus was recovered from the brains of flying foxes (fruit bats) *Pteropus alecto* and *P. scapulatus* found in New South Wales and Queensland, Australia in 1995 and 1996. These are the first reports of enzootic lyssavirus infection in Australia. A fatal human case in a woman who took care of bats occurred in 1996. Preliminary serological studies indicate that the Australian bat lyssavirus is neutralized by rabies antibody, although it is not identical to rabies in its reactivity with monoclonal antibodies. It is provisionally considered a member of the classic rabies serotype 1. A partial sequence is 92% homologous with rabies virus at the amino acid level.

Charles Calisher and his colleagues in 1989 tested 89 rhabdoviruses for antibody crossreaction by immunofluorescence and found that 19 reacted with the known rabies-related viruses or with rhabdoviruses that in turn reacted with the known rabies-related viruses. Other than rabies, Mokola, Lagos bat, Duvenhage virus and European bat lyssavirus, none of these rabies relatives has been implicated as a cause of rabies-like disease in people or domestic animals. Here, the properties of the rabies-related lyssaviruses that cause rabies in domestic animals and/or people are described, whereas kotonkan, Obodhiang, and the 19 other rhabdoviruses that have distant serological relationships to members of the genus *Lyssavirus* are not detailed.

Taxonomy and Classification

The rabies-related viruses belong to the genus *Lyssavirus* of the family *Rhabdoviridae* of RNA viruses. These viruses have nonsegmented negative-strand RNA genomes enclosed in a lipid envelope derived from the host cell. The particles are covered by glycoprotein (G), a layer of spikes contained in the envelope, which endows the particles of at least two of the viruses, Mokola and Lagos bat, with the ability to agglutinate avian red blood cells. Beneath the lipid envelope is a membrane phosphoprotein designated P associated with a helical complex containing the

nucleoprotein (N) and the polymerase protein (L). A matrix (M) protein forms the inner lining of the lipid envelope. The viruses in the genus are bullet-shaped; Obodhiang and kotonkan resemble bovine ephemeral fever virus with mostly cone-shaped particles whereas the other members are indistinguishable from rabies particles. They have parallel sides, one flat end and one hemispherical end, and are approximately 180 nm in length and 65 nm in diameter.

The lyssaviruses are classified in five serotypes: serotype 1, rabies; serotype-2, Lagos bat (Lag); serotype-3, Mokola (Mok); serotype-4, Duvenhage (Duv) and serotype-5, European bat lyssavirus (EBL). These types were historically distinguished by neutralization test using polyclonal sera. Serotypes are currently typed and subtyped using a battery of anti-N monoclonal antibodies by immunofluorescence, and anti-G monoclonal antibodies by neutralization test. The serotypes correspond to genotypes except that EBL has two recognized genotypes, EBL1 and EBL2.

Geographic and Seasonal Distribution

Rabies-related viruses are geographically limited to Africa, except for European bat lyssavirus which is found in Europe. European Bat lyssaviruses are recognized throughout Europe from Spain to Russia and Ukraine. The rabies-related virus distribution is almost certainly a function of the range of reservoir hosts. There is no pattern of seasonality recognized.

Host Range and Virus Propagation

The reservoir hosts of Lagos bat, Mokola, Duvenhage, and European bat viruses are inferred from knowledge of sources of virus in nature. Lagos bat virus has been isolated at least nine times from bats. Where the bats were identified, these were fruit-eating bats: *Eidolon helvum* in Nigeria and Senegal, *Micropterus pusillus* in the Central African Republic, and *Epomophorus wahlbergi* in South Africa. The virus has presumably spilled over into domestic animals. It caused rabies in cats in South Africa and Zimbabwe, and in a dog in Ethiopia.

The reservoir of Mokola virus is believed to be the shrew, *Crocidura* spp., from which it has been isolated in Nigeria and Cameroon. A single isolation was made from a rodent, *Lophuromys sikapusi* in Central African Republic. The virus was recovered from rabid dogs in Zimbabwe and Ethiopia, and from rabid cats in Zimbabwe and South Africa indicating spillover into the domestic animal population. A fatal human case was recorded in Nigeria.

Insectivorous bats are implicated as the reservoir host of Duvenhage virus. The virus has been isolated repeatedly from *Eptisicus serotinus* throughout Europe, and once from *Nycteris thebaica* in Zimbabwe. The bat that bit the lip of the South African farmer who died of Duvenhage virus infection in 1970 was not identified.

European bat virus has been isolated repeatedly from *Eptisicus serotinus* (genotype EBL1) and *Myotis dasycneme* and *M. daubentonii* (genotype EBL2).

Lagos bat, Mokola, and Duvenhage viruses kill baby and adult mice following intracerebral inoculation. The three viruses induce plaques in Vero cells, and Duvenhage virus was readily adapted to form plaques in BHK-21 cells.

Wild-caught *Crocidura flavescens manni*, the African giant shrew, were inoculated in the laboratory with Mokola virus. Many of these became infected, including shrews exposed orally. Virus was recovered from brain, salivary glands, kidney, pancreas, lung and mouth swab. Virus was also sometimes detected in blood. Some animals became sick. They tended to save their food without eating it, to be more aggressive than uninfected animals, and to develop flaccid hind-limb paralysis. Shrews attack other animals in nature. Both sick and apparently healthy infected shrews transmitted Mokola virus to laboratory mice by bite.

Rhesus monkeys and beagle dogs were inoculated experimentally with Lagos bat and Mokola viruses. Animals inoculated intracerebrally invariably developed encephalitis indistinguishable from rabies and either died, or were killed. Dogs inoculated intramuscularly (i.m.) survived without illness as did the majority of monkeys inoculated i.m. One of five monkeys inoculated i.m. with Lagos bat virus developed unilateral paresis, but survived. One of five monkeys inoculated i.m. with Mokola virus developed tremors and died on day 19 postinoculation. Thus, Mokola and Lagos bat viruses are less pathogenic after i.m. inoculation than street rabies virus.

Mokola virus was passaged sequentially in baby mice, *Aedes albopictus* mosquito cells, and Vero cells. This passage material was found to infect *Aedes aegypti* mosquitoes by intrathoracic inoculation and was maintained by sequential passage in mosquitoes for 340 days. The virus was found in salivary glands, but in higher titer in nervous tissue of the mosquitoes. Mokola virus was transmitted transovarially in the mosquito, but infection of mice by mosquito bite was not demonstrated. Whether Mokola virus can be transmitted by arthropods in nature is still not known.

Genetics

The RNA of Mokola virus was cloned and the cDNA sequenced. The genome is very similar in length and

organization to rabies virus. The length is estimated at 12 000 nucleotides. The genes, designated by the proteins they code, are arranged as follows: 3′-N-P-M-G-L-5′. The 3′ and 5′ end sequences of Mokola virus are highly conserved when compared to the PV strain of rabies virus, and the Mokola end sequences appear to be complementary.

Evolution

Rabies and the other lyssaviruses are postulated to have evolved initially on the African continent. This hypothesis is based on the known African distribution of nearly all lyssaviruses described. Sequence analysis of the gene coding for the N protein of the European bat lyssavirus shows that EBL1 and EBL2 have probably evolved from a progenitor on the African continent. The worldwide distribution of rabies virus can be explained by transport with people of domesticated dogs which were infected and carried extensive distances by sailing ships from Africa during the long incubation period of the virus. For this hypothesis to be credible, one also needs to postulate that rabies virus spread in the Americas, Asia, and Australia from dogs to sylvatic animals such as the skunk, fox, raccoon and bat, and that further divergent evolution occurred in these hosts.

Serologic Relationships and Variability

Serologic relationships among lyssaviruses have been measured by complement fixation, neutralization and immunization challenge tests. The N protein plays a major role in complement fixation. By this method, rabies, Mokola, Lagos bat and Duvenhage viruses differ among themselves by two- to 16-fold in each direction in reciprocal tests. This degree of closeness indicates a relative conservation of the N protein antigens.

The neutralization test measures the surface G antigen. When undiluted hyperimmune sera are tested against various dilutions of infectious virus in the baby mouse, the four lyssaviruses are very closely related. However, the same test done with constant virus dose and various dilutions of antibody shows almost no relationship among the viruses except with undiluted antibody. When the test is done in cell culture using plaque reduction and varying dilutions of antibody, Duvenhage virus is most closely related to rabies, and Mokola virus is quite distinct.

The immunization challenge test is the most likely method to indicate the utility of a rabies vaccine to protect against challenge with other lyssaviruses. Mokola and Lagos bat crossreact minimally with rabies virus; a rabies vaccine with solid homologous protection, yields only a 1.5 log protection index against Duvenhage virus challenge. If these same relationships hold in human infection, there is some rationale for use of rabies vaccines to protect against Duvenhage infection, but the efficacy would not be expected to be optimal.

Batteries of monoclonal antibodies are used to determine the variability in reactivity by immunofluorescence of the G and N antigens. The monoclonal antibodies reactive with the G antigens have also been tested for neutralization. On the basis of reactivity with these batteries, subtypes of lyssaviruses have been recognized. Lagos bat virus has three proposed subtypes (Lagos, Central African Republic and South Africa). Duvenhage and Mokola viruses have only one each, but there is some evidence that the Ethiopian Mokola virus is different from the Nigerian. European bat lyssavirus is still under study, but is considered at this time to be a single antigenic subtype (with two genotypes).

Epidemiology

Lyssaviruses related to rabies are maintained in transmission cycles of terrestrial wildlife or bats. Each serotype is found in one or a relatively limited number of related species. It is believed that transmission to humans or domestic animals represents spill-over from the wildlife cycle. These spill-over events are rare. They may occur when people intrude into wildlife habitats, such as a Finnish spelunker who was infected in 1985 with EBL, or when the wildlife enter houses such as may have happened in Ibadan, Nigeria in 1971 when a 6-year-old girl was infected fatally with Mokola virus.

There is no evidence that epidemics or epizootics occur. Animals and people may survive infection with Duvenhage, Lagos bat and Mokola viruses. Survival of experimentally infected dogs and monkeys following Lagos bat and Mokola virus infection is documented. Mokola virus was reportedly isolated from a child that recovered from poliomyelitis-like disease in Nigeria, although the patient did not develop antibody during convalescence and the validity of the isolation is in doubt. The seasonality, carrier rates, sex and age susceptibility are not known for animals or people.

Transmission and Tissue Tropism

Experimentally, Mokola virus is transmitted by bite of shrews, and there is anecdotal evidence that a bat transmitted Duvenhage virus to a person by bite. Aerosol transmission has not been eliminated as a possible mechanism of spread. Mokola virus was

adapted to mosquitoes in the laboratory, but there appears to be no need to postulate mosquito transmission to explain maintenance in nature.

The limited data available from autopsy and infection of experimental animals indicate that Mokola, Lagos bat and Duvenhage viruses are neurotropic. The brain is the prominent target organ, as in rabies, and virus is also found in salivary glands late in infection.

Pathogenicity and Clinical Features of Infection

The range of pathogenicity of different strains of rabies-related viruses is not known. The clinical features of Mokola and Duvenhage virus infections are recorded in three patients; all presented with central nervous system disease. Duvenhage is associated with Guillain–Barré ascending paralysis and radiating pain in the arm and neck, followed later by agitation, increased respiration rate, muscle spasms, then coma and death. Diabetes insipidus with polyuria complicates the course. The child who died of Mokola virus infection had a prodromal illness of fever and vomiting, then flaccid paralysis of the limbs, progressing to deep coma before death.

Pathology and Histopathology

Specific lesions in the child who died of Mokola virus infection after a 9-day illness were limited to the brain. There were lymphocytic perivascular cuffing, neuronal changes of chromatolysis, eosinophilic necrosis, and nuclear pyknosis most marked in the midbrain and basal ganglia. Eosinophilic cytoplasmic inclusion bodies were found clustered in degenerating neurons. There were several lytic changes in the brain of the Finnish case of EBL. Rabies-reactive antigen was found in central nervous system neurons postmortem.

Immune Response

The very limited observations of patients infected with Mokola and Duvenhage viruses failed to show a specific serological response. The Finnish case of EBL infection was followed through 23 days of illness.

Prevention and Control of Disease Caused by Rabies-related Viruses

Prevention of human infection with rabies-related viruses is based on elimination of exposure in Europe and Africa, contact with bats should be avoided. Sick animals or those with abnormal behavior should be handled only with protective gloves. There is no vaccine available for Mokola or Lagos bat viruses. Commercial rabies vaccine and immune globulin may give limited protection against Duvenhage and EBL infection. Thus persons with bat bites and other bat exposures should receive classic rabies postexposure treatment, but should not expect complete protection. Persons whose occupations lead to exposure to bats should receive pre-exposure rabies vaccination.

Vaccines prepared from Mokola, Duvenhage and European bat lyssaviruses would be useful biologicals, but with the very low attack rates, it is not likely that such vaccines will be developed commercially.

Future Perspectives

The finding in Zimbabwe and Ethiopia of dogs and cats infected with Lagos bat and Mokola viruses is cause for concern. Human exposure and disease may be prevalent and not diagnosed. Active surveillance in Africa for Lagos bat, Mokola and Duvenhage viruses is needed.

Likewise, the threat posed by European bat lyssavirus is not yet completely appreciated. The scientific community needs to maintain vigilance for possible human cases, and needs increased research on the prevalence in bats, the possible spread to species other than serotine bats, and the extent to which terrestrial wildlife and domestic animals are exposed and infected.

See also: **Rabies virus (*Rhabdoviridae*); Rhabdoviruses (*Rhabdoviridae*): Ungrouped mammalian, bird and fish rhabdoviruses.**

Further Reading

Swanepoel R (1994) Rabies: rabies-related viruses. In: Coetzer JAW, Thomson GR and Tustin RC (eds.) *Infectious Diseases of Livestock,* vol. I, p. 520. Cape Town: Oxford University Press.

RECOMBINATION OF VIRUSES

Jozef J Bujarski, Northern Illinois University, Plant Molecular Biology Center, Montgomery Hall, DeKalb, Illinois, USA

Introduction

Biochemically, recombination is a process of creating new genomic molecules by combining or substituting pieces of nucleic acids. Genetically, recombination could be defined as physical exchange of fragments among the parental genetic material. The results of recombination are progeny genomes that contain genetic information in nonparental combinations. Recombination was recognized as an important factor producing the genetic diversity upon which natural selection can operate.

Recombination events can occur in both RNA and DNA viruses. Since the molecular events behind DNA and RNA recombination differ in many aspects, they are described separately below.

Recombination in DNA Viruses

Recombination in many DNA viruses is believed to be accomplished by cellular enzymatic activities. There are two general types of genetic DNA recombination in the cell: homologous recombination (general recombination) and nonhomologous recombination. Nonhomologous or site-specific recombination occurs relatively rarely and requires special proteins that recognize specific DNA sequences to promote recombination. Homologous recombination occurs between two DNA sequences that are the same or very similar in the region of crossovers. Homologous recombination probably occurs in every DNA-based organism and it happens much more often than nonhomologous recombination.

There is plenty of information on the biochemical pathways responsible for crossovers in DNA. Besides sequence identity the requirements of general recombination include complementary base-pairing between double-stranded DNA molecules, recombination enzymes and the formation of heteroduplex within the regions of complementary base-pairing between the two recombining DNA molecules. Studies of the enzymology of DNA recombination in bacteria (and in particular of RecA, RecBCD proteins of *Escherichia coli*) have led to a large amount of literature, including many general reviews. Related recombination activities have been found and studied in eucaryotic sources, including yeast, insect, mammalian and plant cells.

Some DNA viruses encode their own proteins that function during recombination processes. In fact some DNA viruses serve as model systems for the study of recombination. For instance, certain bacteriophages encode the recombination pathways in order to avoid dependence on host systems. Such recombination can be used for repairing damaged phage DNA and for exchanging DNA between related phages to increase their diversity. High-frequency illegitimate recombination was observed at the replication origin of bacteriophage M13 in the *E. coli* host. The crossovers occurred at the nucleotide adjacent to the nick at the replication origin, by joining to a nucleotide elsewhere in the genome. This implied a breakage-and-reunion mechanism of illegitimate recombination, operating in *E. coli*.

Many of the phage recombination activities are analogous to those present in the host bacteria and studies on bacterial recombination systems were influenced by studies on phage systems. These pathways include Rec proteins of phages T4 and T7 (analogous to host RecA, RecG, RuvC or RecBCD proteins), RecE pathway in the rac prophage of *E. coli* K-12, or the phage 1 *red* system. In phage lambda there is another recombination system that can substitute for the RecF pathway components in *E. coli*. Models illustrating functions of RecA protein, RecBCD enzyme and Ruv proteins are shown in **Fig. 1**. A cartoon of the correlation of different stages of DNA recombination with transcription and DNA replication during bacteriophage T4 growth cycle is shown in **Fig. 2**.

Analogies between selected phage and *E. coli* host recombination functions are shown in **Table 1**.

Recombination between viral DNA and host genes was first observed in transducing bacteriophages in procaryotes and for retroviruses in eucaryotes. In some cases this represents a useful way for DNA viruses to acquire cellular genes. Among interesting examples of acquisition of cellular genes by DNA viruses are tRNA genes present in bacteriophage T4. These genes contain introns indicating that bacteriophage T4 must have passed through a eucaryotic host during evolution.

Genetic methods that rely on the use of mutants have been one of the most popular approaches for studying genetic recombination of DNA viruses. In viruses that have a single-component DNA genome,

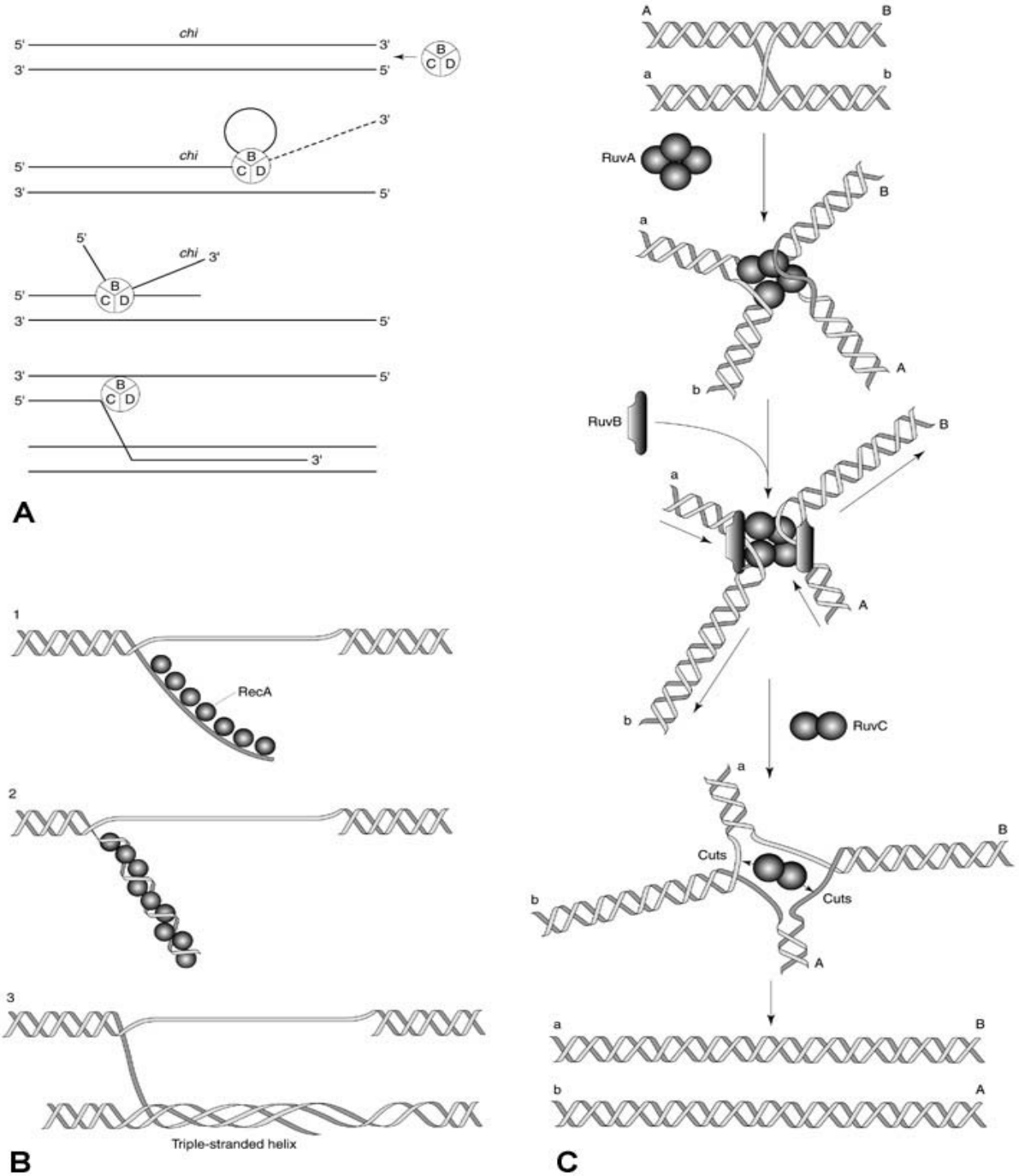


Figure 1 (**A**) Model for promotion of recombination initiation at a *chi* site by the RecBCD enzyme. The RecBCD enzyme loads on to the DNA at a free end or at a double-strand break internal to the DNA. It then moves along the DNA, displaces a loop, and the 3′ end is degraded by the exonuclease activity of the RecBCD enzyme (dotted line). The exonuclease activity is inhibited at a *chi* site, the 3′ end is no longer degraded and can thus invade another DNA molecule. (**B**) Synapse formation between two homologous DNAs by RecA protein. In steps 1 and 2, the RecA protein binds to the single-stranded end and forces it into an extended helical structure. In step 3, the helical single-stranded DNA can pair with a homologous double-stranded DNA in its major groove to form a stable extended triple helix. (**C**) Model for the mechanism of action of the Ruv proteins. RuvA binds to the Holliday junction. Note that the figure starts with one turn of blue-gray heteroduplex. Then RuvB binds to RuvA, and the junction migrates, deriving energy from ATP cleavage. RuvC cleaves two strands of the Holliday junction to resolve the junction into separate DNA molecules. Note the three turns of heteroduplex after junction migration. (Reproduced with permission from *Molecular Genetics of Bacteria* (1997) ASM Press.)

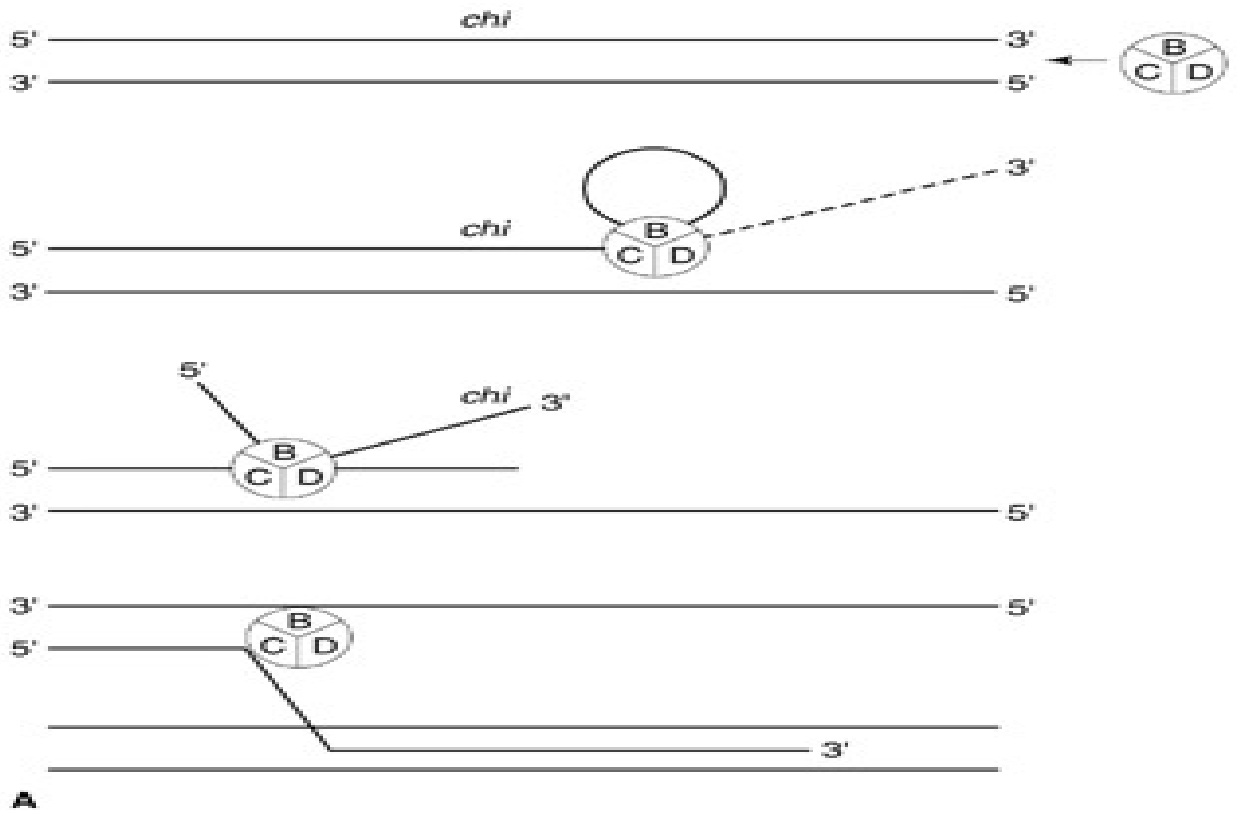


Figure 2 Cartoon showing the correlation between T4 recombination, and transcription and replication processes during bacteriophage T4 life cycle. The upper panel shows early (a), middle (b), and late (c) promoters recognized by forms of the host RNA polymerase that is modified by ADP-ribosylation of the α subunits and by T4-encoded accessory proteins. (d) shows a ribosome-binding site (RBS) of the mRNA for a late protein (Endo VII) that can be sequestered in a long early transcript but is free to initiate protein synthesis from a late transcript. The lower panel illustrates different stages of DNA replication and recombination in the context of the transcriptional program. (e) represents two T4 DNA molecules infecting a bacterium. (f) depicts bidirectional origin of replication cycle. (g) shows a replication–recombination pathway that becomes essential when the RNA polymerase modifications prevent further origin initiation. It is hypothesized that late after infection proteins required to initiate Okazaki pieces become limiting and that some join-cut-copy recombination requiring endonuclease VII occurs (h and i). Packaging proteins finally compete with replication and early recombination proteins for the recombinational intermediates, the concatamers are debranched, cut to headfull lengths, and packaged. (Reproduced with permission from *Molecular Biology of Bacteriophage T4* (1994) ASM Press.)

recombination can involve the exchange of DNA fragments. In contrast, in segmented DNA viruses, in addition to exchanges within each DNA component, the genome segments can be reassorted. This has implications for the recombination behavior observed among mutants. Recombination analyses are most easily performed using conditional-lethal type. The cells are mixedly infected with two mutants at permissive conditions and then the nonpermissive conditions are applied to select for recombinants. The so called two-factor crosses are performed through simple pairwise infections. For single-segmented DNA viruses this approach allows the mutants to be ordered into complementation groups and the relative positions of mutations to be placed on a linear map. For segmented genome DNA viruses, however, due to the reassortant factors, the data obtained in two-factor crosses do not allow mutants to be ordered on to a linear map. Three-factor crosses involve the use of three mutations, with one of them being kept unselected during selection of recombinants between the other two. These crosses are useful for determining linkage relationships between mutants and for establishing the order of marker mutations. Due to reassortment the three-factor crosses are of less use in segmented viruses.

Recombination in DNA viruses of eucaryotes was observed for both animal and plant viruses. The recombination frequencies among pairs of temperature-sensitive mutants (two-factor crossings) were studied in herpes simplex virus. A linear dependence upon the distance between mutations and the fre-

Table 1 Functional analogy between phage and *E. coli* recombination proteins.

Phage	*E. coli*
T7 gene 3	RuvC, RecG
T4 UvsX	RecA
T4 gene 49	RuvC, RecG
T4 genes 46 and 47	RecBCD
I ORF in nin region	RecO, RecR, RecF
Rac recE gene	RecJ, RecQ

ORF, open reading frame.

quency was observed, suggesting the lack of specific signal sequences responsible for crossover events in this virus.

Three-factor crosses were performed using herpes simplex virus. Here, a syncytial plaque morphology mutation was used as an unselected marker for ts mutations. In the case of adenoviruses the host range determined by the helper function of two mutations has been used as a third marker between ts mutants.

Epstein–Barr virus (EBV), a member of the gamma herpesvirus family, is a DNA virus with a long double-stranded DNA genome which shows a high degree of variation among strains. This variation takes the form of single base changes, restriction site polymorphism, insertions and deletions. It was found that some EBV variants arose by DNA recombination events.

Homologous genetic recombination was observed in vaccinia virus (VV) and other poxviruses. This is evident from the high frequency of intertypic crossovers, the ease of the marker rescue and the isolation of viral recombinants. Recombination of VV DNA occurs both intra- and intermolecularly, and is dependent on DNA target size. A function of the viral DNA polymerase or viral DNA replication itself has been implicated and viral proteins with DNA strand transfer activity have been identified.

Intertypic crosses between ts mutants were selected for adenoviruses. Analysis of the segregation patterns of DNA fragments and their restriction enzyme polymorphism has allowed the specific ts mutations to be mapped on adenovirus genomes.

General recombination in somatic cells was observed for SV40 (a papovavirus). The authors tested recombination events from artificially constructed recombinant circular oligomers. While this type of recombination was high, homologous recombination in this type of DNA tumor viruses was rare.

The geminiviruses are a unique group of single-stranded plant DNA viruses. Intermolecular recombination between geminivirus DNAs has frequently been observed using various combinations of mutants. Likewise, intramolecular homologous recombination between tandem repeats of a geminivirus genome was found in agro-infected tobacco plants. Both homologous recombination model and replication-based recombination model were proposed to explain the observed events. In addition, deletions, insertions and other rearrangements have frequently been detected in geminivirus infections. These illegitimate recombination processes may rely on aberrant breakage–fusion events as well as errors of DNA replication, and may be inter- or intramolecular in nature.

Cauliflower mosaic virus (CaMV) is a plant DNA virus. It belongs to the pararetroviruses, which replicate through a reverse transcription step. A high recombination rate was observed *in vivo* for CaMV. The replication cycle of CaMV offers a variety of possibilities for recombination: recombination at the DNA level, which occurs in the nucleus, and at the RNA level, which occurs during reverse transcription in the cytoplasm. In general, it is difficult to decide by which recombination route a CaMV recombinant is obtained. However, such features as recombinational hot spots and apparent mismatch repair might be indicative. Namely, the presence of hot spots reflects replicative (RNA) recombination, while mismatch repair can occur during formation of heteroduplex intermediates and is thus indicative of DNA recombination. Recombination between CaMV strains and CaMV transgene mRNA was observed. It is believed that this type of recombination represents an RNA–RNA recombination event (during reverse transcription).

Recombination in RNA Viruses

RNA viruses utilize RNA as their genetic material. The potential for variation of the RNA genome is very large owing to a high mutation rate (during copying by RNA-dependent RNA polymerase) and to recombination. The terms of classic population biology do not describe RNA viruses. Instead, a term of quasispecies was proposed to reflect the nature of RNA virus populations. The processes of genetic recombination in plus-stranded RNA viruses probably do occur at the RNA level, as these viruses most likely do not go through DNA steps in their replication cycles. RNA recombination processes are generally categorized as either homologous or nonhomologous. In 1992, Lai postulated three classes of RNA recombination: homologous, aberrant homologous and nonhomologous. Homologous recombination occurs between two related RNA molecules at corresponding sites, although homologous RNA

recombination can also occur within a common region shared by otherwise unrelated RNA sequences. Aberrant homologous recombination involves crossovers between related RNAs, but does not occur at corresponding sites, leading to sequence insertions or deletions. Nonhomologous recombination occurs between unrelated RNA molecules. Slightly different definitions, based on mechanistic models and considerations, were proposed recently by several authors.

Although genetic recombination in RNA viruses such as influenza virus and poliovirus has been described, it has not been found in Newcastle disease virus. The complete nucleotide sequences of the genomic RNAs of a large number of RNA viruses belonging to different virus groups have been obtained. This revealed the relatedness of various animal, plant and other RNA viruses and allowed the definition of sequence rearrangements in the viral RNA genome.

Sequence rearrangements were found in the following animal plus-strand RNA viruses: picornaviruses: poliovirus and foot-and-mouth-disease virus (FMDV); in coronaviruses: mouse hepatitis coronavirus (MHV); Sindbis alphavirus (SIN); flock house nodavirus (FHV); and in bacteriophages $Q\beta$, and MS-2. Recombinants were found in bunyaviruses. Genetic rearrangements were also observed in other types of RNA viruses, including influenza virus, a minus-strand RNA virus, in retroviruses, and in double-stranded $\Phi 6$ bacteriophage. The following genomes of plant RNA viruses reveal RNA rearrangements: alfalfa mosaic virus (AlMV), beet necrotic yellow vein virus (BNYVV), bromoviruses (see below), hordeiviruses, luteoviruses, nepoviruses, tobamoviruses, tobraviruses, tombusviruses and turnip crinkle carmovirus (TCV).

Recombination by reassortment was observed for multisegmented animal RNA viruses, including influenza virus and double-stranded reoviruses and orbiviruses. The reassortment mechanism functioning in reoviruses is reflected by the fact that the interpretation of two-factor crosses (using for instance temperature-sensitive mutations) appears to be difficult: the mutants cannot be ordered on to a self-consistent linear map and quite often no linkage between mutants could be detected.

The examples in which host-derived sequences have recombined with viral RNAs include an ubiquitin-coding sequence of bovine diarrhea virus, a sequence from 28S rRNA inserted in the hemagglutinin gene of an influenza virus, and a tRNA sequence in Sindbis virus RNA. For plant viruses, several potato leafroll virus isolates contain sequences homologous to an exon of tobacco chloroplast. Acquisition of chloroplast sequences during RNA recombination was observed for brome mosaic virus.

The use of transgenic plants expressing viral RNA sequences has confirmed that plant RNA viruses are able to recombine with host mRNAs. This was shown for cowpea chlorotic mottle bromovirus (CCMV), red clover necrotic mosaic virus (RCNMV), potato virus Y potexvirus (PVY) and plum pox potyvirus (PPV).

Studies on the molecular mechanism of RNA recombination have progressed when experimental systems that supported the high frequency of crossovers were established. The available data on rearrangements in picornaviruses suggest a mechanism of template switching that occurs during minus-strand RNA synthesis. These rearrangements may be facilitated by the existence of identical or completely dissimilar signal sequences between the recombining RNA substrates.

Certain RNA viruses can produce both homologous and nonhomologous RNA recombinants. The molecular mechanism involved in the formation of homologous and nonhomologous recombinants was tested using an efficient recombination system of brome mosaic virus (BMV). A partially debilitating BMV RNA3 mutant was repaired *in vivo* by exchanges with the sequences of other BMV RNA components. Low recombination frequency was overcome by construction of RNA3-based recombination vectors, where recombinationally active sequences could be inserted and analyzed. It appeared that short base-paired regions between the two BMV RNA recombination substrates can target efficient nonhomologous recombination crossovers. A model invokes the formation of local RNA–RNA heteroduplexes to be responsible for targeting the RNA crossovers as a result of: (1) bringing the RNA substrates into a close proximity; and (2) slowing down or stalling the approaching replicase enzyme complex (**Fig. 3**).

Similarly, homologous RNA recombination was studied by inserting a BMV RNA2-derived sequence into the recombination vector. Both precise and imprecise crossovers were observed. Other RNA sequences revealed that the frequency of RNA2–RNA3 homologous crossovers depends upon sequence composition and tends to occur at hot spot regions that contain stretches of GC-rich alternating with AU-rich sequences. Such nucleotide composition may act as recombination activators during switching between RNA templates by the RNA replicase enzyme (**Fig. 4**). Overall, the data on BMV RNA recombination suggest that molecular mechanisms involved in the two types of crossovers in BMV differ from each other.

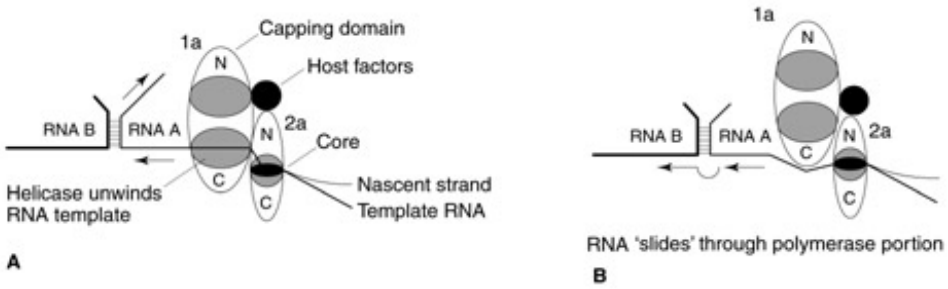


Figure 3 The model of strand switching by BMV replicase. The replicase is composed of the interacting host (smaller black circles) and viral (represented by ellipses) proteins. Functional domains of nucleotidyl transferase (capping enzyme), helicase and core RNA polymerase on proteins 1a and 2a are represented by smaller shaded ellipses. At the replication mode (**A**) the enzyme copies the original template through the double-stranded region (represented by a short 'ladder') due to the helicase action. During recombinational mode (**B**), the polymerase 'slides' under the double-stranded regions and changes the templates from RNA A to RNA B. The arrows indicate the direction of replicase migration. (Reproduced from Bujarski and Nagy (1994) Genetic RNA–RNA recombination in positive-stranded RNA viruses of plants. In: Paszkowski J (ed.) *Homologous Recombination and Gene Silencing in Plants.* Kluwer Academic.)

The term 'recombinosome' was proposed to describe a complex between the recombining RNAs, the replicase proteins and other (putative) factors involved in template switching events. The participation of replicase proteins of BMV in recombination was studied using a temperature-sensitive 1a of BMV protein mutant. This revealed a *5′* shift in crossover sites within the RNA1–RNA3 heteroduplex, suggesting that the helicase domain of 1a participates in heteroduplex-mediated crossovers. Likewise, a single amino acid mutation within the core domain of 2a protein and mutations within the N-terminal portion of 2a, the polymerase component of the replicase, inhibited the frequency of nonhomologous recombination in BMV. These studies confirm the participation of replicase proteins in recombination.

The role of replicase enzyme in RNA recombination was also studied in TCV, a small single component RNA virus that is associated with a number of subviral RNAs, such as satellite RNA D and chimeric RNA C. High frequency recombination was observed *in vivo* between RNAs C and D. A template switching model was proposed where viral replicase utilizes the nascent plus-strand of RNA D to reinitiate RNA elongation at a hairpin structure on the acceptor minus-strand RNA C template (**Fig. 5**).

The participation of TCV replicase in RNA recombination was studied *in vitro* with a TCV replicase preparation and a chimeric RNA template containing the *in vivo* hot spot region from RNA D joined to the hot spot region from RNA C. This demonstrated roles for a priming stem sequence in the RNA C portion and the TCV RNA-dependent RNA polymerase (RDRP) binding hairpin, also from the RNA3 portion. It probably reflects such late steps of the *in vivo* RNA recombination as strand transfer and primer elongation.

For coronaviruses, the animal RNA viruses containing a large RNA genome, recombination has been demonstrated between coronavirus genomes and defective-interfering RNAs, and it was postulated to account for the diversity in the genomic structure of these viruses. The mechanistic considerations suggest the nonprocessive nature of the coronavirus RNA polymerase, which might be responsible for recombination. Similarly, RNA recombination in nodaviruses, two-partite RNA viruses, occurs between RNA segments at a site, where the nascent strand could form a base-paired region with the acceptor template. Such factors as template secondary structure and the similarity of the crossover sites to an origin of replication seem to influence the choice of recombination site. A model of recombination where the polymerase interacts directly with the acceptor nodavirus RNA was postulated.

A copy-choice template switching mechanism was also suggested for recombination in a double-stranded bacteriophage Φ6. Here, the crossovers occur inside the virus capsid structure. Apparently, the crossovers can occur in regions that share little sequence similarity and the frequency of recombinants can be enhanced by conditions that prevent the minus-strand synthesis.

The bacteriophage Qβ has emerged as a unique RNA virus system for the study of RNA recombination both *in vivo* and in cell-free systems. It was demonstrated for the first time in this virus that RNA recombination can occur not by polymerase template switching events but rather via a splicing-type RNA recombination mechanism.

Genetic RNA recombination has been observed in retroviruses. Here, the efficient recombinant jumpings are secured by reverse transcriptase. In fact, the retrovirus system represents a well-established model

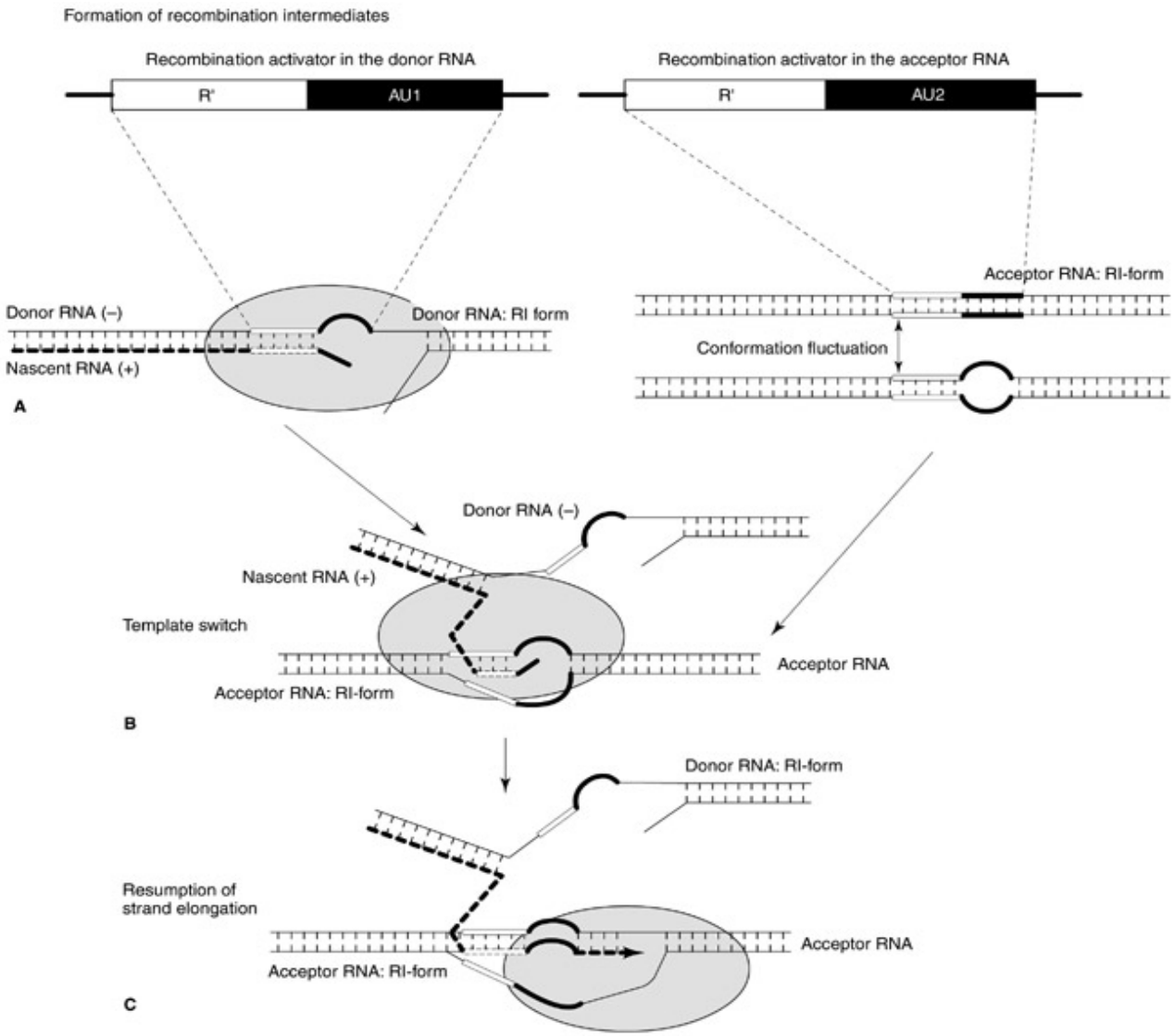


Figure 4 Processive template switching model explaining the formation of homologous recombination hot spots within the recombination activator sequences. (**A**) Template switching of the BMV replicase occurs during positive-strand synthesis. Although localized double-stranded replicative intermediates (RIs) are shown, the existence of single-stranded RNAs with negative polarity is also possible (not shown). The weak base pairing within the AU-rich region (shown on the left side of the diagram with a black line) can facilitate the release of the 3′ end of the incomplete nascent RNA. The weak base pairing within the AU-rich region can also facilitate the temporary formation of a bubble structure in the RI of the acceptor strand (gray line on the right side). The replicase (large shadowed ellipse) pauses on the donor strand at the UA-rich region, and the very 3′ end of the nascent strand disengages from the original template strand. (**B**) The released 3′ end of the nascent strand hybridizes to the acceptor strand facilitated by the bubble structure. Hybridization of the upstream located R′ (shown by empty boxes) stabilizes the recombination intermediate. (**C**) The viral replicase resumes chain elongation on the acceptor strand (shown by an arrowhead). This leads to the formation of homologous recombinant RNA3s. (Reproduced with permission from a paper by Nagy and Bujarski (1997) *J. Virol.* 71(8): 3808.)

of the polymerase/template switching reactions both *in vivo* and *in vitro*. Apparently, the virally encoded reverse transcriptases are evolutionarily selected to secure jumping during reverse transcription reactions. The recombinant jumpings are responsible for both inter- and intramolecular template switching, and for

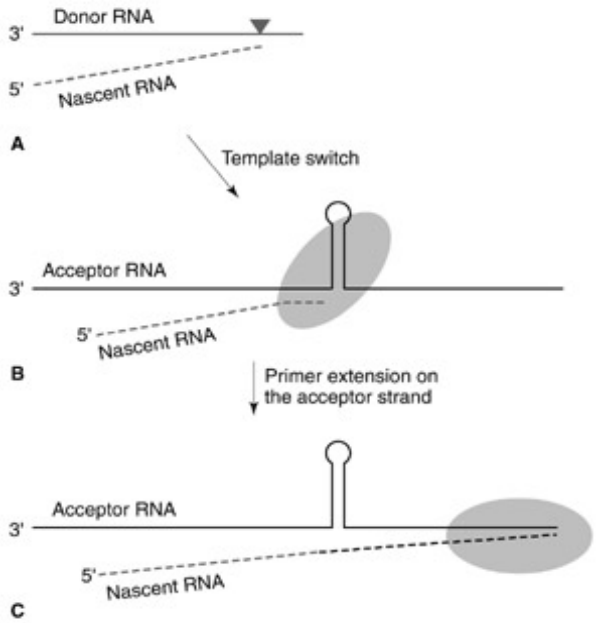


Figure 5 Model for RNA recombination in the turnip crinkle virus (TCV) system. (**A**) The TCV RDRP copying minus-strand sat-RNA D either reaches the natural 5′ end or pauses at some positions (mainly 13 nucleotides from the 5′ end, as indicated by a triangle) likely due to the presence of a protein-binding site. (**B**) The TCV RDRP, which is still associated with the nascent sat-RNA D strand, switches to the acceptor template (minus-strand sat-RNA C or TCV) facilitated by either the motif I or motif III hairpins. Hybridization between the nascent strand and the acceptor strand may stabilize the recombination intermediate. (**C**) The TCV RDRP reinitiates RNA synthesis using the 3′ end of the nascent sat-RNA D as a promoter. Further copying of the acceptor RNA by the RDRP results in a recombinant RNA molecule. (Reproduced with permission from Simon *et al* (1996) *Semin. Virol.* 7: 373.)

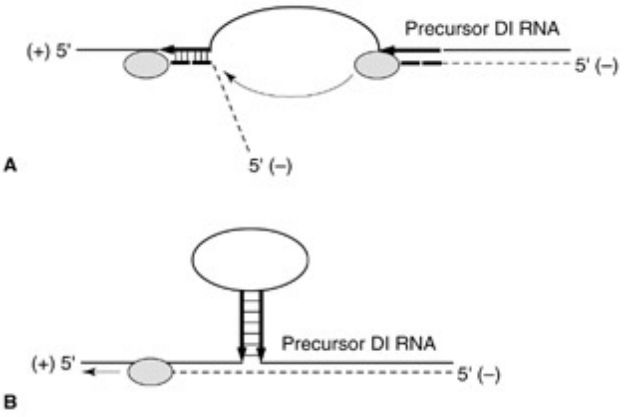


Figure 6 Proposed replicase-mediated model for deletion of segments from precursor DI RNAs. RNA templates are depicted as solid lines while nascent strands are shown as dashed lines. The viral replicase is represented by shaded ovals and its path is indicated by thin arrows. Deletion of segments from precursor DI RNA molecules may be facilitated by tracts (bold arrows) of sequence (**A**) identity or (**B**) complementarity, as discussed in the text. (Reproduced with permission from White A (1996) Formation and evolution of Tombusvirus defective interfering RNAs. *Semin. Virol. 7:* 409.)

the formation of defective retroviral genomes. They contribute significantly to genetic variability of retroviruses.

Defective-Interfering RNAs

Defective-interfering (DI) RNAs are subviral RNA molecules derived from the helper virus genomic RNA and typically interfere with helper virus accumulation and affect symptoms produced by the helper virus. Paul von Magnus was first (in 1954) to report DI RNAs in influenza virus. Later, DI RNAs were observed in a majority of animal and in many plant RNA virus infections. Naturally-occurring DI RNAs have been identified during infection with several coronavirus species. These molecules appear to arise by a polymerase strand-switching mechanism. In fact the DI RNAs were used in coronavirus research, to study the mechanism of high-frequency, site-specific RNA recombination events that progress through leader acquisition during RNA replication, and as vehicles for the generation of targeted recombinants of the parental virus genome.

For plant tombusviruses and carmoviruses the DI RNAs reveal a consistent pattern of rearranged mosaic-type sequences flanked by unmodified terminal regions. Analysis of these DI RNAs suggests that in some cases the base-pairing between an incomplete replicase-associated nascent strand and the acceptor template can mediate the selection of the rearrangement sites (**Fig. 6**).

Single deletion-type DI RNAs were isolated from infections with several viruses, including beet necrotic yellow vein furovirus (BNYVV), soil-borne wheat mosaic furovirus (SBWMV), peanut clump furovirus (PCV), clover yellow mosaic potexvirus (CYMV), sonchus yellow net rhabdovirus (SYNRV), and tomato spotted wilt tospovirus (TSWV). Such factors as the length of the defective RNA or its coding capacity seem to affect the selection of DI RNAs during infection.

For broad bean mottle bromovirus (BBMV) the naturally existing DI RNAs were found to be derived by single deletions in the RNA2 component. A secondary structure-mediated model for BBMV DI RNAs, where local complementary regions bring the remote parts of RNA2 together to facilitate the crossover events, has been proposed (**Fig. 5**). Similar to BBMV, single deletion-type DI RNAs have been found associated with cucumoviruses. Overall, it

seems likely that the mechanisms leading to DI RNA generation are similar to those of RNA recombination.

The closteroviruses are the RNA viruses of plants that have the longest known RNA genome. Recently, multiple species of citrus tristeza closterovirus (CTV) defective RNAs, which resulted from the recombination of a subgenomic RNA with distant parts from the 5′ end of the viral genomic RNA, were identified. This suggests that closteroviruses can utilize subgenomic RNA as factors for the modular exchange and rearrangement of their genomes.

Defective-interfering particles were also found in negative-strand RNA viruses. Namely, for vesicular stomatitis virus (VSV) *cis*-acting terminal sequence elements play an important role during RNA replication. The formation and accumulation of certain classes of DI RNA molecules were observed: the majority of DI RNAs were of the 5′ copy-back class, reflecting the mechanisms involved in DI RNA generation.

In the case of RNA viruses both homologous and nonhomologous recombination events were observed. Various types of copy-choice mechanisms were proposed to explain the formation of RNA recombinants, and the role of special RNA signal sequences and viral proteins in recombination was observed. In some cases a mechanism of RNA breakage and religation cannot be excluded and was experimentally demonstrated for bacteriophage Qβ. Further studies are required to obtain a more general picture of genetic recombination pathways available for viruses, especially for RNA viruses.

See also: **Bromoviruses (*Bromoviridae*); Defective interfering viruses; Genetics of animal viruses; History of virology: Bacteriophages, General; Pathogenesis: Animal viruses, Plant viruses; Phage Homologous Recombination; Replication of viruses; Taxonomy and classification – general.**

Conclusions

Genetic recombination can be found in many groups of DNA and RNA viruses. Both the observed natural sequence rearrangements and the data obtained with the use of experimental systems demonstrate that recombination plays an important role in providing the genetic diversity during virus infections. The molecular mechanisms involved in genetic recombination depend on the class of viruses. DNA viruses utilize mechanisms of homologous (general) recombination available in the host cells, although some DNA viruses encode their own recombination proteins. Also, site-specific (nonhomologous) recombination events were found for certain classes of DNA viruses.

Further Reading

Bujarski J (ed.) (1996 and 1997) RNA recombination and defective RNA formation. *Semin. Virol.* 7/6 (Part I) and 8/2 (Part 2).

Gibbs AJ, Calisher CH and Garcia-Arenal F (eds) (1995) *Molecular Basis of Virus Evolution.* Cambridge: Cambridge University Press.

Holland JJ (ed.) (1992) *Genetic Diversity of RNA Viruses.* Berlin: Springer.

Karam JD (ed.) (1994) *Molecular Biology of Bacteriophage T4.* Washington: ASM Press.

Roossinck MJ (1997) Mechanisms of plant virus evolution. *Annu. Rev. Phytopathol.* 35: 191.

Snyder L and Champness W (1997) *Molecular Genetics of Bacteria.* Washington: ASM Press.

REOVIRUSES (*REOVIRIDAE*)

Contents

General Features

Kenneth L Tyler, Neurology Department, B-182, University of Colorado Health Sciences Center, Denver, Colorado, USA

History

In 1959, AB Sabin proposed the designation reovirus (respiratory enteric orphan virus) for a subgroup of respiratory and enteric viruses not known to be associated with human disease (hence orphan) with particular distinguishing characteristics. The distin-

guishing features of reoviruses included: (1) their size, which at ≥75 nm was larger than other known enteric viruses; (2) their capacity to produce cytoplasmic inclusions in monkey kidney cells in tissue culture; (3) their pathogenicity for newborn but not adult mice; and (4) their capacity to hemagglutinate human type O erythrocytes. All of the viruses that would subsequently be designated the prototypes for the three reovirus serotypes (see below) were originally isolated in the 1950s from the stools of children. The first reovirus was isolated by NF Stanley and colleagues in 1951 from an aboriginal child and subsequently named hepatoencephalomyelitis virus. An enteric virus isolated by M Ramos-Alvarez and AB Sabin in 1953 from the stool of a baby named Lang initially became the prototype virus for ECHO 10, and subsequently for reovirus serotype 1 (reovirus serotype 1 strain Lang). The prototype for reovirus serotype 2 was isolated by Ramos-Alvarez and Sabin from the stool of a child named Jones with a summer diarrheal illness (reovirus serotype 2 strain Jones) in 1955. Viruses isolated by Ramos-Alvarez and Sabin in 1955 from the stool of a child named Dearing and by L Rosen in 1957 from an anal swab from a baby named Abney became the prototype strains for serotype 3 (reovirus serotype 3 strains Dearing and Abney).

Taxonomy and Classification

The genus *Orthoreovirus*, of which the mammalian reoviruses are the prototypes, is one of the nine genera belonging to the family *Reoviridae*. Viruses belonging to this family are nonenveloped spherical viruses of 60–85 nm diameter, whose genome consists of 10–12 discrete segments of double-stranded RNA (dsRNA) that is contained in two or three concentric protein capsids with icosahedral symmetry. Members of different genera can be distinguished by differences in relative size, capsid number and structure, genome segment number, nature and number of structural proteins, and patterns of reactivity with antisera. Viruses belonging to different genera also differ substantially in their host range and pathogenesis. (*See also*: **Orbiviruses and Coltiviruses, Phytoreoviruses, Rotaviruses.**) Nucleotide sequence data indicate that members of the *Orthoreovirus* genus are highly divergent from other *Reoviridae* genera (e.g. *Orbivirus, Rotavirus*) including those known to infect humans and other mammals. Members of the *Orthoreovirus* genus have 10 segments of dsRNA contained in two concentric protein capsids of approximately 85 nm diameter (**Table 1**).

The mammalian orthoreoviruses, which form the subject of this chapter (subsequently referred to as 'reoviruses'), are further classified into three serotypes (designated serotypes 1, 2, 3) on the basis of their patterns of reactivity with typing antisera and differences in their capacity to hemagglutinate human and bovine erythrocytes. Classically, serotyping was performed by hemagglutination–inhibition assay on human type O erythrocytes using serotype-specific antisera. Serotyping can also be accomplished by evaluating the capacity of serotype-specific antisera to produce neutralization as measured in plaque reduction neutralization assays. Genetic studies with reassortant viruses derived from crosses between the prototype strains for different serotypes indicate that the $\sigma 1$ protein, which is encoded by the S1 dsRNA segment, is the major serotype-specific determinant. Accurate serotyping can also be accomplished by evaluating the capacity of serotype-specific sigma 1 monoclonal antibodies to neutralize infectivity of test isolates. The most accurate information concerning the relationship between different reovirus strains can be accomplished by comparison of nucleotide and amino acid sequence variability between their cognate gene segments and encoded proteins. Detailed phylogenetic analyses of the evolutionary relationship between a number of reovirus strains based on the nucleotide sequence of their S1, S2, S3 and S4 gene segments have now been published (**Fig. 1**). Interestingly, the phylogenetic trees of individual viral strains differ depending on the dsRNA segment chosen for comparison, indicating that reassortment of reovirus gene segments occurs under natural circumstances.

Other members of the *Orthoreovirus* genus include a large group of viruses isolated from birds, the avian reoviruses, and several currently incompletely categorized viruses isolated from the flying fox (Nelson Bay virus), baboons and several species of snakes. These viruses all have basic similarities in the nature of their genomic organization (10 dsRNA segments), protein coding pattern and general virion structural organization. Reassortment between these various groups of reoviruses has not been demonstrated and their cognate gene segments and corresponding proteins are extremely divergent in their nucleotide and protein sequences. It is likely that mammalian reoviruses, avian reoviruses, and perhaps some of these other currently uncharacterized reoviruses are independent quasispecies.

Geographic and Seasonal Distribution

Reoviruses are ubiquitous in their geographic distribution. Studies of variation in the seasonal pattern of infection are extremely limited. As noted, many of the initial isolates of reoviruses from humans were made from infants and children with summer diarrheal illnesses. Studies of the prevalence of child-

Table 1 Properties of the mammalian reoviruses

Genome
Double-stranded RNA
10 gene segments in three size classes (L, M, S)
Total size 23 500 bp
Gene segments encode either one or two proteins each
Gene segments are transcribed into full-length mRNAs
Plus strands of gene segments have 5′ caps
Nontranslated regions at segment termini are short
Gene segments can undergo reassortment between virus strains
Particles
Spherical, with icosahedral (5:3:2) symmetry
Nonenveloped
Total diameter 85 nm (excluding σ1 fibers)
Two concentric protein capsids: outer capsid subunits in $T = 13$ lattice, arrangement of inner capsid subunits unknown
8 structural proteins: 4 proteins in outer capsid (λ2, μ1 (mostly as cleavage fragments μ1N and μ1C), σ1, and σ3) and 4 proteins in inner capsid (λ1, λ3, μ2, and σ2)
Subviral particles (ISVPs and cores) can be generated from fully intact particles (virions) by controlled proteolysis.
Cell-attachment protein σ1 can extend from the virion and ISVP surface as a long fiber.
Protein λ2 forms pentamers that protrude from the core surface.
Replication
Fully cytoplasmic
Sialic acid can serve as cell surface receptor for recognition by cell-attachment protein σ1.
Proteolytic processing of outer capsid proteins σ3 and μ1/μ1C is essential to infection and can occur either extracellularly or in endo/lysosomes.
Uncoating of parent particles is incomplete: genomic dsRNA does not exit particles to enter the cytoplasm.
Transcription and capping of viral mRNAs occur within particles and are mediated by particle-associated enzymes.
Segment assortment and packaging involves mRNAs.
Minus-strand synthesis occurs within assembling particles.
Mature virions are inefficiently released from infected cells by lysis.

Reproduced with permission from Nibert *et al* (1996).

hood illnesses associated with reovirus type 2 infection suggested an increased incidence in summer months (June–September).

Host Range and Viral Propagation

Evidence of infection as documented by virus isolation or the presence of antireovirus antibodies, has been found in an enormous variety of animal species including humans, a wide variety of nonhuman primates, swine, horses, cattle, sheep, goats, dogs, cats, rabbits, rats, mice, guinea pigs, voles, bats, a large number of marsupials and several avian species. The clinical syndrome produced by natural reovirus infection in animals includes predominantly respiratory, enteric and neurological disease. Among the more commonly described diseases are conjunctivitis, rhinitis, other upper respiratory tract infections, and pneumonia in horses, cattle, sheep and pigs. Dogs and cats can develop neurological illnesses including encephalitis and 'ataxia' in addition to respiratory and enteric illness. In birds, infection may produce arthritis, enteritis and hepatitis.

Reoviruses can be propagated in a variety of cultured cells. Cytopathic effects (CPE) can be seen in a wide variety of cell types derived from different animal species and tissues including mouse strain L clone 929 cells ('L cells' 'L929 cells', ATCC CCL1), human HeLa cells (ATCC CCL2), African green monkey kidney cells (CV-1, ATCC CCL70), and human embryonal kidney cells (HEK). Growth of virus for preparation of viral stocks, purification of virions, and quantification of viral titer by plaque assay is most commonly performed in L929 cells.

Genetics

The genome of reoviruses consists of equimolar quantities of ten discrete segments of dsRNA which can be divided into three size classes: large (dsRNA

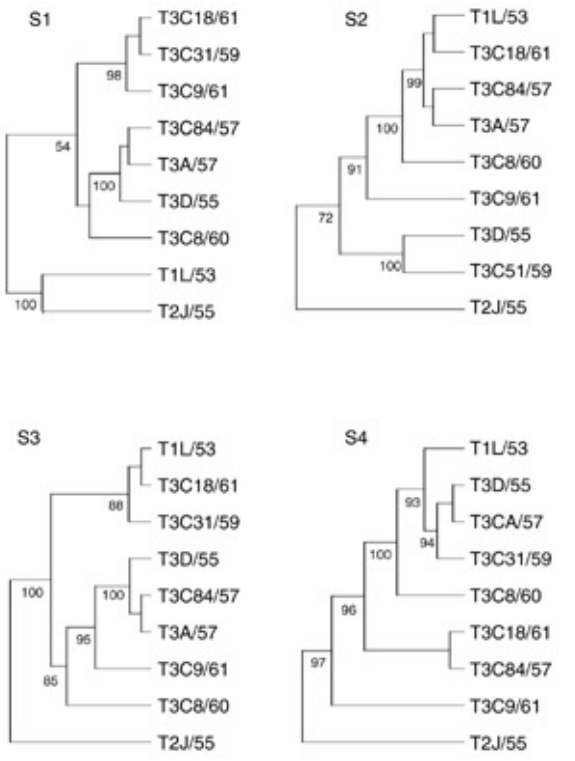


Figure 1 Phylogenetic trees indicating the potential evolutionary relationship and degree of diversity or relatedness between reovirus strains based on the nucleotide sequences of the reovirus S1, S2, S3, and S4 dsRNA segments. Each tree is rooted at the midpoint of its longest branch. (Reproduced with permission from Virgin *et al* (1997).)

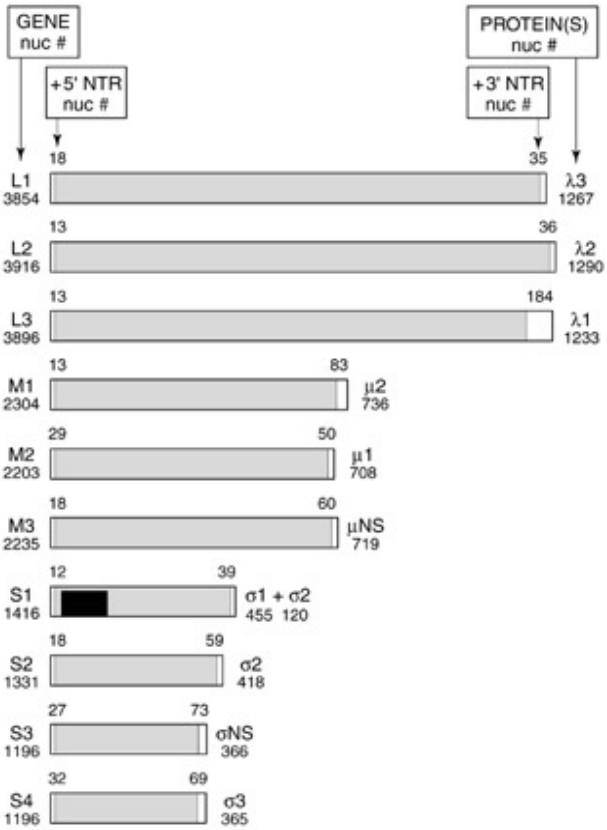


Figure 2 Coding strategies of the ten reovirus gene segments. The names of the individual reovirus dsRNA segments are shown on the left, along with their length in nucleotides. The name and size of the protein(s) encoded by each dsRNA segment are shown on the right. The lengths of the nontranslated regions (in nucleotides) are also indicated. Note that each dsRNA segment encodes a single protein with the exception of the S1 dsRNA segment. The S1 dsRNA segment encodes two proteins from different but overlapping open reading frames. (Reproduced with permission from Nibert *et al* (1996).)

segments L1, L2, L3), medium (M1, M2, M3) and small (S1, S2, S3, S4). dsRNA segments belonging to the three size classes can be distinguished on the basis of their different mobility patterns when subjected to polyacrylamide gel electrophoresis (PAGE). The cognate dsRNA segments of different reovirus strains often have different mobility patterns. Differences in the PAGE mobility pattern of gene segments derived from reoviruses belonging to different strains or serotypes has facilitated identification of the derivation of individual genes in reassortant viruses derived from co-infection of cells or animals with two distinct reoviruses (see below).

Nucleotide and derived amino acid sequence information is available for each of the ten dsRNA segments from at least one strain of reovirus (**Fig. 2**). The nucleotide sequence of the genome of the prototype strain T3D is 23.55 kilobase-pairs (kbp) in length. Individual dsRNA segments range in size from 1196 bp (S4) to 3916 bp (L2). The 5′-nontranslated regions (NTR) are extremely short, the largest being only 32 nucleotides. The longest 5′-NTR, that of the L3 segment, is 184 nucleotides. All other dsRNA segments have 5′-NTR of $\leq$83 nucleotides. The gene segments encode protein ranging in size from 365 amino acids (S4) to 1290 amino acids (L2). dsRNA segments are typically transcribed into a full-length mRNA from a single open reading frame (ORF). Each mRNA is translated into a single protein species. An exception to this scenario involves the S1 dsRNA segment which encodes two proteins (σ1, σ1s) from separate initiator sites in different, but overlapping, reading frames. Further details on the properties of the reovirus genome and reovirus replication are contained in the following entry on Reoviruses: Molecular Biology.

A number of mutants of reovirus have been identified or created in the laboratory. Temperature-sensitive (ts) mutants, defined by their reduced capacity to replicate at nonpermissive (39°C) com-

pared to permissive (31°C) temperatures, have been created by chemical mutagenesis with nitrous acid, nitrosoguanidine, or proflavin. These ts mutants have been classified into ten ts groups (tsA–tsJ) representing mutations involving each of the ten reovirus dsRNA segments. Several intra- and extragenic suppressor mutants resulting in reversion of the ts− to the ts+ phenotype have also been identified. Additional reovirus mutants have been generated and selected based on their resistance to inactivation by physicochemical agents such as ethanol, or by their resistance to neutralization by monoclonal antibodies specific for the $\sigma 1$ protein. Reovirus mutants, including deletion mutants, can also be identified in high passage viral stocks. These deletion mutants typically contain deletions involving the L1, L2, L3 and M1 dsRNA segments. Mutant viruses have also been selected based on their capacity to generate persistent infections (PI viruses), by selecting revertant viruses from hemagglutination-negative (HA−) T3 strains which have regained the capacity to bind to bovine erythrocytes or murine erythroleukemia (MEL) cells, and by serial passage through the brains of neonatal mice.

Evolution

Comparison between the complete nucleotide and derived amino acid sequences from the S1, S2, S3 and S4 dsRNA segments of a large number of reovirus strains has allowed the construction of phylogenetic trees indicating potential evolutionary relationships between various reovirus strains (**Fig. 2**). Of these four gene segments, the S1 dsRNA segment shows the most extreme diversity between viruses belonging to different serotypes. Within strains belonging to a particular serotype the degree of diversity in the S1 dsRNA segment is considerably less than the diversity between serotypes. It has been suggested that there were originally three 'progenitor' S1 genes representative of the current three viral serotypes, which have subsequently diverged to produce the differences seen between strains of virus belonging to the same serotype.

Comparison of the evolutionary relationships between reovirus strains differ strikingly depending on which dsRNA segment is used for analysis (**Fig. 2**). This provides strong evidence that reoviruses can and have reassorted their dsRNA segments in nature. Reovirus reassortants can be easily generated *in vitro* by co-infection of susceptible cells with two distinct strains of virus. Reassortant viruses can also be isolated from mice simultaneously co-infected with two different reovirus strains. These studies provide experimental support for the feasibility of reassortment.

In distinction to the evidence supporting reassortment of dsRNA segments between reovirus strains is the absence of evidence indicating that recombination occurs between either homologous or heterologous dsRNA segments.

Serologic Relationships and Variability

As discussed above, reoviruses are classified into three serotypes on the basis of reactivity with type-specific polyclonal or monoclonal antibodies in reactions including hemagglutination inhibition and plaque-reduction neutralization. Each serotype is represented by one or more prototype strains including reovirus serotype 1 Lang (T1L), serotype 2 Jones (T2J), and serotype 3 Dearing (T3D) and Abney (T3A). In addition to the prototype viruses, many additional strains have been isolated, characterized to varying degrees, and classified according to serotype. Variability between viruses belonging to different serotypes and/or strains has been analyzed based on variability in the nucleotide and derived amino acid sequence of their cognate dsRNA segments (see above).

Epidemiology

The majority of individuals have developed detectable serum antireovirus antibodies against all three reovirus serotypes by late childhood. Seroepidemiological studies suggest that less than 25% of children ≤ 1 year-old are seropositive for reovirus antibodies, but that by ≥ 3 years of age over 70% of individuals are seropositive. The majority of cases of reovirus infection in humans appear to be sporadic in nature, although outbreaks of infection caused by reovirus serotype 1 have been described. Age-related susceptibility to reovirus infection has also been observed in both natural and experimental infections in non-human animals. Calves, foals, piglets, and neonatal mice all appear more susceptible to reovirus infection than their adult counterparts. Experimental studies in mice indicate that the host's immune status is another important factor in determining the nature and outcome of reovirus infection. Immunocompetent adult mice develop an immune response but do not show clinical or pathological evidence of disease following reovirus infection. By contrast, after reovirus infection SCID (severe combined immunodeficient) mice develop prominent and often lethal hepatic disease. SCID mice, and mice with targeted disruptions of the transmembrane exon of IgM (i.e. antibody and B cell deficient mice), show altered

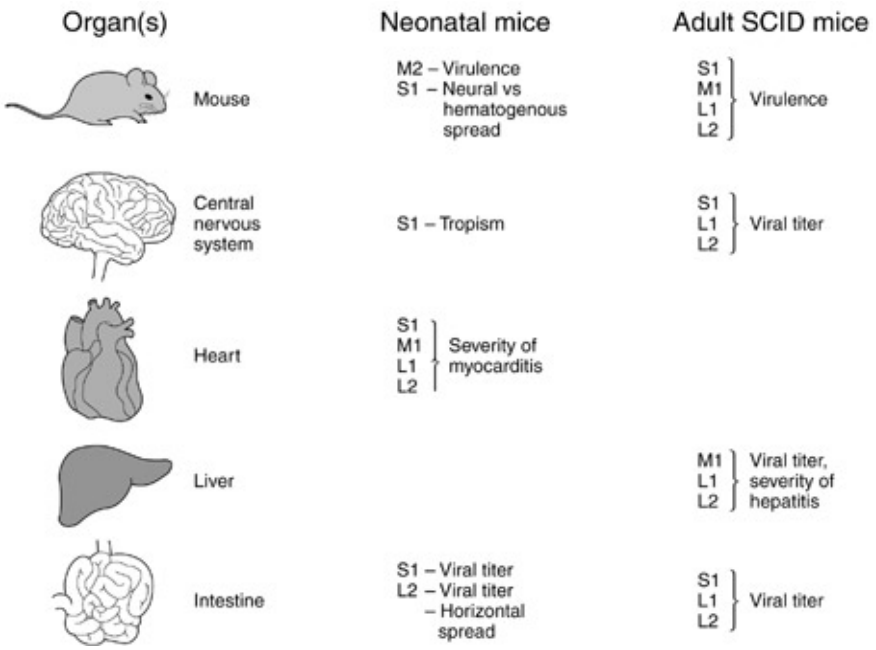


Figure 3 Reovirus dsRNA segments shown to have a role in determining organ-specific virulence in mice. (Reproduced with permission from Virgin *et al* (1997).)

patterns of viral clearance following peroral inoculation of reovirus.

Transmission and Tissue Tropism

Reoviruses are respiratory and enteric viruses whose transmission ('horizontal spread') under natural circumstances involves respiratory aerosols and secretions and fecal–oral transmission. Following oral inoculation in mice reovirus strains differ in their capacity to grow in intestinal tissue. There is an excellent correlation between the capacity of reoviruses to grow in the intestine, the amount of virus subsequently shed in the stool, and the efficiency with which an infected animal transmits disease to its uninfected litter mates. Genetic studies using reovirus reassortants derived from strains exhibiting high and low transmission efficiency indicate that the viral *L2* gene, which encodes the core spike protein λ 2, is the primary determinant of the efficiency of viral transmission following peroral inoculation. Both the *L2* and the *S1* genes influence growth and survival of reovirus in intestinal tissue (**Fig. 3**).

Transmission is also influenced by the capacity of the virus to survive in the environment after being shed from an infected host. Most reoviruses are generally stable at temperatures below room temperature, although at higher temperatures strain-specific differences in thermostability become apparent. One index of thermostability is the time required for a viral stock to lose 50% of its infectivity at 37°C. Reovirus T1L has $t_{1/2}$ of 19 h compared to 2.6 h for T3D. Reoviruses are also stable in aerosols, especially in the presence of high relative humidity. Studies of the genetic basis of physicochemical inactivation of reoviruses indicate that the viral outer capsid proteins are the major determinants of virion stability.

The basic steps in the pathogenesis of reovirus infection have been extensively studied in experimental animals including mice and rats. After peroral or intratracheal inoculation reoviruses adhere to the surface of epithelial M (microfold) cells. These cells overlie collections of lymphoid tissue in the small intestine and bronchi that form part of the systems of gut-associated lymphoid tissue (GALT) and bronchus-associated lymphoid tissue (BALT). In the intestinal lumen virions are partially digested by proteases into intermediate subviral particles (ISVPs). ISVPs lack the major outer capsid protein σ3, contain a partially cleaved form of the major outer capsid protein μ 1C, and contain the cell attachment protein σ1 in an extended conformation. It appears that, at least in the intestine, ISVPs are the form of virus particles that bind to M cells. After binding to M cells ISVPs and/or virions are transported across these cells to the underlying intestinal lymphoid tissue. Studies

of intestinal infection suggest that replication may occur in macrophages within mucosal lymphoid tissue.

Spread of virus from the site of primary infection to distant tissues and organs results in systemic disease. Reoviruses are capable of spreading in the host by means of the lymphatic system, through the bloodstream, or via axoplasmic transport within neurons. Reovirus strains differ both in their capacity to generate and sustain viremia and the efficiency with which they utilize neuronal transport. Following footpad or intramuscular infection in neonatal mice, reovirus T1L spreads to the central nervous system (CNS) primarily through the bloodstream, whereas T3D spreads predominantly through neural pathways. Studies of this pathway of spread using T1L × T3D derived reassortant viruses indicate that the viral *S*1 gene determines the pathway of spread used by reoviruses in the infected host (**Fig. 3**). Similarly, studies of the spread of reoviruses to extraintestinal organs following peroral inoculation into mice also indicate that the viral *S*1 gene is the major determinant of the extent of extraintestinal spread in this experimental model.

Depending on the viral strain, the route of inoculation and host factors such as the age and immune status of the host, reoviruses can produce injury in a variety of target tissues. Among the most extensively studied targets of viral infection in murine model systems are the CNS, the lung, the heart, the hepatobiliary system and the gastrointestinal tract. The specific pathology induced in these various organ systems is discussed extensively in the references included at the end of this chapter.

Reovirus strains often show striking differences in their pattern of organ and tissue tropism. These differences are particularly, but not exclusively, evident in reovirus infection of the CNS. For example, the prototype T3D strains infects neurons and retinal ganglion cells whereas the prototype T1L strain infects ependymal cells, and cells in the anterior lobe of the pituitary gland. Using T1L × T3D reassortant viruses it has been shown that differences in the tropism of reoviruses in the brain, the pituitary gland and the retina are all determined by the viral *S*1 gene. This gene encodes the virus cell attachment protein as well as a small nonstructural protein (σ1S). Studies with monoclonal antibody resistant σ1 variants of T3D, indicate that even single amino acid substitutions within this protein are sufficient to alter the neurovirulence, CNS growth and pattern of CNS tropism of reoviruses.

Tropism is obviously influenced by the nature and distribution of viral receptors on host cells. The nature of the reovirus receptor(s) on cells is only incompletely understood, and the nature of the reovirus receptor(s) on host cells remains controversial. Reoviruses appear to utilize different receptors on different types of cells. Sialic acid residues appear to be important for the binding of reoviruses to a variety of cultured cells and for their capacity to hemagglutinate erythrocytes. Studies with anti-idiotypic antibodies have suggested that reovirus binds to a surface receptor complex that contains components of M_r 65 kDa (p65) and 95 kDa (p95). It has been suggested that the p65 receptor component has structural similarity to the family of receptors that include the β-adrenergic receptor and that the p95 component either has or is associated with a tyrosine kinase activity. Interestingly, the state of tyrosine kinase activity in target cells has also been shown to influence the severity of reovirus-associated cytopathic effects in certain target cells. It is clear that if reoviruses bind to members of the β-adrenergic receptor family they do so without triggering any of the usual pharmacologic activities associated with β-adrenergic receptor binding (e.g. stimulation of adenylate cyclase activity). Reoviruses can also bind to the epidermal growth factor receptor (EGF-R), and the activity of this receptor may influence the efficiency of reovirus infection in certain cultured cells. Additional candidate reovirus receptor proteins that appear to be distinct from either sialic acid residues or the p65/p95 receptor complex have been identified on some cells.

Clinical Features and Infection

Reoviruses remain as much human orphan viruses as when they were first described (**Table 2**). Seroepidemiological studies indicate that human infection occurs during early childhood. The clinical correlates of this infection are difficult to determine accurately. It would appear that the overwhelming majority of human infections are either asymptomatic or produce mild symptoms of upper respiratory or intestinal infection or in some cases exanthema with fever. The predominant symptoms in children during an outbreak of T1 infection included rhinorrhea (81%), pharyngitis (56%) and diarrhea (19%), although the extent to which these were attributable exclusively to the reovirus infection itself is unclear. Over half the children shed reovirus in the stool for at least one week, and 21% shed virus for at least two weeks. The longest reported duration of stool shedding of virus was five weeks. Deliberate inoculations of adult human volunteers with reoviruses produces similar patterns of infection to those that appear to occur under natural circumstances. Reovirus seronegative volunteers nasally inoculated with T1L are typically

Table 2 Reovirus as a possible human pathogen

Probable associations
Enteritis in infants and children
Upper respiratory infections
Possible associations (unproven)
Exanthema
Neonatal biliary atresia
Neonatal hepatitis
Isolated cases
Meningoencephalitis
Pneumonia
Myocarditis
Keratoconjunctivitis
Speculative or doubtful associations
Chronic neuropsychiatric illnesses
Adult cholestatic liver disease
African Burkitt's lymphoma

From Tyler and Fields (1996), with permission.

asymptomatic. Approximately one-third of individuals develop symptomatic infection lasting 4–7 days and beginning 24–48 h after viral challenge. The predominant symptoms include fever, headache, coughing, sneezing, rhinorrhea and generalized malaise. Infection is associated with shedding of virus in the stool and seroconversion. Challenge with T2J has not been associated with clinical illness in volunteers, but is associated with seroconversion and shedding of virus in stools. Individuals challenged with T3D are also generally asymptomatic or develop mild rhinitis. Infected volunteers shed virus in the stools and show seroconversion. In general, individuals with preexisting antireovirus antibody prior to challenge with reoviruses do not develop signs of clinical disease and do not shed significant amounts of virus in stool.

Reovirus infection of mice produces a disease whose clinical and pathological features resemble human extrahepatic biliary atresia (EHBA). Attempts to link reovirus infection to human EHBA have produced conflicting results. Some serologic studies show a higher frequency or higher titers of antireovirus antibodies in children with EHBA as compared to controls, whereas other studies do not. Reovirus has not been directly isolated from pathological specimens obtained at biopsy, surgery or autopsy from patients with EHBA nor has reovirus antigen been detected in liver or biliary tissues of EHBA patients by immunocytochemistry. However, reovirus nucleic acid corresponding to the L1 gene segment is detectable by polymerase chain reaction (PCR) with significantly greater frequency in liver and biliary tissues of patients with extrahepatic biliary atresia and choledochal cysts compared with liver and biliary tissues from patients with other liver diseases.

There are occasional reports of an association between reovirus infection and human diseases including meningitis, encephalitis, keratoconjunctivitis, and pneumonia. These cases are sufficiently rare and unusual as to suggest that if reovirus is involved in the production of these diseases it must be an exceedingly unusual event. It is important to recognize that currently available studies suggest that reoviruses are only responsible for a vanishingly small percentage of the total number of cases of these various illnesses.

Pathology and Histopathology

Pathological studies of material from humans infected with reovirus are exceedingly rare. Most of our current knowledge of the pathology of reovirus infection comes from studies of experimental infection in animals, notably mice. A detailed description of the pathology of reovirus infection in different organs is beyond the scope of this chapter, and interested readers should consult the references for further reading. As noted, in mice the brunt of reovirus infection and injury involves the CNS, the lungs, the heart, the liver and biliary tree, and the intestinal tract. The nature and severity of injury depends on the strain of virus, route of inoculation, and host factors include the age and immune status of the infected animals. The pattern of injury shows considerable variation. In some cases virus induces significant destruction of tissue with minimal or absent early inflammatory response. This pattern of infection can be seen in the CNS after intracerebral inoculation of T3D, and in the heart after intramuscular inoculation of reovirus 8B. Conversely, reovirus can also generate more prominent perivascular and parenchymal inflammation with less tissue injury. This pattern is often seen in skeletal muscle following intramuscular inoculation of T1L.

Recent studies indicate that reoviruses can produce apoptosis in infected L929 and MDCK cells in culture. Reovirus strains differ in this capacity with T1L strains producing less apoptosis than T3 strains. Studies using reassortant viruses derived from apoptotic-inducing (APO+) and non-inducing (APO−) strains (e.g T1L × T3D or T1L × T3A) indicate that the viral *S1* gene is a major determinant of the capacity of these viruses to induce apoptosis. Recent studies indicate that apoptosis also occurs *in vivo* in the CNS following intracerebral inoculation with T3D. Virus-infected (antigen positive) and apoptotic cells (TUNEL positive) colocalize to the regions of the brain that show the brunt of neuropathological

injury. At a cellular level, colocalization studies suggest that apoptotic cells may be either virus-infected or 'bystander' cells in proximity to virus-infected cells.

Immune Response

Following natural or experimental reovirus infection antibodies specific for a variety of reovirus structural and nonstructural proteins are produced. The bulk of both the immunoglobulin (Ig)A and IgG antibody response appears to be directed against viral structural proteins. There is no evidence that reovirus infection results in production of antibodies against nonprotein viral antigens (e.g. nucleic acid). The majority of the antibody response is not serotype specific, as would be expected by the high degree of homology between most reovirus proteins between viruses belonging to different serotypes. Serotype-specific immune responses are directed against the reovirus $\sigma 1$ protein, which is the least conserved of the reovirus proteins. Currently available monoclonal antibodies directed against the $\sigma 1$ protein (e.g. 9BG5, 5C6) are serotype-specific and show minimal if any crossreactivity between viruses belonging to different serotypes. Monoclonal antibodies directed against the reovirus outer capsid proteins $\sigma 3$ and $\mu 1$ are all crossreactive between reoviruses belonging to different serotypes, although in some cases they may show differing degrees of reactivity to viruses belonging to different serotypes or to individual viral strains within a serotype.

The nature of the reovirus-specific antibody response is influenced by the route of viral inoculation. Following peroral inoculation of reovirus T1L there is an increase in the number of reovirus-specific IgA-producing cells in intestinal Peyer's patches and in the spleen. Enteric infection is also associated with the induction of IgG antibody, predominantly of the IgG_{2a} and IgG_{2b} subclasses. Intradermal inoculation of reovirus does not result in significant IgA production, but instead induces predominantly reovirus-specific antibody belonging to the IgG_{2a} and IgG_{2b} subclasses. Variations in the dominant reovirus-specific IgG antibody subclass are influenced both by the route of virus inoculation and the strain of mouse: for example, IgG_1 antibodies are induced in some strains of mice following intradermal inoculation of virus.

T cell responses are induced during reovirus infection. Following peroral inoculation of reovirus, virus-specific major histocompatibility complex (MHC)-restricted cytotoxic T lymphocytes (CTLs) can be found in Peyer's patches and among the intraepithelial intestinal lymphocyte population. These cells are CD8+, bear the alpha/beta T cell receptor (TCR), and are capable of MHC-restricted lysis of reovirus-infected target cells. The number of these reovirus-specific intraepithelial CTLs increases dramatically after intestinal infection with reoviruses. Studies of $V\beta$ TCR usage indicate that reovirus infection is associated with increases in CTLs bearing $V\beta$ 7, 12, 14 and 17 TCRs, suggesting that reovirus infection is associated with oligoclonal expansion of specific TCR subpopulations.

Both serotype-specific and nonspecific CTL responses occur following reovirus infection. Serotype-specific CTL responses are directed against products of the *S*1 gene. As noted, this gene encodes the viral cell attachment protein $\sigma 1$ and a small nonstructural protein $\sigma 1S$. Nonserotype-specific CTL responses are presumably directed against epitopes on proteins other than $\sigma 1$ or on conserved epitopes within the $\sigma 1$ protein. The exact peptide epitopes recognized by MHC class I or class II restricted reovirus-specific T-cells have not yet been identified.

The role of antibody and T-cells in protection against reovirus infection has been investigated by passive transfer experiments, by selective depletion of B cells and T-cell subsets in mice, and in immunodeficient mice. Both antibody and reovirus-specific lymphocytes can protect mice against challenge with a variety of reovirus strains and from infection by a variety of different routes. Although direct comparisons are difficult, it appears that passively transferred reovirus-specific immune cells are more effective than antibody in controlling viral replication at primary sites, although systemic IgG antibody does permit viral clearance from mucosal surfaces. Conversely, antibody may be more effective than immune cells in controlling growth and spread of virus within certain tissues or organs including the CNS.

Studies with monoclonal antibodies indicate that passive protection can be conferred by antibodies specific for each of the reovirus outer capsid proteins ($\sigma 1$, $\mu 1C$, $\sigma 3$). *In vitro* studies suggest that protective antibodies may act at a variety of steps in the reovirus replication cycle including inhibition of viral binding to cell surface receptors, penetration of host cell plasma membranes, and inhibition of the intralysosomal proteolytic processing of virions to ISVPs. Similarly, *in vivo* protection can be associated with inhibition of viral replication at primary sites, spread of virus through nerves or the bloodstream to critical target tissues such as the CNS, and growth and spread of virus within these tissues.

Studies with passively transferred reovirus-specific T cells indicate that both CD4 and CD8 cells are required for optimal production and that depletion of either subset results in a significant loss of protective

capacity of transferred cells. There is currently no evidence that intestinal intraepithelial γ/δ TCR+ T cells, as opposed to α/βTCR+ T cells, play a significant role in immunity to reovirus infection. As noted earlier, both SCID mice, and antibody and B cell-deficient mice show increased susceptibility to reovirus infection and diminished capacity to clear viral infection. Adult SCID mouse, unlike their congeneic immunocompetent counterparts, develop lethal hepatitis after peroral infection with reovirus T1L or T3 clone 9. Similar results have been found in immunocompetent neonatal mice depleted of CD4 and/or CD8 T cells. This suggests, that at least in certain organs, T cells play a critical role in controlling reovirus infection.

Studies of experimental reovirus-induced pulmonary infection in rats indicate that there are serotype-specific differences in the capacity of reoviruses to induce cytokine gene expression and cellular inflammatory responses. T3D is associated with significantly greater neutrophil influx into the lung than T1L. T3D-induced pulmonary neutrophilia correlates with the capacity of T3D to induce expression of tumor necrosis factor alpha (TNFα) and macrophage inhibitory protein 2 (MIP 2) mRNAs and proteins. Peroral and intradermal infection with reovirus T1L in mice is associated with the production of interferon gamma (IFN-γ), and low levels of interleukin 5, although the magnitude of these responses varies significantly among different strains of mice. Reovirus strains differ in their capacity to induce IFN-γ following infection of cultured cells, with T3D typically inducing higher levels than T1L. In mice, the level of IFN-γ induction also varies with the strain of mouse infected. Induction of IFN-γ is associated with enhanced MHC I expression.

Reovirus strains differ in their sensitivity to IFN-γ, and this effect may vary in different cell types. Reovirus T3D appears to be generally more sensitive to the effects of IFN-γ than T1L. Therefore the amounts of IFN-γ induced and the effects of this induction on reovirus replication, involve a complex interplay between the nature of the infecting viral strain, the species of animal, and the particular tissue involved. Given this complexity it is perhaps not surprising that the role of IFN-γ in the pathogenesis of reovirus infection *in vivo* remains to be established. In the case of reovirus-induced myocarditis, there is an excellent correlation between the myocarditic potential of reovirus strains and the level of viral RNA produced by these strains in infected cells. Since dsRNA is a potent inducer of IFN-γ, this raises the possibility that differences in the amount of IFN-γ induction could potentially play a role in determining the severity of reovirus-induced myocarditis.

Prevention and Control

Because reoviruses are not important human pathogens, investigations of the prevention and control of reovirus infection are limited. In experimental models of infection protection can be conferred by passive transfer of both reovirus specific antibodies and immune cells. Several reovirus vaccines have been developed for veterinary use and appear to be reasonably effective in inducing reovirus-specific antibody responses and in preventing symptomatic infection. Most veterinary vaccines against reovirus utilize either formalin or β-propiolactone-killed virus. Protease inhibitors, which can prevent the conversion of reovirus virions into ISVPs, can protect against reovirus infection after peroral inoculation. In this experimental model, ISVPs appear to be the critical form of virus responsible for initiating infection and blockage of the digestion of virions into ISVPs by intestinal proteases prevents infection. Reovirus infection *in vitro* can be inhibited by ribivarin, acivicin and cicloxolone; but none of these agents has been tested against reovirus infection *in vivo*. These agents have a variety of mechanisms of action. For example, ribivarin is a guanosine analogue, acivicin is a glutamine analogue and cicloxolone is a monesin-like Golgi apparatus inhibitor.

Future Perspectives

Future studies of reoviruses are likely to provide more detailed information at the atomic level of the structure of the reovirus particle and the mechanisms by which the various structural proteins interact. Many aspects of the reovirus replication cycle remain obscure, particularly as they relate to the methods by which equimolar quantities of the ten dsRNA segments are correctly packaged in the virion. Increased understanding of the viral replication cycle may facilitate the development of experimental systems that allow gene segments to be modified and reinserted into virions. Many fundamental aspects of the interaction between reovirions and host cells remain to be delineated. This includes clarification of the nature of the reovirus receptor(s) on specific host cells, the nature of and mechanisms by which reovirus infection affects host cell signal transduction pathways, and the precise biochemical processes that lead to reovirus-induced inhibition of host cell macromolecular synthesis and ultimately cell death. A major theme of future reovirus research will certainly continue to be the effort to understand the molecular and genetic basis for specific steps in viral pathogenesis. Many areas of reovirus biology and pathogenesis remain to be explored. For example, the nature of the

age-related susceptibility to reovirus infection remains unknown. Many aspects of the fundamental immunology of reovirus infection remain obscure, such as the exact nature of the specific peptide epitopes recognized by reovirus-specific antibodies and immune cells, and the role played by cytokines in host defense against reovirus infection. Finally, the role, if any, of reoviruses as agents of human disease remains as mysterious now as it was 40 years ago.

See also: **Immune response: Cell mediated immune response, General features; Orbiviruses and coltiviruses (*Reoviridae*): General features, Molecular biology; Phytoreoviruses (*Reoviridae*); Reoviruses (*Reoviridae*): Molecular biology; Rotaviruses (*Reoviridae*): General features, Molecular biology.**

Further Reading

Nibert ML, Schiff LA and Fields BN (1996) Reoviruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 1557. Philadelphia: Lippincott–Raven.

Tyler KL and Fields BN (1996) Reoviruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 1597. Philadelphia: Lippincott–Raven.

Tyler KL and Oldstone MBA (eds) (1998) *Reoviruses. Current Topics in Microbiology and Immunology*, vols 223 and 224. Heidelberg: Springer-Verlag.

Virgin HW IVth, Tyler KL and Dermody TS (1997) Reovirus. In: Nathanson N (ed.) *Viral Pathogenesis*, p. 669. Philadelphia: Lippincott–Raven.

Molecular Biology

WK Joklik, Duke University Medical Center, Durham, North Carolina, USA

Introduction

Reoviruses (*Re*spiratory entero) are members of the genus *Orthoreovirus* of the family *Reoviridae,* which also includes five other genera, namely, *Rotavirus* and *Orbivirus,* both of which are also viruses of vertebrates, *Cypovirus* of insects and *Phytovirus* and *Fijivirus,* both of which are plant viruses. The common characteristic features of all these genera are a basically similar particle morphology and size, and double-stranded RNA (dsRNA)-containing genomes that comprise 10, 11 or 12 genome segments.

Reoviruses are ubiquitous among mammals in which they usually do not cause overt disease. Mammalian reoviruses fall into three serologic subgroups or serotypes (ST1, ST2 and ST3), members of each of which are not neutralized by antisera against the other two.

Reoviruses have also been isolated from other vertebrates such as birds and reptiles, as well as from invertebrates, such as insects and molluscs. None of these viruses have been studied in biochemical or molecular terms except the avian reoviruses which fall into five serotypes. In contrast to mammalian reoviruses, they cause disease [the viral arthritis syndrome (VAS) and the stunted growth syndrome (transient digestive system disorder, TDSD)], as well as pericarditis/myocarditis, hepatitis and nephritis.

Most of our knowledge concerning the biochemistry and molecular biology/genetics of orthoreoviruses derives from studies of the mammalian reoviruses.

The Reovirus Particle

Reovirus particles consist of a core some 55 nm in diameter which is surrounded by an outer capsid shell that is about 12.5 nm thick (**Fig. 1**). Since the thickness of the core shell is about 7.5 nm, the central cavity accounts for about 12.5% of the reovirus particle volume, and the core for about 33%.

Both the outer capsid and the core shell are composed of capsomers which are arranged with icosahedral symmetry. The reovirus particle surface reveals 600 protrusions, presumably capsomers, arranged with $T = 13l$ symmetry in the form of shared hexamer rings around 120 holes. The core shell also possesses $T = 13l$ symmetry elements, but the arrangement of capsomers in them is difficult to discern (**Fig. 2**). However, cores possess 12 icosahedrally located columnar projections or spikes which are about 10 nm in diameter, possess central channels 5 nm in diameter, and project about halfway through the outer capsid shell in which they are visible as depressions or craters. The spikes, which can be removed from cores by incubating them at pH 11.4 at 4°C for 15 min, are pentamers of protein $\lambda 2$ and appear to be partially covered by 3 nm-thick 'lids' which appear to be trimers of protein $\sigma 1$ (see below).

The outer capsid shell is stable at high and physiological salt concentrations, but loses capsomers on storage at low ionic strength. It is readily digested by chymotrypsin to which the core shell is completely resistant. Virus particles and cores are readily separated by density gradient centrifugation; their sedimentation coefficients are about 630 and 470S, respectively, and their densities in cesium chloride density gradients are 1.36 and 1.43 g ml^{-1}, respectively.

age-related susceptibility to reovirus infection remains unknown. Many aspects of the fundamental immunology of reovirus infection remain obscure, such as the exact nature of the specific peptide epitopes recognized by reovirus-specific antibodies and immune cells, and the role played by cytokines in host defense against reovirus infection. Finally, the role, if any, of reoviruses as agents of human disease remains as mysterious now as it was 40 years ago.

See also: **Immune response: Cell mediated immune response, General features; Orbiviruses and coltiviruses (*Reoviridae*): General features, Molecular biology; Phytoreoviruses (*Reoviridae*); Reoviruses (*Reoviridae*): Molecular biology; Rotaviruses (*Reoviridae*): General features, Molecular biology.**

Further Reading

Nibert ML, Schiff LA and Fields BN (1996) Reoviruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 1557. Philadelphia: Lippincott–Raven.

Tyler KL and Fields BN (1996) Reoviruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 1597. Philadelphia: Lippincott–Raven.

Tyler KL and Oldstone MBA (eds) (1998) *Reoviruses. Current Topics in Microbiology and Immunology*, vols 223 and 224. Heidelberg: Springer-Verlag.

Virgin HW IVth, Tyler KL and Dermody TS (1997) Reovirus. In: Nathanson N (ed.) *Viral Pathogenesis*, p. 669. Philadelphia: Lippincott–Raven.

Molecular Biology

WK Joklik, Duke University Medical Center, Durham, North Carolina, USA

Introduction

Reoviruses (*Re*spiratory entero) are members of the genus *Orthoreovirus* of the family *Reoviridae,* which also includes five other genera, namely, *Rotavirus* and *Orbivirus,* both of which are also viruses of vertebrates, *Cypovirus* of insects and *Phytovirus* and *Fijivirus,* both of which are plant viruses. The common characteristic features of all these genera are a basically similar particle morphology and size, and double-stranded RNA (dsRNA)-containing genomes that comprise 10, 11 or 12 genome segments.

Reoviruses are ubiquitous among mammals in which they usually do not cause overt disease. Mammalian reoviruses fall into three serologic subgroups or serotypes (ST1, ST2 and ST3), members of each of which are not neutralized by antisera against the other two.

Reoviruses have also been isolated from other vertebrates such as birds and reptiles, as well as from invertebrates, such as insects and molluscs. None of these viruses have been studied in biochemical or molecular terms except the avian reoviruses which fall into five serotypes. In contrast to mammalian reoviruses, they cause disease [the viral arthritis syndrome (VAS) and the stunted growth syndrome (transient digestive system disorder, TDSD)], as well as pericarditis/myocarditis, hepatitis and nephritis.

Most of our knowledge concerning the biochemistry and molecular biology/genetics of orthoreoviruses derives from studies of the mammalian reoviruses.

The Reovirus Particle

Reovirus particles consist of a core some 55 nm in diameter which is surrounded by an outer capsid shell that is about 12.5 nm thick (**Fig. 1**). Since the thickness of the core shell is about 7.5 nm, the central cavity accounts for about 12.5% of the reovirus particle volume, and the core for about 33%.

Both the outer capsid and the core shell are composed of capsomers which are arranged with icosahedral symmetry. The reovirus particle surface reveals 600 protrusions, presumably capsomers, arranged with $T = 13l$ symmetry in the form of shared hexamer rings around 120 holes. The core shell also possesses $T = 13l$ symmetry elements, but the arrangement of capsomers in them is difficult to discern (**Fig. 2**). However, cores possess 12 icosahedrally located columnar projections or spikes which are about 10 nm in diameter, possess central channels 5 nm in diameter, and project about halfway through the outer capsid shell in which they are visible as depressions or craters. The spikes, which can be removed from cores by incubating them at pH 11.4 at 4°C for 15 min, are pentamers of protein $\lambda 2$ and appear to be partially covered by 3 nm-thick 'lids' which appear to be trimers of protein $\sigma 1$ (see below).

The outer capsid shell is stable at high and physiological salt concentrations, but loses capsomers on storage at low ionic strength. It is readily digested by chymotrypsin to which the core shell is completely resistant. Virus particles and cores are readily separated by density gradient centrifugation; their sedimentation coefficients are about 630 and 470S, respectively, and their densities in cesium chloride density gradients are 1.36 and 1.43 g ml^{-1}, respectively.

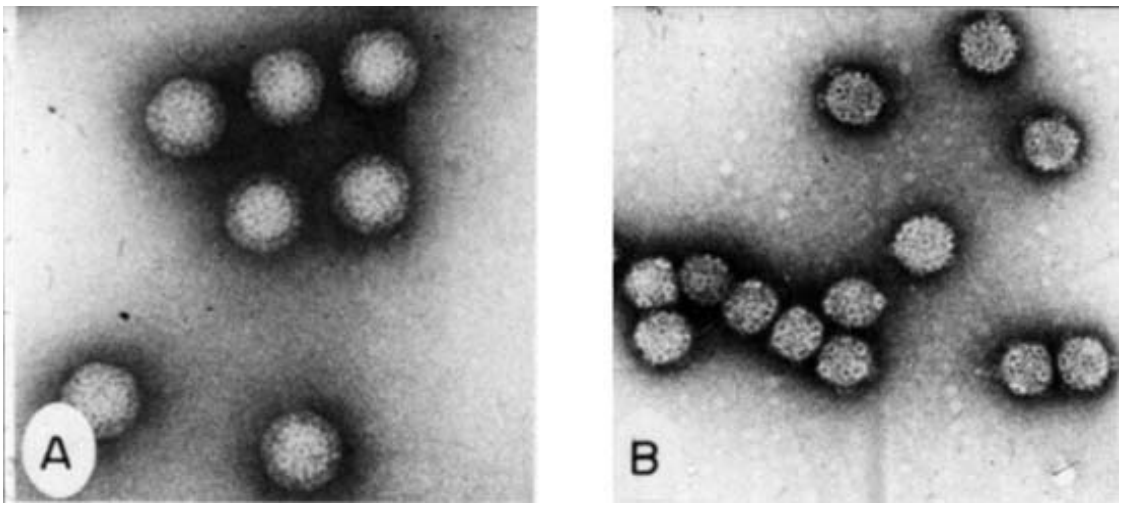


Figure 1 (**A**) Reovirus particles. Note double-capsid shell. The arrangement of capsomers is discernible at the periphery. (**B**) Reovirus cores. Note the large spikes, located as if situated at the 12 vertices of an icosahedron. Magnification for both ×120 000. (Courtesy of Dr R. B. Luftig.)

The reovirus genome

The reovirus genome consists of ten segments of dsRNA, all of which have been cloned and sequenced (**Table 1**). They fall into three size classes termed L, M and S, members of which are about 3900, 2250 and 1300 bp long, respectively. The total length of the reovirus genome is 23 549 bp; it is one of the largest and therefore most complex of RNA-containing genomes. Small-angle x-ray diffraction studies indicate that the RNA segments exist within the central cavity in tight well-ordered packing arrangement with adjacent helices aligned locally parallel to each other rather like the DNA in T even bacteriophages.

Each of the ten reovirus genome segments possesses a major open reading frame (ORF) which varies in length from 365 to 1289 codons. Most of them also contain short ORFs in other reading frames at least one of which, in S1, is translated in infected cells (see below). The 5′-untranslated regions are all short (12–32 nucleotides); those at the 3′-ends are longer, but still (with one exception, L3 (182 nucleotides)) less than 85 nucleotides long. The four 5′-terminal and five 3′-terminal base pairs of all ten genome segments are identical (5′-GCUA- and -UCAUC-3′ for the plus-strands).

Reovirus oligonucleotides

Reovirus particles also contain about 3200 oligonucleotides of which about 2400 are 5′-G-terminated and are the products of abortive reiterative transcription catalyzed by the reovirus transcriptase (see below), and about 800 are oligoadenylates from 2 to 20 residues long. The latter may represent either an untemplated polymerase activity of the reovirus transcriptase committed to transcribe but unable to move along its template, or slippage transcription of the three genome segments (L2, M3 and S3) the 3′

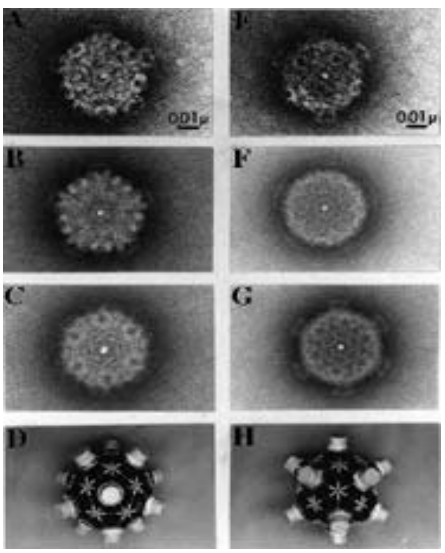


Figure 2 Two reovirus core particles the central axis of which passes through either a presumptive fivefold (**A**) or threefold (**E**) vertex. Enhancement of the five peripheral spikes of (**A**) was achieved by an $n = 5$ rotation (**B**), but not by an $n = 6$ rotation (**C**). (**D**) Model that depicts the spike orientations when the central axis is through a fivefold vertex. Enhancement of the six peripheral spikes (**E**) was exhibited with an $n = 6$ rotation (**G**), but not an $n = 5$ rotation (**F**). (**H**) Model with the central axis through a threefold vertex. The particles were stained with 2% uranyl acetate. (From Luftig RB, Kilham S, Hay AJ, Zweerink HJ and Joklik WK (1972) *Virology* 48: 170.)

Table 1 The reovirus serotype 3 genome segments and the proteins that they encode

Genome segment			*Protein*				
Segment	*Length (bp)*	*Protein*	*Number of amino acids*	*Number of molecules/particle*	*Percent of viral protein*	*Location in particle*	*α-helix/ β-sheet ratio*
L1	3854	λ3	1267	120	15	Core	0.9
L2	3916	λ2	1289	60	8	Core	0.5
L3	3896	λ1	1223	12	1.5	Core	0.9
M1	2304	μ2	687	12	1	Core	1.2
M2	2203	μ1	708	<60	1.5	OCS[a]	0.9
		μlC	666	600	39	OCS	
		μlN	42	?		OCS	
M3	2235	μNS	719	—	—	—	2.5
		μNSC	678	—	—	—	
S1	1416	σ1	455	36	1.5	OCS	0.5
		σ1S	120	—	—	—	
S2	1331	σ2	418	120–180	10	Core	0.5
S3	1198	σNS	366	—	—	—	1.8
S4	1196	σ3	365	600	22	OCS	0.9

[a] OCS, outer capsid shell.

ends of the minus-strands of which are CGAUUU- (see below).

Enzymes in reovirus cores

Since host cells do not contain enzymes capable of generating mRNA from dsRNA templates, reovirus particles contain the enzymes necessary for this purpose. Since reovirus mRNAs are capped at their 5′ ends, this involves possession of a transcriptase or RNA polymerase, an RNA triphosphatase to convert the 5′-terminal ppp groups generated by the transcriptase to the pp groups required by the guanylyltransferase, the guanylyltransferase itself and two methyltransferases to methylate the 7 position of the cap G and the 2′-O position of what was the 5′-terminal ribose of the uncapped RNA.

In core form the transcriptase is very stable and functions for long periods of time (more than 24 h) at elevated temperatures (up to 50°C), transcribing the genome segments many times.

The reovirus proteins

The various reovirus proteins are listed in **Table 1**. All reovirus proteins except μ1 have been isolated in native form from cells infected with recombinant vaccinia viruses with TK (thymidine kinase) genes containing reovirus genome segments inserted under the control of powerful promoters like the T7 polymerase promoter or the cowpox virus A-type inclusion body protein gene promoter.

The core shell components λ1 and σ2 The core shell consists of an inner layer of 120 or 180 molecules of protein σ2 and an outer layer of 120 molecules of protein λ1. The two proteins associate with each other *in vitro* and in cells infected with recombinant vaccinia viruses expressing them, core-like particles are formed. Protein λ1 possesses a nucleotide-binding site –TKGKSSG– starting at residue 8; a zinc finger motif centered around residue 194 (λ1 is a zinc-binding metalloprotein); and a weak dsRNA-binding site upstream of the zinc finger. It possesses two enzymic activities; it is a helicase and, as suggested by genome segment reassortant analysis, an RNA triphosphatase/ATPase. This enzyme activity may function in preparing transcripts for capping or to provide energy for the helicase-mediated unwinding of the dsRNA genome segments required for the transcription of their minus strands. Protein σ2 also binds dsRNA weakly and possesses a region that bears a striking similarity to a region in the β subunit of *Escherichia coli* DNA-dependent RNA polymerase.

The spike component λ2 Protein λ2, in the form of pentamers, is the reovirus spike. It is also the reovirus guanylyltransferase, that is, it is the capping enzyme. Protein λ2 exhibits strong epitopes that are exposed on the reovirus particle surface, are identical or very similar on all three (ST1, ST2 and ST3) λ2 proteins, and elicit the formation of group-specific antibodies. It possesses strong affinity for proteins σ1, λ1 and λ3. There is some evidence that is possesses SAM-

utilizing methyltransferase activity, but such an activity has not yet been demonstrated for isolated protein λ2.

The minor core components λ3 and μ2 Reovirus cores contain about 12 copies of each of two proteins, λ3 and σ2, that possess no apparent structural functions, but play very important roles in two key enzymic activities. One is protein λ3 which possesses several amino acid sequences or motifs that are present, and similarly spaced, in all positive strand- and dsRNA-dependent RNA polymerases, such as DxxxxxD, GxxxTxxx(N/E)(S/T) and GDD; the genome segment that encodes it, namely L1, controls the pH optimum of the transcription of dsRNA genome segments into mRNAs by cores; and is a poly(C)-dependent poly(G) polymerase. It is very likely, therefore, that it is the catalytic component of the reovirus RNA polymerase/transcriptase, specificity being supplied by another component, most likely μ2. It should be pointed out, however, that these two proteins exhibit no affinity for each other *in vitro*. However, it is also true that the intracore concentrations of these two proteins are very high (of the order of 0.1 M), so that high affinity may not be required for them to be capable of interacting. The second enzymic activity is the nucleoside triphosphatase activity. As pointed out above, this activity has been linked to protein λ1; but genome segment reassortant analysis has also linked it to protein μ2. Either there are two such activities in cores, or protein λ1 is its catalytic subunit and protein μ2 provides specificity or some other required function.

The outer capsid shell The reovirus outer capsid shell consists of 600 μ1C–σ3 complexes, with the former -SS- bonded and internal to the latter. The cleavage of μ1, which is myristoylated at its N-terminus, to μ1C and μ1N, its 42 amino acid-long myristoylated N-terminal fragment, occurs when μ1 associates with σ3 well before the insertion of the resulting complexes into the outer capsid shell; most μ1 in cells is free, whereas most μ1C is associated with σ3. Interestingly, most μ1N remains associated with μ1C and is also present in virus particles, as is a small amount of μ1 (about one-twentieth of the amount of μ1C). The presence of myristoyl groups in the reovirus outer capsid shell raises interesting questions concerning their function in the assembly and structural stability of the outer capsid shell, as well as during the uptake and entry of reovirus particles into cells (in analogy with the presence of myristoyl groups on picornavirus and other viral proteins).

Protein μ1C accounts for almost 40% of the reovirus protein complement. Antibodies against it neither precipitate virus particles, nor neutralize infectivity. Not surprisingly, protein μ1C plays a major role in specifying how reovirus particles interact with their environment. On the one hand, it controls sensitivity to chemical reagents like ethanol and phenol; on the other, it controls some aspects of tissue tropism because susceptibility of μ1C to proteolytic cleavage controls activation of the transcription of mRNA as the first step of the reovirus replication cycle (see below). It therefore specifies the extent to which reovirus replicates in the intestine, which in turn determines the extent to which it spreads to the central nervous system; it thus controls neurovirulence. It also plays a role in inducing serotype-specific immunologic tolerance and delayed-type hypersensitivity responses. Finally, there is evidence that μ1 is cytotoxic, apparently damaging membranes; and that it is one of the two reovirus proteins, the other being σ1, that inhibits cellular DNA replication and induces apoptosis in infected cells.

The second major component of the reovirus outer capsid shell is protein σ3 which has a variety of functions. First, it possesses a Zn-binding site in a zinc finger that encompasses residues 45–72 inclusive. Interestingly, residues 45–53 inclusive are very similar to a region in picornavirus proteases. Since σ3 is strongly implicated in the cleavage of μ1 to μ1C (see above), it was at first thought that this sequence might be involved; but it turns out that μ1 is cleaved even when it complexes with σ3 variants that lack this sequence. Rather the association of σ3 with μ1, which does not proceed in the absence of the zinc finger, causes conformational changes in μ1 that cause the cleavage to be autocatalytic. Second, σ3 possesses a strong dsRNA-binding site centered around residue 265, a remarkable property for an outer capsid shell component. However, sigma 3 also binds to ssRNA transcripts in vivo very soon after they are formed (see below), which suggests that its affinity is not for dsRNA per se, but for regions of ssRNA with dsRNA character such as hairpins (stem-loops) or intermolecular complementary regions.

Protein σ1 Protein σ1 is a reovirus particle component that exists in the form of 12 trimers inserted into the spikes that are composed of pentamers of λ2 (see above). Normally σ1 appears to form a lid that covers the channel of these spikes, but mild heat causes σ1 to assume the form of 48 nm-long 4–6 nm-wide fibers topped by 9.5 nm-diameter globular heads that extend from the surface of the virus particles, the heads containing the cell attachment site. Most of the N-terminal one-third of σ1 is made up of a series of about 20 tandemly arranged heptads in which the first and fourth amino acids are hydrophobic. This type of

sequence results in an alpha-helical coiled coil type structure in which the hydrophobic residues form the interfaces beween alpha-helices. It is these sequences that cause the trimerization of $\sigma 1$ which in turn generates the signals for its association with insertion into the spike channels.

Although present in reovirus particles to the extent of 36 molecules (only 3 of which are essential, that is, virus particles that possess only one $\sigma 1$ trimer are about one-half as infectious as virus particles that possess all twelve), protein $\sigma 1$ plays an extremely important role in specifying the interactions between reovirus particles with host cells and intact hosts. Protein $\sigma 1$ is the cell attachment protein (and the hemagglutinin); thus is specifies tissue tropism and is therefore the major factor in reovirus pathogenesis. It also elicits the formation of three forms of neutralizing antibodies that crossreact minimally, if at all; that is, $\sigma 1$ is the reovirus type-specific antigen. Protein $\sigma 1$ also induces delayed-type hypersensitivity, generates suppressor T cells and cytolytic T lymphocytes, and is recognized by cytotoxic T lymphocytes. Finally, genome segment reassortant analysis suggests that $\sigma 1$ inhibits cellular DNA synthesis and induces apoptosis in infected cells, and it plays a crucial role in the maintenance of persistment infections.

The nonstructural reovirus proteins Reovirus encodes three nonstructural proteins that are not virus particle components. Two are encoded by genome segments M3 and S3 and are produced in large amounts. The former, muNS, is produced in two forms, one of which is translated from the entire M3 ORF, whereas the other, designated muNSC because it was at first thought to be a cleavage product of muNS, is translated in the same reading frame but starting at AUG at codon position 42. Protein muNS has a very high alpha-helix content and in its C-terminal region shares a periodic sequence similarity pattern with various myosins. It rapidly complexes with newly-transcribed mRNAs with which it remains associated until they are transcribed into minus-strands to form progeny genome segments (see below). It possesses affinity for elements of the cytoskeletal framework which may play an important role in morphogenesis.

Protein σNS is another ssRNA-binding protein. Like proteins muNS and $\sigma 3$ it forms complexes with reovirus mRNA molecules very soon after they are transcribed. Proteins muNS and σNS possess affinity for each other as demonstrated by the fact that antibodies against either also precipitate the other.

The third nonstructural reovirus protein is the basic protein σ1S, encoded by the minor ORF in S1, which is formed in infected cells in small amounts. It localizes to the cytoplasm and to nucleoli where it could interfere with both DNA and RNA synthesis and/or expression. Its actual function may only be discovered by examining the phenotypes of knock-out mutants.

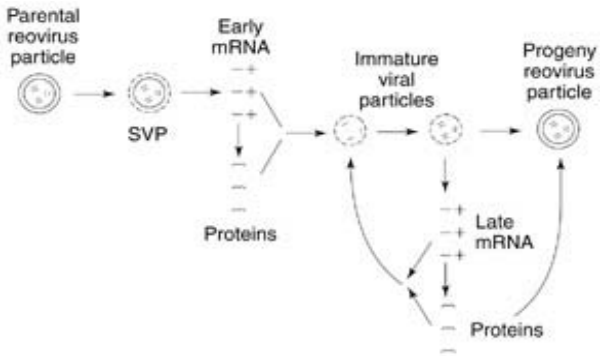


Figure 3 The reovirus multiplication cycle. SVP, subviral particle.

Reovirus Replication

Strategy

Reovirus multiplication exhibits two unusual features, both consequences of the fact that the reovirus genome is dsRNA, which means that a virus associated polymerase is required to generate mRNA, and the fact that it is segmented. As a result, parental genomes are not uncoated to naked RNA, and a highly complex mechanism functions to assort the ten genome segments into complexes that contain one, and one only, of each (**Fig. 3**).

Reovirus particles adsorb to specific receptors (see below) and internalized in endoplasmic vesicles that fuse with lysosomes within which the reovirus capsid shell is extensively degraded: a 12 kD fragment is cleaved from the C-terminal portion of μ1C to generate the 60 kDa protein δ, and proteins $\sigma 3$ and $\sigma 1$ are lost. The resultant particles, which are cores covered by an outer shell that consists only of protein δ and which are known as viral particles (SVPs), are liberated into the cytoplasm. The major functional difference between reovirus particles and SVPs is that the former genome segments are not transcribed into mRNA, whereas in the latter they are transcribed. The mRNAs are then translated into the various reovirus proteins and, after a certain interval, generally at about 3 h after infection, they begin to be transcribed into minus-strands with which they remain associated, thereby generating the progeny dsRNA-containing genome segments; and at the same time equimolar amounts of them are assorted into complexes. These complexes, or particles, then

transcribe the dsRNA molecules back into plus-strands, which are again either translated and/or used as templates for the generation of further ds genome segments. This cycle represents the multiplication phase of reovirus replication. As more dsRNA-containing complexes (dsRCCs) are generated and more reovirus proteins are formed, the protein complement of the dsRCCs is modified in stepwise fashion to generate first core-like particles and eventually complete, mature reovirus particles.

The nature of the reovirus receptor

There are several reovirus receptors that differ in their affinity for the cell-attachment sequences on the $\sigma1$ proteins. Protein $\sigma1$ itself possesses highest affinity for a 67 kDa glycoprotein and somewhat lower affinity for several other glycoproteins. The reovirus receptor on erythrocytes is glycophorin. The minimal essential receptor is the sialic acid residue at the ends of the carbohydrate prosthetic groups of glycoproteins. Presumably the remainder of the carbohydrate moiety and the protein modulate affinity and specificity.

All this evidence suggests a relatively nonstringent requirement for reovirus receptor recognition which is consistent with its very wide host range and its ability to bind to and infect a variety of cell types in the body.

The activation of the reovirus transcriptase

Reovirus particles do not transcribe mRNA; SVPs do so. This is not due to the fact that the transcriptase is inactive in virus particles; on the contrary, transcription initiation proceeds normally in virus particles, but transcription ceases before transcripts are more than four to six residues long and then reinitiates. The products of this abortive reiterative transcription are 5′-triphosphorylated – and also often capped – oligonucleotides, the sequence of most of which is GCUA$_n$ or GCUAU$_n$ where n is 1–4, and which remain associated, at least temporarily, with reovirus particles, in which they make up the bulk of the approximately 2400 5′-G-terminated oligonucleotides that they contain (see above).

SVPs, by contrast, are capable of transcribing full-length mRNA molecules, although for them also the majority (of the order of 90%) of transcripts are abortive transcripts. Clearly, what is activated is not the transcriptase, but rather the movement of template segments relative to the transcriptase catalytic site. Exactly what is involved in this release of movement is not known. Physicochemical evidence indicates that the conversion of reovirus particles to SVPs or cores triggers a conformational change in the dsRNA.

Transcription and translation of reovirus mRNA

Under optimal conditions of NTP and Mg^{2+} concentration, reovirus cores transcribe all ten genome segments at the same rate, that is, in amounts inversely proportional to their sizes. In infected cells, the relative proportions of the various species of mRNAs that are formed generally differ in two respects: there is usually a deficiency in the relative amounts of the *l* size class species, and during the early phase of the multiplication cycle, before progeny dsRNA genome segments have been formed, several species of mRNA (*l*1, *m*3, *s*3 and *s*4) appear to be formed in amounts larger than the rest. The basis of this effect, which is variable – often it is the *m*2, *s*3 and *s*4 species that are formed in relatively higher proportions, and often the effect is not limited to the early period – is not known.

Whereas these effects on the relative transcription frequencies are variable and quantitatively minor (that is, the excess transcription efficiencies are no more than two- to threefold), the relative translation efficiencies of the ten species of reovirus mRNAs differ enormously. The most efficiently translated mRNA species is usually species *s*4, followed by *m*2 (relative translation efficiency 0.67), *s*2 and *s*3 (slightly less than 0.5), *m*3, *l*2 and *l*3 (0.25–0.33), *s*1 (0.1) and *l*1 and *m*1 (0.01). These differences in relative translation efficiencies are due to differences in the sequences that surround and lie upstream of the initiation codons. Not only does the well-known Kozak rule apply, namely, positions −3 and +4 relative to the first nucleotide of the initiation codon must be G or A, but the nature of nucleotides at least as far upstream as position −8 also profoundly affects translation efficiency, depending on the nature of the nucleotides in positions −1 to −3. There is also an optimal length of the 5′-untranslated region (about 14 nucleotides), and there are also secondary structure constraints: the 5′-upstream sequence, including the initiation codon, must not be part of a stable stem-loop, and the 5′-cap must be accessible, that is, it also must not be part of or too close to, a stem-loop.

The fact that reovirus cores contain enzymes that catalyze the entire capping reaction indicates that capping is very important for reovirus mRNA translation. Some studies have suggested that late reovirus mRNAs are uncapped and that reovirus infection modifies the host cell translational machinery, so that late uncapped viral mRNAs are translated preferentially over capped cellular mRNAs, or that a factor in reovirus-infected cells stimulates translation of late uncapped reovirus mRNAs. Other studies have found that reovirus-infected cells can translate both uncapped and capped mRNAs, which confirms earlier studies that indicated that possession of a cap facili-

tates translation, but is not essential. It has also been suggested that the preferential translation of reovirus mRNAs in infected cells is mediated at the level of competition between mRNAs for a limited amount of a message-discriminatory factor. The resolution of all this conflicting evidence may lie in the finding that, like infection with many other viruses, infection with reovirus activates, via the generation of dsRNA or of short sequences in ssRNA with a locally dsRNA-like character, a cellular protein kinase that phosphorylates the α subunit of protein synthesis initiation factor eIF-2, thereby inactivating it and inhibiting protein synthesis, including the synthesis of reovirus proteins; and that protein $\sigma 3$, by virtue of its affinity for dsRNA (see above), prevents this activation. Thus protein $\sigma 3$ itself may be the factor that is essential for the efficient translation of late reovirus mRNAs.

The assortment of genome segments into reovirus genomes

The mechanism responsible for assorting the ten genome segments into genomes containing one of each is one of the most fascinating problems of reovirology; and very little is known about it. Since it is much easier to imagine how ssRNA molecules can be recognized by each other and by proteins than dsRNA molecules, it has always been assumed that assortment proceeds at the level of ssRNA. However, such ssRNA-containing complexes (ssRCCs) cannot be found. Rather it appears that the plus-strands associate with three viral proteins very soon after they are formed: the nonstructural protein μNS, the nonstructural protein σNS and $\sigma 3$. The resultant complexes contain one molecule of RNA and 15–30 molecules of these three proteins, depending on their length; most of them contain μNS, as well as σNS and/or $\sigma 3$. Presumably the binding is sufficiently reversible not to interfere with the RNAs being translated. The relative amounts of the various ssRNA species in the populations of these complexes reflect the relative frequencies with which they are transcribed (see above). Very significantly, however, even the first double-stranded RNA-containing complexes (dsRCCs) that can be detected (and which contain $\lambda 2$ as a major component as well as, presumably, $\lambda 3$) contain strictly equimolar amounts of all ten genome segments. This suggests that the generation of dsRNA genome segments and their assortment into genome sets are functionally linked and concomitant events; and it focuses attention on the RNA polymerase $\lambda 3$ as a key effector of assortment.

Infectious reovirus RNA

Since the only molecular links between parental and progeny virus particles are the plus-strands transcribed by SVPs, infectious reovirus should in theory be formed in cells into which the ten species of plus-stranded RNA are introduced. Conditions have indeed been found recently under which reovirus RNA is 'infectious' in this sense. The basic system consists of lipofecting into cells all ten species of ST3 ssRNA together with rabbit reticulocyte lysates in which all ten species of ST3 ssRNA have been translated for 60 min, and infecting these cells 4–8 h later with ST2 reovirus. When analyzed for their virus content 24 h later, ST3 virus is found to be present in these cells to the extent of 10^3 to 10^4 plaque-forming units (PFU)/10^6 cells. If ST3 dsRNA is also lipofected at the same time, the virus yields are 100 times higher; and most of the increased amount of virus can be shown to be the progeny of the ssRNA, not the dsRNA, the function of which is therefore to enhance the infectivity of the former.

This system is very important because it permits identification of the signals that are required for the introduction of heterologous, that is, novel, genetic information into the reovirus genome. Clearly there must be recognition signals on incoming, that is, to-be-accepted, genome segments because orbivirus or rotavirus genome segments, for example, are not accepted. What is very unexpected, however, is that there are also acceptance signals: thus the reovirus ST3 genome will not accept novel genome segments like, for example, ST2 genome segments, unless the S4 genome segment of the accepting ST3 genome is a variant of the normal wild-type S4 genome segment that contains two point mutations, one of which causes an amino acid change, whereas the other does not. The discovery of these acceptance signals permits the introduction of novel genetic information into the reovirus genome, permits identification of the nature of recognition signals, permits the construction of reovirus strains with desired properties such as, for example, highly efficient and nonpathogenic vaccine virus strains (this would be of great importance for orbiviruses and rotaviruses which, in contrast to reoviruses, include important human and domestic animal pathogens and to which this technology should be transferable), and opens the way for the development of the nonpathogenic reovirus as a highly efficient expression vector for clinical application in the fields of gene therapy, on the one hand, and cancer therapy, on the other.

Effect of reovirus infection on infected cells

There is no special mechanism for the release of reovirus progeny; reovirus particles are released when cytopathic effects have progressed sufficiently for cell necrosis to result in cell lysis. In cells infected with

reovirus, masses of granular material develop in areas scattered throughout the cytoplasm which eventually move toward the nucleus and coalesce, forming characteristic inclusions or 'viral factories'. These inclusions are easily identified with fluorescein-labeled antibodies against reovirus proteins; they represent the areas where viral assembly proceeds, and quasicrystalline arrays of reovirus particles are often associated with them. Microtubules appear to extend throughout these viral factories and appear to be covered with viral protein; in particular, proteins $\sigma 1$ and μNS possess affinity for elements of the cytoskeleton (CSK), which suggests that they may play a role in facilitating or mediating reovirus morphogenesis. However, microtubules *per se* are not essential, since colchicine does not inhibit reovirus multiplication or assembly. Like infection with all lytic viruses, reovirus infection causes progressive disruption of the CSK organization, but it is not known which reovirus protein(s) cause(s) this effect.

See also: **Pathogenesis: Animal viruses, Plant viruses; Reoviruses (*Reoviridae*): General features; Replication of viruses.**

Further Reading

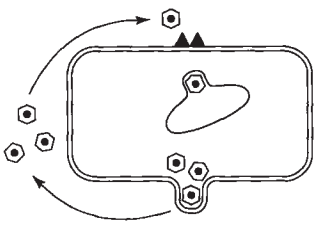
Dryden KA, Wang G, Yeager M *et al* (1993) Early steps in reovirus infection are associated with changes in supramolecular structure and protein conformation: analysis of virions and subviral particles by cryoelectron microscopy and image reconstruction. *J. Cell Biol.* 122: 1023.

Joklik WK and Roner MR (1996) Molecular recognition in the assembly of the segmented reovirus genome. *Prog. Nucleic Acids Res. Mol. Biol.* 53: 249.

Nibert ML, Schiff LA and Fields BN (1996) Reoviruses and their replication. In: Fields BN, Knipe DM and Howley PM (eds) *Fields Virology*, 3rd edn. New York: Raven Press.

REPLICATION OF VIRUSES

V Gregory Chinchar, Department of Microbiology, University of Mississippi Medical Center, Jackson, Mississippi, USA



Introduction

Viruses are obligate intracellular parasites that replicate only within living animal, plant, or bacterial cells. Of the 71 taxonomically defined virus families, 24 contain members that infect vertebrates, and these families will be the focus of this overview. Among the smallest vertebrate viruses, the virion consists only of the viral genome and a closely associated protein coat (nucleocapsid), whereas larger viruses possess, in addition to the nucleocapsid, a variety of catalytic, regulatory and structural proteins, and in some cases a host-derived lipid membrane (envelope) containing one or more virus-encoded glycoproteins. In animal cells, virus replication is complete within several hours to at most a few days, and results in the synthesis of 10^3–10^5 virions per cell. Virus replication will be discussed in three stages: (1) early events (attachment to susceptible cells, penetration and uncoating), (2) viral biosynthetic events (replication of the viral genome, transcription and translation) and (3) virion assembly. However, because of space limitations, early events and mechanisms of virion assembly will be dealt with briefly so that viral biosynthetic strategies can be considered in greater detail.

Early Events

Attachment

Infection begins with the attachment of a virion, via capsid or envelope proteins, to specific cell-surface macromolecules (viral receptors). Because of the specificity of this interaction, the host range (tropism) of a given virus is determined primarily by the presence of viral receptor molecules on the cell surface. As a group, viruses utilize a variety of proteins, lipids and oligosaccharides as receptors. One class of receptors includes cellular macromolecules involved in ligand binding, endocytosis and cell recognition. For example, the receptors for poliovirus, human rhinovirus (ICAM-1), human immunodeficiency virus type 1 (CD4) and Epstein–Barr virus (CD21) are members of the immunoglobulin superfamily of proteins. In contrast, the receptor for Mahoney leukemia virus is an amino acid transporter and sialic acid-

reovirus, masses of granular material develop in areas scattered throughout the cytoplasm which eventually move toward the nucleus and coalesce, forming characteristic inclusions or 'viral factories'. These inclusions are easily identified with fluorescein-labeled antibodies against reovirus proteins; they represent the areas where viral assembly proceeds, and quasicrystalline arrays of reovirus particles are often associated with them. Microtubules appear to extend throughout these viral factories and appear to be covered with viral protein; in particular, proteins $\sigma 1$ and μNS possess affinity for elements of the cytoskeleton (CSK), which suggests that they may play a role in facilitating or mediating reovirus morphogenesis. However, microtubules *per se* are not essential, since colchicine does not inhibit reovirus multiplication or assembly. Like infection with all lytic viruses, reovirus infection causes progressive disruption of the CSK organization, but it is not known which reovirus protein(s) cause(s) this effect.

See also: **Pathogenesis: Animal viruses, Plant viruses; Reoviruses (*Reoviridae*): General features; Replication of viruses.**

Further Reading

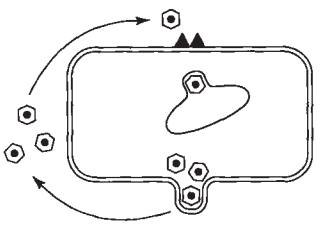
Dryden KA, Wang G, Yeager M *et al* (1993) Early steps in reovirus infection are associated with changes in supramolecular structure and protein conformation: analysis of virions and subviral particles by cryoelectron microscopy and image reconstruction. *J. Cell Biol.* 122: 1023.

Joklik WK and Roner MR (1996) Molecular recognition in the assembly of the segmented reovirus genome. *Prog. Nucleic Acids Res. Mol. Biol.* 53: 249.

Nibert ML, Schiff LA and Fields BN (1996) Reoviruses and their replication. In: Fields BN, Knipe DM and Howley PM (eds) *Fields Virology*, 3rd edn. New York: Raven Press.

REPLICATION OF VIRUSES

V Gregory Chinchar, Department of Microbiology, University of Mississippi Medical Center, Jackson, Mississippi, USA



Introduction

Viruses are obligate intracellular parasites that replicate only within living animal, plant, or bacterial cells. Of the 71 taxonomically defined virus families, 24 contain members that infect vertebrates, and these families will be the focus of this overview. Among the smallest vertebrate viruses, the virion consists only of the viral genome and a closely associated protein coat (nucleocapsid), whereas larger viruses possess, in addition to the nucleocapsid, a variety of catalytic, regulatory and structural proteins, and in some cases a host-derived lipid membrane (envelope) containing one or more virus-encoded glycoproteins. In animal cells, virus replication is complete within several hours to at most a few days, and results in the synthesis of 10^3–10^5 virions per cell. Virus replication will be discussed in three stages: (1) early events (attachment to susceptible cells, penetration and uncoating), (2) viral biosynthetic events (replication of the viral genome, transcription and translation) and (3) virion assembly. However, because of space limitations, early events and mechanisms of virion assembly will be dealt with briefly so that viral biosynthetic strategies can be considered in greater detail.

Early Events

Attachment

Infection begins with the attachment of a virion, via capsid or envelope proteins, to specific cell-surface macromolecules (viral receptors). Because of the specificity of this interaction, the host range (tropism) of a given virus is determined primarily by the presence of viral receptor molecules on the cell surface. As a group, viruses utilize a variety of proteins, lipids and oligosaccharides as receptors. One class of receptors includes cellular macromolecules involved in ligand binding, endocytosis and cell recognition. For example, the receptors for poliovirus, human rhinovirus (ICAM-1), human immunodeficiency virus type 1 (CD4) and Epstein–Barr virus (CD21) are members of the immunoglobulin superfamily of proteins. In contrast, the receptor for Mahoney leukemia virus is an amino acid transporter and sialic acid-

containing glycoproteins serve as receptors for paramyxo- and orthomyxoviruses.

Penetration and uncoating

Following attachment, the virion must enter the cell (penetration) and release its genome (uncoating). The process by which many viruses accomplish this dual task is termed receptor-mediated endocytosis and is the same mechanism used by the cell to import growth factors and other large molecules to which the plasma membrane is not permeable. Virions, bound to their cognate receptors, are transported laterally within the plasma membrane to clathrin-coated pits and enter the cell as the clathrin-coated pit invaginates. Subsequently, this vesicle fuses with an endosome and, within this acidic compartment, uncoating takes place. The acidic pH of the endosome is critical and agents that raise the intraendosomal pH (e.g. NH_4Cl, chloroquine, etc.) block virus uncoating. For enveloped viruses, uncoating involves fusion of the viral envelope with the endosomal membrane followed by release of the nucleocapsid into the cytoplasm. In the case of influenza virus, it is thought that low pH changes the conformation of the hemagglutinin (HA) allowing the hydrophobic fusion peptide to interact with target cell membranes. In addition, among negative-stranded viruses, the acidic environment within the endosome promotes release of the matrix (M) protein from the nucleocapsid, a step necessary for subsequent transcription. Nonenveloped viruses also appear to utilize receptor-mediated endocytosis, although here uncoating does not involve membrane–membrane fusion. For example, following attachment of poliovirus to target cells, one of the capsid proteins (VP4) is released exposing hydrophobic residues buried inside the virion. Interaction of these residues with the endosomal membrane may provide a pore through which viral RNA is extruded into the cytoplasm. In the adenovirus system, low endosomal pH induces conformational changes in the capsid which rupture the endosomal membrane at virion–membrane contact points. After its release into the cytoplasm, adenovirus is transported via microtubules to nuclear pores where viral DNA enters the nucleus. In contrast to the above mechanism, several viruses (e.g. paramyxoviruses, herpesviruses and human immunodeficiency virus type 1 [HIV]) do not require an acidic environment for uncoating and enter cells by fusion at the plasma membrane.

Although the presence of viral receptors is a primary determinant for infectivity, not all cells carrying the appropriate receptor are susceptible to infection. In several 'restrictive' systems, the synthesis of infectious progeny is blocked at a postattachment step. For example, some mammalian cell lines bind influenza virus and support the synthesis of all viral macromolecules, yet do not generate infectious virions because they lack the protease required to cleave the hemagglutinin precursor (HA_0) and generate activated (i.e. fusion-competent) HA_1 and HA_2. Conversely, some cells that lack the appropriate viral receptor can nonetheless support a productive infection if the viral genome is introduced into the cell by transfection.

Synthesis of Virus-specific Macromolecules

The 24 families of vertebrate viruses, although differing in genomic make-up, virion morphology, and their repertoire of viral-encoded enzymes, can be ordered on the basis of replicative mechanisms. However, before examination of different replication strategies, several common themes need to be addressed.

Viral transcription and genome replication

Viral nucleic acid synthesis is catalyzed by both viral and host enzymes, the relative contribution of which is determined by the type of virus and the specific molecule. Viruses with RNA genomes, except for the retroviruses, synthesize mRNA and replicate their genomes using virus-encoded RNA-dependent RNA polymerases. In contrast, retroviruses synthesize a double-stranded complementary DNA (cDNA) copy of their single-stranded RNA genome using a virion-encoded RNA-dependent DNA polymerase (reverse transcriptase). In subsequent steps, the retroviral cDNA is integrated into the host chromosome and transcribed by host-encoded DNA-dependent RNA polymerase II (pol II) to yield viral messages and genomic RNA. DNA viruses, except for poxviruses, also use host-encoded pol II to transcribe their messages. Poxviruses, because they replicate in the cytoplasm and do not have access to pol II, assemble a novel transcriptase composed of multiple poxvirus-specific (and possibly one or more host-derived) subunits. Most DNA virus families (e.g. *Poxviridae, Iridoviridae, Herpesviridae, Adenoviridae*) synthesize a virus-encoded DNA-dependent, DNA polymerase. However, two families (i.e. *Parvoviridae* and *Papovaviridae*) utilize host DNA polymerase, and the *Hepadnaviridae* replicate viral DNA through an RNA intermediate using a virus-encoded reverse transcriptase.

Gene regulation

Viruses have evolved a variety of mechanisms to control gene expression and maximize efficiency. In

some systems, viral gene expression is divided into temporal phases in which catalytic and regulatory proteins are synthesized early in infection, whereas the synthesis of structural proteins is limited to late times. Alternatively, the expression of viral genes may be controlled by differences in the transcription rate of specific genes (e.g. rhabdoviruses and paramyxoviruses), the translational efficiency of different viral messages (e.g. reovirus) or the replication of transcriptional templates (e.g. influenza virus). Moreover, it is likely that, even within a single virus family, multiple mechanisms regulate gene expression. At the molecular level, gene expression is controlled by both *cis*- and *trans*-acting signals. In some cases, the nucleotide sequence of viral messages and transcriptional templates may be the primary factor in determining how efficiently a given sequence is translated or transcribed. For example, the differential synthesis of the various coronavirus mRNAs is thought to be controlled by interaction between *trans*-acting coronavirus leader RNA and *cis*-acting sequences located at the beginning of each gene. Furthermore, transcription and genome replication among DNA viruses (and retroviruses) is regulated by the (often) combined action of *trans*-acting viral- and host-encoded factors with *cis*-acting viral nucleotide sequences. For example, herpesvirus immediate-early gene transcription requires, aside from pol II, both host- (OTF-1) and virus-encoded (α-TIF) transcription factors. Lastly, in several families (e.g. *Orthomyxoviridae, Poxviridae, Herpesviridae* and *Iridoviridae*), there are hints that viral gene expression is also regulated at post-transcriptional and translational levels.

Viral protein synthesis

Viral protein synthesis is completely dependent on the cell's translational machinery (i.e. ribosomes, tRNAs, initiation factors, etc.). Reflecting that dependence, viral mRNAs, despite some prominent exceptions (e.g. picornaviruses), are similar in overall structure to host messages, i.e. they are capped and methylated at their 5′ terminus and polyadenylated at their 3′ end. Viral mRNAs are monocistronic and are translated as are other eucaryotic transcripts. However, in some systems, viral proteins are synthesized as part of a larger precursor (polyprotein) which is cleaved to generate the final products. This mechanism overcomes the inability of eucaryotic ribosomes to translate polycistronic messages and allows one viral mRNA to code for several proteins. Viruses have also developed several ways to utilize the same nucleotide sequence to encode one or more proteins:

1. HIV-1 and influenza A virus use alternative splicing to generate additional transcripts encoding novel proteins;
2. measles virus and other paramyxoviruses generate a novel P-related protein (V) by RNA editing, a process in which one or more nontemplated nucleotides are added at a site within the 3′ end of some P transcripts;
3. Sendai virus synthesizes five proteins from its P transcript by using alternative translational initiation codons;
4. retroviruses use frameshifting or read-through mechanisms to circumvent a stop codon lying between the capsid and polymerase coding regions of the gag-pol transcript.

Finally, following their translation, viral proteins, like their cellular counterparts, are post-translationally modified (e.g. glycosylated, phosphorylated, etc.) using cellular enzymes.

As infection progresses, viral protein synthesis often supplants cellular translation. In some cases, this simply reflects the increased abundance of viral messages, whereas in others viral messages appear to initiate translation at a higher rate than host messages. Alternatively, virus infection may actively inhibit host translation by (1) proteolytically inactivating or covalently modifying initiation factors required solely or preferentially by cellular messages, (2) selectively degrading host messages, or (3) altering the intracellular ionic environment to favor viral over host translation. Furthermore, because infection can lead to the phosphorylation and functional inactivation of eucaryotic initiation factor 2 (eIF-2), several virus families (*Poxviridae, Reoviridae, Orthomyxoviridae, Adenoviridae* and *Picornaviridae*) have evolved mechanisms to block eIF-2 phosphorylation. Virus infection also blocks host cell RNA and DNA synthesis. Although transcriptional shut-off may be the direct result of inactivating specific transcription factors, the inhibition of cellular DNA synthesis is likely due to the earlier inhibition of protein synthesis.

Cytoskeleton

In addition to providing the biochemical components required for replication, the cell also supplies the virus with an intracellular highway to facilitate infection and assembly. There is growing evidence that the transport of infecting virions to the nucleus and the transport of viral proteins into assembly sites takes place along the various fibers of the cellular cytoskeleton.

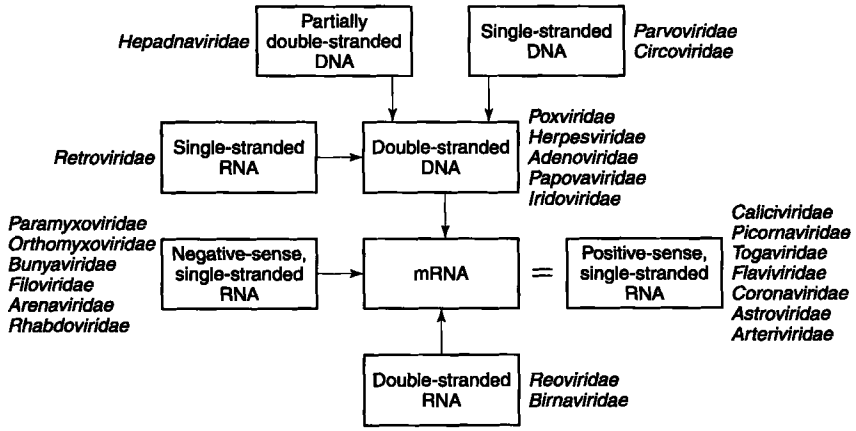


Figure 1 Strategies for the production of viral mRNA utilized by 24 families of viruses infecting humans and other animals. (Updated from Baltimore D (1971) *Bacteriol. Rev.* 35: 235.)

RNA Viruses

RNA-containing viruses are discussed in the light of four basic transcriptional strategies (**Fig. 1**). These strategies encompass viruses with message-sense (positive-strand viruses) and antisense genomes (negative-strand viruses), viruses that package their replicative form as genome (double-stranded RNA viruses) and viruses that utilize 'reverse transcription'. Although this approach is conceptually useful, not all viruses within a class conform precisely to the prototypic replication strategy. Despite this caveat, representative examples will be cited to illustrate the replication mechanism.

Positive-strand RNA viruses

Positive-strand viruses contain a single-stranded, message-sense RNA genome which is translated immediately after uncoating. To simplify this discussion, poliovirus (family *Picornaviridae*; genus *Enterovirus*) will be used as a prototype because it is the most extensively studied positive-strand virus and provides a clear view of this strategy (**Fig. 2**).

Following uncoating, the poliovirus genome is translated to yield an ~200 000 mol. wt polyprotein. Initial cleavage of the polyprotein occurs cotranslationally and is mediated by the autocatalytic activity of a virus-encoded protease, polypeptide 2A. Subsequent cleavages, catalyzed by viral protease 3C, yield structural (capsid) and catalytic (polymerase) proteins. As infection proceeds, translation of capped host mRNAs is blocked due to the virus-induced degradation of the large subunit (eIF-4G) of the mRNA cap recognition factor (eIF-4F). In contrast, viral messages, which are uncapped and possess a highly structured 5′-nontranslated region (5′-NTR), escape shut-off because the 40S ribosomal subunit binds to an internal sequence within the 5′-NTR. The ability of the 40S ribosomal subunit to bind internally, in contrast to the usual scenario in which the 40S ribosome binds at the 5′ end of the message and 'scans' until a start codon is encountered, is termed

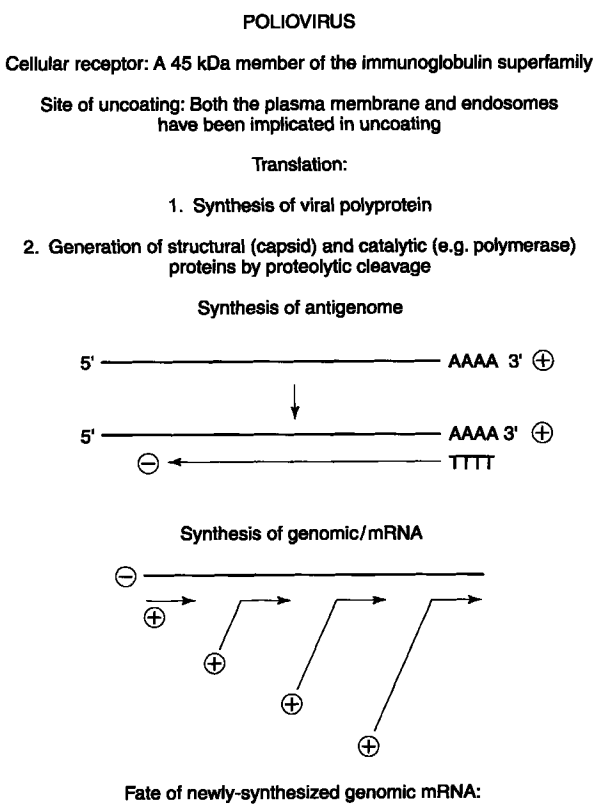


Figure 2 Replication strategy of a representative positive-stranded RNA virus.

'internal ribosome entry'. Thus cap-independent initiation and internal ribosome entry allow poliovirus messages to be selectively translated under conditions where translation of capped messages is progressively compromised.

Following its synthesis, the viral RNA-dependent RNA polymerase catalyzes the synthesis of a full-length negative-sense copy of the genome. Subsequently, the negative-strand serves as template and directs the synthesis of multiple plus-strands. Early in infection, when the concentration of viral structural proteins is low, newly synthesized positive-strands most likely are translated and serve to amplify the synthesis of viral proteins. Later when the concentration of virion precursors is high, newly synthesized plus-strands are encapsidated to generate infectious virus particles. A small virus-encoded protein (VPg) is covalently linked to the 5′ end of all picornavirus RNAs except message. VPg is thought to play a role in RNA synthesis, but it is unclear whether VPg functions as a primer or in some other capacity.

Negative-strand RNA viruses

Because the genome of negative-sense RNA viruses cannot be translated, the first virus-specific biosynthetic event following uncoating is the synthesis of viral mRNA by a virion-associated RNA-dependent RNA polymerase using the viral genome as template. Negative-stranded viruses are divided into two classes: those with unsegmented (monopartite) genomes (i.e. the order *Mononegavirales* containing the families *Paramyxoviridae, Rhabdoviridae* and *Filoviridae*) and those with segmented (multipartite) genomes (i.e. the families *Orthomyxoviridae, Bunyaviridae* and *Arenaviridae*). Although each class uses the negative-strand strategy, they possess unique attributes and will be dealt with separately.

The replication of monopartite viruses is discussed using vesicular stomatitis virus (VSV), a rhabdovirus, as the prototype (**Fig. 3**). Immediately after uncoating, the VSV genome is transcribed to yield a short nontranslated 'leader' RNA followed, in decreasing molar amounts, by five capped, methylated and polyadenylated viral mRNAs. Transcription occurs within the nucleocapsid, a structure containing the viral genome and multiple copies of three virus-encoded proteins. Aside from the nucleocapsid (N) protein, which tightly encloses the genome and is present in ~2000 copies/nucleocapsid, two catalytic proteins are also present within the nucleocapsid. The polymerase, polypeptide L (mol. wt ~200 000), is present in about 50 copies per nucleocapsid and catalyzes transcriptional initiation and elongation, as well as capping, methylation and polyadenylation.

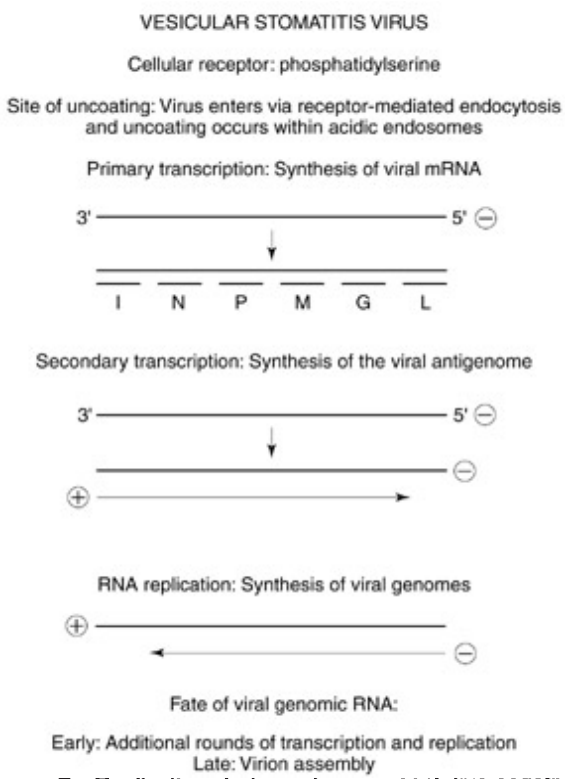


Figure 3 Replication strategy of a representative negative-stranded RNA virus.

The phosphoprotein P, present in about 500 molecules per nucleocapsid, plays a variety of roles in RNA synthesis. It binds L to the nucleocapsid, maintains the solubility of free N and may function in chain elongation. The viral transcriptase binds genomic RNA at its 3′ terminus and initiates transcription. At each intergenic junction (with the exception of the leader-N junction), a poly(A) tract is added to the newly synthesized mRNA by repetitive copying ('stuttering') of an oligo(U) sequence present at the end of the gene. After synthesis of the poly(A) tract, transcription terminates, releasing newly synthesized mRNA, but maintaining the transcriptase on its template. Re-initiation at the next gene downstream occurs via a conserved start sequence present at the beginning of each gene. However, because re-initiation does not take place every time, downstream genes (i.e. those coding for the envelope and polymerase proteins) are transcribed less frequently than upstream ones encoding the nucleocapsid, phosphoprotein and matrix proteins. Thus viral gene expression is controlled by transcriptional polarity.

Viral genome replication, i.e. the synthesis of a full-length positive-sense copy of the genome and the subsequent generation of progeny negative-strands, is catalyzed by the same polymerase that directs transcription. The switch between the transcriptive

and replicative modes appears to be controlled by the concentration of the nucleocapsid protein. When N reaches a critical concentration, it binds newly synthesized RNA within the leader sequence and allows the polymerase to readthrough intergenic regions and synthesize full-size positive-strands (i.e. antigenomes) which will serve as templates for virion RNA synthesis.

Segmented viruses encode their genetic information in multiple molecules of negative-sense RNA. In the case of influenza A virus (*Orthomyxoviridae*), the genome is composed of eight unique segments of virion RNA. In contrast to most RNA virus families, orthomyxoviruses require a functional cell nucleus for replication. This requirement reflects the fact that the orthomyxovirus polymerase complex can neither initiate transcription *de novo* nor cap and methylate viral mRNAs. Instead, the complex 'pirates' the capped and methylated 5′ terminus from a selected set of newly synthesized host messages and uses these to prime viral transcription. Once initiated, transcription continues until an oligo(U) tract, about 22 nucleotides from the end of virion RNA, signals addition of the 3′ poly(A) tail by repetitive copying. Because of this, viral genomic and mRNAs are not completely complementary, but differ at both their 5′ and 3′ ends. As with unsegmented viruses, the trigger controlling the transition from transcription to replication may be the concentration of nucleocapsid protein.

Other negative-stranded viruses possess additional molecular surprises. Some bunyaviruses (tripartite genome) and all arenaviruses (bipartite genome) possess 'ambisense' genomic RNA, in which non-overlapping subgenomic messages are transcribed from the 3′ ends of both virion RNA and its full-length complement. Furthermore, in what may be the prototype of a new family within the *Mononegavirales*, Borna disease virus replicates and transcribes its genome within the nucleus and utilizes RNA splicing to generate its messages.

Double-stranded RNA (dsRNA) viruses

Animal viruses with dsRNA genomes are segmented and can be viewed as a variant of the negative-sense strategy in which the virion encapsidates the replicative form of the genome. Genomic dsRNA is transcribed within partially uncoated ribonuclease-resistant viral cores by the virion-associated polymerase to yield viral mRNAs. Early in infection, some progeny plus-strands function as translational templates whereas others associate with nonstructural proteins and form complexes which are transcribed once to yield dsRNA. Newly synthesized dsRNA serves as template for the synthesis of additional viral mRNA which amplifies the replication cycle. Later, as the concentration of core and capsid proteins increases, the dsRNA–protein complex exchanges nonstructural for structural proteins and forms mature virus particles.

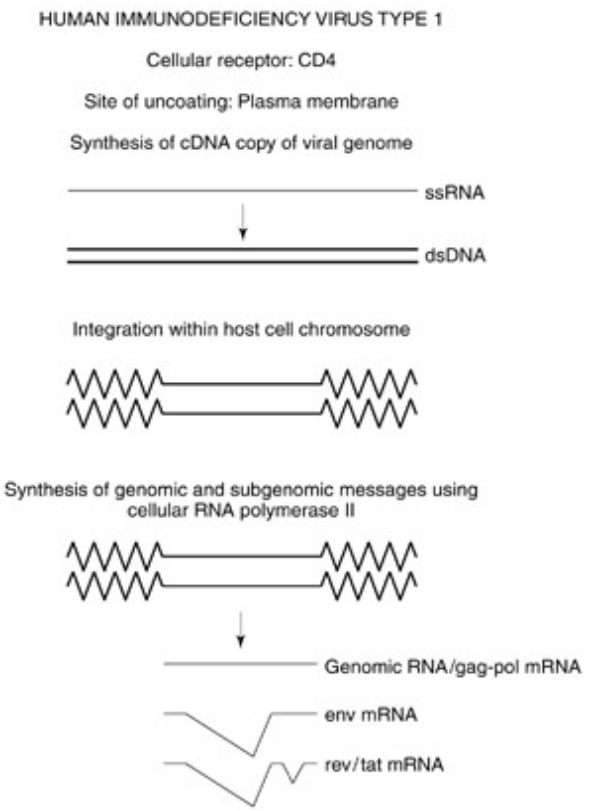


Figure 4 Replication strategy of a complex retrovirus.

RNA viruses that utilize a reverse transcription strategy

Retroviruses replicate their genome and transcribe mRNA using a dsDNA copy of viral genomic RNA as template (**Fig. 4**). This unconventional mechanism, in which single-stranded virion RNA is used as a template for dsDNA synthesis, is catalyzed by a virion-associated, RNA-dependent DNA polymerase (reverse transcriptase). After viral entry, the virion capsid is partially uncoated and a complementary DNA copy of the RNA genome is synthesized using reverse transcriptase. An endonucleolytic activity, integral to the reverse transcriptase, degrades the RNA template and second-strand DNA synthesis begins. Completion of second-strand synthesis results not only in a dsDNA copy of virion RNA, but also generates a unique structure termed the long terminal

repeat (LTR). The LTR flanks the viral cDNA and is composed of unique sequences from the 5′ and 3′ ends of the genome and a repeat element common to both ends. Following cDNA synthesis retrovirus DNA is integrated into the host chromosome, and, in this form, is termed the 'provirus'. Subsequently, the provirus is transcribed by pol II to yield full-length progeny RNA and one or more subgenomic mRNAs. The upstream LTR plays a very important role in retrovirus gene expression because it contains enhancer elements which regulate pol II-catalyzed transcription. (The downstream LTR is not involved in viral gene expression, but may activate host oncogenes and play a role in cell transformation.)

Full-length genome-sized RNA can either be packaged within virions or serve as messenger for the capsid and catalytic viral proteins. Translation of retrovirus genomic RNA yields two classes of polyproteins. The majority (~95% of the total) encode the capsid, core and matrix proteins and result when translation terminates immediately after the coding region of the nucleocapsid gene. However, a minor population, encoding the aforementioned structural proteins as well as the protease, reverse transcriptase, and integrase results when the stop codon at the end of the capsid/core region is bypassed either by frameshifting or read-through. Envelope glycoproteins are translated from a singly-spliced subgenomic mRNA containing sequences primarily from the 3′ end of the genome, whereas lentiviruses, such as HIV-1, utilize doubly spliced subgenomic mRNAs to direct the synthesis of TAT, REV and several other regulatory proteins.

TAT and REV are the two best-studied of the HIV-1 regulatory proteins. TAT is a *trans*-acting protein that binds to a sequence present at the 5′ end of all HIV-1 mRNAs and enhances HIV-1 gene expression by relieving a block in transcriptional elongation or by increasing transcriptional initiation. REV mediates the switch between the synthesis of regulatory proteins (i.e. TAT and REV) and the generation of structural and catalytic proteins by binding to *cis*-acting sequences within viral mRNA and directing the transport into the cytoplasm of unspliced genomic RNA and singly-spliced envelope message.

DNA Viruses

With the exception of parvoviruses and hepadnaviruses, the genomes of which are respectively single-stranded and partially double-stranded, DNA-containing animal viruses possess a dsDNA genome. However, even in these two families, viral mRNA is ultimately transcribed from a dsDNA template using cellular DNA-dependent RNA polymerase (**Fig. 1**). In place of a detailed discussion of each family, broader issues of viral DNA replication will be discussed. To begin with, DNA viruses differ greatly in their genetic content ranging in size from 5 kbp (*Parvoviridae*) to greater than 120 kbp (*Herpesviridae, Poxviridae* and *Iridoviridae*). Thus the small DNA viruses are about as genetically complex as a typical RNA virus, whereas the larger DNA viruses encode 100 or more proteins. Not unexpectedly, the degree to which virus replication is dependent on cellular functions reflects the genetic complexity of the virus. Thus, parvoviruses and papovaviruses require extensive host involvement to support viral biosynthetic events (including DNA synthesis), whereas other families are progressively more independent.

Among herpes-, pox- and iridoviruses, viral genes are expressed in a coordinated temporal sequence of immediate early, early and late genes. Generally immediate early genes code for proteins required to initiate virus replication, early genes encode catalytic functions (e.g. the viral DNA polymerase), and late genes specify structural proteins. Furthermore, immediate early genes activate early and late gene transcription, whereas specific early and late genes downregulate immediate early and early gene expression respectively. Aside from specific regulatory proteins, full late gene expression also requires viral DNA synthesis, thus inhibitors of viral DNA replication block late gene expression despite the presence of functional immediate early and early activators.

Because DNA polymerase requires a primer with an available 3′-OH to initiate DNA synthesis, all viruses with a linear DNA genome have evolved specialized features that allow them to maintain intact termini during replication. For example, adenoviruses solve the 'end-problem' by using a nucleotide-linked terminal protein to initiate DNA replication, herpesviruses replicate through a rolling circle mechanism, and poxviruses and parvoviruses utilize a self-priming mechanism to ensure replication of their termini. In contrast to other DNA viruses, hepadnaviruses possess a circular, partially single-stranded DNA genome that is replicated through an RNA intermediate using virus-encoded reverse transcriptase. Upon entry into the cell the gaps are repaired and dsDNA is transcribed in the nucleus by host polymerase to yield viral mRNAs and pregenomic RNA. The latter is encapsidated and transcribed into complementary DNA using virus-encoded protein P both as the primer and the reverse transcriptase. As with retroviruses, the RNA template is degraded and second-strand DNA synthesis takes place. However, before its completion, the virion is exported from the cell leaving genomic DNA partially single-stranded.

Unlike other DNA viruses, poxviruses replicate solely within the cytoplasm in morphologically distinct viral 'factories'. Reflecting their metabolic independence from the host cell, poxviruses synthesize unique DNA and RNA polymerases, and their virions contain all the proteins needed to transcribe the earliest class of viral mRNAs. Furthermore, viral transcriptional promoters and termination sequences are unique and are regulated by virus-specific factors.

Iridoviruses, occupying a taxonomic middle-ground between poxviruses and the nuclear DNA viruses, possess several distinctive features. Viral DNA replication takes place in two distinct compartments (genome-length progeny DNA is synthesized in the nucleus, followed by the synthesis of concatemeric DNA in the cytoplasm), whereas virion assembly is confined to cytoplasmic viral 'assembly sites'. Viral DNA is highly methylated with nearly 25% of cytosine residues present as methylcytosine. Methylation is catalyzed by a virus-encoded enzyme and, as in some bacteriophage systems, appears to function as part of a restriction–modification system. Surprisingly, despite the high content of methylcytosine, host RNA polymerase II has been implicated in at least the early rounds of viral transcription. However, it is not known whether unmodified pol II transcribes viral DNA late in infection or whether viral-encoded proteins modify pol II and alter its specificity.

Virus Assembly

Once sufficient stores of viral nucleic acid and protein have accumulated in the infected cell, nucleocapsid formation and virion assembly begin and continue as long as the cells are metabolically competent. Despite the large number of vertebrate virus families, only three types of nucleocapsids are found: complex, helical and icosahedral (spherical). The nucleocapsids of poxviruses do not conform to the geometric symmetry found among the helical and icosahedral viruses and are considered to be 'complex'. Little is known about the molecular mechanisms controlling their assembly. Helical nucleocapsids (which, among vertebrate viruses, enclose only RNA genomes) form as viral proteins bind to nascent RNA transcripts and encapsidate them. During virus assembly, helical nucleocapsids migrate to cellular membranes where viral glycoproteins have concentrated. There, through concerted interaction between the nucleocapsid and viral glycoproteins, the nucleocapsid is enveloped by the cellular membrane in a process termed 'budding'. Host proteins are excluded from the membrane and the resulting envelope contains only virus-encoded glycoproteins. Moreover, because envelopment is not a precise process, dual infections with different strains of the same multipartite virus (e.g. influenza virus A) lead to high-frequency genetic reassortment. Although virion envelopment takes place commonly at the plasma membrane (e.g. among the *Paramyxoviridae, Orthomyxoviridae* and *Rhabdoviridae*), intracellular membranes (e.g. those of the Golgi, endoplasmic reticulum and, in the case of DNA viruses, the nucleus) are used by other virus families. In contrast to viruses with helical nucleocapsids, icosahedral nucleocapsids enclose both DNA and RNA genomes. It is thought that nucleocapsids form spontaneously when the concentration of capsid proteins reaches a critical level. In some families, nucleocapsids are not enveloped (i.e. virion equals nucleocapsid), whereas in others nucleocapsids are enveloped as described above. Enveloped icosahedral viruses are released from infected cells by budding, whereas non-enveloped icosahedral virions are liberated by cell lysis.

See also: **Cell structure and function in virus infections; Virus–host cell interactions.**

Further Reading

Cann AJ (1997) *Principles of Molecular Virology*, 2nd edn. San Diego: Academic Press.

Ehrenfeld E (1993) Translational regulation in virus-infected cells. *Semin. Virol.* 4: 199.

Fields BN, Knipe DM, Howley PM *et al* (eds) (1996) *Fields Virology*, 3rd edn. New York: Lippincott–Raven.

Joklik WK, Willet HP, Amos DB *et al.* (eds) (1992) *Zinsser Microbiology*, 20th edn. Norwalk, CT: Appleton and Lange.

Levy JA, Fraenkel-Conrat H and Owens RA (1994) *Virology*, 3rd edn. Englewood Cliffs, NJ: Prentice-Hall.

Wimmer E (1994). *Cellular Receptors for Animal Viruses.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

RESPIRATORY SYNCYTIAL VIRUS – HUMAN (*PARAMYXOVIRIDAE*)

Peter L Collins, Laboratory of Infectious Disease, National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, Maryland, USA

History

Human respiratory syncytial virus (RSV) was isolated in 1956 from a laboratory chimpanzee with upper respiratory tract disease. Shortly thereafter, an apparently identical virus was recovered from children ill with pneumonia or croup and was identified as a virus of humans. RSV is now recognized as the leading viral agent of pediatric respiratory tract disease worldwide. It also is gaining recognition as a significant cause of disease in the elderly and in immunocompromised individuals. Its poor growth *in vitro* and instability have impeded research. RSV lacks an approved vaccine or highly effective antiviral therapy.

Taxonomy and Classification

RSV is a member of the Order *Mononegavirales*, the nonsegmented negative-strand RNA viruses (**Table 1**). These are enveloped viruses that have as genome a single strand of protein-coated negative-sense RNA. Vesicular stomatitis and Sendai viruses are the most extensively characterized members of the group.

RSV is the type species for genus *Pneumovirus* in the subfamily *Pneumovirinae* of the family *Paramyxoviridae*. There also are bovine (BRSV), caprine (CRSV) and ovine (ORSV) versions which are related to human RSV. BRSV is one of several respiratory tract pathogens associated with shipping fever, an important disease of cattle. Pneumonia virus of mice (PVM) is a somewhat more distant relative. There is a turkey pneumovirus, formerly called turkey rhinotracheitis virus (TRTV) but now called avian pneumovirus (APV), which is sufficiently distinct (see below) so that it has been placed in a second genus, *Metapneumovirus*.

Virion Structure and Viral Proteins

RSV virions include spherical particles of 80–350 nm in diameter (**Fig. 1**) and filamentous particles of 60–100 nm in diameter and up to 10 μm in length. The nonuniform nature of the particles and the presence of large amounts of cell debris lends uncertainty as to the exact size and shape of the infectious particle. The virion contains a helical nucleocapsid (diameter 12–15 nm, compared to 18 nm for other paramyxoviruses) packaged in a lipoprotein envelope acquired from the host cell plasma membrane during budding. The genome and antigenome (positive-sense replicative intermediate) are found only in nucleocapsid form. The virion surface has spike-like glycoprotein projections of 11–20 nm spaced at intervals of 6–10 nm. RSV lacks a hemagglutinin and a neuraminidase; the former is present in all members of *Paramyxovirinae* and the latter in all but the morbilliviruses. But PVM has a hemagglutinin, and thus its absence is not a characteristic of the pneumoviruses.

RSV encodes 11 proteins (**Fig. 1, Table 2**). Four are nucleocapsid proteins, N, P, L and M2-1. The N protein binds tightly along the entire length of genomic RNA. L is the major polymerase subunit. P is thought to associate with free N and L to maintain them in soluble form and might also participate as a cofactor in RNA synthesis. N, P and L are necessary and sufficient to direct RNA replication (synthesis of antigenome and genome), whereas transcription involves in addition an elongation factor, the M2-1 protein. Although the polypeptide components of the viral polymerase are packaged in the virion, as is characteristic of the Order, virion-associated polymerase activity has not been directly demonstrated.

There are three transmembrane virion proteins, F, G and SH, which assemble separately into homo-oligomers that make up the membrane spikes; F and G assemble into either trimers or tetramers, and SH might be a pentamer. The F glycoprotein mediates membrane fusion, which is responsible for viral penetration and syncytium formation. It has a cleaved N-terminal signal sequence and a membrane anchor near the C-terminus. F is synthesized as a precursor, F_0, which is cleaved intracellularly by a furin-type cellular protease into the two subunits which remain disulfide-linked and constitute the biologically active form: NH_2-F_2-S-S-F_1-COOH.

The G glycoprotein mediates viral attachment. It is anchored in the membrane by a signal/anchor sequence near the N-terminus such that the large C-terminal domain is extracellular. G contains several *N*-linked carbohydrate side chains and approximately 24 or 25 *O*-linked chains (the ectodomain contains

Table 1 Order *Mononegavirales*, the nonsegmented negative-strand RNA viruses

Family	*Subfamily*	*Genus*	*Example(s)*
Rhabdoviridae		*Vesiculovirus*[a]	Vesicular stomatitis virus
Filoviridae		'Marburg-like viruses'	Marburg virus
		'Ebola-like viruses'	Ebola virus
Paramyxoviridae	*Paramyxovirinae*	*Respirovirus*	Parainfluenza virus types 1 and 3, including Sendai virus
		Rubulavirus	Mumps virus and parainfluenza virus types 2 and 4 including simian virus type 5
		Morbillivirus	Measles virus
	Pneumovirinae	*Pneumovirus*	RSV, animal RSVs, and pneumonia virus of mice (PVM)
		Metapneumovirus	Avian pneumovirus (APV) (formerly turkey rhinotracheitis virus)
Bornaviridae		*Bornavirus*	Borna disease virus

[a] Four other genera of rhabdoviruses exist which are not shown.

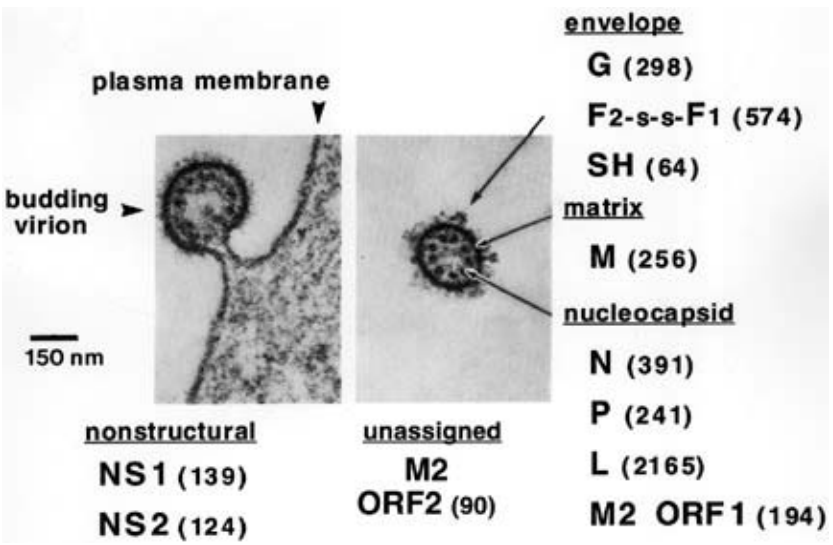


Figure 1 Locations of the RSV proteins, as illustrated on electron micrographs of thin layer sections of (left) a spherical-type RSV virion in the final stage of budding from the plasma membrane of an infected cell, and (right) a free spherical virion. Note the continuity between the envelope of the budding virus and the cell plasma membrane. Virion spikes are visible as a fringe on the exteriors of the virions, and the dense round structures in the virion interior represent cross-sections of the nucleocapsid. The amino acid lengths of the viral proteins are indicated. (The electron micrographs are adapted from Kalica *et al* (1973) *Arch. Gesamte Virusforsch.* 41: 248–258.)

more than 70 potential acceptor sites). It is speculated to have an extended, heavily glycosylated, mucin-like structure. In the middle of the ectodomain there is a predicted disulfide-linked tight turn that coincides with a conserved sequence segment presumed to be a domain important in attachment activity. Remarkably, recombinant RSV lacking the G protein gene can grow in cell culture, implying that an alternative attachment activity exists.

The function of the small SH protein is unknown. Remarkably, recombinant RSV lacking the gene in its entirety is fully viable in cell culture and chimpanzees. SH is anchored in the membrane by a centrally located signal/anchor sequence such that the C-terminal third of the molecule is extracellular. The first two methionine codons in the translational open reading frame (ORF) are alternative translational start sites, and the SH protein accumulates in a variety

Table 2 RSV proteins[a]

Protein	*Function/comments*	*Comments on expression, post-translational modifications*
Nucleocapsid-associated		
N	Binds tightly to genomic and antigenomic RNA	
P	Maintains free N and L proteins in soluble form. Polymerase cofactor	P is the most heavily phosphorylated RSV protein
L	Major polymerase subunit	
M2-1	Transcription elongation factor	Encoded by 5′-proximal ORF of M2 mRNA
Envelope-associated		
G	Transmembrane surface attachment protein. Major neutralization and protective antigen. Antigenic and sequence divergence in G defines the antigenic subgroups. Recombinant RSV lacking the G protein gene can grow in cell culture	Contains several *N*-linked sugar side chains and extensive *O*-linked glycosylation: the M_r of the unglycosylated form is 32.5 kDa and that of the fully-glycosylated form is 90 kDa. Translational initiation at the second methionine in the ORF yields an N-terminally-truncated form which, following additional N-terminal proteolytic trimming, is secreted
F	Transmembrane surface protein. Fusion activity mediates viral penetration and syncytium formation. Major neutralization and protective antigen	Synthesized as an *N*-glycosylated, 70 kDa precursor, F0, which is cleaved by a furin-type intracellular protease into two disulfide-linked subunits, F2 (amino acids 1–130, 19 kDa) and F1 (amino acids 137–574, 50 kDa)
SH	Transmembrane surface protein. Function unknown. Recombinant 'knock-out' virus lacking SH is fully viable	Translational initiation at first and second methionines of the ORF yields full length (SH0) and N-terminally-truncated (SHt) forms. Some of SH0 receives an *N*-linked sugar to form SHg, and some of the SHg is modified by the addition of polylactosaminoglycan to the *N*-sugar to yield SHp
M	Unglycosylated internal protein. Mediates virion assembly	
Nonstructural		
NS1[b]	Putative negative regulatory factor for replication and transcription. Recombinant 'knock-out' virus lacking NS1 is viable in cell culture but exhibits reduced synthesis of progeny virus	
NS2[b]	Recombinant 'knock-out' virus lacking NS2 is viable in cell culture but exhibits delayed synthesis of progeny virus	Unstable, with a half-life of 30 min
Newly-described, unassigned		
M2-2	Putative negative regulatory factor for replication and transcription	Encoded by the second, internal ORF of the M2 mRNA. Much less abundant than the other viral proteins

[a] Proteins known to be virion structural components: N, P, L, M2-1, SH0, SHp, G, F, M.
[b] Although the calculated molecular weight of NS1 (15 567) is greater than that of NS2 (14 674), it typically has a greater electrophoretic mobility.

of forms due to differing degrees of glycosylation. The multiplicity of forms seems to be a conserved feature, but its significance is unknown.

The nonglycosylated matrix M protein is thought to be located on the inner surface of the envelope and to have a central role in organizing the envelope and directing packaging of the nucleocapsid.

NS1 and NS2 are small proteins thought to be nonstructural. NS1 inhibits transcription and RNA replication in a minireplicon system (see below),

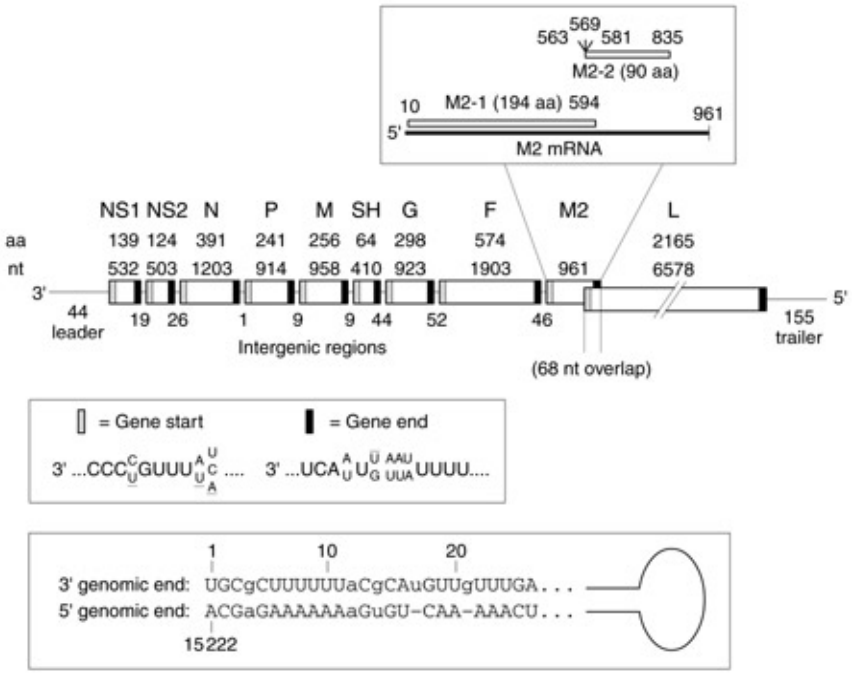


Figure 2 Gene map of RSV strain A2 (not to scale). The 15 222 nt genomic RNA is shown 3′ to 5′. Each box corresponds to an encoded mRNA, with the shaded and filled regions identifying the gene-start and gene-end signals respectively. For the gene-start signal, the first nine genes are identical for the first nine nucleotides of the 10 nt signal, whereas that of the L gene has differences indicated by underlining. The gene-end signal is more variable. For both signals, variable nucleotides (or a gap representing three gene-end sequences which are each one nucleotide shorter) are listed vertically in decreasing order of prevalence. The nucleotide (nt) length of each mRNA (exclusive of nontemplated polyadenylate) is indicated immediately over its box, and the amino acid (aa) length of the encoded protein is indicated above that. An expanded drawing of the M2 mRNA illustrates the two ORFs, shown as open rectangles over the line representing the mRNA, with potential translational start and stop codons numbered according to their positions in the 961 nt M2 mRNA. The extragenic leader, intergenic and trailer regions are drawn as thin lines, with nucleotide lengths shown underneath. The complementarity between the 3′ and 5′ ends is illustrated at the bottom; complementary nucleotides are in capitals and the dashes indicate single-nucleotide gaps introduced to maximize the alignment. These sequences are 81% complementary, after which the degree of relatedness is insignificant.

suggesting that it is a negative regulatory factor. Recombinant RSV lacking the NS1 or NS2 gene is viable in cell culture but replicates less well than wild type. Thus, the NS1 and NS2 genes encode nonessential accessory proteins. The M2 mRNA has a second ORF (**Fig. 2**), encoding the M2-2 protein, a nonabundant species which has been detected in preliminary experiments. Expression of the M2-2 ORF inhibited transcription and RNA replication by an RSV minireplicon, suggesting that M2-2 is a negative regulatory factor.

Genome Organization, Transcription and Replication

The RSV genome is a single, negative strand of RNA that encodes ten mRNAs (**Fig. 2**). Nucleotide lengths for three different RSV genomes sequenced to date are: 15 222 (strain A2 of antigenic subgroup A; see below for a description of the antigenic subgroups), 15 225 (strain B1 of antigenic subgroup B) and 15 133 (BRSV strain A51908). The RSV gene order is conserved within genus *Pneumovirus* but, interestingly, that of APV has two differences: it lacks the NS1 and NS2 genes, and part of its gene order has a different arrangement: 3′-F-M2-SH-G-5′ (**Fig. 3**).

As is the case with the Order in general, RNA replication involves the synthesis of a complete positive-sense replicative intermediate that is called the antigenome and serves as the template for the synthesis of progeny genomic RNA. Genomic RNA contains extragenic 3′ 'leader' and 5′ 'trailer' regions of 44 and 155 nt, respectively (**Fig. 2**). The first 26 nucleotides at the 3′ end of genomic RNA have 81% complementarity with the last 24 nucleotides at the 5′ end (**Fig. 2**). One possibility is that this complementarity allows the two ends to interact. However, an alternative and more likely possibility is that the complementarity reflects sequence conservation at the 3′ ends of the genome and antigenome due to the

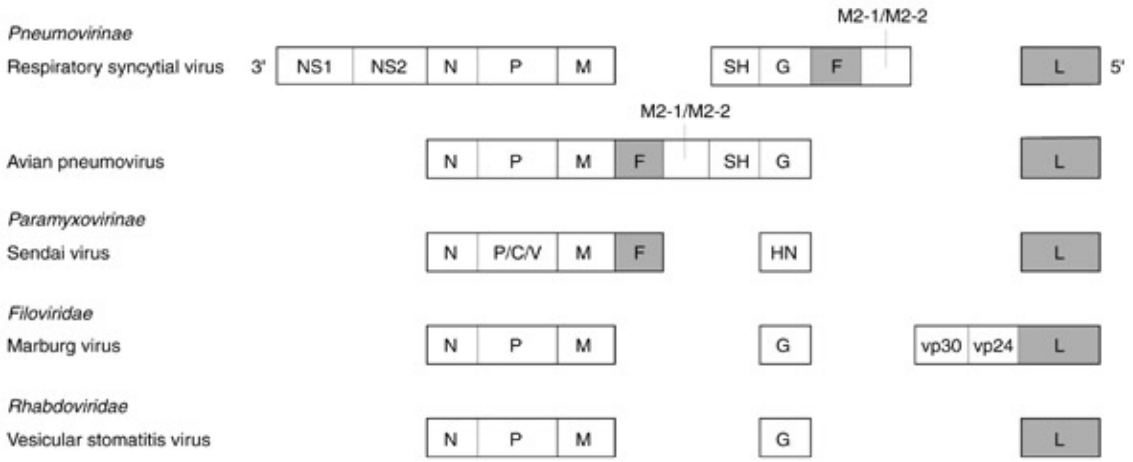


Figure 3 Comparison of the gene maps (not to scale) of the two paramyxovirus subfamilies, *Pneumovirinae* and *Paramyxovirinae*, with those of the filovirus and rhabdovirus families. Each box represents a separate mRNA, with the encoded protein(s) indicated; for mRNAs which contain more than one ORF, the encoded proteins are separated by slashes. The maps were drawn to align analogous proteins vertically when possible. The F and L proteins are shaded to indicate that each exhibits sequence relatedness among the different families. RSV and APV also share sequence relatedness for other proteins, but only F and L have unambiguous relatedness extending beyond the pneumoviruses. The identification of the filovirus P and M proteins is preliminary.

presence of a conserved promoter in each. Also, the conserved 5′ ends might each contain a conserved signal for initiating encapsidation of the nascent RNA. Studies with minireplicons indicate that the RNA signals required for replication are contained in their entirety within the 33–40 nucleotides at the 3′ and 5′ ends of genomic and antigenomic RNA. While certain viruses like Sendai and measles viruses have the remarkable requirement that the genome length be an even multiple of six for efficient replication, there is no such requirement for RSV.

As is characteristic of the Order, RSV transcription initiates at the 3′ genomic promoter and copies the genes by a sequential stop–start mechanism that yields subgenomic mRNAs. Each gene begins with a conserved 10 nt gene-start motif that directs initiation of transcription and encodes the 5′ end of the mRNA, and ends with a 12–13 nt gene-end motif that directs polyadenylation and release of the mRNA (**Fig. 2**). These motifs are self-contained signals that switch the polymerase between transcribing and nontranscribing modes. The first nine RSV genes are separated by intergenic regions, which are of various lengths (1–52 nt for strain A2) and lack apparent conserved sequences. The intergenic regions do not appear to have a significant role in gene expression. In human and animal RSVs, but not in PVM or APV, the last two genes (M2 and L) overlap by 67 (BSRV) or 68 (RSV) nucleotides (**Fig. 2**) and are expressed as separate mRNAs which each contain the overlap sequence. The mechanism for transcribing overlapped genes is not known but does not appear to involve independent internal polymerase entry.

As is typical of this Order, transcription is polar: promoter-proximal genes are transcribed more frequently than downstream ones due to polymerase fall-off. This provides a gradient of transcription thought to be the major factor determining the relative amounts of expression of the various genes. There is no evidence of temporal regulation of gene expression. During transcription, there is a low frequency of readthrough of the gene-end signals, such that 5–10% of the total mRNA consists of readthrough transcripts each representing two or more adjacent genes.

The RSV mRNAs contain a virally-encoded methylated 5′ cap [$m^7G(5')ppp(5')Gp$] and 3′ polyadenylate typical of eucaryotic mRNA, the latter produced by reiterative copying by the viral polymerase on a tract of 4–7 U residues at the downstream side of each gene-end signal. Each mRNA encodes a single major viral protein except for the M2 mRNA, which contains two overlapping ORFs (**Fig. 2**) present in all pneumoviruses. The two ORFs are expressed as separate proteins, although the mechanism for translation of the internal ORF is unknown.

The synthesis of mRNA and antigenome ostensibly occur on the same template, and the factors which regulate the two processes are unknown. The M2-1 elongation factor does not appear to regulate tran-

scription and RNA replication, nor does encapsidation of the nascent RNA by the N protein shift synthesis from that of mRNA to antigenome.

Genetics

Nonsegmented negative-strand viruses in general do not undergo recombination, apart from the polymerase jumping which is involved in the production of short helper-dependent defective-interfering RNA. The differences in the gene orders of RSV and APV (**Fig. 3**) suggest that recombination to yield nondefective infectious genomes can occur, but presumably is very rare.

Negative-sense genomic RNA is not directly infectious alone. However, infectious recombinant RSV can be produced in cultured cells by expression from transfected plasmids of a complete antigenomic RNA and the N, P, L and M2-1 proteins. These components presumably assemble into a nucleocapsid that initiates a productive infection. This method can be used to introduce predetermined changes into infectious virus through the cDNA intermediate.

An alternative genetic system that is very useful for basic studies involves short, internally-truncated, cDNA-encoded genome or antigenome analogues, or minireplicons, in which the viral genes have been replaced with one or more reporter genes under the control of RSV transcription signals. When complemented in *trans* by the appropriate mix of plasmid-encoded RSV proteins, the minireplicons are transcribed, replicated and packaged into virus-like particles which can be passaged to fresh cells. Identification and analysis of the functions of *cis*-acting RNA signals and *trans*-acting proteins using these systems is an active area of research.

Antigens and Antigenic Subgroups

Postinfection human or animal serum contains antibodies to various viral proteins, notably the F, G and N proteins. The F and G proteins are by far the major antigens for neutralizing antibodies, but have markedly different antigenic properties. Many of the available F-specific monoclonal antibodies (MAbs) efficiently neutralize RSV *in vitro,* whereas those for the G protein neutralize weakly or not at all. However, polyclonal antibodies to G neutralize efficiently. The high content of *O*-linked carbohydrate in the G protein is likely to be a factor in its antigenic properties, and indeed the sugars have been shown to be important, directly or indirectly, for the binding of many, but not all, MAbs. Since the sugar side chains are host-specific, they might mask the virus-specified polypeptide chain. Microheterogeneity in the placement of side chains among the many potentially available acceptor sites might result in subpopulations of molecules among which epitopes are variably altered or masked.

RSV is monotypic serologically, with up to four-fold differences between disparate strains in cross-neutralization *in vitro* by postinfection serum. However, binding studies with MAbs showed that RSV isolates can be segregated into two distinct antigenic subgroups designated A and B. Epitopes for F MAbs tend to be conserved, whereas those for G are not, such that antigenic relatedness between the two subgroups is greater than 50% for the F protein, compared with only 5% for the G protein. Comparably distinct antigenic subgroups also have been described for BRSV and APV.

The amino acid sequences of the various RSV proteins are 87% or more identical between the two subgroups, except for the extracellular domains of the SH and G proteins which are only 50% and 43% conserved, respectively. Potential acceptor sites in the G protein for *N*- and *O*-linked sugars also are poorly conserved. These sequence differences provide a structural basis for the observed antigenic dimorphism. The amount of sequence variation between strains within a given subgroup is considerably less, although the G protein is more variable than the others, with as much as 12% amino acid difference. While the presence of antigenic dimorphism in circulating virus is thought to be one mechanism for mitigating host immunity, RSV has not been observed to undergo significant antigenic divergence on the time scale of months or a few years.

RSV infection also generates virus-specific CD4+ helper T cells (Th) and CD8+ cytotoxic T cells (CTLs). Depending on the host and genetic background, various viral proteins have been shown to be T cell antigens. The G protein seems to be a relatively poor antigen for CTLs, perhaps a consequence of its high content of sugar side chains.

Antigenic and Sequence Relatedness of RSV to Other Viruses

Antigenic crossreactivity has been observed between RSV and BRSV for most of the proteins, and between RSV and PVM for the N and P proteins. The amount of sequence divergence between BRSV and RSV is approximately two times greater than between the two RSV subgroups. (For example, the N and F proteins are, respectively, 7% and 19% divergent between RSV and BRSV, compared with 4% and 9% divergence between the RSV subgroups.) As would be expected, the BRSV G protein is the most divergent relative to RSV, being 70% divergent with RSV,

compared with 47% divergence between the G proteins of the RSV subgroups. As an example of the divergence of PVM and APV relative to RSV, the PVM or APV N protein is 40% or 59% divergent, respectively, from its RSV counterpart.

RSV lacks antigenic relatedness with paramyxoviruses outside of its subfamily. The F protein has low but significant sequence relatedness with the F proteins of the other paramyxoviruses. The L protein has low but significant relatedness with L proteins within the Order and contains sequence motifs conserved among a wide range of polymerases and thought to represent catalytic domains. Although the other RSV proteins lack obvious, unambiguous sequence relatedness with their nonpneumovirus counterparts, it is likely that functional similarity (and evolutionary relatedness) exists between these viruses for proteins such as N, P and M (**Fig. 3**).

Virus Infection in Cell Culture and Animals

RSV initiates infection by binding to a cellular receptor(s) which remains to be identified, and the viral envelope fuses with the plasma membrane. Genome expression and replication are entirely cytoplasmic. Progeny virus buds at the plasma membrane in areas of coalesced viral envelope proteins.

RSV can be grown in a variety of cultured cells of human, simian or bovine origin, and can also be adapted to grow in chick cells. The human epidermal HEp-2 or the African green monkey kidney Vero cell lines are the most commonly used. During infection with strain A2 at an input multiplicity of 5, the intracellular production of viral proteins and nucleic acids can be detected by 6–10 h postinfection and reaches maximum at 15–24 h. Virus release begins by 10–16 h and is maximal by 29 h; syncytia become evident by 20–30 h, and extensive cytopathology and destruction of the monolayer occurs at 30–48 h. Host cell macromolecular synthesis does not appear to be inhibited except by the indirect effects of cytopathology. Although most cells infected in culture are killed, persistent infection *in vitro* can be readily established. Much of the progeny virions remain cell-associated and are released by freeze–thawing or sonication. RSV is very labile to inactivation during unfrozen storage or freeze–thawing, although stability can be greatly improved if the harvested culture supernatants are adjusted to pH 7.5 and to contain 0.1 M magnesium sulfate. Virus yield is 10^5 to 5×10^8 PFU ml^{-1} depending on the cell, strain and growth conditions. A yield of 10 PFU per cell is typical, indicating that the production of infectious virus is inefficient indeed. The instability and size heterogeneity of the virion reduces the efficiency of purification and concentration.

The only hosts that are fully permissive for RSV are the human and the chimpanzee. RSV also can replicate in the respiratory tract of several species of monkey as well as in hamsters, guinea pigs, ferrets, mice, and cotton rats. But in these animals the infection is semipermissive: the titer of recoverable virus is 100–1000-fold lower than in the fully-permissive chimpanzee, and disease either does not occur or is greatly reduced in severity.

RSV buds from the apical surface of polarized cultured cells. *In vivo*, RSV infection is generally restricted to the superficial layers of the respiratory tract epithelium; however, it can spread to secondary organs under conditions of immunosuppression or immunodeficiency, indicating that systemic spread is normally restricted by host immunity rather than a viral factor. Infection of monocytes and macrophages has been reported but is of unknown significance. While individuals can shed virus for sustained periods, there is no evidence of persistent infection *in vivo*. RSV inoculated intramuscularly undergoes a single cycle of replication without the production of infectious virus.

Epidemiology and Clinical Factors

RSV is worldwide. It causes yearly epidemics centered in the winter months in temperate climates or in the rainy season in the tropics. It is highly contagious. It is an important cause of nosocomial infection. Spread involves inoculation of conjunctival or mucosal surfaces by hand or aerosolized particles containing respiratory secretions. Essentially everyone is infected by 1–2 years of age, with the greatest incidence of serious disease occurring between 6 weeks and 6 months of age. The relative sparing of newborns is thought to be due to the transient protective effects of maternally-derived serum IgG. The higher incidence of serious disease in young infants probably reflects in part the greater susceptibility of smaller airways to obstruction by edema and secretions. RSV regularly accounts for more than 20% of pediatric hospitalizations due to respiratory tract disease. In developed countries, mortality is very low for normal children. However, infants and children with bronchopulmonary dysplasia, congenital heart disease or immunodeficiency are at special risk for serious, life-threatening RSV disease. In these cases, mortality can be as high as 30%. In the USA, RSV causes an estimated 91 000 hospitalizations and 4500 deaths annually. In developing countries the infant death rate from respiratory disease can exceed 2000 per 100 000 births,

and it is estimated that 20–25% of these would be due to RSV, which can be extrapolated to one million deaths per year worldwide.

During natural infection, RSV has an incubation period of 4–5 days. It causes upper respiratory tract disease, with symptoms of a common cold. Between 25 and 40% of primary infections progress to the lower respiratory tract and cause bronchitis, bronchiolitis or pneumonia. Symptoms include rhinorrhea, middle-ear disease, fever, coughing and wheezing. Seriously ill infants have increased coughing and wheezing, rapid respiration and hypoxemia, requiring the administration of humidified oxygen. The duration of illness is 7–12 days. Virus is shed in large amounts (10^4–10^6 PFU ml^{-1} nasal wash) throughout infection and sometimes during recovery.

RSV infection can be diagnosed rapidly and efficiently by the detection of viral antigens by immunofluorescence of exfoliated cells or enzyme-linked immunosorbent assay (ELISA) of respiratory secretions. Other methods in common use include isolation of the virus in cell culture or detection of an increase in RSV-specific antibodies during convalescence.

RSV is unusual in that it can infect young infants despite the presence of maternally-derived virus-neutralizing serum IgG. This reflects the inefficiency with which serum IgG moves by transudation on to the respiratory tract mucosa and indicates the importance of local immunity in restricting virus replication in the upper respiratory tract. It is not clear why RSV is more infectious under these conditions than are other viruses of the respiratory tract. Another striking feature of RSV is its ability to reinfect repeatedly during childhood and throughout life. Certainly the transient nature of local secretory immunity and the relative ineffectiveness of serum antibodies in accessing the luminal surface of respiratory mucosa are factors in frequent reinfections. But it is not clear why reinfections are so much more frequent with RSV than with other viruses of the respiratory tract. The two antigenic subgroups have been described as alternating in local prevalence from year to year, supporting the idea that the degree of antigenic variation can contribute to reinfection. That the G protein, one of the two protective antigens, might be sheathed in sugar, might exhibit microheterogeneity in side-chain location and structure, and is produced in part as a secreted form, presents other possible mechanisms for mitigating the effectiveness of host immunity. Although the virus can reinfect, serious disease is associated mostly with first or second infection. Sparing during subsequent infections is due to immunological restriction of virus replication in the lower respiratory tract, and indicates that immunoprophylaxis is feasible.

Much of the pathogenesis of RSV is the direct result of destruction of epithelial cells by virus replication and the concomitant edema, mucus secretion and influx of lymphocytes and macrophages. Pathogenesis can probably also be influenced by additional immune factors, such as antibody-mediated or cell-mediated hypersensitivity, which remain to be elucidated. In some individuals, airway reactivity in RSV disease might involve an allergic-type reaction mediated by IgE. The ability of immune factors to profoundly influence RSV pathogenesis is illustrated by the enhancement of RSV disease associated with immunization with a formalin-inactivated RSV vaccine (see below). A long-term reduction in pulmonary function is a common sequel to serious RSV disease, but it is not clear whether this is due to the infection or whether such individuals already had underlying pulmonary deficiencies which predisposed them to serious RSV infection.

Immunity

The major mechanisms for resolving primary infection appear to be innate immune mechanisms, CTLs and antibodies. In naive mice, the reduction in pulmonary RSV replication coincided with the appearance of RSV-specific CD8+ CTLs and was inhibited by prior depletion of that subset. Children deficient in cell-mediated immunity experience more serious disease and have difficulty resolving infection. Studies in calves and humans indicated that the appearance of secretory antibodies is coincident with viral clearance. The late appearance of serum antibodies suggests that they are less important in resolving the primary infection.

In experimental animals immunized with individual RSV proteins, antigens which elicited predominantly either (1) neutralizing antibodies or (2) RSV-specific CTLs could induce resistance to subsequently challenge virus replication. However, the resistance induced by CTL antigens was short-lived compared with that afforded by neutralizing antibodies. This suggests that, while CTL clearly have an important role in resolving infection, the long-term resistance to virus replication that develops from prior infection or immunization is more likely to be mediated by antibodies. The F and G proteins are the major RSV antigens which induce virus-neutralizing antibodies and protection. Secretory IgA antibodies are important mediators of resistance in the upper respiratory tract but are relatively short-lived. Virus-neutralizing serum antibodies alone can restrict virus replication in the lower respiratory tract, an effect due to their transudation, albeit inefficient, on to the respiratory epithelium.

Immunoprophylaxis and Treatment

Because natural RSV infection does not provide complete, long-lasting resistance to reinfection, it seems unlikely that a vaccine will do so. None the less, early immunization could reduce the incidence of serious disease associated with the first one or two infections of life. It is likely that natural infections would not be prevented, but their consequences would be reduced in severity and would have the desirable effect of boosting immunity. Very young infants, who would be the targets for immunization, have been shown to have reduced immune responses to RSV infection owing to immunologic immaturity. Also, maternally-derived serum antibodies can suppress the induction of antibodies and CTLs, although this effect can be abrogated to a considerable extent by direct immunization of the respiratory tract. Recent vaccine trials suggest that very young infants can mount a satisfactory immune response against live attenuated RSV administered intranasally.

Vaccine safety is a major consideration. A vaccine made from formalin-inactivated, concentrated RSV and tested in 1966 failed to prevent natural infection and, paradoxically, primed the vaccinees for an increased frequency and severity of disease upon subsequent natural infection. The lack of protective efficacy was probably due to denaturation of neutralization epitopes, and the disease enhancement appears to be due to an exaggerated Th response, perhaps one imbalanced in favor of a Th2-type response instead of the Th1-biased response typical of natural infection. The elucidation of this phenomenon, and the testing of RSV vaccines in general, is complicated by the semipermissive nature of RSV infection in convenient experimental animals like the mouse.

Live attenuated RSV strains for immunization by intranasal infection are under development and might represent the safest, most effective vaccine. Recombinant vaccinia and adenoviruses which express the F or G glycoproteins have been tested in experimental animals as prototype vaccines but, to date, have not been sufficiently immunogenic. Purified F and G glycoproteins for intramuscular immunization have been produced from cultured mammalian cells infected with RSV or from cultured insect cells infected with recombinant baculoviruses. The noninfectious nature of a subunit vaccine might be especially appropriate for immunization of the very young. But these purified antigens appear to induce antibodies with low neutralizing activity, perhaps due to denaturation during preparation, and there are indications that they, like the formalin-inactivated vaccine, prime for immune-mediated pathology upon subsequent infection.

Ribavirin treatment of hospitalized normal or high-risk infected children is well established, although debate as to its level of efficacy has continued. The administration of aerosolized ribavirin to children who were seriously ill and supported by mechanical respirators is associated with improvement and reduced hospital stay. As another approach, the systemic or topical application of RSV-neutralizing antibodies (either polyclonal serum antibodies or murine MAbs) to infected experimental animals was shown to be efficacious both in immunoprophylaxis and therapy. This concept led to the recent development of a strategy in which a pooled human serum antibody preparation with high RSV-neutralizing activity (RespiGam^{TM}) is administered intravenously to high-risk children as a method of passive immunoprophylaxis. A further improvement will be to increase the specific antiviral activity by replacing the preparation of serum antibodies, of which only a small component is RSV-specific, with humanized or human RSV-specific MAbs. Also, application of antibodies directly to the respiratory tract of infected individuals might be effective in curtailing infection and disease.

See also: **Parainfluenza viruses (*Paramyxoviridae*) – Human; Respiratory viruses; Sendai virus (*Paramyxoviridae*); Defective interfering viruses; Vesicular stomatitus viruses (*Rhabdoviridae*).**

Further Reading

Collins PL, Hill MG, Cristina J and Grosfeld H (1996) Transcription elongation factor of respiratory syncytial virus, a nonsegmented negative strand RNA virus. *Proc. Natl. Acad. Sci. USA* 93:81.

Collins PL, McIntosh KM and Chanock RM (1996) Respiratory syncytial virus. In: Fields BN, Knipe DM, Howley PM et al (eds) *Fields Virology* 3rd edn, p. 1313. New York: Lippincott-Raven.

Hall CB (1994) Prospects for a respiratory syncytial virus vaccine. *Science* 265: 1393.

Murphy BR, Hall SL, Kulkarni AB (1994) An update on approaches to the development of respiratory syncytial virus (RSV) and parainfluenza virus type 3 (PIV3) vaccines. *Virus Res.* 32: 13.

Pringle CR and Easton AJ (1997) Monopartite negative strand RNA genome. *Semin. Virol.* 8: 49.

Wright PF (1997) Respiratory diseases. In: Nathanson N, Ahmed R, Gonzalez-Scarano F *et al* (eds) *Viral Pathogenesis,* p. 703. New York: Raven Press.

RESPIRATORY VIRUSES

David O White and **Lorena E Brown**, Department of Microbiology and Immunology, The University of Melbourne, Parkville, Victoria, Australia

Introduction

The respiratory tract is a major portal of entry for pathogenic organisms; infections at this site are the most common afflictions of humans. Children contract up to half a dozen respiratory illnesses each year, adults perhaps two or three, and most of these are caused by viruses. Though trivial colds and sore throats account for the majority of respiratory disease, their impact on communities is significant; millions of lost working hours and a considerable proportion of all visits to family physicians can be attributed to this type of infection. More serious lower respiratory tract infections tend to occur at the extremes of life, and in those with pre-existing pulmonary conditions. The most important human respiratory viruses are influenza and respiratory syncytial viruses (RSV), the former killing mainly the aged and the latter the very young. Of the estimated 5 million deaths from respiratory infections in children annually worldwide, at least one million are viral in origin.

Altogether, there are over 200 human respiratory viruses, falling mainly within six families: orthomyxoviruses, paramyxoviruses, picornaviruses, coronaviruses, adenoviruses and herpesviruses (see corresponding entries elsewhere in this text). Here we shall confine ourselves to those that enter the body via the respiratory route and cause disease confined largely to the respiratory tract. Many other 'respiratory' viruses become disseminated via the bloodstream to produce a more generalized disease, as is the case with most of the human childhood exanthems such as measles, rubella and varicella, or rinderpest and foot-and-mouth disease in cattle. Yet other viruses, entering by nonrespiratory routes, can reach the lungs via systemic spread, and pneumonia may represent the final lethal event, e.g. in overwhelming infections with herpesviruses or adenoviruses in immunocompromised neonates or patients with the acquired immune deficiency syndrome (AIDS).

Epidemiology

By definition, respiratory viruses are transmitted via the respiratory route. Virions are shed from the respiratory tract of an infected human or animal, particularly during sneezing, coughing, talking or barking. A sneeze generates an aerosol comprising up to a million tiny droplets less than 10 μm in diameter that quickly evaporate to yield droplet nuclei which remain suspended in the air for several minutes. This particulate material containing virions transmits infection following inhalation by someone nearby. Larger droplets (up to 100 μm) contain more virions but fall to ground within seconds. They are a danger to anyone directly in the line of fire. Alternatively, respiratory infections can spread by direct contact, e.g. kissing, or by transfer of nasal or oral secretions via hands to nose or mouth. At the height of a common cold such secretions are particularly copious and readily find their way on to handkerchiefs, towels, toothbrushes, eating utensils, doorknobs and so on, as well as hands.

Enveloped viruses such as orthomyxoviruses, paramyxoviruses and coronaviruses tend to be rather susceptible to inactivation by desiccation or by summer temperatures, but icosahedral viruses such as adenoviruses and picornaviruses are more stable; for example, certain outbreaks of foot-and-mouth disease have been attributed to virus carried in the wind for 100 km.

We associate respiratory infections with cold, wet weather but there is no evidence that the winter incidence of the common cold, or indeed any other respiratory disease, is attributable to cold or wet *per se*. Colds are not common in Arctic or Antarctic explorers, for example. It seems more likely that the striking winter peaks of respiratory disease caused by influenza and RSV (**Fig. 1**) are a reflection of our predilection during that season for avoiding the invigorating outdoor climate and shutting ourselves away in ill-ventilated centrally-heated buildings and vehicles, in close apposition to others of like mind. This hypothesis is supported by the observation that in the tropics, where summer and winter are replaced by 'wet' and 'dry' seasons, respiratory infections are more prevalent during the monsoonal rains when people spend more time indoors, exchanging parasites in crowded, often squalid conditions. An additional factor in the less developed world is the domestic air pollution (smoke) generated by the ever-present fire, lit for cooking and warmth inside poorly ventilated huts. Outbreaks of influenza often occur in boarding

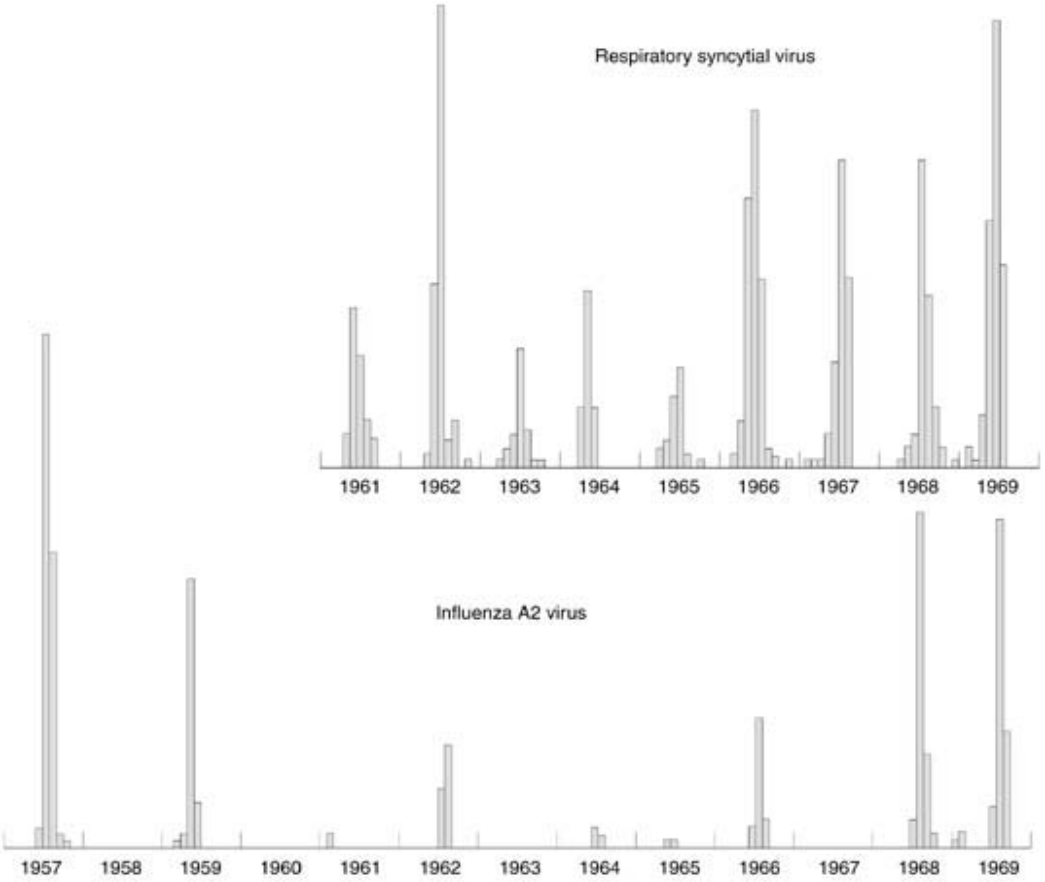


Figure 1 Epidemic occurrence of influenza A and respiratory syncytial viruses. The histograms show the monthly isolations of these two viruses from patients admitted to the Fairfield Hospital for Infectious Diseases, Melbourne, over the periods indicated. Compare the regular winter epidemics of RSV (causing significant disease mainly in infants) with the less regular winter epidemics of influenza. There were major peaks of influenza in 1957 (first appearance of the new human influenza subtype H2N2, known as 'Asian flu') and then again in 1968 (marking the emergence of the novel subtype H3N2, known as 'Hong Kong flu'). (Data courtesy of Drs A. A. Ferris, F. Lewis and I. D. Gust. From Fenner F and White DO (1976) *Medical Virology* 2nd edn. New York: Academic Press.)

schools, army camps, nursing homes, etc.; similarly, nosocomial spread of RSV is common in hospital nurseries. Livestock such as cattle are particularly vulnerable when crowded together in feed-lots or transport vehicles, e.g. shipping fever.

Respiratory viruses spread with great facility and speed, albeit not with the explosive onset that characterizes certain 'common source' outbreaks of enteric viruses when feces contaminate food or water supplies. First, respiratory diseases have a very short incubation period, usually 2–7 days. Second, very large numbers of virions (10^3–10^9 per ml of respiratory secretions) are shed, commencing even before symptoms develop, and peaking around the time the patient is coughing or sneezing with greatest abandon. A single infectious particle may infect a susceptible contact. Typically, a young child picks up the latest virus at school, brings it home and passes it on to the rest of the family and perhaps to the neighbours' children. Within 2 or 3 months up to half the population of a city may have contracted the infection and developed immunity to the virus. As the proportion of uninfected susceptibles in the community falls, the epidemic burns itself out (**Fig. 1**).

Respiratory viruses may evolve quite rapidly in the field. RNA viruses in particular display a very high rate of mutation, because their RNA polymerase is error prone and lacks the error-correcting capability that accompanies DNA replication. Any spontaneously arising mutant that is capable of replicating in the presence of antibody against the wild-type virus will have a growth advantage. Eventually mutants emerge that contain amino acid substitutions in most or all of the immunodominant antigenic domains on the critical surface protein of the virion. Such multiple mutants, no longer neutralizable by wild-type antibody, are designated a new strain and may initiate another epidemic. This phenomenon is known as

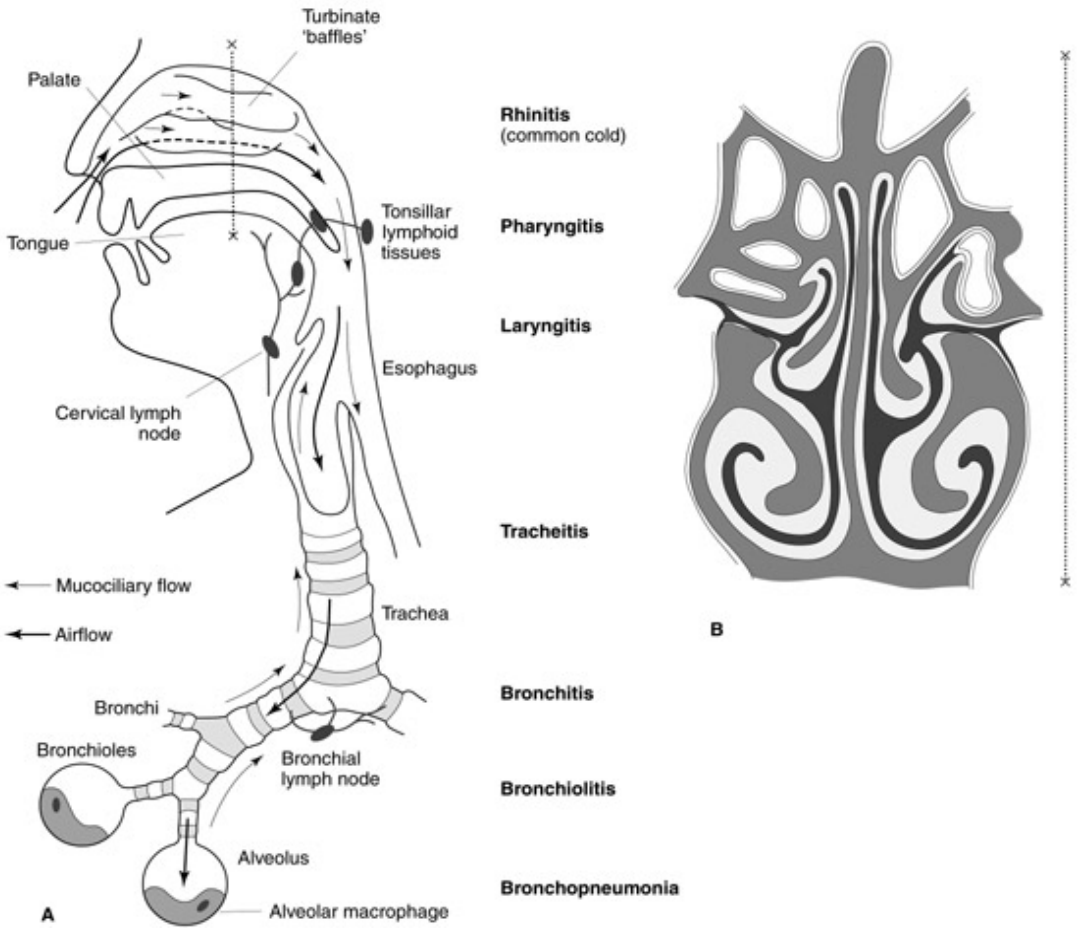


Figure 2 (**A**) Pathways of infection and mechanical protective mechanisms in the respiratory tract. On the right, clinical syndromes produced by infection at various levels of the respiratory tract. (**B**) Section of the turbinates (X—X) magnified 2.5 times, showing the narrow and complicated pathway of inspired air, and thus the ease with which slight swelling 'blocks the nose'. (From White and Fenner (1994), as modified from Mims CA and White DO (1984) *Viral Pathogenesis and Immunology*. Oxford: Blackwell Scientific.)

antigenic drift. While best characterized for influenza, it is presumably the mechanism that has also given rise to the dozens of known serotypes of rhinoviruses, enteroviruses, adenoviruses, and so on. A second, more dramatic type of change, known as antigenic shift, is observed in RNA viruses with segmented genomes that can undergo genetic reassortment. Novel subtypes of human influenza A viruses, capable of initiating pandemics that can infect the majority of the world's population within a year, arise every generation or so as a result of reassortment following co-infection of, say, a pig with a human and an avian influenza virus simultaneously.

Pathogenesis and Immunity

Inhaled droplets of more than 10 μm in diameter are trapped in the turbinates of the nose (**Fig. 2**), whereas those measuring 5–10 μm often reach the trachea and bronchioles. Many of these particles become trapped in the layer of mucus that blankets the ciliated epithelium and are carried by ciliary action to the pharynx, where they are swallowed or coughed out. Smaller particles still can be inhaled directly into the lung and some may reach the alveoli. Here, virus may be phagocytosed and destroyed by alveolar macrophages (although some viral species undergo an abortive cycle of replication and others have developed the capacity to replicate in macrophages). A few virions will succeed in attaching to susceptible epithelial cells via the appropriate ligand–receptor pairing and thereby initiate infection. Progeny virions will be released a few hours later, often by budding from the apical surface of the cell into the lumen of the respiratory tract, and then initiate a second cycle of infection in adjacent or more distant cells. Some of the enveloped species of respiratory viruses are dependent upon a particular cellular protease to

cleave the appropriate viral envelope glycoprotein, e.g. influenza HA, or RSV F protein, otherwise the progeny are noninfectious. Moreover, mucus contains glycoprotein inhibitors which can neutralize the infectivity of certain viruses, e.g. influenza. Mannose-binding lectins such as those in lung surfactant can also neutralize virus. Interferon, synthesized by and secreted from virus-infected cells, binds to interferon receptors on nearby uninfected cells and protects them by inhibiting viral replication.

If specific neutralizing antibodies of the IgA class are already present in the mucus coating the respiratory tract as a result of previous infection or vaccination, they will bind to the corresponding epitopes on the surface of the virion and neutralize its infectivity by blocking attachment or fusion of viral envelope with plasma membrane or endocytic vesicle, thus preventing uncoating of the viral genome. In the absence of pre-existing antibody, however, infection can progress because primary antibody synthesis does not become significant for several days. Additional mechanisms are brought into play to control viral replication during this early period, with the first cells to be mobilized being the natural killer (NK) cells from bone marrow, which become activated by interferon (IFN) to lyse virus-infected cells. Shortly thereafter, the relevant clones of T lymphocytes are activated. Helper T (Th) cells (CD4+) recognize peptides generated from endocytosed virions by proteolysis and presented in the peptide-binding groove of the class II major histocompatability complex (MHC) molecules on the surface of antigen-presenting cells (dendritic cells and macrophages) and are triggered to proliferate and to secrete a range of lymphokines that mediate inflammation by attracting macrophages and other leukocytes to the site, and by upregulating macrophages, B cells and T cells. Cytotoxic T cells (CD8+), on the other hand, see endogenous viral peptides generated by proteolysis of newly synthesized viral proteins and bound to class I molecules on the surface of infected cells.

A major mechanism of recovery from viral infection is lysis of infected cells by activated CD8+ T lymphocytes, preferentially but not exclusively by a perforin/granzyme-mediated process. Children with a congenital T cell deficiency may die from measles or RSV infection; conversely, influenza virus-infected athymic mice may be saved by adoptive transfer of virus-specific CD8+ T cells. CD4+ T cells also contribute to the process of recovery by secretion of cytokines such as IFN-γ and through provision of help to B cells in the developing humoral response. Specific antibodies may act by (1) neutralizing virus, (2) complement-mediated lysis of infected cells, or (3) antibody-dependent cell-mediated cytolysis (ADCC). In addition, IgA, during its active passage through respiratory epithelial cells, may combine with viral proteins produced within these cells, resulting in reduced output of infectious progeny.

Whereas the immune response to respiratory infection is instrumental in recovery, it can also, paradoxically, exacerbate the disease itself. CD4+ Th1 cells may induce such a strong inflammatory response (delayed-type hypersensitivity or DTH) as to cause lethal consolidation of the lung (pneumonia). Furthermore, responses orchestrated by the type 2 cytokines IL-4 and IL-5, namely IgE production and eosinophil degranulation, can precipitate a life-threatening attack of asthma in a young infant infected with RSV. Other factors may also contribute to RSV bronchiolitis: virus infection not only enhances bronchial reactivity to antigen but also destroys the ciliated epithelial cells responsible for mucociliary clearance, thus allowing the infant's narrow bronchioles to become plugged with mucus, inflammatory cells and necrotic cell debris, while bronchoconstriction may also be triggered by vagal nerve reflexes or by release of mediators by inflammatory cells. Blockage of airways causes hypoxia and a pathophysiologic cascade that leads to acidosis and uncontrollable fluid exudation into airways.

Superinfection with bacteria, typically *Streptococcus pneumoniae, Haemophilus influenzae* or *Staphylococcus aureus*, often complicates viral pneumonitis, and without chemotherapy can lead to a fatal outcome. The very young and very old are particularly at risk, as are the immunocompromised, and premature or malnourished infants.

Systemic viral infections such as measles generate a strong memory response and prolonged production of IgG antibodies, which protect against reinfections for life. In contrast, viruses that cause infection localized to the respiratory tract, with little or no viremia, e.g. RSV or rhinoviruses, induce only a relatively short-lived mucosal IgA antibody response, and reinfections with the same or a somewhat different strain can recur repeatedly throughout life. In addition, numerous strains arising by antigenic drift may cause sequential episodes of the same disease in a single individual.

Viral Diseases of the Human Respiratory Tract

While some viruses have a predilection for one particular part of the respiratory tract, most are capable of causing disease at any level and the syndromes to be described below overlap somewhat (**Fig. 3**). Nevertheless, for ease of description we will

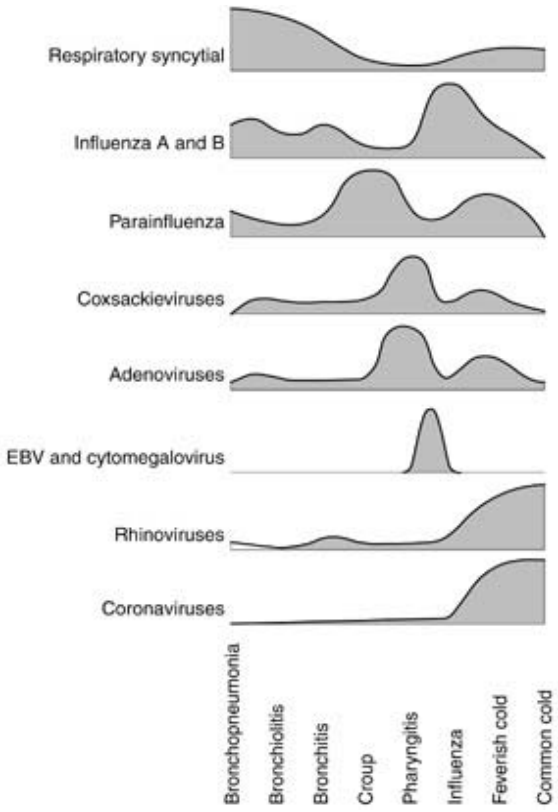


Figure 3 Frequency with which particular viruses produce disease at various levels of the human respiratory tract. (Data courtesy of Dr D. A. J. Tyrrell. From White and Fenner (1994).)

designate six basic diseases of increasing severity as we descend the respiratory tract: rhinitis, pharyngitis, croup, bronchitis, bronchiolitis and pneumonia (Table 1).

Rhinitis (common cold)

The classical common cold (coryza) is marked by copious watery nasal discharge and congestion, sneezing, and perhaps a mild sore throat or cough, but little or no fever. Rhinoviruses are the major cause, several serotypes being prevalent all year round and accounting for about half of all colds. Coronaviruses are responsible for about another 15%, mainly those occurring in the winter months. Certain enteroviruses, particularly coxsackieviruses A21 and A24, and echoviruses 11 and 20 cause febrile colds and sore throats in the summer. In children, RSV, parainfluenza viruses and the lower-numbered adenoviruses are between them responsible for up to half of all upper respiratory tract infections (URTI or URI).

Otitis media or sinusitis sometimes complicate URI. Bacterial superinfection is generally involved, but viruses have also been recovered from the effusion. Respiratory infections with RSV, influenza, parainfluenza, adenovirus or measles viruses predispose to otitis media. Indeed, repeated viral infections can precipitate recurrent middle ear infections, leading to progressive hearing loss.

Pharyngitis

Most pharyngitis is of viral etiology. URI with any of the viruses just described can present as a sore throat, with or without cough, malaise, fever and/or cervical lymphadenopathy. Influenza, parainfluenza and rhinoviruses are common causes throughout life, but other agents are prominent in particular age groups: RSV and adenoviruses in young children; herpesviruses in adolescents and young adults. Adenoviruses, though not major pathogens overall, are estimated to be responsible for about 5% of all respiratory illnesses in young children, often presenting as pharyngoconjunctival fever. Primary infection with herpes simplex virus (HSV), if delayed until adolescence, presents as a pharyngitis and/or tonsillitis rather than as the gingivostomatitis seen principally in younger children; the characteristic vesicles, rupturing to form ulcers, can be confused only with herpangina, a common type of vesicular pharyngitis caused by coxsackie A viruses. Infectious mononucleosis (glandular fever) is usually marked by a very severe pharyngitis, often with a membranous exudate, together with cervical lymphadenopathy and fever; this syndrome is generally caused by Epstein–Barr (EB) virus in 15–25-year-olds, and less commonly by cytomegalovirus.

Laryngotracheobronchitis (croup)

Croup is one of the serious manifestations of parainfluenza and influenza virus infections. A young child presents with fever, cough, inspiratory stridor and respiratory distress, sometimes progressing to complete laryngeal obstruction and cyanosis. Parainfluenza viruses are responsible for about half of all cases, type 1 being more common than type 2. Influenza and RSV are important causes during winter epidemics.

Bronchitis

Influenza, parainfluenza and RSV are the main viral causes of acute bronchitis. There is also evidence that chronic bronchitis, which is particularly common in smokers, may be exacerbated by acute episodes of infection with influenza viruses, rhinoviruses or coronaviruses.

Table 1 Human respiratory viral diseases

	Virus	
Disease	*Common*	*Less common*
Rhinitis (common cold)	Rhinoviruses	RSV, parainfluenza, influenza
	Coronaviruses	Adenoviruses
		Coxsackie A21, 24; echo 11, 20
Pharyngitis	Parainfluenza 1–3	Rhinoviruses
	Influenza	Adenoviruses 1–7
	Herpes simplex	RSV
	EB virus	Cytomegalovirus
	Coxsackie A	
Laryngotracheobronchitis (croup)	Parainfluenza	RSV
	Influenza	
Bronchiolitis	RSV	Influenza
	Parainfluenza 3	
Pneumonia	RSV	Adenoviruses 3, 7
	Parainfluenza 3	Cytomegalovirus
	Influenza	Measles
		Varicella

Bronchiolitis

RSV is the most important respiratory pathogen during the first year or two of life, being responsible, during winter epidemics, for about half of all bronchiolitis in infants. Parainfluenza viruses (especially type 3) and influenza viruses are the other major causes of this syndrome. Breathing becomes rapid and labored, and is accompanied by a persistent cough, expiratory wheezing, cyanosis, a variable amount of atelectasis, and marked emphysema visible by x-ray. The disease can develop with remarkable speed and is one of the causes of sudden infant death syndrome (SIDS), where an infant may die overnight.

Pneumonia

Whereas viruses are relatively uncommon causes of pneumonia in adults, they are very important in young children. RSV and parainfluenza (mainly type 3) are between them responsible for 25% of all pneumonitis in infants in the first year of life. Influenza also causes a considerable number of deaths during epidemic years. Adenoviruses 3 and 7 are less common but can be severe; long-term sequelae such as obliterative bronchiolitis or bronchiectasis may permanently impair lung function. Up to 20% of pneumonitis in infants has been ascribed to perinatal infection with cytomegalovirus (CMV). CMV may also cause potentially lethal pneumonia in immunocompromised patients, as may measles, varicella and adenoviruses. Moreover, viral pneumonia not uncommonly develops in adults with varicella, and in military recruits involved in outbreaks of adenovirus 4 or 7, while measles is often complicated by bacterial pneumonia, especially in malnourished children in Africa and South America. In the elderly, and particularly in those with underlying pulmonary or cardiac conditions, influenza is a major cause of death, either via influenza pneumonitis or more commonly via secondary bacterial pneumonia.

Viral pneumonitis often develops insidiously following URI and the clinical picture may be atypical. The patient is generally febrile, with a cough and a degree of dyspnea, and auscultation may reveal some wheezing or moist rales. Unlike typical bacterial lobar pneumonia with its uniform consolidation, or bronchopneumonia with its streaky consolidation, viral pneumonitis is usually confined to diffuse interstitial lesions. The radiological findings are not striking; they often show little more than an increase in hilar shadows or, at most, scattered areas of consolidation.

Space does not permit a discussion of veterinary diseases, but **Table 2** lists some of the most important respiratory viral diseases of farm and companion animals. Most of these agents are relatively specific to a single animal species, or to closely related species, and do not infect humans. Influenza is occasionally transmitted to humans following close contact with

Table 2 Major respiratory viral diseases of animals

Host	*Disease (virus)*	*Virus family*
Cattle	Infectious bovine rhinotracheitis	*Herpesviridae*
Cattle	Respiratory syncytial virus	*Paramyxoviridae*
Cattle, swine	Foot-and-mouth disease	*Picornaviridae*
Horse	Equine rhinopneumonitis	*Herpesviridae*
Cat	Feline calicivirus	*Caliciviridae*
Cat	Feline rhinotracheitis	*Herpesviridae*
Dog	Canine laryngotracheitis	*Adenoviridae*
Dog	Canine distemper	*Paramyxoviridae*
Chicken	Infectious laryngotracheitis	*Herpesviridae*
Chicken	Avian infectious bronchitis	*Coronaviridae*
Birds	Newcastle disease	*Paramyxoviridae*
Birds, horses, swine	Influenza	*Orthomyxoviridae*

pigs or birds infected with a swine or avian influenza virus respectively, but generally fails to spread beyond the first human case. At the time of writing an avian influenza strain has caused a small number of deaths in Hong Kong residents recently in contact with infected chickens. The greater danger in such a situation is the possibility of genetic reassortment occurring between the avian strain and a human strain already well adapted to human–human transmission. A recently discovered paramyxovirus, known as equine morbillivirus because it was responsible for the death from pneumonia of several racehorses as well as their well-known trainer, now appears to be native to Australian fruit-eating bats.

Laboratory Diagnosis

The etiology of respiratory viral infection can be established in the laboratory by identifying the virus itself, viral antigen or the viral genome. The most appropriate specimen is generally a throat swab or, better still, mucus aspirated from the nasopharynx, taken early in the disease. Because enveloped respiratory viruses with helical nucleocapsids are notoriously labile, the specimen is kept cold and moist, transported promptly to the laboratory and processed as soon as practicable.

Enzyme immunoassay (EIA) is the method of choice for the rapid detection of antigen in respiratory secretions. Diagnostic kits, based on appropriate monoclonal antibodies for antigen-capture and detection respectively, and often incorporating a biotin–avidin readout system, are now available for all common human respiratory viruses and many of the important animal pathogens. EIA is replacing immunofluorescence (application of fluorescein-labeled monoclonal antiviral antibody to infected cells aspirated from the throat). Multiple target (multiplex) polymerase chain reaction (PCR) for the amplification of the viral genome, followed by its identification using an appropriate nucleic acid probe, is fast becoming popular as a diagnostic technique for respiratory infections and in epidemiologic investigations.

Most of the known respiratory viruses can quite readily be cultivated in appropriate cell lines from the corresponding host species, e.g. diploid lung fibroblasts or HUT-292 cells for human viruses. Growth of the virus is detected by cytopathic effects and/or hemadsorption or immunofluorescence, and the virus recovered from the supernatant is then typed using any of a variety of serological techniques. However, this sequence is so time consuming and expensive that isolation and identification of virus is today undertaken mainly by reference laboratories requiring a large supply of the virus for further characterization, for research, or for antigen or vaccine production. Viral culture nevertheless remains a vital first step in the identification of agents responsible for newly emerging diseases.

'Serology', i.e. identification and quantification of antibody in the patient's serum, is also far too slow to be of value in influencing the management of the patient, hence it is used principally in seroepidemiological surveys to assess the immune status of populations. IgM-capture EIA does provide a rapid diagnosis but fills only a limited role in the routine diagnostic laboratory.

Vaccines and Chemotherapy

Very successful live attenuated vaccines are in general use against certain 'respiratory' viruses like measles, mumps and rubella, which, though naturally trans-

mitted via the respiratory route, are absolutely dependent upon viremic spread to their target organs elsewhere in the body. In contrast, it is a much more challenging assignment to develop effective vaccines against viruses whose pathogenicity is essentially confined to the respiratory tract. The major reasons for this are that (1) secretory IgA memory is relatively short-lived, and (2) numerous antigenically distinct strains or serotypes are capable of causing the same clinical syndrome. Thus, a common cold vaccine might need to contain dozens of different serotypes of rhinoviruses (± coronaviruses). An inactivated vaccine is used to protect the aged and other risk groups against the currently prevalent strains of influenza, but its composition must be updated regularly to keep abreast of antigenic drift and shift and, even so, its efficacy in the aged is only of the order of 50–80%. The incorporation into vaccines of mucosal adjuvants, e.g. low-toxicity mutants or individual subunits of cholera toxin and *Escherichia coli* heat-labile toxin, is being examined in the hope of stimulating potent antiviral mucosal IgA responses. Improved antigen-delivery strategies, many of which utilize the size and multivalent antigen presentation properties of the viruses themselves, are also being investigated. Immunostimulatory complexes or 'ISCOMs', which are small adjuvant- and lipid-containing particles into which viral proteins can be inserted, already form the basis for an equine influenza vaccine. Live 'cold-adapted' mutants, for many years licensed as influenza vaccines in Russia, replicate only at the low temperature of the nose and are generally avirulent. Although it has proven difficult to walk the tightrope between genetic stability of avirulence on the one side, and adequate replication (hence immunogenicity) on the other, there is currently considerable interest in re-exploring the potential of such viral mutants for intranasal vaccination in other parts of the world. Another potentially exciting vaccine strategy is the use of recombinant DNA encoding the vaccine antigen, delivered in the form of a plasmid by syringe or 'gene gun'. Such vaccines, encoding the nucleoprotein and hemagglutinin of influenza A virus, have successfully elicited protective responses in animals. Subsequent efforts in this area include methods for enhancing the efficiency of transfection of host cells to increase the feasibility of application in humans.

In the long term another source of hope may lie with antiviral chemotherapeutic agents (see Antivirals), although significant barriers still remaining are (1) the multiplicity of viruses involved and the specific antiviral spectrum of available agents; (2) difficulties with drug delivery, efficacy and toxicity; (3) the fact that high titers of virus may be produced even before the onset of symptoms; and (4) emergence of drug-resistant mutants. Since the discovery of interferon in 1957, the field of antiviral chemotherapy has moved slowly. Although IFN-α is effective when given intranasally shortly before or after virus exposure, its use is not practical because of cost of production, frequency of doses required and problems with local bleeding and discharge. The only agents currently in use against respiratory viruses are (1) amantadine and its methylated derivative rimantadine, which display activity against influenza A if given prophylactically and act by interfering with the ion flux mediated by the M2 channel-forming protein of the virus, thereby inhibiting its uncoating; and (2) ribavirin, a nucleoside analogue, which may be of value in severely ill infants with RSV bronchiolitis/pneumonia when administered as a small-particle aerosol. More recently, thanks to x-ray diffraction and cryoelectron microscopy techniques, sites on viral proteins essential for interaction with the host cell receptor or otherwise critical to the infectious process can be visualized. From this has blossomed the age of computer-aided drug design for the development of antivirals. Among the compounds already in clinical trial are those that block the enzyme active site of influenza neuraminidase, a glycoprotein whose role is to facilitate the release of progeny virions from infected cells and prevent self-aggregation of the virus. Others target a small hydrophobic pocket within the canyon region of the coat proteins of rhinoviruses; these drugs stabilize the coat proteins, thereby preventing uncoating and release of viral RNA for replication. Viral mutants resistant to these drugs can be derived in the laboratory but their significance *in vivo* remains to be established.

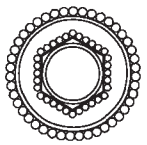
See also: **Adenoviruses (*Adenoviridae*): Animal viruses, General features, Malignant transformation and oncology, Molecular biology; Coronaviruses (*Coronaviridae*); Diagnostic techniques: Detection of viral antigens, nucleic acids and specific antibodies, Isolation and identification by culture and microscopy; Influenza viruses (*Orthomyxoviridae*): General features, Molecular biology, Structure of antigens; Parainfluenza viruses (*Paramyxoviridae*) – Human; Rhinoviruses (*Picornaviridae*); Vaccines and immune response; Interferons: General features, Therapy of aids and cancer; Immune response: Cell mediated immune response, General features; Antivirals.**

Further Reading

Brown LE, Hampson AW and Webster RG (eds) (1996) *Options for the Control of Influenza III*. Elsevier: Amsterdam.

Fenner F, Gibbs EPJ, Murphy FA *et al* (1993). *Veterinary Virology*, 2nd edn. San Diego: Academic Press.
Fields BN, Knipe DM, Howley PM *et al* (eds) (1996) *Fields Virology*, 3rd edn. Philadelphia: Lippincott-Raven.
Mims CA and White DO (1984) *Viral Pathogenesis and Immunology*. Oxford: Blackwell.
White DO and Fenner FJ (1994). *Medical Virology*, 4th edn. San Diego: Academic Press.

RETICULOENDOTHELIOSIS VIRUSES (*RETROVIRIDAE*)



Radmila Hrdlickova, **Jiri Nehyba** and **HR Bose**, Department of Microbiology and Institute for Cellular and Molecular Biology, University of Texas, Austin, Texas, USA

Introduction

The reticuloendotheliosis (RE) viruses constitute a small group of avian retroviruses which are genetically and immunologically unrelated to the avian leukosis-sarcoma viruses (ALSV) complex. Interesting aspects of RE viruses include their evolution, tumorigenicity, and the immunosuppression they induce.

Taxonomy, Classification and Evolution

RE viruses belong to the *Retroviridae*, a large family of RNA viruses that replicate through a DNA intermediate. Seven genera of retroviruses are recognized and the RE viruses belong to the same genus (*Betaretrovirus*) as the murine leukemia viruses (MLV). The RE group includes the acutely transforming reticuloendoendotheliosis virus, strain T (REV-T), its helper virus, reticuloendotheliosis associated virus (REV-A), spleen necrosis virus (SNV), chicken syncytial virus (CSV), duck infectious anemia virus (DIAV), and other isolates from chickens, ducks, geese, pheasants and turkeys. A total of 26 replication-competent RE virus isolates have been biologically cloned. Only one acutely transforming replication-defective member (REV-T) has been identified. Three antigenic subtypes of RE viruses are distinguished on the basis of their reactivity with monoclonal antibodies, but all RE viruses belong to a single interference group. No endogenous RE viruses have been recognized.

Nucleotide sequence analysis has demonstrated that the RE viruses are more related to mammalian retroviruses than to the other avian retroviruses. The *gag* and *pol* sequences of RE viruses are closely related to gibbon ape leukemia virus (GALV), a primate type C retrovirus, whereas, the nucleotide sequences of *env* gene are approximately 50% identical at the amino acid level to those of simian type D retroviruses and two type C retroviruses – feline endogenous virus (RD114) and baboon endogenous virus (BAEV). All of these viruses share a common receptor. However, the *env* sequence of RE viruses also shares 30% identity at the amino acid level with the *env* sequence of GALV. The receptor of GALV appears to be a ubiquitous permease protein. It is likely, therefore, that the receptor for RE and primate type D oncoviruses is also a permease.

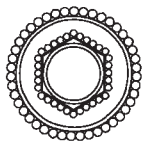
It has been proposed that a common ancestor of BAEV and RE viruses existed and was related to GALV (**Fig. 1**). This ancestor evolved an envelope protein which recognizes a new cellular receptor. From this retrovirus arose the RE virus group as a result of adaptation to avian species. The subgroup D primate viruses evolved by a recombination event between the proposed common ancestor of BAEV and the RE virus which provided the *env* gene and a subgroup B retrovirus. This recombination explains why the subgroup D primate viruses and RE viruses have a similar *env* sequence and share a common receptor.

Virion Structure, Genome and Proteins

RE virus particles have C-type particle morphology resembling MLV more closely than ALV virions. The viral particles are about 100 nm in diameter and are covered with surface projections approximately 6 nm long and 10 nm in diameter. The density of RE virions in sucrose is 1.16–1.18 g ml^{-1}, and RE viruses can be distinguished from those of the ALSV complex by morphology and by density gradient centrifugation. Cell-free stocks of RE viruses may be stored for long periods at −70°C. These viruses are relatively stable

Fenner F, Gibbs EPJ, Murphy FA *et al* (1993). *Veterinary Virology*, 2nd edn. San Diego: Academic Press.
Fields BN, Knipe DM, Howley PM *et al* (eds) (1996) *Fields Virology*, 3rd edn. Philadelphia: Lippincott-Raven.
Mims CA and White DO (1984) *Viral Pathogenesis and Immunology*. Oxford: Blackwell.
White DO and Fenner FJ (1994). *Medical Virology*, 4th edn. San Diego: Academic Press.

RETICULOENDOTHELIOSIS VIRUSES (*RETROVIRIDAE*)



Radmila Hrdlickova, **Jiri Nehyba** and **HR Bose**, Department of Microbiology and Institute for Cellular and Molecular Biology, University of Texas, Austin, Texas, USA

Introduction

The reticuloendotheliosis (RE) viruses constitute a small group of avian retroviruses which are genetically and immunologically unrelated to the avian leukosis-sarcoma viruses (ALSV) complex. Interesting aspects of RE viruses include their evolution, tumorigenicity, and the immunosuppression they induce.

Taxonomy, Classification and Evolution

RE viruses belong to the *Retroviridae*, a large family of RNA viruses that replicate through a DNA intermediate. Seven genera of retroviruses are recognized and the RE viruses belong to the same genus (*Betaretrovirus*) as the murine leukemia viruses (MLV). The RE group includes the acutely transforming reticuloendoendotheliosis virus, strain T (REV-T), its helper virus, reticuloendotheliosis associated virus (REV-A), spleen necrosis virus (SNV), chicken syncytial virus (CSV), duck infectious anemia virus (DIAV), and other isolates from chickens, ducks, geese, pheasants and turkeys. A total of 26 replication-competent RE virus isolates have been biologically cloned. Only one acutely transforming replication-defective member (REV-T) has been identified. Three antigenic subtypes of RE viruses are distinguished on the basis of their reactivity with monoclonal antibodies, but all RE viruses belong to a single interference group. No endogenous RE viruses have been recognized.

Nucleotide sequence analysis has demonstrated that the RE viruses are more related to mammalian retroviruses than to the other avian retroviruses. The *gag* and *pol* sequences of RE viruses are closely related to gibbon ape leukemia virus (GALV), a primate type C retrovirus, whereas, the nucleotide sequences of *env* gene are approximately 50% identical at the amino acid level to those of simian type D retroviruses and two type C retroviruses – feline endogenous virus (RD114) and baboon endogenous virus (BAEV). All of these viruses share a common receptor. However, the *env* sequence of RE viruses also shares 30% identity at the amino acid level with the *env* sequence of GALV. The receptor of GALV appears to be a ubiquitous permease protein. It is likely, therefore, that the receptor for RE and primate type D oncoviruses is also a permease.

It has been proposed that a common ancestor of BAEV and RE viruses existed and was related to GALV (**Fig. 1**). This ancestor evolved an envelope protein which recognizes a new cellular receptor. From this retrovirus arose the RE virus group as a result of adaptation to avian species. The subgroup D primate viruses evolved by a recombination event between the proposed common ancestor of BAEV and the RE virus which provided the *env* gene and a subgroup B retrovirus. This recombination explains why the subgroup D primate viruses and RE viruses have a similar *env* sequence and share a common receptor.

Virion Structure, Genome and Proteins

RE virus particles have C-type particle morphology resembling MLV more closely than ALV virions. The viral particles are about 100 nm in diameter and are covered with surface projections approximately 6 nm long and 10 nm in diameter. The density of RE virions in sucrose is 1.16–1.18 g ml^{-1}, and RE viruses can be distinguished from those of the ALSV complex by morphology and by density gradient centrifugation. Cell-free stocks of RE viruses may be stored for long periods at −70°C. These viruses are relatively stable

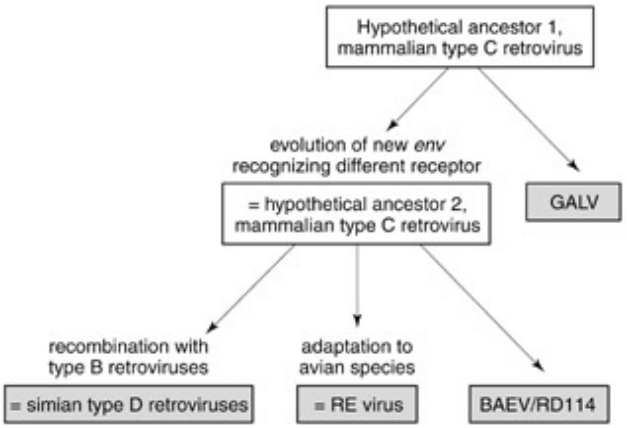


Figure 1 The suggested evolutionary relationship of RE viruses with GALV, BAEV/RD114 and simian type D retroviruses. The shaded boxes indicate currently existing retroviruses. The white boxes indicate hypothetical viral ancestors.

at 4°C but 50% of the infectivity is lost within 20 min when incubated at 37°C.

The genomic RNA of RE viruses consists of a 60–70S complex composed of two identical single-stranded (ss) RNA subunits about 3.9×10^6 Da. The genome of replication-competent RE viruses is approximately 8–9 kilobases (kb) and consists of *gag*, *pol* and *env* genes. The *gag* gene of RE viruses encodes five structural proteins, p10, p12, pp18/pp20 and p30. The *pol* gene encodes a protease (p15), a reverse transcriptase (p84) which is related to the Mn^{2+}-dependent group C mammalian retroviruses and an integrase which facilitates proviral integration (p44). The *env* gene encodes two glycoproteins – a gp90 surface unit and a gp20 transmembrane peptide. REV-T, which is replication-defective, has a genome about 5.7 kb since it contains extensive deletions in the *gag*, *pol* and *env* genes. The transforming gene of REV-T, v-*rel*, has been inserted into *env* sequences.

Replication

Entry of RE viruses is mediated by the envelope glycoproteins which bind to specific receptors on the cell surface. Entry of SNV is pH independent and may involve the direct fusion between the viral envelope and the cell membrane. Shortly after attachment and penetration of the virions, viral DNA is synthesized in the cytoplasm of the infected cell by the reverse transcriptase in uncoated viral cores. Linear as well as circular DNA copies of the retroviral RNA genome are found in the infected cells and the linear DNA serves as the immediate precursor for proviral integration. Two size classes of RNA – genomic-length and spliced subgenomic – are transcribed from the integrated provirus. Translation of genomic-length viral RNA results in the synthesis of two polyprotein precursors: the *gag* and *gag-pol* polyproteins (**Fig. 2**). The *gag* and *pol* genes are in the same reading frame and separated by a stop codon. Expression of the *gag-pol* polyprotein is dependent on stop codon suppression. The *gag* and the *gag-pol* polyproteins are proteolytically cleaved into mature *gag* and *pol* proteins by the virally encoded p15 protease. *Env* proteins are expressed from spliced viral RNA. The primary polyprotein precursor $gPr77^{env}$ is glycosylated and myristoylated and is converted into a second polyprotein precursor $gPr115^{env}$, which is rapidly proteolytically processed by a cellular protease to form gp90 and gPr22(E). A final modification of gp22(E), the transmembrane glycoprotein precursor, to mature gp20 occurs after its incorporation into the virion. The nucleocapsid is assembled in the cytoplasm as a ribonucleoprotein complex and acquires an envelope by budding through the plasma membrane. Release of virus particles begins approximately 24 h after infection.

Hosts and Tissue Tropism

RE viruses have been isolated from chickens, ducks, geese, Japanese quail and turkeys. They can replicate in a wide range of avian cells but specific species or a tissue tropism has not been described. RE viruses are also able to infect certain mammalian cells, including primate cells (HeLa and COS-7). Human cells transfected by SNV produce low levels of infectious virus. Although antibodies against p30 have been detected in human tissue, the health significance of these findings remains to be evaluated.

Immune Response

RE viruses are generally strongly immunosuppressive and the immunosuppression they induce may contribute to their virulence (see below, *Nonneoplastic diseases*). However, most birds are able to develop a strong immune response which efficiently controls the infection.

Humoral immunity

Birds infected at hatching or later develop a 'non-tolerant' infection characterized by a transient viremia and antibody synthesis. Some birds develop a persistent virus infection which can be detected in peripheral blood lymphocytes in the absence of a viremia. Infection of embryos with RE viruses results in a 'tolerant' infection characterized by the lack of antibody production and by a viremia that can persist for 2 years. Also, neonatal bursectomy leads to the

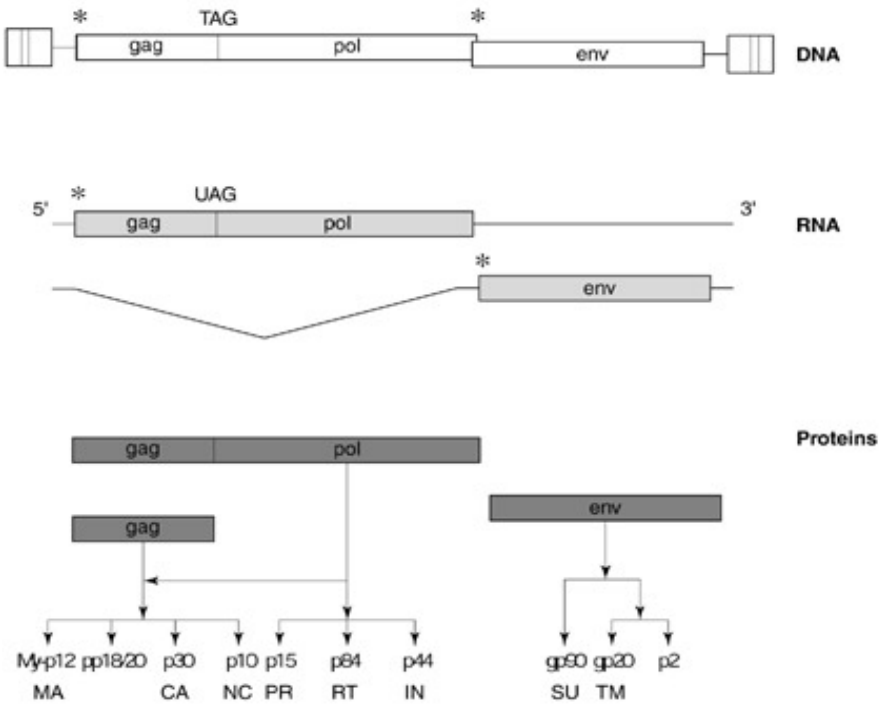


Figure 2 Genome structure, RNA splicing pattern and polyprotein processing pattern of RE viruses. White boxes indicate the genomic regions encoding proteins. Lightly colored boxes represent coding regions in genomic and spliced RNA and dark boxes represent translated proteins. Asterisks indicate the ATG/AUG codons known to be used in initiation of translation. The *gag* stop codon TAG/UAG is shown. The polyproteins and mature processed proteins derived from the *gag, pol* and *env* genes of RE viruses are shown. Proteins are designated according to molecular weight (kDa). The following designations are used: My-p, myristoylated protein; p, protein; pp, phosphoprotein, gp, glycoprotein; MA, *gag* matrix protein; CA, *gag* capsid protein; NC, *gag* nucleocapsid protein; PR, protease; RT, reverse transcriptase; IN, integrase; SU, *env* surface unit; TM, *env,* transmembrane protein. pp18/20 proteins are two differentially post-translation modified proteins encoded by the same *gag* sequence.

establishment of a tolerant infection, which suggests that B lymphocytes are involved in the elimination of the viremia. Tolerance to RE viruses does not depend on immunodepression. In contrast to nontolerantly infected chicken, tolerantly infected dams transmit the virus to their progeny but at low efficiency.

Cell-mediated immunity

Thymectomy increases the mortality of REV-T-infected birds suggesting the involvement of cell-mediated immunity in the elimination of RE virus-infected cells. Cytotoxic T lymphocytes (CTL) lyse virus-infected cells which present viral antigens in conjunction with the major histocompatibility complex (MHC) class I molecules. CTL are induced 7 days after infection with RE viruses and may persist at least 21 days. The CTL response against RE viruses is mediated by $\gamma\delta$ T CD8+, CD4− cells which express MHC class I and II molecules. RE viruses do not appear to activate natural killer cells.

Transmission, Diagnosis, Prevention and Control

Despite the fact that RE viruses are widespread in commercial poultry flocks, they do not create a real economical problem. However, the contamination of vaccines against Marek's disease virus (MDV) by RE viruses has been associated with a runting disease and chronic neoplasia. In addition, occasional outbreaks of neoplastic diseases in turkeys have also been described.

Both vertical and horizontal routes of transmission have been observed but at low levels in the viremic birds. Vertical infections may occur when viruses released from the mother contaminate the egg resulting in the infection of the embryo. Alternatively, infectious virus may be transmitted to the offspring from the semen of a viremic male. RE viruses are transmitted horizontally by contact and may also be transmitted by insects.

RE virus epidemics are prevented by testing commercial vaccines for contamination by these

viruses. RE viruses can be detected by the presence of the viral genome, viral antigens, reverse transcriptase or the induction of a cytopathic effect by some strains. The sensitive polymerase chain reaction (PCR) and reverse transcriptase PCR can be used for detection of viral genomes. RE viruses specific antigens may be detected by immunofluorescence, complement fixation assays or enzyme immunoassays. Antibodies to RE viruses may be monitored by immunofluorescence, virus neutralization, agar-precipitation, Western blot analysis, enzyme immunoassays and pseudotype neutralization tests.

A commercial vaccine is not available since RE viruses do not create a significant economic impact. It is, however, feasible to develop an effective vaccine against RE viruses. Fowlpox virus recombinants expressing the *env* gene of SNV induce neutralizing antibodies and reduce the viremia and runting disease in infected chickens.

Pathogenesis

Nonneoplastic diseases

All replication-competent RE viruses induce to some degree a rapid and severe immunosuppression, in which T cells fail to undergo a blastogenic response to antigens or mitogens. This immunosuppression probably facilitates expression of the oncogenic potential of the virus and is responsible for the increased susceptibility of RE virus-infected birds to other pathogens. Other nonneoplastic disease syndromes include a runting disease syndrome, enlarged peripheral nerves, abnormal feather development, bursal and thymic atrophy and anemia. Several of these syndromes may be the direct or indirect consequence of the immunosuppression. These syndromes develop during the first month after infection and may either be transient or result in death.

The induction of nonneoplastic diseases by RE viruses correlates with their ability to induce a cytopathic response in cultured cells. The replication-competent RE viruses induce an acute infection characterized by an extensive cytopathic effect and cell killing. Large amounts of unintegrated and integrated proviral DNA accumulate in the infected cell during this acute phase. Surviving cells which replicate and become persistently infected contain much lower levels of both unintegrated and integrated proviral DNA. The correlation between the transient accumulation of large numbers of proviral DNA and the transient development of a cytopathic effect suggests that cell death results from the toxic effect of this DNA or proteins coded by this DNA.

The primary determinants of the runting disease syndrome have been mapped to distinct regions in the structural genes of RE viruses by comparing highly pathogenic REV-A with the less pathogenic CSV. Both *env* and *gag* sequences were necessary for the full expression of the pathogenic effect of REV-A. The *env* genes of REV-A and CSV encode the immunosuppressive peptide (ISP) which is conserved in a number of mammalian retroviruses including human T-cell leukemia virus (HTLV) I and II, feline leukemia virus, MLV and GALV. The murine env protein which contains the ISP, suppresses the proliferation of an interleukin-2-dependent cytotoxic T lymphocytes cell line, the mixed lymphocyte response of T and B cells, the respiratory burst of monocytes, immunoglobulin production, the cytolytic activity of natural killer cells, monocyte-mediated tumor cell killing and the production of γ-interferon by peripheral blood lymphocytes. Interestingly, ISP is an inhibitor of protein kinase C and this may explain its profound suppressive effect on immune cells. However, the sequence of ISP is conserved in both CSV and REV-A and, therefore, the other regions of the *env* genes of REV-A and CSV must be responsible for the difference in pathogenicity of these viruses. These *env* sequences may influence how the ISP immunosuppressive region is exposed on the virion surface accounting for the difference in pathogenicity between REV-A and CSV. The *gag* sequences may directly influence the replication efficiency of the virus. The matrix region of *gag* is responsible for the increased replication efficiency of REV-A in some cell types relative to other replication-competent RE viruses. Therefore, the cytopathogenicity and immunosuppressive ability of RE virus is most likely determined by *env*. By increasing replication ability, the *gag* sequence of REV-A also enhances the virulence of the virus.

Syncytia formation has also been observed in certain cell lines infected by RE viruses. The viral envelope glycoproteins expressed on the surface of the infected cell may interact with cellular receptors of neighboring cells, resulting in fusion and syncytia formation. This phenomenon may contribute to the pathology induced by RE viruses.

Chronic neoplasia

Two types of chronic neoplasia induced by replication-competent RE viruses have been described. In the first type, RE viruses induce B cell lymphomas after a relatively long latent period (17–35 weeks). Tumor induction may be prevented by surgical or chemical bursectomy. This type of disease is indistinguishable from lymphoid leukosis induced by ALV. As is the

case with ALVs, RE proviruses are integrated upstream from the second exon (the first exon is a noncoding exon) of the c-*myc* locus in the same transcriptional orientation in these tumor cells. Invariably these RE proviruses have deleted the 5′ LTR and/or large parts of the viral genome. When the 5′ LTR is lacking, the 3′ LTR becomes transcriptionally active leading to constitutive expression of c-*myc* RNA.

The second type of neoplasia induced by replication-competent RE viruses is a T cell lymphoma which develops after a shorter latent period (6 weeks) and does not involve the bursa. This lymphoma involves the thymus, but also the liver, spleen, heart and peripheral nerves. Induction of this T cell lymphoma is also associated with insertional activation of c-*myc*, but one half of the proviruses are oriented in the opposite transcriptional direction from the c-*myc* gene. Whereas some of the proviruses in these T cell lymphomas use the 3′ LTR promoter to transcribe the downstream c-*myc* gene, others apparently activated a cryptic promoter located in the first intron of c-*myc*. This insertion pattern contrasts with that observed in B cell lymphomas induced by the same virus. The proviral integration pattern in these T cell lymphomas induce lower levels of c-*myc* and it has been suggested that T cell lymphomas can be induced at lower levels of c-*myc* than the B cell lymphomas.

Interaction with herpesvirus

Recently, the recombination of RE viruses with Marek's disease virus (MDV) an avian herpesvirus, has been described. This interaction represents the first example of genetic recombination between an RNA and a DNA virus. This phenomenon has important biological consequences for the transmission of RE viruses into cells which would otherwise not be susceptible to RE virus infection. The presence of RE viral sequences in MDV also alters MDV gene expression and pathogenesis. Co-infection of avian cells with MDV and RE viruses frequently leads to the integration of RE proviral sequences within the MDV genome. Although usually only the LTR sequences become stably integrated into the MDV genome, a complete infectious RE provirus has been detected in one case.

REV-T induced neoplasia

REV-T was initially isolated from a lymphoma of a turkey in 1958. Subsequently, it was discovered that the original isolate contains both REV-T which is a replication-defective virus encoding the v-*rel* oncogene and a replication-competent helper virus REV-A. In experimentally infected avian species REV-T induces tumors in the liver, spleen and other visceral organs and causes death within 2 weeks. REV-T transforms a number of cell types at different stages of differentiation including B and T cells, macrophage-like cells and dendritic cells and birds die from a lymphoproliferative disease. REV-T can transform these cell types *in vitro* and also morphologically transforms avian fibroblasts. REV-T-transformed fibroblasts have a distinct morphology, prolonged life-span and induce sarcomas in susceptible birds. REV-T(REV-A) can infect mammalian cells but fails to transform them. Transgenic mice which constitutively express the v-*rel* gene under the control of the *lck* promoter in T cells develop thymic lymphomas in approximately 10 months. However, these murine T cell tumors grow poorly in culture and they do not induce tumors in syngenic mice.

The role of v-*rel* mutations in transformation REV-T induces an acute neoplastic disease due to the expression of the v-*rel* oncogene. v-*rel* arose as a result of the transduction of the c-*rel* proto-oncogene into envelope sequences of REV-A. c-Rel is a member of the Rel/NF-κB family of transcription factors, a ubiquitously expressed group of proteins which participate in the control of cell proliferation, differentiation and apoptosis. The transduction of c-*rel* resulted in the deletion of 2 N-terminal and 118 C-terminal amino acids. Since c-*rel* was transduced into envelope sequences, translation of the v-*rel* subgenomic RNA results in the formation of an oncogene fusion protein with 11 amino acids of envelope protein at the N-terminus and 18 out-of-frame envelope sequences at the C-terminus. Two critical functions of c-Rel were lost or modified during the initial transduction event. The loss of the C-terminal amino acids of c-Rel removed sequences responsible for cytoplasmic anchoring allowing v-Rel increased nuclear access. The C-terminal deletion also removed a significant portion of the sequences involved in transactivation. v-Rel, therefore, activates transcription of some genes less efficiently than c-Rel. One of the genes immediately activated when Rel/NF-κB complexes translocate to the nucleus is IκB-α. IκB-α inhibits the ability of Rel/NF-κB complexes to regulate target genes through cytoplasmic retention and displacement of Rel/NF-κB complexes from DNA. v-Rel, because it has deleted a portion of its transactivation domain induces the transcription of IκB-α much more weakly and with reduced kinetics relative to c-Rel. Decreased IκB-α synthesis may lead to the constitutive activation of v-Rel in transformed cells.

Following the initial transduction event, v-*rel* sustained multiple mutations many of which altered the amino acid sequence of v-Rel and contribute directly to its oncogenicity. These mutations are

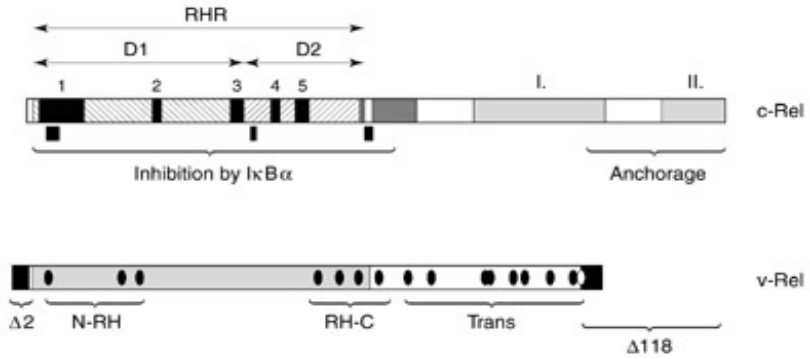


Figure 3 The functional domains of c-Rel and the position of mutations in v-Rel. The Rel homology region (RHR) consists of two domains D1 and D2. The five loops in the RHR that contact DNA directly are indicated by black boxes and numbered above. Loop 1 contains the major DNA recognition motif RxxRxRxxC. A hinge-like loop 3 connects domains D1 and D2. The region flanking D2 on the C-terminal side (white diagonals on black background) contains the nuclear localization signal indicated by the white box. The region of c-Rel that is both necessary and sufficient for inhibition of DNA binding of c-Rel by IκB-α is shown (Inhibition by IκBα). Small black boxes identify the amino acids believed to be in contact with IκB-α. Two transactivation domains (light gray boxes) and a cytoplasmic anchorage region (Anchorage) are present in the C-terminus. The RHR of v-Rel is represented by an intermediate color pattern. Mutated amino acids in v-Rel are indicated. Deletions of 2 N-terminal and 118 C-terminal amino acids resulting from the transduction of c-*rel* into REV-A are indicated below (Δ2, Δ118). The black boxes represent amino acids encoded by *env*-derived sequences which are fused to the truncated N- or C-termini. Internal mutations are identified by black or white ellipses. These mutated amino acids can be divided into three groups: N-RH, RH-C and Trans mutation clusters. The groups contain mutations that may affect a similar structural/functional domain. The N-RH and RH-C mutation clusters are situated at N- and C-termini of the RHR and contain, respectively, three and four single amino acid substitutions. A group of mutations designated Trans contains six single amino acid substitutions and three internal deletions of one or two amino acids. The majority of the Trans mutations are situated in the transactivation domain. (Adapted from Nehyba J, Hrdlickova R and Bose HR, Jr (1997) Differences in κB DNA-binding properties of v-Rel and c-Rel are the result of oncogenic mutations in three distinct functional regions of the Rel protein. *Oncogene* 14, 2881.)

located in several functional regions of v-Rel (**Fig. 3**). Two clusters of mutations are located in the N-terminus (N-RH) and C-terminus (RH-C) of the Rel homology region (RHR). The RHR is conserved among all members of the Rel/NF-κB family of transcription factors. This region contains the sequence responsible for DNA binding, dimerization, nuclear localization and IκB-α association. A third cluster of mutations (Trans) maps to the sequences in the C-terminus of v-Rel with the majority of them present in the transactivation region. The mutated amino acids in v-Rel are located in regions normally involved in the regulation of c-Rel. The functions of c-Rel including DNA-binding activity, the ability to form dimeric complexes as well as transcriptional activity are required for v-Rel to be transforming. The conversion of c-Rel into a highly oncogenic protein is due, therefore, to alterations which modify rather than eliminate the functional domains of c-Rel which are responsible for interacting with other proteins and/or DNA. The amino acid substitutions in the N-terminal part of RHR inside of or near the DNA contact motif are responsible for the altered DNA-binding specificity of v-Rel. Amino acid changes in the C-terminal portion of RHR, in the region responsible for dimerization, enables v-Rel to escape IκB-α-mediated inhibition of DNA binding. Finally, alteration in the amino acid sequence in the extreme C-terminal region of v-Rel may be responsible for altered transcriptional activity or may modulate the DNA-binding activity of v-Rel. These changes created an oncogenic protein which is no longer responsive to normal cellular regulatory constraints.

Altered gene expression in v-*rel* transformed cells Disruption of the ability of v-Rel to bind DNA abolishes its oncogenicity indicating that v-Rel must bind κB sites and directly alter gene expression in order to induce transformation. Although the role of some v-Rel mutations in the activation of the transformation potential of c-*rel* are known, how altered regulation of gene expression by v-Rel results in transformation remains largely unknown. Numerous genes have been identified in which the expression pattern is altered in cells transformed by REV-T. To date, all the genes which display an altered pattern of transcription in REV-T transformed cells are regulated by the Rel/NF-κB family members. These genes are listed in **Table 1**.

A few of the genes altered by v-*rel* expression are known to contribute to the transformed phenotype of v-Rel infected cells. The expression of the gene

Table 1 Genes induced by v-Rel. The genes are grouped into classes with similar function of their protein products. All genes were detected in avian cells unless indicated otherwise

Functional groups	*Genes induced by v-Rel*
Rel/NF-κB family	*nfkb1, nfkb2, Ikba*
Cell surface receptors	The MHC genes class I and II, DM-GRASP[a], the interleukin-2 receptor, p75 membrane protein, *sca-2*
Cytokines	*mip-1β*, *ctca*, CEF-4 (avian homologue of interleukin-8), interleukin-6[b], tumor necrosis factor-α[b]
Transcription factors	c-*fos*, c-*jun*, *fra*-2, interferon response factor 1 and 3, interferon consensus sequence-binding protein, the high-mobility-group protein 14b
Inhibitors of apoptosis	ch-IAP1
Structural proteins	δ-crystallin, vimentin, type I collagen α, β-tubulin
Proteins of oxidative metabolism	Mitochondrial cytochrome *b*
Chaperons	GRP-78

[a] Upregulated in T cell lymphomas in transgenic mice expressing v-Rel under the control of the *lck* promoter and in avian v-Rel transformed cells.
[b] Upregulated in T cell lymphomas in transgenic mice expressing v-Rel under the control of the *lck* promoter, not tested in avian v-Rel transformed cells.

encoding the cell surface receptor DM-GRASP and the cytokine *mip-1β* are altered in v-Rel transformed cells. Antibody against the DM-GRASP inhibits the *in vitro* proliferation of v-*rel*-transformed cells. The overexpression of *mip-1β* induces limited colony formation in soft agar and prolongs the life span of avian fibroblasts. Activation of an inhibitor of apoptosis, ch-IAP1, contributes to the immortalization of lymphocytes transformed by v-Rel. Expression of exogenous ch-IAP1 in temperature-sensitive v-Rel mutant transformed lymphocytes inhibits apoptosis of these cells at nonpermissive temperature. The fos/jun family of transcription factors also plays an important role in the Rel transformation pathway. v-Rel binds to the c-*jun* promoter inducing the constitutive expression of c-*jun* in transformed cells. Sup*jun*, a dominant inhibitor of c-*jun* significantly reduces the transformation efficiency by oncogenic Rel proteins.

v-Rel directly up- or downregulates genes which are normally under the control of c-Rel, but with different efficiencies. For example, the c-*jun* promoter is transactivated by v-Rel more efficiently than by c-Rel. The *Ikba* promoter is transcriptionally upregulated by both v-Rel and c-Rel, however, v-Rel is a much weaker inducer than c-Rel. To date, no genes have been identified which are activated by c-Rel but inhibited by v-Rel or the reverse. v-Rel, therefore, functions as constitutively activated c-Rel but modulates gene expression in a qualitatively different way than c-Rel. The differences in the DNA-binding specificity of v-Rel versus c-Rel may be partly responsible for this behavior. This suggestion is in agreement with the fact that v-Rel binds to the c-*jun* κB site with a higher affinity than c-Rel.

Retroviral Vectors

REV-A and SNV-based retroviral vectors have been successfully used to study retrovirus replication, recombination, cell transformation and to establish transgenic chickens. Moreover, several properties of RE viruses make them candidates for human gene therapy. The use of retroviral vectors for human therapy must address potential safety concerns. It is desirable to avoid homologous recombination which could reconstitute a replication-competent virus. Therefore, RE viruses are produced from packaging cell lines which express genes encoding structural retroviral proteins under the control of nonretroviral promoters. After transfection by the plasmid form of the retroviral vector which encodes only the desired therapeutic protein controlled by its own promoter, the viral RNA is packaged into virions. Unlike other retroviral vectors, RE vectors have no homology with structural genes coded by the packaging cell lines, therefore, replication-competent viruses are not produced. Also, it is essential that the retroviral vectors used in gene therapy are unable to undergo homologous recombination with endogenous viruses in target cells. Human cells do not contain endogenous RE virus sequences. The integration of the strong retrovirus LTR promoter/enhancer element into the DNA of target cells which could influence the transcription of neighboring genes must also be avoided. The construction of SNV-derived self-

inactivating vectors which produce proviruses lacking LTRs addresses this safety concern.

The delivery of retrovirus vectors into specific cell types is also required for human therapy. The envelope protein of RE viruses contains multiple binding domains and, therefore, can tolerate the extensive modification associated with the introduction of cell specific targeting sequences. Retroviruses that recognize specific target cells have been generated by the incorporation of sequences encoding antigen binding sites into the *env* gene of SNV. These targeted SNV-derived vectors are very efficient at infecting specific human cell types.

See also: **Avian type C retroviruses (*Retroviridae*); Gibbon ape leukemia virus (*Retroviridae*); Murine leukemia viruses (*Retroviridae*); Retroviral Oncogenes; Recombination of viruses; Retroviruses – type D (*Retroviridae*); Vectors: Animal viruses.**

Further Reading

Coffin JM (1996) Retroviridae and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology,* 3nd edn, p. 1767. New York: Lippincott-Raven.

Dornburg R (1995) Reticuloendotheliosis viruses and derived vectors. *Gene Ther.* 2: 301.

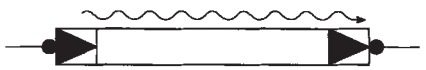
Gilmore TD (1995) Malignant transformation of cells by the v-Rel oncoprotein. In: Cooper GM, Temin RG and Sugden B (eds) *The DNA Provirus: Howard Temin's Scientific Legacy,* p. 105. Washington, DC: American Society for Microbiology.

Johnson ES (1994) Poultry oncogenic retroviruses and humans. *Cancer Detect. Prev.* 18: 9.

Payne LN (1992) Biology of avian retroviruses. In: Levy JA (ed.) *The Retroviridae,* vol. 1, p. 299. New York: Plenum.

Witter RL (1991) Neoplastic diseases/reticuloendotheliosis. In: Calnek BW, Barnes HJ, Beard CW, Reid WM and Joder HW, Jr (eds) *Diseases of Poultry,* 9th edn, p. 439. Ames: Iowa State University Press.

RETROTRANSPOSONS OF FUNGI



Jef D Boeke, Department of Molecular Biology and Genetics, Johns Hopkins University, Baltimore, Maryland, USA

History

Transposable elements in eucaryotes can nearly all be classified into three basic types. The first type, typified by the Ac elements of plants and the P elements of *Drosophila,* resemble bacterial transposons in that they bear short inverted repeat termini; the available evidence strongly suggests that this type of element transposes directly via a DNA intermediate. However, most eucaryotic transposons differ from the bacterial elements in that they encode a reverse transcriptase (RT) or RT-like protein. Two basic types of these 'retrotransposons' are known – the LTR (long terminal repeat)-containing type, which are structurally highly reminiscent of retroviruses, and the poly(A)-type, which lack LTRs and usually (but not always) contain an oligo(A), poly(A) or similar sequence tract at their extreme 3′ end. These two types of retrotransposon are shown in **Fig. 1.** Both classes of retrotransposons are now known from organisms as phylogenetically distinct as fungi, trypanosomes, insects and mammals. Hence this brief review will focus on what is currently known about fungal retrotransposons, principally the retrotransposons that are more retroviral-like and are distinguished by the presence of LTRs. Thus, only a small and highly selective glimpse of the total picture of retrotransposons is provided.

Structural Features

The structural features of the known fungal retrotransposons are summarized in **Table 1.** LTR-containing retrotransposons isolated from fungi resemble retroviral proviruses in structure. They contain LTR sequences of a few hundred base pairs long flanking a central coding region that contains one or two open reading frames (ORFs), called *gag* and *pol* by analogy to the retroviral counterparts. As is the case with proviruses and DNA-based transposons, target site duplications of a fixed length flank the elements. These vary greatly in sequence and are presumably generated during the integration process

inactivating vectors which produce proviruses lacking LTRs addresses this safety concern.

The delivery of retrovirus vectors into specific cell types is also required for human therapy. The envelope protein of RE viruses contains multiple binding domains and, therefore, can tolerate the extensive modification associated with the introduction of cell specific targeting sequences. Retroviruses that recognize specific target cells have been generated by the incorporation of sequences encoding antigen binding sites into the *env* gene of SNV. These targeted SNV-derived vectors are very efficient at infecting specific human cell types.

See also: **Avian type C retroviruses (*Retroviridae*); Gibbon ape leukemia virus (*Retroviridae*); Murine leukemia viruses (*Retroviridae*); Retroviral Oncogenes; Recombination of viruses; Retroviruses – type D (*Retroviridae*); Vectors: Animal viruses.**

Further Reading

Coffin JM (1996) Retroviridae and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology,* 3nd edn, p. 1767. New York: Lippincott-Raven.

Dornburg R (1995) Reticuloendotheliosis viruses and derived vectors. *Gene Ther.* 2: 301.

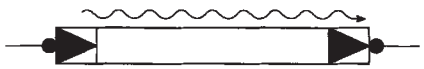
Gilmore TD (1995) Malignant transformation of cells by the v-Rel oncoprotein. In: Cooper GM, Temin RG and Sugden B (eds) *The DNA Provirus: Howard Temin's Scientific Legacy,* p. 105. Washington, DC: American Society for Microbiology.

Johnson ES (1994) Poultry oncogenic retroviruses and humans. *Cancer Detect. Prev.* 18: 9.

Payne LN (1992) Biology of avian retroviruses. In: Levy JA (ed.) *The Retroviridae,* vol. 1, p. 299. New York: Plenum.

Witter RL (1991) Neoplastic diseases/reticuloendotheliosis. In: Calnek BW, Barnes HJ, Beard CW, Reid WM and Joder HW, Jr (eds) *Diseases of Poultry,* 9th edn, p. 439. Ames: Iowa State University Press.

RETROTRANSPOSONS OF FUNGI



Jef D Boeke, Department of Molecular Biology and Genetics, Johns Hopkins University, Baltimore, Maryland, USA

History

Transposable elements in eucaryotes can nearly all be classified into three basic types. The first type, typified by the Ac elements of plants and the P elements of *Drosophila,* resemble bacterial transposons in that they bear short inverted repeat termini; the available evidence strongly suggests that this type of element transposes directly via a DNA intermediate. However, most eucaryotic transposons differ from the bacterial elements in that they encode a reverse transcriptase (RT) or RT-like protein. Two basic types of these 'retrotransposons' are known – the LTR (long terminal repeat)-containing type, which are structurally highly reminiscent of retroviruses, and the poly(A)-type, which lack LTRs and usually (but not always) contain an oligo(A), poly(A) or similar sequence tract at their extreme 3′ end. These two types of retrotransposon are shown in **Fig. 1**. Both classes of retrotransposons are now known from organisms as phylogenetically distinct as fungi, trypanosomes, insects and mammals. Hence this brief review will focus on what is currently known about fungal retrotransposons, principally the retrotransposons that are more retroviral-like and are distinguished by the presence of LTRs. Thus, only a small and highly selective glimpse of the total picture of retrotransposons is provided.

Structural Features

The structural features of the known fungal retrotransposons are summarized in **Table 1**. LTR-containing retrotransposons isolated from fungi resemble retroviral proviruses in structure. They contain LTR sequences of a few hundred base pairs long flanking a central coding region that contains one or two open reading frames (ORFs), called *gag* and *pol* by analogy to the retroviral counterparts. As is the case with proviruses and DNA-based transposons, target site duplications of a fixed length flank the elements. These vary greatly in sequence and are presumably generated during the integration process

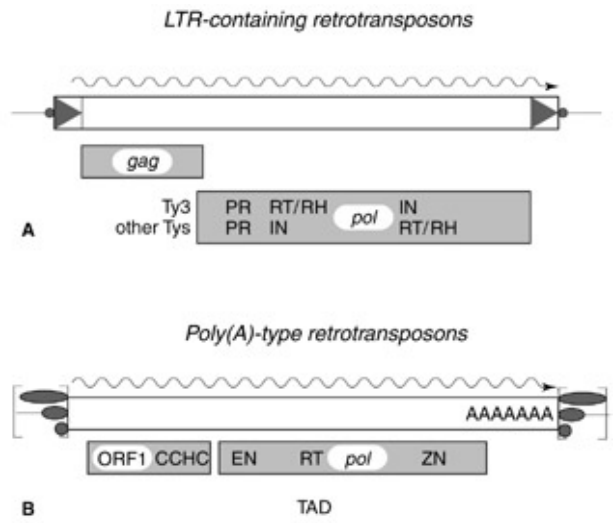


Figure 1 Retrotransposon types in fungi. All elements found to date in the yeasts, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* are LTR-containing transposons (**A**). PR, protease; IN, integrase; RT, reverse transcriptase; RH, RNase H. Boxed triangles represent the LTRs; wavy line represents the transcript; shaded circles represent fixed-length target site duplications. Note that the order of functional domains differs between Ty3 (*Metaviridae*) and the other yeast elements (*Pseudoviridae*). (**B**) Two elements of the 'poly(A)' type have been found in the filamentous fungi (see **Table 1**). Note that unlike almost all members of this class of elements, TAD does not contain the 3′ poly(A) tract, but instead contains an AT-rich sequence. Abbreviations and symbols as above except: CCHC, retroviral Gag Zn-finger-like sequence; EN, endonuclease domain; FZN, zinc-finger-like domain found in *pol* genes of this class of elements; bracketed shaded ovals represent variable-length target site duplications.

by a transposon-encoded integrase function. In the cases where the RNA has been examined, it resembles retroviral genomic RNA in that it extends from LTR to LTR and is terminally repetitious, allowing definition of U3, R and U5 regions of the LTR sequence in a manner formally analogous to that used by retrovirologists. The elements with two ORFs clearly contain the equivalents of *gag* and *pol*, but no analogue of retroviral *env*. The elements with single ORFs, Tf1 and Tf2, (and possibly also Ty5) apparently translate a Gag/Pol fusion protein only. In all cases, the primary translation products are then cleaved into smaller final products by the element encoded aspartyl protease.

The elements with LTRs fall into two basic classes, distinguishable by the order of the functional domains in pol (see **Fig. 1**). In the class typified by Ty3, the RT domain precedes the IN domain, as it does in retroviruses. This family of elements has recently been classified as the *Metaviridae*. In the other type, typified by Ty1 and the other yeast elements except Ty3, the order of these domains is reversed. This family of elements has recently been classified as the *Pseudoviridae*.

Thus far there is only one report in the literature of a fungal retrotransposon that lacks LTRs, the TAD element from the filamentous fungus *Neurospora crassa* Adiopodoume strain, although structurally similar elements have now been isolated from other filamentous fungi. As these elements are much less retroviral-like, they are not reviewed here.

Transposition Mechanism

There is probably more known about the LTR-containing retrotransposons of fungi than about those of any other species. By far the most heavily studied elements are the Ty1 and Ty3 elements of *Saccharomyces cerevisiae*, and the Ty5 elements of the related species *Sac. paradoxus*, and the Tf1 elements of *Schizosaccharomyces pombe*. The life cycles of these

Table 1 Fungal retrotransposons

Host[a]	*Element name*	*Type*	*ORFs*	*Target site duplication (bp)*	*LTR length (bp)*	*Primer (−) strand*
Sac. cerevisiae	Ty1	LTR	2	5	334	$tRNA^{Met}_i$
Sac. cerevisiae	Ty2	LTR	2	5	334	$tRNA^{Met}_i$
Sac. cerevisiae	Ty3	LTR	2	5	340	$tRNA^{Met}_i$
Sac. cerevisiae	Ty4	LTR	2	5	371	$tRNA^{Asn}$
Sac. paradoxus	Ty5	LTR	1	5	250	$tRNA^{Met}_i$
Sch. pombe	Tf1,2	LTR	1	5	358	self-priming
N. crassa	TAD	polyA	2	14, 17	N.A.	?
M. grisea	MAGGY	LTR	2		253	
A. fumigatus	Afut	LTR	2	5	282	

[a] Generic abbreviations: *M., Magnaporthe; Sac., Saccharomyces; Sch., Schizosaccharomyces; N., Neurospora; A., Aspergillus.*

are quite similar. In contrast, except for the presence of a cytoplasmic transposition intermediate, and evidence for reverse transcription during its transposition, relatively little is yet known about TAD transposition. This is because these poly(A)-type elements are generally less well known (and retroviral analogies are uncertain at best). Thus the discussion below applies to yeast Ty elements specifically, and to LTR retrotransposons generally.

A transposon copy (usually studied in the laboratory in the form of a *GAL* promoter/Ty element fusion) produces a transcript that extends from a point in the 5′ LTR to a different, downstream point in the 3′ LTR. Thus, a terminally redundant RNA is generated. This RNA is polyadenylated and exported to the cytoplasm, where it can have two different fates; it can serve (1) as an mRNA for Gag and Gag-Pol protein products and/or (2) as genetic material for transposition.

Translation of Ty elements is somewhat unconventional. Gag is produced directly by conventional translation of this mRNA, whereas Pol is expressed as a Gag-Pol fusion protein (readthrough protein); this 'frameshifting' process is mediated at a special sequence within the region of overlap of *gag* and *pol*. Both the Ty1 and Ty3 frameshifts differ from those of more conventional retroviruses in that they are +1 frameshifts rather than −1 frameshifts, and the intrinsic mechanism used to effect the frameshift is different. In the −1 frameshifts used by retroviruses, coronaviruses, and the yeast killer double-stranded RNA virus, a 'slippery site' allows for a simultaneous slip of the ribosome during the translational step in which the ribosomal P and A sites are simultaneously occupied. Stem–loop structures or pseudoknots that are often found just downstream of the slippery site are thought to cause ribosomal pausing and perhaps even to induce the ribosome to slip backwards. In contrast, the Ty1 element appears to utilize a completely different mechanism. There appears to be no requirement for any special RNA secondary structure; rather the frameshifting appears to be sequence-mediated, because a seven nucleotide sequence from the overlap region readily confers frameshifting on a heterologous reporter gene. Ribosomal pausing, thought to be required for frameshifting, is apparently caused by limiting amounts of a rare tRNA, $tRNA^{Arg}_{CCU}$. The stalled ribosome, which unlike the retroviral case, has an empty A site, has the P site occupied by a specific $tRNA^{Leu}$ that recognizes all six Leu codons. The stall caused by low $tRNA^{Arg}$ levels allows time for the slippage event to occur; the $tRNA^{Leu}$ slips to an overlapping Leu codon in the +1 (*pol*) frame. This then exposes a Gly codon recognized by an apparently abundant tRNA in the A site, allowing translation to continue in the *pol* frame. This mechanism results in an efficiency of frameshifting that varies somewhat with context, but on average is about 10–20%.

Once sufficient Ty protein products, in the form of intact Gag and Gag-Pol readthrough proteins, are produced, an assembly process that is not yet well understood begins. What follows is a working model for this assembly process, although this is based largely on interpretations of the limited experiments that have been done on Ty assembly and by analogy with retroviral systems and models. Ty RNA is apparently selectively packaged, together with at least one specific tRNA, the primer tRNA, into a capsid initially consisting of a coassemblage of unprocessed Gag and Gag-Pol proteins. Presumably, these coassemble via Gag-Gag interactions, and these are made in such a way that the C-termini of the proteins, and the RNA, reside in the internal cavity of the virus-like particle (VLP). The aspartyl protease encoded within *pol*, presumably activated by a dimerization process facilitated by the high protein concentration involved in the assembly process, then cleaves the precursor Gag proteins and Gag-Pol proteins to their mature, presumably physiologically relevant forms. This results in a change in the morphology of the VLPs, as well as an apparent activation of the endogenous reverse transcriptase activity.

Once reverse transcriptase is activated, the initial DNA products, corresponding to the left end of the transposon are synthesized, using a cellular tRNA as primer. This primer was recently shown to be $tRNA^{Met}_{i}$ for Ty1. Eventually, a full-length double-stranded (ds) DNA is made through a series of priming and DNA strand transfer events. In the Ty5 element, the same tRNA is apparently cleaved to form a half-molecule; the 5′ half-molecule is then used to prime reverse transcription. The identity of the enzyme that effects this cleavage is unknown.

In the Tf elements of *Sch. pombe*, a different type of reverse transcriptase priming occurs – self priming. In these unusual elements, the primer for reverse transcription is a piece of the retrotransposon RNA itself. The 5′ end of the RNA folds into a complex secondary structure, and an 11 nucleotide (nt) fragment is then released, apparently by the RNase H activity associated by the reverse transcriptase. A number of other retroelements from fungi and other organisms are thought to use a similar mechanism.

The dsDNA remains associated with Gag and Pol proteins such as reverse transcriptase inside the cell. These DNA-containing VLPs (isolated as a mixture of RNA-containing and DNA-containing VLPs) have been shown to contain all the macromolecular factors needed to carry out an *in vitro* transposition

(integration) reaction. Like retroviral core particles, which have this same activity, these VLPs require only a divalent cation for activity. The Ty DNA is apparently synthesized inside VLPs inside the cytoplasm. It is interesting to consider how and in what form this DNA is delivered to the nucleus. This is a particularly interesting question to ask in yeast, because fungi, unlike mammalian cells, undergo a 'closed mitosis' in which the nuclear membrane does not break down and reform during each mitosis, but apparently remains intact. Recent experiments implicate a small basic amino acid sequence at the C-terminus of the integrase in this process. This 'nuclear localization signal' may be responsible for delivering not only the integrase but also the DNA to the nucleus.

Once the Ty DNA and Ty integrase enter the nucleus, a concomitant cleavage of host DNA and joining to transposon ends similar to that occurring during retroviral integration occurs. Ty3 is extremely selective for its integration, and apparently inserts only at the transcription initiation sites for RNA polymerase III. Ty1 also targets tRNA genes, but probably by recognizing a unique chromatin structure associated with their 5′ ends. Ty5 elements appear to target 'silenced' chromatin. All of these mechanisms tend to ensure that these Ty elements will not destroy host genes, as most of the Ty targets do not encode genes, or at least not essential ones.

Virus-like Particles: Evolutionary Vestige or Transposition Intermediates?

The presence of VLPS in Ty elements has often raised the question of the evolutionary relationship between retroviruses and LTR-containing retrotransposons. Is the VLP a degenerate leftover of some decaying retrovirus? Or are LTR-containing retrotransposons a family of modern-day descendants of the precursor of the retroviruses? Presumably all retroelements descended ultimately from a 'cellular reverse transcriptase gene' as originally proposed by Temin. This gene may be ancient, and its original product may have been the molecule that archived the genetic information of the RNA world into DNA. Examination of the spectrum of modern-day retroelements from this perspective, reveals a natural progression from the simple to the very complex, as follows: starting with a simple RT gene (perhaps represented by modern-day telomerase?), to the poly(A)-type retrotransposons, which contain a second ORF in addition to RT, to the LTR-containing retrotransposons, which have the above plus LTRs, to the simple retroviruses, which have acquired a third ORF, *env*, and on to the most elaborate of all, the lentiviruses, with multiple additional regulatory reading frames in addition to the basic three. Since all LTR-containing retrotransposons studied appear to involve a VLP intermediate (that is, all examined to date have many properties of a transposition intermediate), the implication is that, as is the case in all complex biological reactions, a structure is built to ensure (1) high local concentration of numerous macromolecules required for the reaction and (2) appropriate orientations/conformations of these macromolecules to allow the reactions to proceed appropriately. The strongest support for this idea comes from studies on Ty1, which suggest that the VLP is a direct, functional transposition intermediate. Thus, if LTR-containing retrotransposons predated retroviruses, and their VLP structure evolved in response to selection for this organizing function, it does not stretch the imagination too far to suggest that these elements were 'preadapted' for subsequent selection for infectivity. In fact, one may see such transitional forms within the *gypsy* family of LTR-containing retrotransposons, in which some family members resemble the Tys in having two ORFs, but a few elements have

Table 2 Host genes affecting Ty1

Gene name	*Normal function/product*	*Function for Ty1*	*Found by*
Transcriptional effect genes			
SPT3, 7, 8	Transcription factor	Transcription initiation	Suppression of Ty- or LTR-induced mutation
SPT4, 5, 6	Chromatin factors	Transcription initiation	Suppression of Ty- or LTR-induced mutation
SPT10, 21	Repressor and activator	Repress 3′ LTR	Suppression of Ty- or LTR-induced mutation
Post transcriptional effect genes			
IMT1-4	Translation initiation, $tRNA^{Met}_{i}$	Prime reverse transcription	Intentional mutagenesis of genes to reveal interaction
(none)	$tRNA^{Arg}_{CCU}$	Low level causes ribosome stalling, frameshifting	Search for genes which interfere with transposition when overexpressed
DBR1	Debranch intron lariats (2′-5′ phosphodiesterase)	Unknown	Search for chromosomal mutations that interfere with transposition

a third *env*-like ORF in the appropriate genomic position.

Host Functions in Retrotransposition

The Ty1 system has provided some insights into the roles of host-encoded proteins and RNAs on the retrotransposition process, and it is anticipated that many more remain to be discovered. A large number of host genes that play roles in Ty and host gene transcription have been uncovered genetically; these are called *SPT* genes because they were originally identified by mutations that *sup*pressed the effect of *T*y or LTR insertions. Although some of these affect Ty transcription, they do not affect the production of GAL/Ty mRNA. The development of sensitive assays for transposition of GAL/Tys *in vivo* has led to the identification of a number of host factors that are important for transposition at a post-transcriptional level; these are reviewed in **Table 2.**

See also: **Coronaviruses (*Coronaviridae*); Retroviruses – type D (*Retroviridae*); Yeast RNA viruses (*Totiviridae*).**

Further Reading

Boeke JD and Sandmeyer SB (1991) Yeast transposable elements. In: Broach J, Jones E and Pringle J (eds) *The Molecular and Cellular Biology of the Yeast Saccharomyces*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Devine SE and Boeke JD (1996) Integration of the yeast retrotransposon Ty1 is targeted to regions upstream of genes transcribed by RNA polymerase III. *Genes Dev.* 10: 620.

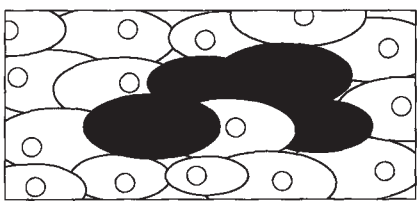
Eichinger DJ and Boeke JD (1988) The DNA intermediate in yeast Ty1 element transposition copurifies with virus-like particles: cell-free Ty1 transposition. *Cell* 54: 955.

Kinsey JA (1990) *Tad*, a LINE-like element of *Neurospora*, can transpose between nuclei in heterokaryons. *Genetics* 126: 317.

Levin HL, Weaver DC and Boeke JD (1990) Two related families of retrotransposons from *Schizosaccharomyces pombe*. *Mol. Cell. Biol.* 10: 6791.

Levin HL (1995) A novel mechanism of self-primed reverse transcription defines a new family of retroelements. *Mol. Cell. Biol.* 15: 3310.

RETROVIRAL ONCOGENES



Paula J Enrietto and **Gabriela Maldonado-Codina**, Genomica Corporation, Boulder, Colorado, USA

Michael J. Hayman, Department of Molecular Microbiology and Genetics, SUNY at Stony Brook, Stony Brook, New York, USA

Introduction

The role of viruses in the etiology of cancer was suggested by the work of Peyton Rous who isolated the first acutely transforming retrovirus, Rous sarcoma virus (RSV), from a chicken sarcoma. These viruses are highly oncogenic and induce malignant transformation of cells because of the presence of cellular sequences, oncogenes (v-*onc*) within the viral genome. The cellular sequences from which viral oncogenes are derived, proto-oncogenes, are cellular genes converted into oncogenes by mutation, rearrangement or deletion. Retroviral oncogenes are generally mutated versions of their respective proto-oncogene. These changes result from the transduction process and additional changes are acquired during viral replication. Consequently, the oncogenic potential of the viral oncogene is significantly enhanced when compared to the cellular oncogene.

In this entry we will focus on retroviral oncogenes, which will be discussed in functional groups. The description of oncogenes within each group is meant to be broad. Specific details have been excluded but can be found in the references listed at the end.

Nuclear Oncogenes

ErbA

V-*erbA* is found in the avian erythroblastosis virus, AEV-ES4 and -R strains, isolated in 1934 by Englebreth-Holm. AEV transduced two different oncogenes, v-*erbA* and v-*erbB*. V-*erbB* is a homologue

a third *env*-like ORF in the appropriate genomic position.

Host Functions in Retrotransposition

The Ty1 system has provided some insights into the roles of host-encoded proteins and RNAs on the retrotransposition process, and it is anticipated that many more remain to be discovered. A large number of host genes that play roles in Ty and host gene transcription have been uncovered genetically; these are called *SPT* genes because they were originally identified by mutations that suppressed the effect of Ty or LTR insertions. Although some of these affect Ty transcription, they do not affect the production of GAL/Ty mRNA. The development of sensitive assays for transposition of GAL/Tys *in vivo* has led to the identification of a number of host factors that are important for transposition at a post-transcriptional level; these are reviewed in **Table 2.**

See also: **Coronaviruses (*Coronaviridae*); Retroviruses – type D (*Retroviridae*); Yeast RNA viruses (*Totiviridae*).**

Further Reading

Boeke JD and Sandmeyer SB (1991) Yeast transposable elements. In: Broach J, Jones E and Pringle J (eds) *The Molecular and Cellular Biology of the Yeast Saccharomyces*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Devine SE and Boeke JD (1996) Integration of the yeast retrotransposon Ty1 is targeted to regions upstream of genes transcribed by RNA polymerase III. *Genes Dev.* 10: 620.

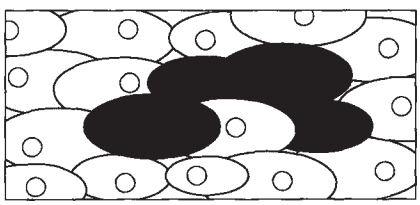
Eichinger DJ and Boeke JD (1988) The DNA intermediate in yeast Ty1 element transposition copurifies with virus-like particles: cell-free Ty1 transposition. *Cell* 54: 955.

Kinsey JA (1990) *Tad*, a LINE-like element of *Neurospora*, can transpose between nuclei in heterokaryons. *Genetics* 126: 317.

Levin HL, Weaver DC and Boeke JD (1990) Two related families of retrotransposons from *Schizosaccharomyces pombe*. *Mol. Cell. Biol.* 10: 6791.

Levin HL (1995) A novel mechanism of self-primed reverse transcription defines a new family of retroelements. *Mol. Cell. Biol.* 15: 3310.

RETROVIRAL ONCOGENES



Paula J Enrietto and **Gabriela Maldonado-Codina**, Genomica Corporation, Boulder, Colorado, USA

Michael J. Hayman, Department of Molecular Microbiology and Genetics, SUNY at Stony Brook, Stony Brook, New York, USA

Introduction

The role of viruses in the etiology of cancer was suggested by the work of Peyton Rous who isolated the first acutely transforming retrovirus, Rous sarcoma virus (RSV), from a chicken sarcoma. These viruses are highly oncogenic and induce malignant transformation of cells because of the presence of cellular sequences, oncogenes (v-*onc*) within the viral genome. The cellular sequences from which viral oncogenes are derived, proto-oncogenes, are cellular genes converted into oncogenes by mutation, rearrangement or deletion. Retroviral oncogenes are generally mutated versions of their respective proto-oncogene. These changes result from the transduction process and additional changes are acquired during viral replication. Consequently, the oncogenic potential of the viral oncogene is significantly enhanced when compared to the cellular oncogene.

In this entry we will focus on retroviral oncogenes, which will be discussed in functional groups. The description of oncogenes within each group is meant to be broad. Specific details have been excluded but can be found in the references listed at the end.

Nuclear Oncogenes

ErbA

V-*erbA* is found in the avian erythroblastosis virus, AEV-ES4 and -R strains, isolated in 1934 by Englebreth-Holm. AEV transduced two different oncogenes, v-*erbA* and v-*erbB*. V-*erbB* is a homologue

of the epidermal growth factor receptor tyrosine kinase (see below).

The two *erb* genes are inserted between *gag* and *env* and are separated by an *erbB* intron sequence, which encodes the C-terminal four amino acids and termination codon of v-*erbA*. The 11 preceding Erb-A amino acids are encoded by *env* sequence. At the N-terminus a substitution of 254 amino acids encoded by the viral *gag* gene is also apparent. Giving rise to the protein product of v-ErbA, $p75^{gag\text{-}erbA}$, v-ErbA is derived from c-ErbA, the thyroid hormone receptor α (THRA1). V-ErbA is a mutated form of chicken THRA1. In addition to deletions resulting from transduction (nine amino acid N-terminal deletion and a 13 amino acid C-terminal deletion), V-ErbA has two point mutations in the DNA-binding domain and 11 in the hormone-binding domain. These changes result in the loss of hormone-binding capacity but do not abrogate sequence-specific DNA binding. The $p75^{gag\text{-}erbA}$ protein is localized to the nucleus. $P75^{gag\text{-}erbA}$ is phosphorylated on Ser16/17 by protein kinase A or protein kinase C. Mutation of the Ser16/17 phosphorylation site releases the block of erythroid differentiation exerted by v-ErbA.

THRA1 is a zinc-finger transcription factor, which binds to the response element TCAGGTCATGACCTGA, repressing its promoter activity. V-ErbA suppresses transcription of several genes, including avian erythrocyte anion transporter (band III), carbonic anhydrase II and β-globin genes. Transcriptional repression of erythrocyte-specific genes correlates with the biological properties of v-Erb-A and requires formation of heterodimeric complexes with retinoid X receptor (RXR).

V-ErbA is not tumorigenic but cooperates with v-Erb-B to cause avian erythroleukemia and fibrosarcomas. *In vitro*, v-ErbA blocks erythroid differentiation in cooperation with v-ErbB and other sarcoma-inducing oncogenes in transformation. V-ErbA stimulates chicken embryo fibroblast (CEF) growth and enhances the tumorigenicity of v-ErbB transformed fibroblasts.

Ets

The avian retrovirus E26, which carries the *ets* oncogene, was isolated in 1962 at the Bulgarian Academy of Sciences from a case of avian erythroblastosis. The E26 genome consists of deleted viral *gag* and *env* genes, a truncated version of *myb*, and *ets* (δgag-v-myb-v-ets-δenv). The *myb-ets* junction was created by an aberrant splice between a cryptic donor in *myb* and the normal splice acceptor in *ets* exonα. The result is the generation of five additional amino acids at this junction (HGTSE). Comparison of c-Ets (ETS-1) with v-Ets reveals amino acid substitutions at Ala285 and Ile445, which are replaced by Val in v-Ets. The 13 C-terminal residues of c-Ets have been replaced by 16 amino acid residues in v-Ets, 13 of which are derived from an inverted Ets-1 sequence. This results in a contiguous Gag-Myb-Ets open reading frame encoding a protein of 135 kDa, $p135^{gag\text{-}myb\text{-}ets}$ that is in the nucleus.

V-Ets is a member of a family of transcription factors that bind to DNA motifs containing (C/A)GGA (A/T) through a conserved 85 amino acid region called the Ets domain. V-Ets has less stringent target sequence requirements than c-Ets and binds to a broad spectrum of DNA sequence motifs, suggesting that v-Ets transforms cells by altering expression of tightly regulated genes with nonconsensus Ets binding sites.

The predominant disease induced by E26 in chickens is erythroblastosis. *In vitro*, both myeloid and erythroid cells can be transformed by E26, reflecting the fact that E26 also contains the myeloid-specific oncogene, v-Myb (see below). E26 transforms quail fibroblasts, NIH-3T3 cells, immature erythroid cells, and stimulates proliferation of CEF.

Fos

The *fos* oncogene is carried by the Finkel–Biskis–Jinkins (FBJ-MSV) and Finkel–Biskis–Reilly (FBR-MSV) murine osteogenic sarcoma viruses. Dr Miriam Finkel isolated both viruses from mice with ^{90}Sr-induced osteosarcomas. A virus isolated from an avian nephroblastoma, NK24, also contains the *fos* gene. The genome structures of FBJ and FBR-MSV differ significantly. FBJ-MSV contains v-*fos* and has no additional viral structural gene information, whereas FBR-MSV v-*fos* is fused to *gag* and non-*fos* sequences from the *fox* gene.

FBJ-MSV has five amino acid changes compared to c-Fos. A 104 bp deletion shifts the reading frame resulting in a C-terminus with 49 novel amino acids. Comparison with c-Fos reveals that FBJ-MSV v-Fos has lost 24 amino acids at the N-terminus (replaced with 310 gag amino acids) and 98 C-terminal amino acids (replaced with eight amino acids from fox). There are two C-terminal in frame deletions of 13 and 9 amino acids. The NK24 virus expresses an unaltered Fos protein linked to the gag encoded sequence.

The FBJ-MSV Fos protein product is 39 kDa, whereas the FBR-MSV fusion protein is $p75^{gag\text{-}fos\text{-}fox}$. Fos is a member of the helix–loop–helix/leucine zipper transcription factor superfamily. Each member of the family contains a basic domain, a leucine zipper, two homology boxes (HOB1 and HOB2) within a transac-

tivation domain, and a *trans*-repression domain. FBJ-MSV and FBR-MSV both lack the *trans*-repression domain at the C-terminus. Both FBJ and FBR-MSV v-Fos proteins are localized in the nucleus.

FBR-MSV v-Fos, but not FBJ v-Fos, is myristylated at the N-terminus. Both proteins are phosphorylated on serine and threonine residues but not to the same extent as c-Fos, as the predominant phosphorylation sites on c-Fos are within the C-terminal sequences deleted from v-Fos. A basic motif, KCR, is conserved in the Fos family and redox-regulation of the cysteine residue mediates DNA binding. All Fos proteins display DNA binding activity at a site (TGAc/GTCA) termed either activating protein-1 site (AP-1) or TPA response element (TRE) and form heterodimers with Jun proteins. These function as positive or negative transcription regulators binding to DNA via the basic domain.

FBJ, NK24 and FBR-MSV transform fibroblasts *in vitro*. FBR immortalizes murine cells in culture, while FBJ does not. Transforming ability appears to correlate with the presence of a C-terminal transactivation domain.

Jun

Avian sarcoma virus 17 (ASV17), which contains V-Jun, was isolated from a chicken sarcoma in 1987; 220 viral Gag residues (p19 and δp10) are joined in frame to 296 Jun-encoded amino acids. Comparison to c-Jun reveals a 27 amino acid deletion in the N-terminus of v-Jun and three nonconservative substitutions in the C-terminal half; two of which are in the DNA binding domain. The v-Jun protein product $p65^{gag\text{-}jun}$ is localized in the nucleus and has several domains: the A1 activator domain, two homology box regions, HOB1 and HOB2, the ε region and δ region (the region of 27 amino acids deleted in v-Jun), A2 activation domain, and a Pro/Gln-rich ancillary DNA-binding domain. The primary DNA-binding domain, a basic region and leucine zipper, are in the C-terminal 110 residues. This region and the activation domain are necessary for transformation by Jun. V-Jun forms heterodimers with members of the Fos family and Fos-related antigens (FRA1 and FRA2) and binds with high affinity to a TPA response element (TGAc/gTCA), TRE.

Oncogenic activation of c-Jun results from 27 amino acid deletion in the N-terminus. This region contains a MAP kinase site allowing for control of c-Jun activity by phosphorylation. V-Jun is not subject to control by phosphorylation. Two of the three nonconservative amino acid substitutions may be important functionally. A Ser222 to Phe mutation prevents the glycogen synthase kinase-3 phosphorylation of a negative regulatory site. Mutation of Cys248 to Ser disrupts the oxidation of Cys248, inactivating DNA binding.

V-Jun induces tumors when injected into the wing web of young chicks. *In vitro,* v-Jun transforms chicken embryo fibroblasts.

Maf

The v-*maf* oncogene was identified in an avian retrovirus, AS42, isolated from a spontaneous musculoaponeurotic fibrosarcoma of chicken. The v-*maf* gene is inserted into the viral genome in the *gag* gene and v-*maf* encodes a 42 kDa basic region/leucine zipper protein, which is localized in the nucleus. The coding region of v-*maf* has only two structural changes when compared to *c-maf*.

V-Maf and other Maf family members form homodimers and heterodimers with each other and Fos and Jun. The DNA target sequence to which Maf binds is termed the Maf response element (MARE). It is a 13 or 14 bp element that contains a core TRE or CRE palindrome.

In vitro v-Maf transforms avian fibroblasts. *In vivo* the virus induces musculoaponeurotic fibrosarcomas.

Myb

The v-*myb* oncogene is found in two acutely transforming avian retroviruses, avian myeloblastosis virus (AMV) and E26. AMV v-*myb* replaces 26 codons at the 3′ end of *pol* and most of *env*. There are 6 *gag*-coded amino acids at the N-terminus and 11 *env* amino acids at the C-terminus. E26 v-*myb* is fused in frame with v-*ets*, see above. Comparison of *AMV* v-*myb* with c-*myb* revealed extensive 5′ (71 N-terminal amino acids) and 3′ (198 C-terminal amino acids) deletions and 11 point mutations. E26 v-Myb lacks 80 N-terminal and 278 C-terminal amino acids of c-Myb and has one point mutation.

The AMV v-Myb protein product is $p45^{v\text{-}myb}$. The E26 v-Myb protein product is $p135^{gag\text{-}myb\text{-}ets}$. Both proteins are nuclear. C-Myb contains a DNA-binding domain with three repeat regions, R1, R2, R3, transactivation domain, leucine zipper, *trans*-repression domain and a conserved domain in the C-terminus. N-terminal deletions in AMV v-Myb remove most of the R1. The C-terminal conserved region is also deleted. E26 v-Myb lacks almost all of R1, the leucine zipper region, the MAPK/CDC2 site, and the conserved domain. All Myb related genes contain a Cys residue at position 65.

Phosphorylation of a casein kinase II site at the N-terminus decreases c-Myb DNA binding. This site is absent in both E26 and AMV v-Myb. A MAPK/CDC2 site in the C-terminus of c-Myb is not

phosphorylated in AMV v-Myb. V-Myb binds directly to double stranded DNA and regulates transcription through a consensus site YAACT/(C)/GGYCA. V-Myb regulates the expression of mim-1 and c-Myb.

AMV induces myeloid leukemia in chickens. *In vitro,* AMV transforms macrophage precursors (monoblasts). E26 transforms fibroblasts, myeloid and, as a result of the presence of ets, erythroid cells.

Myc

The v-*myc* gene was first identified in avian myelocytomatosis virus, MC29, but was subsequently found in four other virus isolates, CMII, OK10, MH2 and FH3. All the v-*myc* genes are highly homologous and contain only eight single nucleotide changes when compared with c-*myc*. MC29 contains deleted *gag*, 422 v-*myc* amino acids, no *pol* and a complete *env* gene. CMII contains deleted *gag*, 421 v-*myc* amino acids, and complete *pol* and *env* genes. OK10 contains all of *gag*, 426 v-*myc* amino acids, deleted *pol* and *env* genes. $P58^{\delta gag\text{-}myc}$ is a second Myc protein product translated from a subgenomic RNA, which contains a small piece of *gag* sequence at the N-terminal end. MH2 contains deleted *gag* and *env* genes and 417 v-*myc* amino acids and a second oncogene, *mil*, which is homologous to v-*raf*. FH3 contains deleted *gag* gene and 421 *myc* amino acids.

All v-*myc* genes are expressed as fusion proteins with viral structural information. MC29: $P110^{\delta gag\text{-}myc}$; CMII: $P90^{\delta gag\text{-}myc}$; OK10: $P200\text{-}gag^{\delta pol\text{-}myc}$, $P58^{\delta gag\text{-}myc}$; MH2: $P57^{\delta gag\text{-}myc}$; FH3: $P145^{\delta gag\text{-}myc}$. Several functional domains have been defined in the Myc family. A central acidic region influences the transforming host range of v-Myc proteins. Two nuclear localization signals have been defined. The C-terminus contains elements essential for DNA binding, a basic region, a helix–loop–helix motif and a leucine zipper. All v-Myc proteins are localized to the nucleus except $P200^{gag\text{-}\delta pol\text{-}myc}$, which is found in the cytoplasm and the nucleus. The Ser62 is phosphorylated by MAP kinases, lies in a highly-conserved proline-rich region and carries transcriptional activation capacity.

Myc forms a sequence specific (CACGTG) DNA binding heterodimer with a helix–loop–helix protein, Max. The leucine zipper and the helix–loop–helix region are required for heterodimer formation. Both positive and negative regulation of gene expression has been associated with Myc family members.

In vivo, MC29, CMII or FH3 cause myelocytomatosis. MC29, MH2 or OK10 cause liver and kidney carcinomas. *In vitro,* MC29, CMII, OK10, MH2 or FH3 transform immature macrophages or fibroblasts. Primary fibroblasts are immortalized by v-Myc but are rendered tumorigenic only when an activated Ras is also expressed.

Rel

V-*rel*, the oncogene in avian reticuloendotheliosis virus strain T (REV-T), was isolated from a turkey with lymphoid leukosis. V-Rel is a member of the NF-κB family of transcription factors. Incorporation of c-*rel* into the viral genome resulted in truncation of *gag*, *pol* and *env*. Using the *env* initiation codon, v-*rel* begins with 12 *env* amino acids and ends with 18 amino acids which are out of frame with respect to *env*. Comparison of v-Rel and turkey c-Rel revealed that v-Rel has lost two N-terminal amino acids and 118 amino acids at the C-terminus. V-Rel has 14 amino acids changes, ten of which are nonconservative, and three sites of deletion where a total of five amino acids are deleted.

The protein product of v-*rel* is $p59^{v\text{-}rel}$, which is localized to both the cytoplasm and nucleus of infected cells. At the N-terminal end of v-Rel is the Rel homology domain, a DNA-binding domain conserved in all members of the NF-κB family. This region also specifies nuclear localization and protein–protein interaction. C-Rel contains sequences in the C-terminus important for cytoplasmic retention and transcriptional activation which are deleted from v-Rel. In the cytoplasm, v-Rel exists in a complex with several other cellular proteins, including the inhibitor IκB. Dissociation of IκB from this complex allows translocation of v-Rel to the nucleus. V-Rel forms heterodimers with other members of the NF-κB family and binds to NF-κB motifs ($NGGNN^{A}$/TTTCC). In most cells, v-Rel represses gene expression; however, in transformed avian cells v-Rel activates transcription of MHC class I, HMG 14b, NF-κB, macrophage inflammatory protein-1 and an interleukin (IL)-8 related gene.

In vivo, REV-T causes acute reticuloendotheliosis. *In vitro,* v-Rel transforms a bone marrow-derived dendritic cell precursor, spleen-derived lymphoid cells, and fibroblasts.

Qin

The *qin* oncogene is the cell-derived insert in the genome of the avian sarcoma virus, ASV31. V-Qin is fused to Gag sequences at the N-terminus and eight cell coded amino acids link the cellular *qin* coding domain with the viral *gag* domain. Comparison of v-Qin with chicken c-Qin demonstrated several differences between the two. There are two non-conservative amino acid substitutions in the Qin coding region, a truncation in the C-terminus of the

viral protein due to a premature stop codon. V-Qin is a nuclear protein.

V-Qin contains two domains: a winged helix domain and a repression domain. Regions between residues 74–141 and 383–395 are required for transformation. Qin is a member of the winged helix transcription factor family which function as important regulators of embryonal development and tissue differentiation in vertebrates and invertebrates, regulating expression of a number of genes. It is most closely related to brain factor-1. Qin also functions as a transcriptional repressor.

In vivo ASV31 induces fibrosarcomas in chickens. *In vitro,* the virus transforms avian fibroblasts.

Ski

V-ski is the oncogene found in the Sloan–Kettering viruses, SKV. Inially two molecular weight forms of v-Ski proteins were described. A 125 kDa form, in which the *ski* sequence was inserted into the *gag* gene, and a 110 kDa form, which was derived from the former via deletion of *gag* sequence 3′ to the *ski* gene resulting in a Gag-Ski-Pol fusion protein. Both proteins are located in the nucleus of infected cells. By comparison to the c-Ski protein, v-Ski is truncated at both the N- and C-terminus. Both v-Ski and c-Ski can transform fibroblasts and so these truncations do not seem to be essential for oncogenic activity.

Ski has several protein motifs, including two amphipathic helices which are required for transforming activity. Ski can both transform cells and induce differentiation of muscle cells. The exact function of ski is unknown. However it appears to be capable of acting as a repressor of retinoic acid-induced transcription and this may be important for hematopoietic cell transformation. It can also activate transcription and this may be important for its effects on differentiation.

In vivo ski can effect the differentiation of muscle cells. It can also cause fibrosarcomas and stem cell leukemia when coexpressed with the v-*sea* oncogene. *In vitro* it can transform fibroblasts and hematopoietic cells.

Growth Factors

Sis

The v-*sis* oncogene is the transforming oncogene of the simian sarcoma virus. The oncogenic sequences of the Parodi–Irgens (PI) FSV are homologous with the v-*sis* sequences of the simian sarcoma virus but very little is known about this feline virus-encoded protein. The v-*sis* oncogene product is a protein of 28 kDa, which shares 92% homology with the platelet-derived growth factor (PDGF) B chain. *V-sis* is presumed to transform cells via autocrine activation of the endogenous PDGF receptor. The location of these autocrine interactions between the v-Sis protein and PDGF receptors remains somewhat controversial; however, it is thought that internal autoactivation of PDGF receptors may be essential for transformation by v-Sis. V-Sis can transform fibroblasts in culture and causes fibrosarcomas *in vivo*.

Protein Tyrosine Kinases

Abl

The v-*abl* oncogene was originally found in the Abelson murine leukemia virus. Subsequently the Hardy–Zuckerman 2, HZ2, feline sarcoma virus was also found to have transduced the feline *abl* gene. The protein encoded by the murine v-*abl* oncogene is a 160 kDa fusion protein, p160, which contains N-terminal gag sequences and is a truncated version of the cellular *c-abl* gene. A 120 kDa protein encoded by a variant Abelson MuLV has been described, in which 263 codons have been deleted from the middle of the p160 sequence. The feline v-Abl protein is a fusion protein of 110 kDa, which is comprised of N-terminal Gag sequences followed by Abl sequences and then sequences from the viral *pol* gene. The c-Abl protein is composed of several structural motifs. An SH3 domain is located close to the N-terminus, followed by an SH2 domain, then the tyrosine kinase domain, and a DNA-binding domain followed by an F-actin-binding domain has been located at the C-terminus. The murine p160 v-Abl protein contains all of these domains, apart from the N-terminal SH3 domain. In contrast, the feline v-Abl protein contains the SH3, SH2 and kinase domains, but lacks the C-terminal DNA-binding and actin-binding domains. A model has been proposed in which association of the N-terminal SH3 domain with C-terminal sequences keeps the tyrosine kinase activity of the c-Abl protein in an inactive state. This model would predict that the murine v-Abl protein would be an active kinase due to the loss of the SH3 domain, and the feline v-Abl protein would also be active owing to the loss of the C-terminal sequences. Consistent with this model, both v-Abl proteins are constitutively active tyrosine kinases and this activity is essential for their transforming abilities.

The v-Abl protein is located at the plasma membrane of the cell by virtue of the myristylated N-terminus provided by the Gag sequences. This is in contrast to the cellular Abl protein, which is primarily found in the nucleus of the cell. These myristylation sequences not only direct membrane localization but

also activate tyrosine phosphorylation and are necessary for cell transformation. *In vivo* the murine Abelson MuLV causes lymphocytic leukemias, whereas the feline HZ2 virus causes multicentric sarcomas. *In vitro* v-Abl can transform fibroblast cell lines and also transforms hematopoietic cells of the myeloid and lymphoid lineages. The transformation of these hematopoietic cells is of particular interest as the *c-abl* gene is rearranged by a balanced translocation between chromosomes 9 and 22, (t9;22)[q34;q11], in over 95% of the human malignancy chronic myelogenous leukemia (CML). This translocation forms a fusion protein between Bcr and Abl that gives rise to a constitutively active tyrosine kinase similar to the v-Abl proteins.

ErbB

The v-*erbB* oncogene was originally identified in the replication-defective avian erythroblastosis virus AEV-ES4. This retrovirus also contains the *erbA* oncogene (see above, Nuclear Oncogenes). The AEV-H strain of avian erythroblastosis virus also contained the *erbB* oncogene but did not contain any other oncogenes. This identified *erbB* as the oncogene that was primarily responsible for the induction of disease. In addition to erythroblastosis, AEV-ES4 and AEV-H also caused fibrosarcomas. A specific strain of chickens, L15, is very susceptible to erythroblastosis induced by avian leukosis virus, and analysis of these diseased birds demonstrated that the *erbB* oncogene was activated by retroviral insertions into the *c-erbB* gene. Furthermore the *erbB* sequences were frequently transduced into the retroviral genomes, giving rise to new strains of avian erythroblastosis viruses that encoded v-ErbB proteins. These proteins have sustained the N-terminal truncations but not the C-terminal truncations. Upon further passage, AEVs acquire the ability to cause other tumors in addition to erythroblastosis, for example fibrosarcomas and angiosarcomas. This increase in oncogenic potential is associated with point mutations and deletions within the cytoplasmic region of the ErbB protein.

The *erbB* oncogene is a truncated version of the epidermal growth factor receptor (EGFR). The EGFR is a member of the tyrosine kinase family of growth factor receptors. In comparison to the EGFR, the different forms of ErbB have been truncated at the N-terminus and frequently they are also truncated at the C-terminus. The N-terminal truncations all involve deletion of the ligand-binding domain of the EGFR and this results in constitutive activation of tyrosine kinase activity. The v-ErbB proteins of AEV-ES4 and AEV-H are glycoproteins of approximate molecular mass 74 kDa, which are located at the plasma membrane. Kinase activity and membrane localization are necessary for full oncogenic potential. The EGFR can induce mitogenic signals, which are accompanied by tyrosine phosphorylation of several cellular substrates. Presumably the constitutive activation of v-ErbB leads to the activation of similar substrates. V-ErbB is a much more powerful oncogene than the EGFR, thus the possibility exists that the mutations have somehow altered the specificity of v-ErbB for some substrates. To date no novel substrates for v-ErbB have been identified. Potential substrates identified in v-ErbB transformed include caldesmon, the catenin-like protein p120cas, Shc and Stat5b. In addition, the Grb2 adapter protein binds to v-ErbB and the mitogen-activated protein kinase pathway is constitutively active.

Eyk (ryk)

The v-*eyk* oncogene was found in the avian retrovirus RPL30. It was originally termed v-*ryk*, but the name was changed to avoid confusion with another gene that had been previously termed *ryk*. The v-*eyk* sequences are 1.39 kb in length and code for a tyrosine kinase domain that is fused in frame to the gp37 Env sequences. Unlike other acute transforming retroviruses, the transduction of the v-*ryk* gene did not result in the loss of viral sequences. The v-Eyk protein is first synthesized as a 150 kDa precursor Env-Eyk fusion protein. This protein undergoes the normal proteolytic processing of the *env* gene product to release the Env protein gp85 and a gp69 fusion protein containing gp37 and Eyk sequences. The gp69 protein is located in the membrane of transformed cells and possesses tyrosine kinase activity. C-Eyk encodes a receptor-type tyrosine kinase of 974 amino acids, which is a member of the UFO/Axl/Ark subfamily of receptors. The v-Eyk sequences are truncated by comparison to c-Eyk and have lost the regions that encode the extracellular and transmembrane domains; in addition there are two amino acid changes. RPL30 causes fibrosarcomas when injected into chickens and the cloned *eyk* gene can transform chicken embryo fibroblasts *in vitro*.

Fes/fps

The *fes/fps* oncogene has been isolated from both avian and feline sarcoma viruses. It was identified in the avian viruses: Fujinami sarcoma virus, Poultry Research Center viruses II and IV, PRCII, PRCIV, University of Rochester virus 1, UR1 and 16L. The name *fps* was derived from *F*ujinami-*P*RCII *S*arcoma, as these were the first two viruses described to contain this gene. The cognate oncogene *fes* was found in the Snyder–Theilen, Gardner–Arnstein and Hardy–

Zuckerman 1 strains of feline sarcoma viruses and the name is derived from *f*eline *s*arcoma. The c-*fps/fes* oncogene encodes a cytoplasmically located 98 kDa protein tyrosine kinase that contains an SH2 domain. All of the viral Fps/Fes forms are fusion proteins between N-terminal Gag sequences and fps sequences. The v-Fps/Fes sequences have lost the N-terminus of Fps/Fes but retain the SH2 domain and the tyrosine kinase domain. The v-Fps/Fes proteins are of varying sizes: Fujinami sarcoma virus v-Fps is 140 kDa; PRCII v-Fps is 105 kDa; PRCIV v-Fps is 150 kDa; 16L v-Fps is 142 kDa; UR1 v-Fps is 150 kDa; ST-FSV v-Fes is 85 kDa; GA-FSV v-Fes is 95 kDa; and HZ1-FSV v-Fes is 100 kDa.

The fusion of the Fps/Fes sequences to Gag is sufficient to activate the transforming potential. This fusion seems to be important for the activation of the tyrosine kinase and membrane location of the virally-encoded proteins. Although the feline proteins have lipid modification, there is no evidence of myristylation for any of the avian proteins. *In vitro* the v-Fps proteins can transform fibroblasts and *in vivo* these viruses cause fibrosarcomas and/or myxosarcomas. The tyrosine kinase activity of the Gag-fusion proteins is essential for transformation, and the identity of several cellular substrates is known. These include the GTPase-activating protein, the Shc adapter protein and fibronectin receptor proteins. A key pathway involved in transformation appears to be the Ras pathway, as microinjection of anti-Ras antibody blocks Fps-induced transformation. Interestingly the Bcr protein, which is fused to the Abl oncoprotein in CML, has recently been identified as a substrate of the Fps/Fes kinase. Phosphorylation of BCR provides a docking site for Grb-2 and in this way links Fps/Fes to Ras activation.

Fgr

The v-*fgr* oncogene was first identified in the Gardner–Rasheed feline sarcoma virus, GR-FSV and was subsequently found in the Theilen–Petersen feline sarcoma virus, TP1-FSV. Fgr encodes a cytoplasmic tyrosine kinase activity and is a member of the Src family. In the GR-FSV virus the *fgr* sequences are expressed as a fusion between Gag, followed by γ-actin and then the Fgr sequences to generate a 70 kDa protein. In the TP1-FSV the Gag sequences are fused to the Fgr sequence to generate an 83 kDa protein. The Gag sequences are myristylated and this explains the plasma membrane location of the v-Fgr protein. There is no actin domain in the TP1 v-Fgr protein, and in fact deletion of this region from the GR-FSV v-Fgr protein actually increases both tyrosine kinase activity and transformation.

In vitro Fgr has been shown to transform most mammalian fibroblasts. *In vivo* the GR-FSV causes fibrosarcomas and rhabdosarcomas upon infection of kittens. The c-Fgr protein is a 55 kDa protein tyrosine kinase that is most abundantly expressed in hematopoietic cells. c-*fgr* mRNA levels are increased up to 50-fold in Epstein–Barr virus-associated lymphoproliferative disease; however, viral infection does not readily lead to tumors of hematopoietic cells.

Fms

The *fms* oncogene is the transforming oncogene of the Susan McDonough (SM-FSV) and Hardy–Zuckerman 5 strains of feline sarcoma virus. C-Fms is a growth factor receptor tyrosine kinase that is activated by the ligand, macrophage colony-stimulating factor, CSF-1. V-Fms protein is initially synthesized as a Gag-Fms fusion protein in which the Gag sequences are rapidly removed upon cleavage at the Fms-signal peptide sequence. V-*fms* and c-*fms* encode mature glycoproteins of approximately 140 kDa in size that differ in a few scattered amino acids and at the C-terminus, where in the SM-FSV encoded protein the last 50 amino acids of c-Fms have been replaced by 14 residues that come from c-*fms* 3′ untranslated sequences. This substitution has removed a C-terminally-located tyrosine residue, which plays a role in activating the oncogenic potential of v-Fms. V-Fms is a constitutively activate tyrosine kinase and, of the scattered amino acid changes, the substitution of two serine residues in the extracellular domain seems to be particularly important in activating the tyrosine kinase. Membrane localization and tyrosine kinase activity are necessary for transformation. V-Fms-transformed cells contain similar tyrosine phosphorylated substrates to those seen following c-Fms activation, for example PI3-kinase. Interestingly, tyrosine residue 809 is involved in Myc gene expression and this seems to be key for mitogenesis induced by Fms.

In vivo SM-FSV causes fibrosarcomas and does not normally induce hematopoietic malignancies in spite of the fact that v-Fms is derived from the receptor for CSF-1; however *in vitro* v-fms can induce the growth factor-independent growth of CSF-1 dependent cell lines in addition to transforming fibroblast cell lines.

Kit

V-*kit* is the oncogene found in Hardy–Zuckerman 4 feline sarcoma virus, HZ4-FSV. V-Kit is derived from the tyrosine kinase growth factor receptor for stem cell factor, c-Kit. V-*kit* is expressed as a Gag-Kit fusion protein of 80 kDa. V-*kit* contains sequences encoding the tyrosine kinase domain from c-*kit*

(residues 558–925). The N-terminal sequences, which include the extracellular domain and the transmembrane domain, are deleted, and 50 amino acids from the C-terminus of c-Kit are replaced by five unrelated residues. Thus the v-Kit protein is not a transmembrane glycoprotein like its cellular homologue. However the $P80^{gag\text{-}kit}$ protein is myristylated, which suggest that it is membrane associated and it was shown to display a tyrosine-specific autophosphorylation activity.

Comparison of the v-Kit and c-Kit sequences revealed, in addition to the deletions mentioned above, three additional v-Kit mutations: deletion of Tyr569 and Val570, and the exchange of Asp at position 761 to Gly. Examinations of the consequences of the deletion of Tyr569 and Val570 revealed significant enhancement of transformation. Tyr568 and Tyr571 in c-Kit are a potential binding site for Src family members. Thus the repositioning of Y571 by this two-codon deletion has been postulated to play a role in the enhancement of v-Kit oncogenic activity.

In vitro v-Kit can transform the murine NIH3T3 fibroblast cell line. *In vivo* HZ4-FSV has been reported to cause fibrosarcomas in cats.

Ros

Ros is the transforming oncogene of the University of Rochester UR2, replication-defective avian sarcoma virus. It is derived from c-Ros, which is an orphan growth factor receptor that has similarity in overall structure to the drosophila sevenless protein. C-Ros is a large glycoprotein of 260 kDa. In contrast v-Ros is a 68 kDa protein that is expressed as a Gag-fusion protein. V-Ros contains the transmembrane and the tyrosine kinase domain of c-Ros, and has lost C-terminal sequences that have been replaced by 12 amino acids. In addition the transmembrane sequence in v-Ros contains a three amino acid insertion compared to c-Ros which seems to increase the transforming ability of v-Ros. This Gag-Ros fusion protein is a transmembrane protein in which the Gag moiety protrudes extracellularly. This Gag-Ros fusion protein is an active tyrosine kinase, is autophosphorylated and is found in a complex with phospholipase Cγ. In addition the mitogen-activated protein kinase pathway is activated in v-Ros transformed cells.

In vitro the UR2 virus can transform fibroblasts and *in vivo* cause fibrosarcomas.

Src

V-*src* is the transforming oncogene of the avian Rous Sarcoma virus, RSV. RSV is unique amongst acutely transforming retroviruses in being a replication competent virus in which the v-*src* sequence is located between the *env* gene and the 3′ end of the viral genome.

C-Src is a cytoplasmic protein tyrosine kinase and is the founding member of the Src superfamily of kinases, which includes Blk, Fgr, Fyn, Hck, Lck, Lyn, Src and Yes. All of these kinases have been implicated to some extent in oncogenesis and Src, Fgr and Yes have viral homologues. The cellular proteins are N-terminally myristylated and are membrane located. They contain a tyrosine kinase domain and Src-homology 2 and 3 domains (SH2 and SH3). In addition the cellular proteins have a C-terminal Tyr, which is important for regulation of the tyrosine kinase activity. In c-Src this tyrosine is Tyr527 and its phosphorylation *in vivo* inhibits the tyrosine kinase activity. In v-Src this tyrosine is deleted and thus v-Src has a constitutive tyrosine kinase activity which is essential for its transforming ability.

In vivo Src causes fibrosarcomas that are rapid and fatal in young birds. *In vitro* v-Src is capable of transforming several different cell types including fibroblasts, chondrocytes and hematopoietic cells. V-Src transformed cells have very high levels of tyrosine phosphorylation and over 30 different proteins are reported to be phosphorylated on tyrosine. The contributions to the transformed phenotype by these proteins are not always clear. However the activation of proteins in the MAP kinase pathway and cytoskeletal proteins clearly have roles in the uncontrolled growth and morphological changes associated with v-Src transformation.

Sea

V-*sea* is the transforming oncogene of the S13 avian erythroblastosis virus. The cellular c-Sea protein is a member of the hepatocyte growth factor (HGF) receptor family, which is comprised of Met, Ron and Sea. V-Sea is expressed as a fusion protein between the viral *env* gene and the cytoplasmic sequences from the c-*sea* gene. The Env-Sea fusion protein is initially synthesized as a precursor molecular weight of 155 kDa, which is then subject to proteolytic cleavage at the normal site in Env to give rise to a disulfide-linked complex comprising the Env protein gp85 and a gp37-Sea fusion protein called gp70. The Env protein sequences provide the extracellular domain and transmembrane domain and serve to oligomerize this complex. Oligomerization activates the tyrosine kinase activity. Oligomerization, plasma membrane localization and tyrosine kinase activity are all necessary for transformation by v-Sea.

The HGF family of receptors all have two tyrosine located near to the C-terminus of the receptor which are autophosphorylated and serve as a multifunctional binding site for SH2-domain signaling proteins. The v-Sea protein has retained this site and it has been shown to be essential for transformation. *In vitro* v-Sea can transform chicken and rat fibroblasts and also chicken erythroid progenitor cells. *In vivo* infection of young chicks by the S13 virus causes sarcomas, erythroblastosis and anemia.

Yes

V-*yes* is the transforming oncogene of the avian sarcoma viruses Y73 and Esh. The name derives from these two strains, *Y*73 and *Esh*. C-Yes is a member of the Src family of protein tyrosine kinases and encodes a 61 kDa protein. V-*yes* is expressed as Gag-Yes fusion proteins in both Y73 and Esh of molecular weights 90 kDa and 80 kDa respectively. In the Yes sequences in the Y73 virus the C-terminal eight amino acids of c-Yes are replaced by three amino acids, which are encoded by the avian leukemia virus *env* gene. This alteration changes the position and context of a tyrosine residue in $p61^{c\text{-}yes}$. In addition, there are the six amino differences between v-Yes and c-Yes sequences. Based on the importance of the C-terminal sequences in regulating Src protein kinase family members, it is thought that the differences at the C-terminus of v-Yes are important for the activation of the tyrosine kinase. The c-Yes protein is found associated with the plasma membrane and also with the cell cytoskeleton. V-Yes is more diffusely distributed and, in a similar manner to v-Src, is also found associated with adhesion plaques.

Analysis of proteins phosphorylated on tyrosine in v-Yes transformed cells has identified multiple substrates as in Src transformed cells. These substrates include proteins associated with the cytoskeleton proteins and those involved in adhesion, such as integrins, $p130^{cas}$, and focal adhesion kinase as well as PI3 kinase.

In vitro the Y73 and Esh viruses can transform chicken embryo fibroblasts and *in vivo* they cause fibrosarcomas.

Cytokine Receptors

Cytokine receptors do not possess intrinsic tyrosine kinase activity; however, upon ligand activation cytoplasmic tyrosine kinases are activated. Although this is a very large gene family, to date only one member has been found as a retrovirally encoded oncogene, and this is v-*mpl*.

Mpl

V-*mpl* is the transforming oncogene of the murine myeloproliferative virus, MPLV. C-Mpl has been recently identified as being the receptor for the cytokine, thrombopoietin, and plays a role in the growth and differentiation of cells into megakaryocytes. V-*mpl* encodes a transmembrane protein that is expressed as an Env-Mpl fusion gene and is a truncated form of the c-Mpl receptor. The v-Mpl protein contains 100 amino acids of Env followed by 184 amino acids derived from c-Mpl, which contains the transmembrane and cytoplasmic sequences.

In vivo MPLV causes an acute hematological disorder characterized by the rapid growth of cells from several lineages. In contrast to most acutely transforming retroviruses, MPLV does not transform fibroblasts but *in vitro* infection of bone marrow cells will lead to the rapid outgrowth of immortalized cells from several lineages.

Adapter Proteins

Recently a class of proteins has been identified that has no intrinsic biochemical activity but they serve as linker molecules to connect proteins in key signaling pathways. These proteins are known as adapter molecules and they possess structural motifs that allow for protein–protein interaction. They also frequently serve as targets for tyrosine protein kinases, and upon phosphorylation can act as scaffolding molecules that bind to several downstream effector molecules. Two such molecules have been identified in acute transforming retroviruses; these are the *cbl* and *crk* oncogenes.

Cbl

Cbl is the transforming oncogene of the murine Cas NS-1 virus. The c-Cbl protein is a cytoplasmic protein of approximately 120 kDa. V-Cbl is a Gag-fusion protein of 100 kDa, which contains only 355 amino acids from the N-terminus of c-Cbl. V-Cbl is found in the nucleus of transformed cells. C-Cbl is associated with the Grb2 adapter protein and is phosphorylated upon tyrosine residues following growth factor stimulation. C-Cbl is related to the *Caenorhabditis elegans* gene Sli-1, which is a negative regulator of growth factor signaling. The N-terminal region of c-Cbl contains a putative phosphotyrosine binding domain and a ring-finger motif. Cbl is thought to function as a scaffold protein and associates with several SH2 and SH3 domain-containing molecules, including the Crk adaptor family and Vav. The deletion of C-terminal sequences up to the ring-finger

motif, as found in v-Cbl, causes Cbl to become oncogenic. This deletion is suggested to generate a structural alteration, allowing the oncogenic forms of v-Cbl to displace wild-type c-Cbl from the receptor complex and in doing so to abrogate c-Cbl's negative regulatory function.

The Cas NS-1 murine retrovirus induces lymphomas and leukemias *in vivo*.

Crk

V-*crk* is the transforming oncogene of the avian sarcoma viruses, CT10 and ASV-1. C-Crk is an adapter protein of molecular weight 28 kDa that contains both an SH2 and two SH3 domains. Although the CT10 virus was isolated in the 1920s and the ASV-1 virus in 1983, the v-crk sequence in these viruses is virtually identical and is a Gag-fusion protein of 47 kDa. Although v-Crk does not possess a tyrosine kinase domain, fibroblasts transformed by v-Crk have greatly elevated levels of tyrosine phosphorylated proteins. This is thought to be due to the activation of a cellular tyrosine kinase by v-Crk and potentially also the stabilization of tyrosine phosphorylation upon association of tyrosine phosphorylated proteins with v-Crk. Abl is a candidate for a tyrosine kinase that may be regulated by v-Crk, as it forms a stable complex with v-Crk via the SH3 domain of v-Crk. Src-family members have also been implicated as being regulated by v-Crk. V-Crk is associated with several proteins in transformed cells. These include the proteins paxillin and p130Cas, which bind to Crk SH2 domain, and are thought to play roles in adhesion. The C3G protein, a guanine nucleotide exchange protein for Rap1, was shown to be bound to Crk SH3 domains. The SH3 domain of Crk also binds to Sos, and Eps15. The nature of the proteins bound to Crk implies that Crk influences the activities of effector proteins that are included in pathways involving cell morphology and growth.

V-Crk transforms fibroblasts *in vitro* and infection of birds with the AVS-1 and CT10 viruses causes fibrosarcomas.

Serine/Threonine Kinases

Akt

V-*akt* is the transforming oncogene of the acute transforming retrovirus AKT8 which was isolated from a spontaneous mouse T-cell lymphoma. V-*akt* has two human cellular homologues, AKT1 and AKT2, also known as protein kinase B. These genes encode protein kinase C-related serine/threonine kinases.

V-*akt* arose by recombination between MuLV and *c-akt*, 785 nucleotides downstream of the Gag ATG codon and 60 nucleotides upstream of the ATG codon in the 5′ untranslated region of the *c-akt* protooncogene. The 60 base pairs of 5′ *c-akt* noncoding sequence and the entire coding and 3′ untranslated regions are fused to viral Gag giving a 763 amino acid, 105 kDa fusion phosphoprotein of structure (p12, p15, Δp30)-X-*c-akt*, where X is 21 amino acids that result from the translation of the 60 *c-akt* 5′ noncoding nucleotides plus three additional nucleotides inserted at the junction between *Gag* and *c-akt*. The Akt protein sequence of v-Akt is identical to c-Akt. V-Akt and c-Akt contain a pleckstrin homology domain and a serine/threonine homology domain.

C-Akt kinase activity is positively regulated by phosphorylation at a Thr residue in the T-loop and Ser473 in the C-terminal regulatory domain. The PIP3-dependent kinase 1 (PDK1) is responsible for phosphorylation at Thr308. Currently the kinase that phosphorylates the Ser473 residue is unknown. C-Akt is activated by growth factors, including platelet-derived growth factor. Akt interacts directly, via its pleckstrin homology domain, with PI(3,4)P2 and PIP3, promoting a conformational change, a change in localization from the cytoplasm to the plasma membrane, as well as an increase in kinase activity. Although Akt is a serine/threonine kinase, part of its regulatory region is similar to the Src homology domain (SH2), a characteristic of cytoplasmic tyrosine kinases that function in protein–protein interactions. Possible c-Akt targets include glycogen synthase kinase 3 (GSK3), ribosomal protein S6 kinase $p70^{S6K}$ and the Bcl-2 family member BAD, phosphorylation of which makes it unable to inhibit the survival activity of proteins like Bcl-2.

Fusion of Gag sequences to Akt provides a myristylation site at the N-terminus of the v-Akt protein that mimics the pleckstrin homology domain, anchoring the protein to the membrane. Localization of v-Akt is unregulated as dependence on PIP3 formation is lost. As a result of its permanent presence at the plasma membrane, v-Akt kinase activity is unregulated.

Raf

V-*raf* is the transforming oncogene of the murine retrovirus 3611-MSV derived from the mouse *Raf-1* proto-oncogene. The avian homologue of *Raf-1*, v-*mil* (originally termed v-*mht*), occurs in the Mill Hill 2 (MH2) virus isolated from a spontaneous ovarian tumor in chickens.

The 3611-MSV genome structure is 5′ *gag*(Δp15, p12, Δp30)-v-*raf*-Δ*pol*-*env*-3′. The 5′ v-*raf* junction has 12 nucleotides identical to the 3′ end of mouse *Raf-1* exon 9 and differs from MuLV $p30^{gag}$ by only one nucleotide. At the 3′ end, eight nucleotides of v-*raf*, MuLV and mouse *Raf-1* exon 17 are identical. V-*raf* is a truncated version of *Raf-1* starting with a few nucleotides of exon 9 and ending with part of exon 17 coding for amino acids 326–648 from full length Raf-1. The N-terminal regulatory conserved regions 1 and 2 (CR1 and CR2) are deleted in v-*raf*. Therefore, the kinase domain (CR3) is joined to viral *gag* sequences. The resulting protein is cytosolic like its cellular counterpart, and is phosphorylated and myristylated. The v-*mil* oncogene of the MH2 virus begins in exon 7 and encodes the last 380 amino acids of chicken *Mil* (*Raf*) with 19 amino acid substitutions and one deletion. None of these mutations appear critical for transformation.

C-Raf is positively regulated by serine/tyrosine phosphorylation, which occurs mainly in response to mitogenic signals. Serine phosphorylation may also exert a negative effect, as phosphorylation of Ser357 and Ser359 in the ATP-binding domain of Raf-1 may inhibit Raf-1 activity. C-Raf is a serine/threonine kinase, which activates MAP kinase-kinase (MAPK-K), which in turn stimulates the mitogen-activated protein kinases ERK1 and ERK2. V-Raf has constitutive kinase activity and consequently MAPK-K, ERK1 and ERK2 are constitutively active in v-*raf* transformed cells. The ΔGag-v-Raf and ΔGag-v-Mil proteins have autophosphorylating protein kinase activity, which is essential for transforming activity. These oncoproteins *trans*-activate expression of genes driven by AP-1, ETS and NFκB binding motifs.

V-*raf* induces fibrosarcomas, erythroblastosis and occasionally erythroleukemia *in vivo*. Raf-induced tumors are more frequent in the hematopoeitic cell lineages, followed by pancreatic epithelium and connective tissues. Raf and Myc act synergistically to transform cells of all hematopoeitic lineages.

Ras

The v-H-*ras* and v-K-*ras* oncogenes, found in Harvey and Kirsten murine sarcoma virus (Ha-MuSV and Ki-MuSV), are derived from H-*ras* and K-*ras* cellular oncogenes, members of the Ras superfamily of transforming small G proteins. These viruses were isolated from rats infected with mouse leukemia viruses, Mo-MuLV and Ki-MuLV. Following the induction of leukemia, plasma from these animals was injected into BALB/c mice, which rapidly developed solid tumors. BALB-MuSV, AF-1 and Rasheed-MuSV viruses have also acquired Ras genes.

The viral genomes of Ha-MuSV (5.5 kb) and Ki-MuSV (6.5 kb) are composed of three types of nucleotide sequences: sequences homologous to MuLV (0.2 kb at the 5′ end and 1 kb at the 3′ end), sequence homologous to rat retrovirus-like 30S RNA, and the oncogene sequences which are 1.5 kb in Ha-MuSV and 1.75 kb in Ki-MuSV inserted towards the 5′ end of the 30S RNA.

V-H-*ras* and v-K-*ras* both encode full-length proteins with two and four point mutations, respectively. The v-H-Ras point mutations are Lys12 to Gly and Lys143 to Gln. The v-K-Ras point mutations are Gly12 to Ser, Glu37 to Gln, Ala59 to Thr, Ile100 to Leu. The v-H-*ras* and v-K-*ras* oncogenes encode a 189 amino acid protein, $p21^{ras}$. Ras proteins are modified by polyisoprenylation and palmitoylation, and as a consequence bind tightly to the inner surface of the plasma membrane. Ras-encoded proteins are GTPases that are activated by a variety of growth factors. Normal $p21^{ras}$ molecules hydrolyze GTP and exist in an equilibrium between active (GTP bound state) and inactive states (GDP bound). Two other proteins are involved in determining the state of the Ras proteins: GAPs (GTPase activating proteins), which catalyze the GDP bound state; and GEFs (guanine nucleotide exchange factor), which catalyze the release of bound GDP, thereby activating the protein. Three residues are critical for activating oncogenic potential: Gly12, Ala59 and Gln61. Gly12 and Ala59 are assumed to interfere with the action of the Gln61 side chain that forms a complex with the γ-phosphate in GTP. All inhibit GTP hydrolysis by diminishing GTPase activity or modulating the rate of nucleotide exchange (Ala59). Oncogenic mutations allow the GTP.$p21^{ras}$ complex to remain active, thereby stimulating cell growth. Ras activates a cascade of serine/threonine protein kinases that include c-Raf, MAP kinase kinase (MAPK-K) and extracellular signal-regulated kinases (ERKs or MAP kinases). Via this cascade v-Ras activates FOS, JUN and other AP1 components and therefore modulates transcription, which eventually results in DNA synthesis.

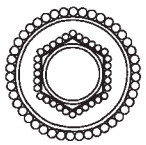
Injection of mice with Ha-MuSV and Ki-MuSV gave rise to sarcomas. It is important to note that activating mutations of Ras have been detected in a wide variety of human tumors. Normal fibroblasts are not transformed by v-Ras unless coinfected with viruses containing immortalizing oncogenes such as Myc. However, cell lines such as NIH3T3 can be transformed by v-Ras. Ras oncogenes can efficiently transform murine erythroid, myeloid and mast cells *in vitro*.

See also: **Transformation: Animal viruses.**

Further Reading

Glover D, Hall A and Hastie N (1994) *Cell Biology of Cancer*. Cambridge: Company of Biologists.
Hesketh R (1994) *The Oncogene Handbook*. London: Academic Press.
Hesketh R (1995) *The Oncogene Facts Book*. London: Academic Press.
Macdonald F and Ford CHJ (1997) *Molecular Biology of Cancer*. Oxford: BIOS Scientific.
Weinberg RA (1989) *Oncogenes and the Molecular Origins of Cancer*. New York: Cold Spring Harbor Laboratory Press.

RETROVIRUSES – TYPE D (*RETROVIRIDAE*)



Maja A Sommerfelt, Department of Microbiology and Immunology, Gades Institute, University of Bergen, Bergen, Norway

Eric Hunter, Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama, USA

History

The first type D retrovirus described was isolated in 1970 from a spontaneous mammary carcinoma of an 8-year-old rhesus macaque. This virus, named Mason Pfizer monkey virus (M-PMV), has since become the prototype for an enlarging family of both endogenous and exogenous viruses (**Table 1**). Although isolated from a mammary carcinoma, M-PMV is not oncogenic; instead, infected primates succumb to a severe and often fatal immunosuppressive disease, which is distinct from that caused by the simian immunodeficiency viruses (SIVs) that are lentiviruses. Simian acquired immune deficiency disease (SAIDS-D) was first defined in 1983 as a disease entity caused by type D viruses. The precise mechanism of type D virus-induced immune suppression is not known, but the syndrome is currently restricted to the genus *Macaca* (subfamily Cercopithecinae) of old world monkey.

Approximately 12 years after the discovery of M-PMV, several additional horizontally transmitted type D viruses were isolated. They were called simian AIDS D-type (SAIDS-D) viruses together with the primate center of origin (for example, the virus from the New England Primate Center was named SAIDS-D/NE; **Table 1**). Based on the observation that the type D viruses could be divided into distinct neutralization groups, a new nomenclature was devised to distinguish these viruses from each other and to differentiate them from the HIV-like AIDS-inducing SIV. Members of the group are now named simian retrovirus (SRV) followed by the serological type. Serotypes 1–3 have all been molecularly cloned and sequenced. SRV-2, isolated at the Washington and Oregon primate centers, in addition to causing immune suppression, is directly associated with a severe retroperitoneal fibromatosis (RF). SRV-1–5 correspond to the exogenous type D viruses. It is presently unclear whether SRVPc isolated from an Ethiopian baboon at the Washington primate center represents an accidental laboratory infection or a new serotype. Two endogenous viruses have been isolated, one endogenous to the New World squirrel monkey *Saimiri sciureus* (squirrel monkey retrovirus SMRV) and the other to the Old World spectacled langur *Presbytis obscurus* (PO-1-Lu). An endogenous virus considered to be ancestral to the exogenous type D retroviruses has been recently identified in baboon genomic DNA. There have been reports of type D viruses isolated from human permanent cells lines: Hep-2V and PMFV, but these appear to be contaminants as only certain cell stocks carried the virus. The host species of origin for the exogenous type D viruses has not yet been defined.

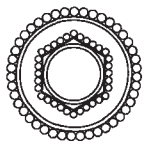
Taxonomy and Classification

Type D retroviruses, classified in the family *Retroviridae*, are members of the *Betaretrovirus* genus. These are positive-strand RNA viruses that replicate by reverse transcription to form a proviral DNA intermediate which can integrate covalently into the host chromosomal DNA. M-PMV has a morphogenesis similar to that of the type B mouse mammary tumor virus (MMTV), in that it preassembles immature capsids (referred to as intracytoplasmic A-type particles (ICAPs)) within the infected cell cytoplasm.

Further Reading

Glover D, Hall A and Hastie N (1994) *Cell Biology of Cancer*. Cambridge: Company of Biologists.
Hesketh R (1994) *The Oncogene Handbook*. London: Academic Press.
Hesketh R (1995) *The Oncogene Facts Book*. London: Academic Press.
Macdonald F and Ford CHJ (1997) *Molecular Biology of Cancer*. Oxford: BIOS Scientific.
Weinberg RA (1989) *Oncogenes and the Molecular Origins of Cancer*. New York: Cold Spring Harbor Laboratory Press.

RETROVIRUSES – TYPE D (*RETROVIRIDAE*)



Maja A Sommerfelt, Department of Microbiology and Immunology, Gades Institute, University of Bergen, Bergen, Norway

Eric Hunter, Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama, USA

History

The first type D retrovirus described was isolated in 1970 from a spontaneous mammary carcinoma of an 8-year-old rhesus macaque. This virus, named Mason Pfizer monkey virus (M-PMV), has since become the prototype for an enlarging family of both endogenous and exogenous viruses (**Table 1**). Although isolated from a mammary carcinoma, M-PMV is not oncogenic; instead, infected primates succumb to a severe and often fatal immunosuppressive disease, which is distinct from that caused by the simian immunodeficiency viruses (SIVs) that are lentiviruses. Simian acquired immune deficiency disease (SAIDS-D) was first defined in 1983 as a disease entity caused by type D viruses. The precise mechanism of type D virus-induced immune suppression is not known, but the syndrome is currently restricted to the genus *Macaca* (subfamily Cercopithecinae) of old world monkey.

Approximately 12 years after the discovery of M-PMV, several additional horizontally transmitted type D viruses were isolated. They were called simian AIDS D-type (SAIDS-D) viruses together with the primate center of origin (for example, the virus from the New England Primate Center was named SAIDS-D/NE; **Table 1**). Based on the observation that the type D viruses could be divided into distinct neutralization groups, a new nomenclature was devised to distinguish these viruses from each other and to differentiate them from the HIV-like AIDS-inducing SIV. Members of the group are now named simian retrovirus (SRV) followed by the serological type. Serotypes 1–3 have all been molecularly cloned and sequenced. SRV-2, isolated at the Washington and Oregon primate centers, in addition to causing immune suppression, is directly associated with a severe retroperitoneal fibromatosis (RF). SRV-1–5 correspond to the exogenous type D viruses. It is presently unclear whether SRVPc isolated from an Ethiopian baboon at the Washington primate center represents an accidental laboratory infection or a new serotype. Two endogenous viruses have been isolated, one endogenous to the New World squirrel monkey *Saimiri sciureus* (squirrel monkey retrovirus SMRV) and the other to the Old World spectacled langur *Presbytis obscurus* (PO-1-Lu). An endogenous virus considered to be ancestral to the exogenous type D retroviruses has been recently identified in baboon genomic DNA. There have been reports of type D viruses isolated from human permanent cells lines: Hep-2V and PMFV, but these appear to be contaminants as only certain cell stocks carried the virus. The host species of origin for the exogenous type D viruses has not yet been defined.

Taxonomy and Classification

Type D retroviruses, classified in the family *Retroviridae*, are members of the *Betaretrovirus* genus. These are positive-strand RNA viruses that replicate by reverse transcription to form a proviral DNA intermediate which can integrate covalently into the host chromosomal DNA. M-PMV has a morphogenesis similar to that of the type B mouse mammary tumor virus (MMTV), in that it preassembles immature capsids (referred to as intracytoplasmic A-type particles (ICAPs)) within the infected cell cytoplasm.

Table 1 Type D retroviruses

Virus	*Full name*	*Previous nomenclature*	*Accession number*[a]	*Exo/Endo*	*Initially isolated from*
SRV-1	Simian retrovirus type 1	SAIDS-D/CA, SAIDS-D/NE	M11841	Exo	*Macaca mulatta*
SRV-2C	Simian retrovirus type 2	SAIDS-D/OR	M16605	Exo	*Macaca nigra*
SRV-2R	Simian retrovirus type 2	SAIDS-D/OR		Exo	*Macaca mulatta*
SRV-2W	Simian retrovirus type 2	SAIDS-D/WA	L38695 (env)	Exo	*Macaca nemestrina*
SRV-3	Simian retrovirus type 3	Mason Pfizer monkey virus	M12349	Exo	*Macaca mulatta*
SRV-4	Simian retrovirus type 4	None		Exo	*Macaca fascicularis*
SRV-5	Simian retrovirus type 5	None		Exo	*Macaca mulatta*
SRVPc	Simian retrovirus Pc	None	U16843 (p27) U16844 (gp20)	Exo	*Papio cynocephalus*
PO-1-Lu	Endogenous langur virus	None	None	Endo	*Presbytis obscurus*
SMRV	Squirrel monkey retrovirus	None	M23385[b]	Endo	*Saimiri sciureus*
SERV	Simian endogenous retrovirus	None	U85505/U85506	Endo	*Papio cynocephalus*

Exo, exogenous; endo, endogenous; SAIDS-D, simian acquired immunodeficiency syndrome D; RF, retroperitoneal fibromatosis; CA, California; NE, New England; W, Washington; OR, Oregon regional primate centers.
[a] DNA sequences are stored at the EMBL Nucleotide Sequence Database, GenBank. Three subtypes of SRV-2 are shown. Accession numbers for two SERV clones are shown.
[b] The accession number for SMRV-H isolated from human cells is shown.

The morphology of the mature extracellular virion is different, in that it has a centrally located nucleoid similar to type C retroviruses and a much less dense fringe of glycoprotein than MMTV. Being of primate origin with these differences, a new morphological class of retrovirus was designated type D.

Properties of the Virion

Type D viruses assemble an immature capsid in the cytoplasm. These intracellular spherical particles, 60–95 nm in diameter, migrate to the plasma membrane, where they acquire, during release by budding, an envelope containing virus-encoded glycoproteins (**Fig. 1A, 1B**). These particles have a diameter of 100–120 nm. Following release, a proteolytic event termed 'maturation' results in a morphological change in the virion from an electron-lucent core to an electron-dense core (**Fig. 1C**). Surface projections corresponding to the viral glycoproteins can be seen in negatively stained preparations (**Fig. 1D, 1E, 1F**). Type D viruses exhibit a buoyant density of $1.17\,g\,ml^{-1}$ in sucrose, and $1.21\,g\,ml^{-1}$ in cesium chloride. The three-dimensional structure of the virion remains to be determined.

Properties of the Genome

The genome of type D retroviruses that have been sequenced to date is composed of two identical positive-sense, single-stranded RNAs of approximately 8 kb in length (7943 nt for M-PMV). The RNA molecules are proposed to be hydrogen-bonded to each other and to a host $tRNA^{lys}$. This diploid structure of about 5.3×10^6 Da (70 S) is denatured by heat (80°C for 2.5 min) or 40% formamide to 2.65×10^6 Da (35 S). Like eucaryotic mRNA, the retroviral RNA genome is capped at the 5′ end with a 7-methyl GTP, polyadenylated at the 3′ end and internally methylated on scattered adenosine residues.

As with any replication-competent retrovirus genome, coding regions of type D viruses are flanked at both ends by 5′- and 3′-terminal sequences that are important for virus replication and regulation of gene expression. The 5′ sequences include a binding site for a host $tRNA^{lys}$ which serves as a primer for the synthesis of negative-strand DNA by the viral reverse transcriptase enzyme, and an untranslated region preceding the coding regions. The RNA splice donor-site (AAGUAAGU) for subgenomic mRNAs is located 21 nucleotides upstream of the *gag* AUG for M-PMV. A packaging signal sequence for encapsidation of genomic RNAs into virions is also present. The 3′-terminal sequences contain at least two transcriptional elements: the AUUAAA signal sequence for poly(A) addition, and the UAUAUAAG sequence corresponding to the TATA box as promoter for viral RNA synthesis.

Properties of the Proteins

Four genes, arranged as 5′-*gag-pro-pol-env*-3′, encode type D virus proteins. The most detailed information is available for the prototype virus M-PMV. Viral

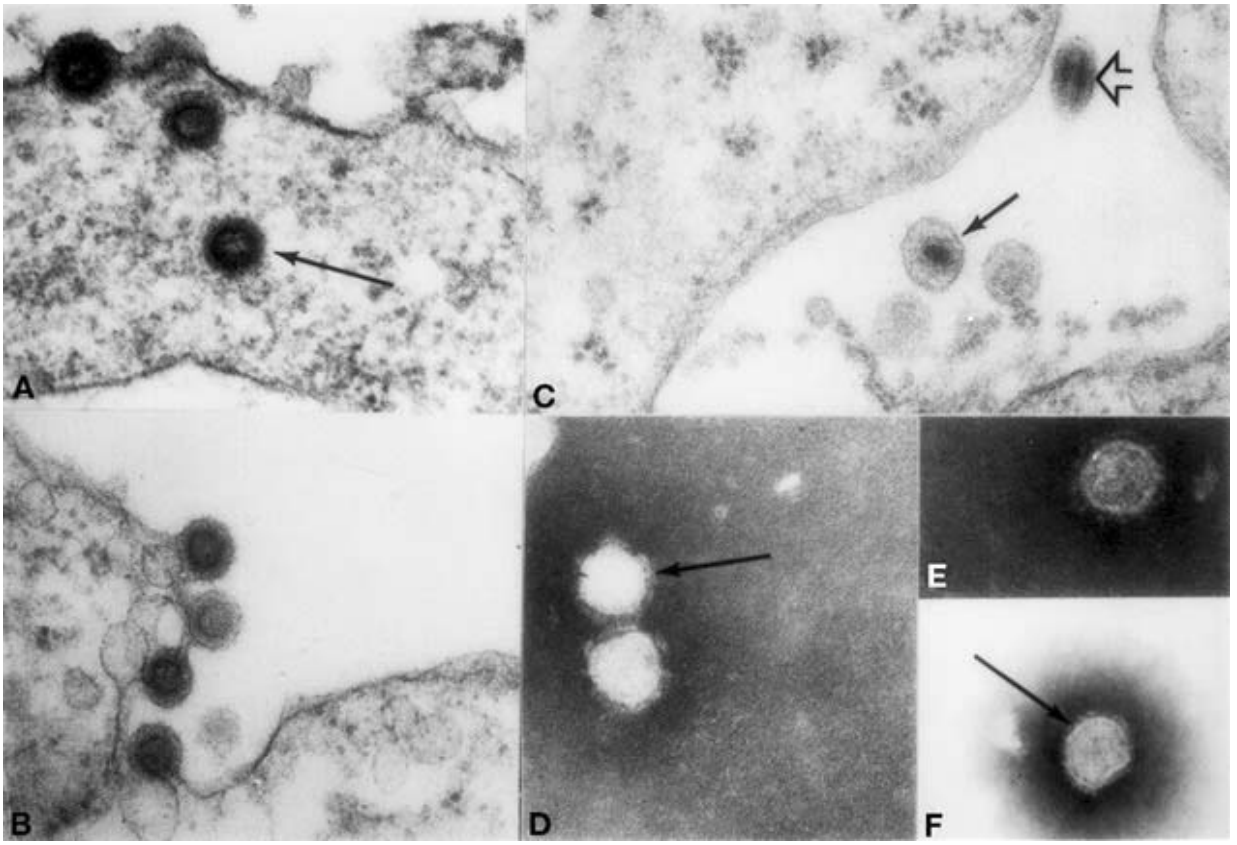


Figure 1 Electron micrographs of M-PMV-infected cells and M-PMV virions. (**A**) Thin section of an M-PMV-infected cell showing immature capsids deep in the cytoplasm (arrow) and in process of budding. (**B**) Immature capsids at a late stage of budding. (**C**) Mature virions showing electron-dense core (arrow) and section through cylindrical core (open arrow). (**D–F**) Negatively-stained virions showing glycoprotein surface projections (arrows).

genes are expressed as polyprotein precursors: Pr78 is encoded by *gag*, and Pr95 by *gag-pro* through a −1 frameshift event at the end of *gag*. The *pol* gene is similarly expressed as a Pr180 *gag-pro-pol* product that is generated from two −1 frameshifts, one at the end of *gag* and a second at the end of *pro*. The envelope glycoprotein is translated from a spliced mRNA as a Pr86 precursor. This polyprotein is comprised of the surface (SU) glycoprotein gp70 that interacts with receptors on susceptible cells, and the transmembrane (TM) glycoprotein gp22, which spans the viral membrane and catalyzes the process of membrane fusion. During virus maturation the gp22 is cleaved to gp20 by the viral protease, a process which appears to activate the fusogenic potential of the glycoprotein complex.

Immature intracytoplasmic A-type particles are composed of Pr78, Pr95 and Pr180 in an approximate 80:15:5% ratio. Expression of the precursor proteins in an *in vitro* translation system results in assembly of these immature capsids in a process that is dependent on ATP hydrolysis. During maturation, the Pr78 precursor is cleaved, by the virus-encoded protease, to yield six internal structural proteins in the order NH_2-p10(MA)-pp24/16-p12-p27(CA)-p14(NC)-p4-COOH (**Fig. 2**). The 10 kDa matrix protein (MA) forms the envelope-associated outer shell of the mature particle and is modified with myristic acid. The 27 kDa polypeptide capsid protein (CA) is the major structural component of the mature capsid, while the 14 kDa nucleic acid binding protein (NC) presumably functions in genomic RNA packaging and dimer formation. The exact functions of the 12 kDa (p12), 4 kDa (p4) proteins and the phosphorylated 24/16 protein remain to be determined. However, the p12 domain of Pr78 is critical for immature capsid assembly both in infected cells and *in vitro*, and a proline-proline-proline-tyrosine (PPPY) motif in pp16/24 is critical for the final stage of virus budding from the cell. SMRV particles contain a major capsid protein p35, which is likely to represent a fusion protein of p12 and p27.

The protease gene of M-PMV could potentially encode a protein of 314 amino acids, fused to the Gag

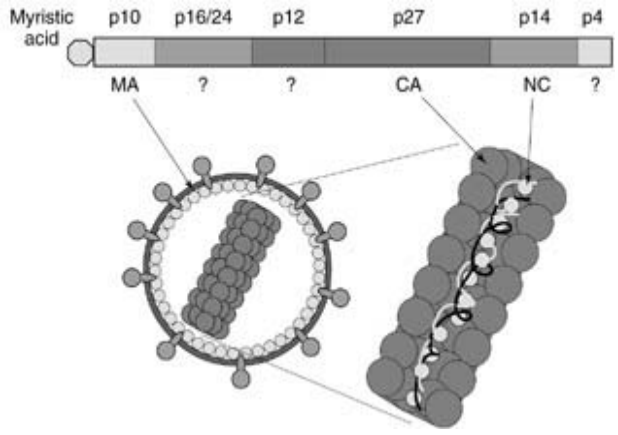


Figure 2 M-PMV *gag* gene-encoded precursor showing the organization of the mature products and their location within the mature virion. A section through the core shows the association of the NC protein with the diploid RNA genome.

precursor that is truncated at the frameshifting site. This *pro*-encoded region is cleaved by the viral protease to two proteins of 17 kDa; the N-terminal protein has dUTPase activity, while the C-terminal portion has protease activity and can be further cleaved to a 12 kDa protein with protease activity. The protease is an aspartyl protease, which remains inert while it is part of the Pr95 and Pr180 precursors within the cell. It is activated by an unknown mechanism following virus release. The importance of dUTPase activity in the virus life cycle is unknown.

The reverse transcriptase enzyme (80–90 kDa) for all the type D retroviruses has a divalent cation preference for magnesium in contrast to manganese, which is utilized by many of the nonhuman mammalian type C viruses. This enzyme also has RNase H and integrase activity.

Physical Properties of the Virion

Type D virions are rapidly inactivated when exposed to high temperature (56°C for 30 min) and are also sensitive to lipid solvents and detergents. The mature virions of M-PMV are easily disrupted in mild detergent concentration (0.5% Triton X-100), whereas immature intracytoplasmic capsid particles remain stable under these conditions. Recent evidence from cryoelectron microscopy indicates that the immature capsids, despite their assembly in the absence of membranes, are not icosahedral and exhibit a broad distribution of sizes.

Although retroviruses are quite resistant to UV light or gamma irradiation, UV irradiation of M-PMV results in inhibition of virus-induced syncytial formation in rhesus monkey embryonic lung cells.

Type D viruses are composed of approximately 60–70% protein, 30–40% lipid, 2–4% carbohydrate and 1% nucleic acid by weight.

Strategy of replication of the nucleic acid

In common with other retroviruses, the type D retroviral RNA genome is reverse transcribed within the infected cytoplasm to form a double-stranded DNA intermediate. The nascent DNA migrates to the nucleus and integrates covalently into the host chromosomal DNA, where it exists as a provirus. Transcription of the provirus results in messenger RNA that will function to produce virus-specific polyprotein precursors, and also genomic RNA that will be packaged into assembling virions.

Characterization of Transcription

Cellular RNA polymerase II transcribes the integrated type D provirus genome into RNA. In the cytoplasm of infected cells, two classes of viral RNA transcripts are detected. A genomic-sized mRNA (8 kb), which is either translated into polyprotein precursors or encapsidated into progeny virus particles, and a 3 kb mRNA representing subgenomic-spliced mRNA which is translated into Env polyproteins. Transport of the unspliced genomic RNA from the nucleus to the cytoplasm is dependent on the presence of a short (~200 nucleotide) element called the constitutive transport element (CTE) that is located between the *env*-gene and the 3′ long terminal repeat (LTR). This element forms a hairpin structure that is presumably recognized by a host protein(s) capable of diverting the genome-length RNA from the splicing machinery and out of the nucleus.

Characterization of Translation

Genome-length RNAs are translated on free polysomes into the Gag polyprotein and, as a result of ribosomal frameshifting, into the Gag-Pro and Gag-Pro-Pol polyproteins. The heptanucleotide sequence motifs for the −1 frameshift have been localized in both M-PMV and SRV-1 genomes: GGGAAAC for Gag-Pro fusion and AAAUUUU for Pro-Pol fusion. Due to the frequency of frameshifting, the majority of precursor proteins are Gag polyproteins, approximately 15% are Gag-Pro polyproteins and around 5% are Gag-Pro-Pol polyproteins.

The spliced subgenomic *env* mRNA is translated on polysomes associated with the endoplasmic reticulum. The cotranslationally glycosylated product is transported via the secretory pathway of the cell to

the plasma membrane, where it is anchored and oriented as a type I glycoprotein.

Post-translational Processing

Cellular enzymes post-translationally modify the Gag-containing polyproteins. The N-terminal methionine residue is cotranslationally removed and myristic acid is added to the new N-terminal glycine residue. The polyproteins are also phosphorylated following translation, but the significance of this modification is not clear.

Following entry into the endoplasmic reticulum of the cell, Env polyproteins form an oligomeric structure (estimated to be a trimer), which is the transport-competent form of the precursor. During transport to the cell surface through the secretory pathway, core oligosaccharide side chains on the SU domain of M-PMV Env polyproteins are processed to complex oligosaccharide chains. The single high-mannose oligosaccharide chain added to the TM domain during translation is maintained in an unmodified form. In a late Golgi compartment the Pr86 polyprotein is cleaved into the two mature glycoproteins, gp70(SU) and gp22(TM), by a furin-like cellular endopeptidase. The gp22 is further processed, by a C-terminal cleavage event, to gp20 during virus maturation by the viral aspartyl protease.

Assembly Site, Release and Cytopathology

Type D viruses are characterized by the assembly of immature intracytoplasmic A type particles (ICAPs). These are assembled from uncleaved precursor proteins and migrate to the plasma membrane, where they are released by budding. They acquire an outer membrane, which is derived from the host cell plasma membrane and contains virus-specific envelope glycoproteins, during this process. The assembly of immature capsids appears to occur at a site in the cytoplasm where precursors congregate in sufficient quantities to allow self-assembly. A signal, which directs the proteins to the intracytoplasmic assembly site, has been identified in the MA protein of M-PMV. Immature capsids appear to contain a full complement of genomic RNA. As with other retroviruses, RNA packaging requires a sequence (psi) found within the 5′ end of genomic RNA. This region has been shown to fold into a specific secondary structure that is presumably recognized by the Gag precursor protein during capsid assembly.

Although the pathway of intracytoplasmic transport has not been defined, the transport of immature capsids to the membrane requires a specific protein-mediated targeting process, in addition to the presence of myristic acid, which is also required for intracellular transport. During the budding process, immature capsids associate with the plasma membrane, presumably via the MA protein, and are extruded from the cell. It has been postulated for M-PMV that there is an interaction between the cytoplasmic domain of the TM glycoprotein, gp22, and the matrix protein, which directs incorporation of envelope glycoproteins into the virion. As with other retroviruses, the viral glycoproteins are not essential for virion release but are required for virus infectivity.

Other Subjects Relevant to Virus Group

Type D viruses are capable of inducing cell fusion *in vitro*. This is mediated by the interaction of virus envelope glycoproteins expressed at the cell surface with available receptors present on adjacent uninfected cells and has provided a method for titrating the virus in culture.

Geographic Distribution

The primates that are infected with exogenous type D viruses primarily comprise the *Macaca* (Cercopithecinae) genus of Old World monkey which naturally inhabit India and Southeast Asia (**Table 2**). Studies of seropositivity to type D retroviruses in feral macaques is limited. At present the disease spectrum of this virus group has mainly been associated with primate research centers in the USA, although seropositivity to type D infection in imported or wild caught monkeys has been noted in primate centers in Europe and China. The spectacled langur, which harbors the endogenous virus PO-1-Lu, inhabits India and Southeast Asia and the squirrel monkey, harboring SMRV, inhabits central and southern America.

Host Range and Virus Propagation

Viruses enter cells following attachment to specific cell surface receptors. These viruses enter cells by a pH-independent pathway, suggesting that they penetrate cells at the plasma membrane. Type D virus receptors appear to be constitutively expressed on human cells. The same receptor moiety appears to be used by all the type D viruses, as well as two related mammalian type C viruses, RD114 (an endogenous virus of cats) and baboon endogenous virus (BaEV), as well as the type C avian reticuloendotheliosis viruses (REV). Viruses within this group exhibit receptor interference where cells chronically infected with one virus will be resistant to superinfection by a second in the group. The receptor gene has been

Table 2 Primates from which exogenous SRVs have been isolated

Genus and species	*Common name*	*Primate center*
Macaca mulatta	Rhesus macaque	California, New England
Macaca arctoides	Stumptail macaque	California
Macaca cyclopsis	Formosan/Taiwanese rock macaque	New England
Macaca fascicularis	Crab-eating macaque	New England, Washington
Macaca nemestrina	Pigtail macaque	Washington
Macaca fuscata	Japanese macaque	Washington
Macaca radiata	Bonnet macaque	New England, California
Macaca nigra	Celebese macaque	Oregon
Macaca assamensis[a]	Assamese macaque	China
Papio cunocephales	Ethiopian baboon	Washington

[a] Seropositivity confirmed by western blotting, no reported virus isolation.

assigned to human chromosome 19q13.1–13.3. The distinct neutralization specificities suggest that the type D retroviruses utilize distinct epitopes of the same cell surface receptor. Exploiting the neutralization epitope of SRV-2, a molecule of 60 kDa has been identified as a putative receptor on Raji cells. Further work is required to confirm a receptor function for this molecule. Type D viruses are not considered to be cytopathic; instead, they integrate into the cell genome and persist as a chronic infection where the cells do not die but continuously shed virus.

The *in vivo* host range of SRV-1–5 is broad, infecting many tissue types, including lymph nodes, salivary gland, spleen, thymus and brain. There have been reports that the SRVs may remain latent in the brain.

The *in vitro* host range of SRV-1–5 is extensive and includes a variety of human lymphoblastoid (H9, Raji) and nonlymphoblastoid cell lines (HeLa and HOS). Mink, chimp lung, African green monkey (COS), horse epithelial and dog thymus cells are also susceptible. Cells that appear to be resistant to productive infection include hamster, rat and mouse.

The endogenous viruses have a more restricted *in vitro* host range. PO-1-Lu infects only human and bat lung cells. It cannot infect langur cells, indicating that it has a xenotropic host range typical of endogenous viruses. SMRV has a broader xenotropic host range infecting cells of dog, chimp, mink, rhesus, human, marmoset, owl monkey and howler monkey, but not baboon. Simian endogenous retrovirus (SERV) has only been studied as cloned DNA. It is present in all *Papio* and *Cercopithec* species, but genomic DNA from African or Asian members of the Colobinae has not yet been analyzed. As no infectious SERV has been isolated, the *in vitro* and *in vivo* host range of this member remains to be determined.

Genetics

The type D retroviruses, in common with other retroviruses, contain two identical RNA genomes; this diploidy allows for genetic recombination.

SRV-1, -2 and -3 have been fully sequenced and show a high degree of both nucleotide and amino acid identity. They have slightly different restriction endonuclease patterns but are uniform within each group. The difference between the SRV genomes probably results from basepair mutations over time rather than large recombinational events. The exogenous SRVs have been described as variants of M-PMV. The major differences are found in regions encompassing the Gag phosphoproteins, the p12 proteins and the envelope glycoproteins. SRV-1 and SRV-3 (M-PMV) appear to be more related than SRV-2, which is also associated with RF.

The viral genome is comprised of four genes in the order *5′-gag-pro-pol-env-3′*, where *gag* encodes the structural components of the capsid, *pro* encodes the viral aspartyl protease, *pol* encodes the polymerase enzyme (reverse transcriptase) and integrase and *env* encodes the surface envelope glycoproteins. The type D retroviruses have neither regulatory genes, found in the immunosuppressive primate lentiviruses, nor oncogenes, found in members of the avian and mammalian type C retroviruses.

Evolution

The type D viruses such as M-PMV exhibit immunological crossreactivity in their envelope glycoproteins to two related endogenous type C retroviruses, namely baboon endogenous retrovirus (BaEV) and RD114. In contrast, the Gag and Pol proteins of the type D viruses are unrelated to these type C viruses.

The genomic organization, reverse transcriptase and Gag proteins of type D viruses are related to those of the B-type MMTV and the intracisternal A-type particles (IAPs). The type D viruses may therefore have arisen as a recombinational event in which the *env* gene was acquired from a virus ancestral to the C-type BaEV, and the capsid and reverse transcriptase genes were derived from a retrovirus ancestral to MMTV or the IAPs.

Early hybridization studies with tissues of different monkey species showed that M-PMV and SRV-1 DNA hybridized more readily to langur DNA (in the subfamily Colobinae) than to rhesus DNA, suggesting that M-PMV may have been derived from a virus similar to that endogenous in the langur monkey. Recently an endogenous type D retrovirus (SERV) genome was isolated from a baboon (*Papio cynocephalus*) genomic library. DNA sequence analysis showed that the *gag*, *pro*, *pol* and *env* (gp20 region) genes were similar to SRV-1, -2 and -3, whereas the gp70-encoding region was more closely related to that of BaEV. No analysis of langur DNA for SERV sequences has yet been undertaken.

The Jaagsiekte sheep retrovirus (JSRV) associated with ovine pulmonary carcinoma (OPC) of sheep, shows both antigenic and proviral DNA sequence similarity to M-PMV in the *gag-pro-pol* genes, while the *env* gene is similar to the prototype type B retrovirus MMTV and may represent a novel recombinant.

The presence of type D retroviral sequences in both Old and New World primates clearly shows that these viruses are ancient. Tracing their evolution is complicated by their potential for extensive recombination and zoonoses.

Serologic Relationships and Variability

There are five different serotypes, each with distinct neutralization epitopes, among the exogenous SRVs. These neutralization epitopes lie in the external envelope glycoproteins, which may show variability because of adaptation to different macaque species. The viruses can also be distinguished using antibodies to the smaller Gag proteins, p10 and p12. In addition to these type-specific determinants, the type D retroviruses demonstrate a high level of antigenic cross-reactivity within the major capsid protein. The type D envelope glycoproteins appear conserved for each serotype and do not show the same degree or kinetics of variation as the human and simian immunodeficiency viruses (HIV/SIV) and SIV. Minor variations are present in each serotype depending on the host species of isolation. Studies addressing sequence variability following infection have not been undertaken.

Epidemiology

Type D retroviruses have been targeted in the development and maintenance of specific-pathogen-free (SPF) colonies of rhesus macaques. Seroepidemiological surveys have shown that up to 20% of captive macaques at US primate centers and commercial suppliers are infected with SRV. Seropositivity to type D retroviruses has also been reported in China in wild-caught macaques (**Table 2**). Since certain infected macaques fail to make detectable antibody, or exhibit a long interval between infection and seroconversion, serologic testing alone is not sufficient for epidemiological studies. The polymerase chain reaction (PCR) provides a rapid means of detecting type D proviral DNA. However, PCR is limited to the detection of viruses for which DNA sequence information is available. Type D retrovirus infection is undoubtedly widespread, although its distribution in primate holding facilities worldwide, in zoos or in the wild has not been fully investigated.

Transmission and Tissue Tropism

Transmission of the exogenous SRVs requires close physical contact and is probably spread by saliva and blood through biting, grooming and fighting – the way such primates establish a social hierarchy. Unlike HIV, SRV transmission does not appear to be via sexual contact. High titers of virus can be found in infected macaque saliva, allowing entry to the bloodstream of a recipient individual following trauma.

The virus can be experimentally transmitted by inoculation of primates, as was done to prove the disease association of these viruses. The *in vivo* tissue tropism shows a broad host range for both lymphoid and nonlymphoid organs. Perinatal transmission and *in utero* transmission of the exogenous type D viruses appears to be infrequent. SRV can be recovered from peripheral blood lymphocytes, saliva, urine, feces and breast milk of viremic animals.

Transmission of the endogenous viruses occurs vertically as inherited genetic elements.

Pathogenicity

SAIDS-D is a naturally occurring, experimentally reproducible infection, where infected rhesus monkeys show a broad spectrum of clinical and pathological abnormalities (**Table 3**). Following infection of juvenile rhesus macaques, the animals may die within 1 year. The course of infection can result in fulminating viremia and death, a relatively mild acute

Table 3 Clinical features of SAIDS-D infection

Immune deficiency
Chronic wasting/ weight loss (>10%)
Persistent diarrhea unresponsive to appropriate therapy
Splenomegaly
Neutropenia
Lymphopenia or histologic lymphoid depletion
Abnormal peripheral blood lymphocytes
Mesenchymal proliferative disorders
Noma
Increased incidence of tumours
Opportunistic infections including:
Disseminated cytomegalovirus infection
Bacterial pneumonia
Progressive generalized lymphadenopathy (PGL)
Anemia
Bone marrow hyperplasia

phase of disease resulting in a chronic carrier state, or recovery from both viremia and latent infection.

The endogenous viruses are not associated with any pathogenic effects in their hosts.

Clinical Features of Infection

Infection of macaques with all of the SRVs isolated to date is associated with a severe and often fatal immune suppression (**Table 3**.) There is a decrease in both T and B cell populations and a low response of peripheral blood lymphocytes to mitogens, together with an increased incidence of tumors. Virus can be isolated from persistently infected animals from saliva, peripheral lymph nodes, plasma, peripheral blood leukocytes and exfoliated cells in milk.

One feature unique to SRV-2 is that this virus is also associated with retroperitoneal fibromatosis, which has been compared to Kaposi's sarcoma in humans. However, RF tissue is infected with SRV-2, unlike Kaposi's sarcoma and HIV. Particularly prevalent in Celebes and pigtailed macaques, RF is a highly vascular mesenchymal proliferative lesion that commonly originates in the subserosa of the intestine at the ileocecal junction and which can aggressively spread throughout the abdominal and thoracic cavity. A cutaneous form of RF has also been recognized in a small number of animals; immunohistochemical studies show a factor VIII-related antigen in endothelial cells and scattered fibroblast-like cells throughout the RF lesions, similar to those described for human Kaposi's sarcoma. RF is restricted to the Oregon and Washington regional primate centers.

Pathology and Histopathology

Typical type D virus particles can be found in the salivary gland acinar cells (secretory cells), mucosal epithelial cells, macrophages, lymph nodes, Langerhans cells, peripheral blood lymphocytes, thymus and spleen, but not in muscle or brain. Viral nucleic acid can be found in brain parenchyma, suggesting that the virus may remain latent in this organ. Neuropathy, however, is not a characteristic of SAIDS-D. Brain cells may thus present a post-transcriptional block to SRV replication. The tissue localization of SRV-1 is also dependent on the severity of the disease. In severe SAIDS-D, virus is present in a higher percentage of cells of the salivary gland, as well as in the sinusoidal cells lining the spleen, and the stellate cells of the thymus. If the primate presents with persistent generalized lymphadenopathy or splenomegaly, virus antigen is not as prevalent, being limited to germinal centers of lymphoid organs and scattered salivary gland acinar cells. There have been reports that some virus may also be found in other secretory cells, e.g. sweat glands, mammary glands and pancreatic acinar cells.

The histology of RF associated with SRV-2 infection reveals thymic atrophy and follicular and paracortical atrophy of the lymph nodes, as well as variable myeloid and lymphoid hyperplasia in the bone marrow.

Immune Response

Infection usually results in an overall decline in both B and T lymphocytes. Unlike in human AIDS, hypergammaglobulinemia is not a feature of SAIDS-D. Impairment of B cell function has been demonstrated by a diminished antibody response to antigenic stimulation, *in vivo* and *in vitro*, and progressively decreasing levels in the serum of all immunoglobulin subclasses. Secondary immune responses may be impaired with no switch from IgM to IgG. Complement C3 and C4 levels remain intact or are slightly elevated through the course of the disease. Antibodies are mounted predominantly to the Gag antigens. Resistance to type D infection corresponds to the presence of high levels of neutralizing antibodies directed against the envelope glycoproteins.

Prevention and Control

Effective vaccines have been generated against SRV-1, SRV-2 and SRV-3. Juvenile macaques inoculated with formalin-inactivated SRV-1 generate neutralizing antibodies that are protective upon challenge. Recombinant vaccinia virus expressing the envelope glycoproteins of SRV-1, SRV-2 and SRV-3 protect

against the respective virus challenge, even years later, demonstrating that the envelope glycoproteins alone are sufficient to elicit a protective immune response. These vaccines have limited crossprotection: SRV-3 vaccinated animals were protected from SRV-1 challenge but no crossimmunity has been observed between the SRV-1 and SRV-2 serotypes. Vaccines are useful for limiting the spread of SRV infection. Control of infection *in vitro* has also been successful using zidovudine (AZT) or 9-(2-phosphonylmethoxyethyl)adenine (PMEA), which inhibit the reverse transcriptase, as well as inhibitors of the HIV proteinase.

Future Perspectives

Although type D virus-induced immunosuppression has been a problem at primate centers in the USA, effective screening measures are possible to identify infected primates. Polyvalent vaccines may eventually be generated to protect primates from all the different serotypes identified to date. A report of the isolation of a type D virus, highly related to M-PMV, from an AIDS patient suffering from lymphoma raised the possibility of human infections by type D viruses; however, human seropositivity to type D antigens remains controversial. Recently, a DNA sequence of 932 bp with similarity to type B/D retroviruses was identified in patients with Sjögren's syndrome. This sequence appears to represent an acquired genome that has been provisionally called human retrovirus 5. Further research is required to establish the significance of this finding.

See also: **Feline immunodeficiency virus (*Retroviridae*); Human immunodeficiency viruses (*Retroviridae*): Molecular biology, Anti-retroviral agents, General features; Mouse mammary tumor virus (*Retroviridae*); Simian immunodeficiency viruses (*Retroviridae*).**

Further Reading

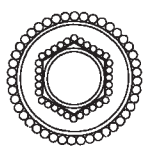
Coffin JM, Hughes SH and Varmus HE (1997) *Retroviruses*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Fine D and Schochetman G (1978) Type D primate retroviruses: a review. *Cancer Res.* 38: 3123.

Hunter E (1994) Macromolecular interactions in the assembly of HIV and other retroviruses. *Semin. Virol.* 5: 71.

Weldon R Jr and Hunter E (1997) Molecular requirements for retrovirus assembly. In: Chiu W, Burnett RM and Garcea R (eds) *Structural Biology of Viruses*, p. 381. New York: Oxford University Press.

RETROVIRUSES OF DROSOPHILA: THE GYPSY PARADIGM



Alain Bucheton, **Alain Pélisson** and **Christophe Terzian**, Institut de Génétique Humaine, CNRS, Gif-sur-Yvette, Montpellier Cedex 5, France

History, Taxonomy and Classification

The *Drosophila melanogaster gypsy virus* has been recently classified in the family *Metaviridae* of the genus *Errantivirus*. However, in context of the earlier classification, this virus is referred to as a retrovirus. Gypsy was first described as a transposable element present in the genome of *Drosophila melanogaster*. Although its structure is very much similar to that of vertebrate retroviruses (see below and **Fig. 1**), it was considered as a long terminal repeat (LTR)-containing retrotransposon. Infectious properties were demonstrated in 1994.

Control of Gypsy by the Host Genome

The transposition and infective properties of Gypsy are controlled by a host gene called *flamenco*. This gene is located on the X chromosome. Restrictive *flamenco* alleles repress Gypsy transposition and infectivity; permissive alleles allow high rates of

against the respective virus challenge, even years later, demonstrating that the envelope glycoproteins alone are sufficient to elicit a protective immune response. These vaccines have limited crossprotection: SRV-3 vaccinated animals were protected from SRV-1 challenge but no crossimmunity has been observed between the SRV-1 and SRV-2 serotypes. Vaccines are useful for limiting the spread of SRV infection. Control of infection *in vitro* has also been successful using zidovudine (AZT) or 9-(2-phosphonylmethoxyethyl)adenine (PMEA), which inhibit the reverse transcriptase, as well as inhibitors of the HIV proteinase.

Future Perspectives

Although type D virus-induced immunosuppression has been a problem at primate centers in the USA, effective screening measures are possible to identify infected primates. Polyvalent vaccines may eventually be generated to protect primates from all the different serotypes identified to date. A report of the isolation of a type D virus, highly related to M-PMV, from an AIDS patient suffering from lymphoma raised the possibility of human infections by type D viruses; however, human seropositivity to type D antigens remains controversial. Recently, a DNA sequence of 932 bp with similarity to type B/D retroviruses was identified in patients with Sjögren's syndrome. This sequence appears to represent an acquired genome that has been provisionally called human retrovirus 5. Further research is required to establish the significance of this finding.

See also: **Feline immunodeficiency virus (*Retroviridae*); Human immunodeficiency viruses (*Retroviridae*): Molecular biology, Anti-retroviral agents, General features; Mouse mammary tumor virus (*Retroviridae*); Simian immunodeficiency viruses (*Retroviridae*).**

Further Reading

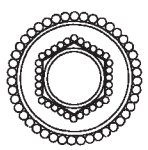
Coffin JM, Hughes SH and Varmus HE (1997) *Retroviruses*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Fine D and Schochetman G (1978) Type D primate retroviruses: a review. *Cancer Res.* 38: 3123.

Hunter E (1994) Macromolecular interactions in the assembly of HIV and other retroviruses. *Semin. Virol.* 5: 71.

Weldon R Jr and Hunter E (1997) Molecular requirements for retrovirus assembly. In: Chiu W, Burnett RM and Garcea R (eds) *Structural Biology of Viruses*, p. 381. New York: Oxford University Press.

RETROVIRUSES OF DROSOPHILA: THE GYPSY PARADIGM



Alain Bucheton, **Alain Pélisson** and **Christophe Terzian**, Institut de Génétique Humaine, CNRS, Gif-sur-Yvette, Montpellier Cedex 5, France

History, Taxonomy and Classification

The *Drosophila melanogaster gypsy virus* has been recently classified in the family *Metaviridae* of the genus *Errantivirus*. However, in context of the earlier classification, this virus is referred to as a retrovirus. Gypsy was first described as a transposable element present in the genome of *Drosophila melanogaster*. Although its structure is very much similar to that of vertebrate retroviruses (see below and **Fig. 1**), it was considered as a long terminal repeat (LTR)-containing retrotransposon. Infectious properties were demonstrated in 1994.

Control of Gypsy by the Host Genome

The transposition and infective properties of Gypsy are controlled by a host gene called *flamenco*. This gene is located on the X chromosome. Restrictive *flamenco* alleles repress Gypsy transposition and infectivity; permissive alleles allow high rates of

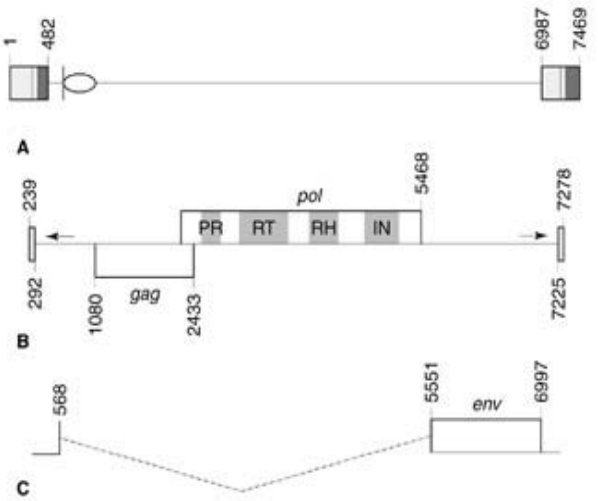


Figure 1 Gypsy proviral structure and transcription (**A**) Structure of a *D. melanogaster* Gypsy provirus. Numerical designations refer to coordinates (in nucleotides) from the first sequence deposited in the databank. Boxes represent the proviral long terminal repeats (LTRs), including U3, R and U5 (light shading, open box and dark shading, respectively). The oval and the vertical bar represent two DNA-binding regions, the SU(HW)-binding site and the downregulating palindrome, respectively. (**B**) Structure of the full-length transcript and its coding potential. The viral RNA is bounded at either end by the short direct repeat R. The U5 and U3 LTR sequences are unique in the transcript. The leftwards and rightwards arrows indicate respectively the positions of the primer binding site (PBS), including nucleotides 482–492, and of the polypurine tract (PPT), including nucleotides 6977–6986. The coordinates of the *gag* first AUG codon and of the *gag* and *pol* stop codons are given. The *pol* open reading frame begins at nucleotide 2363. Position of *pol* domains is as follows: PR, protease; RT, reverse transcriptase; RH, RNAse H; IN, integrase. (**C**) Structure of the *env* subgenomic RNA. The splicing event is figured by the broken line. The vertical bar at position 568 corresponds to the two first nucleotides of the AUG initiator codon which are spliced to a G at position 5551. The 5′ end of this RNA has not been precisely mapped.

transposition and the expression of its infective properties. Transposition only occurs in the progeny of females homozygous for the permissive *flamenco* alleles, indicating that this gene has a maternal effect on Gypsy activity.

Overview of the Strategy of Replication

The typical retroviral replication cycle mainly consists of the following sequence of events: (1) transcription of the provirus; (2) translation; (3) RNA packaging; (4) reverse transcription; and (5) integration as a proviral DNA into the chromosome of the infected host. There is already a wealth of evidence showing that most, if not all, of these steps are involved in the biology of Gypsy. Moreover, like a typical endogenous retrovirus, Gypsy is a germline parasite which can therefore just rely on the chromosome DNA replication machinery for its proviruses to be passively transmitted from generation to generation. Unlike endogenous retroviruses in vertebrates, however, the proviral copy number can dramatically increase in permissive *Drosophila* genotypes. This results from very efficient transfer from the soma towards the germen. Requirement of the infectious potential of Gypsy for this transfer has yet to be demonstrated.

Sequence of the Proviral Genome

Three Gypsy proviruses have been thoroughly sequenced to date in *D. melanogaster*. Despite some differences, these sequences all display the same three open reading frames (ORFs), each of which show at least some similarities to either of the main three genes, *gag, pol* and *env,* shared by all retroviruses (**Fig. 1**). Near the C-terminus of the *gag* homologue gene, an arginine-rich region, conserved in proviruses isolated from two other *Drosophila* species (*D. virilis* and *D. subobscura*), was described as a putative ARM RNA binding motif. The protein putatively encoded by the second ORF is highly homologous to the enzymatic activities, protease, reverse transcriptase, RNAse H and integrase, encoded by other retroviruses. Translation of the third ORF produces a *bona fide* retroviral envelope with a 32 amino acid intracellular domain.

Cis sequences involved in the retroviral replication cycle can also be found: (1) there are two LTRs; (2) adjacent to 5′ LTR, complementarity to $tRNA_1^{Lys}$ discloses a minus-strand primer binding site (PBS); (3) an oligopurine stretch just upstream of 3′ LTR, assumed to be resistant to RNAse H, should be able to prime synthesis of the plus-strand DNA.

Gypsy is not framed by the typical TG...CA nucleotides. Instead, the inverted repeats AGTTA... $TAA^{T}/_{C}T$ are found at the LTR ends.

Transcription

Basal transcriptional activity requires a TATA-less upstream element, located between nucleotides −38 and −24, the TCAGTT Inr sequence at the start site and a downstream sequence between +13 and +60. Polyadenylation of the viral RNA occurs in the 3′ LTR downstream of the Inr sequence, thus leaving short direct repeats (R) which flank unique LTR regions at the 5′ (U5) and 3′ (U3) ends (**Fig. 1B**). A subgenomic RNA (**Fig. 1C**) has been partially characterized in adult females and in Schneider-2 tissue cell cultures; it only seems to differ from the

full-length transcript by the splicing of a 5 kb sequence removing the end of the leader and the *gag* and *pol* ORFs. This splicing event generates a putative initiator codon for ORF 3.

No transcription initiation has ever been described from the 3′ LTR. By contrast, insertion of Gypsy in direct orientation into introns of the *forked* gene may result in mutant phenotypes when polyadenylation of the readthrough transcript occurs in the 5′ LTR before the intron plus the insertion have been spliced.

In addition to the two major transcripts, a rather abundant population of transcripts of various sizes is assumed to originate from the subfamily of defective proviruses, in particular as a result of readthrough transcription in either orientation.

Transcriptional Regulation

The molecular basis of the precise patterns of developmentally regulated Gypsy expression is not yet known. The two following transregulators are currently being studied:

- The product of the *flamenco* gene reduces the accumulation of Gypsy transcripts, especially that of the subgenomic transcript (see below, Transmission and Tissue Specificity). As judged from the ability of a transcriptional fusion to be repressed by *flamenco*, all the relevant *cis*-regulatory sequences must be located within a region encompassing the 5′ LTR and the leader sequence.
- The Gypsy leader region (**Fig. 1A**) contains two elements which bind proteins from nuclear extracts. One is an imperfect palindrome having some homology with the *lac*-operator of *Escherichia coli*. The other contains 12 copies of the binding site ($5'\text{PyPu}^{T}/_{C}\text{TGCATA}^{C}/_{T}\text{PyPu}$) for the *Drosophila* zinc-finger protein SU(HW). When binding of the ubiquitous SU(HW) factor is prevented, Gypsy transcription is uniformly reduced in every tissue. It can act both as an insulator and as a tissue-specific enhancer. This insulating ability is responsible for the SU(HW)-dependent mutant phenotypes induced by Gypsy insertions in the regulatory regions of various genes.

Translation and Post-translational Processing

Very little is known about the ORF 1 and ORF 2 products. From sequence analogy with retroviruses, ORF 2 is assumed to be translated by a −1 frameshifting process. An anti-IN monoclonal antibody failed to detect the GAG-POL fusion protein. Only a 50 kDa polypeptide was lighted up, whereas a 40 kDa IN subunit is expected. In preparations containing extracellular Gypsy particles from the supernatant of *Drosophila* cell cultures (see below, Characteristics of the Viral Particles), a 37 kDa polypeptide was shown to have some specific affinity to Gypsy nucleic acids.

Products of the *env* gene can be detected in ovary extracts of permissive *flamenco* females (see below, Transmission and Tissue Specificity). Treatment by endoglycosidase F reduces the apparent molecular mass of the 66 kDa ENV precursor such that it comigrates with the product of ORF 3 translated *in vitro*. This glycosylated precursor is cleaved into a 34 kDa surface polypeptide (SU) and a 28 kDa transmembrane subunit (TM). Transfection of *Drosophila* cell cultures by the *env* gene under control of the *actin* promoter results in accumulation of the ENV products in the cell membranes.

Characteristics of the Viral Particles

Particles containing Gypsy RNA were first described in the supernatant of *Drosophila* cell cultures (*D. melanogaster* 67j25D and *D. virilis* 79f9 cell lines). Later, fractionation by sucrose density gradients of permissive *flamenco* female extracts produced low-density fractions characterized by the co-occurrence of the ENV product and an endogenous reverse transcriptase activity able to synthetize Gypsy DNA without addition of any Gypsy template. On immunoelectron microscopic and negative staining pictures of these fractions, one can surmise particle-like structures about 100 nm in diameter, the outside of which tends to be decorated by the anti-ENV monoclonal antibodies. In the follicle cells of the same females, much smaller intracytoplasmic particles (40–45 nm in diameter, see **Fig. 2**), lighted up by a Gypsy DNA probe, were found to accumulate close to the membrane domains where ENV is targeted. Neither budding nor enveloped extracellular virions could be observed, as if the replication cycle of this type D-like retrovirus was somewhat abortive in this tissue.

Reverse Transcription

The sophisticated process of reverse transcription of LTR retroelements results in production of DNA with LTRs starting from an RNA template flanked with short terminal repeats. Reconstruction of the complete Gypsy LTR DNA was actually documented in *Drosophila* cultured cells transfected with Gypsy constructs missing the 5′ U3 sequence.

This process is due to the ability of reverse transcriptase to switch templates several times, involving formation of several distinct molecular

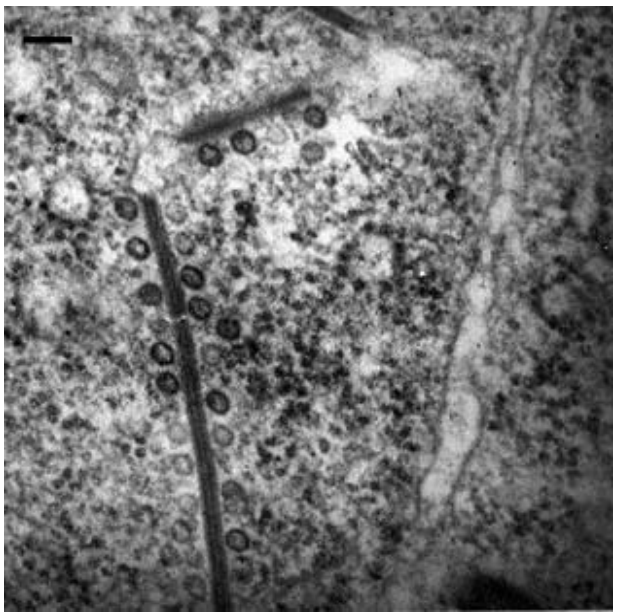


Figure 2 Localization and ultrastructure of Gypsy particles inside *flamenco* permissive follicle cells. In transverse sections, the cellular membranes appear connected together, from place to place, by some junction-like dense material. In immunoelectron microscopic experiments, not shown here, these particular membrane domains were specifically decorated by the anti-ENV antibodies. The picture also shows that these specific membrane domains are covered internally by numerous 40–45 nm particles which, in another experiment, were shown to hybridize with a Gypsy probe. Bar = 100 nm.

forms composed of the template RNA and the nascent DNA strands, organized in a highly specific fashion. Such RNA–DNA hybrids (including the 'minus strong-stop DNA', associated to an RNA primer, the 'plus strong-stop DNA' and full-length minus-strand cDNAs with one or two LTRs), could be actually identified, after oligo(dT) chromatography of nucleic acids extracted from the cell line 67j25d, as permanent cellular ingredients in the absence of exogenous retroviral infection. Similarly, Gypsy strong-stop DNAs are to be found even in females where neither particles nor insertions can be detected, suggesting that they result from the incomplete reverse transcription cycle of the endogenous Gypsy transcripts.

Integration and Excision

In the absence of any external infection, the insertion frequency of germinal proviruses ranges from 10^{-4} to 10^{-1} per haploid genome per active provirus, depending on the host genetic background. In the few insertional mutations analyzed so far, the PyPuPyPu*PyPu consensus sequence – (Py) pyrimidine; (Pu) purine; (*) insertion site – was used as a target, the first four nucleotides being duplicated at both ends of the insertion.

Three different insertional mutations were reported to revert at a frequency of 5×10^{-4} as a result of precise Gypsy excision. The mechanism of this amazing phenomenon is not yet understood.

Transmission and Tissue Specificity

Gypsy is generally transmitted vertically through the germline as a provirus and is not contagious in normal breeding conditions. However, infectious properties of Gypsy were demonstrated by experiments in which crude extracts of pupae issued from a *flamenco* permissive strain containing a high copy number of active Gypsy were put in contact with permissive larvae lacking active Gypsy. Using a genetic assay (based on the screening of mutations due to *de novo* insertion of Gypsy), new sites of Gypsy insertion were observed in the progeny of the flies subjected to the extracts. These results suggest that Gypsy can be efficiently transmitted to the germline of permissive individuals.

The regulation of Gypsy by the *flamenco* gene is tissue-specific. The Gypsy RNAs and ENV proteins accumulate in the ovaries of *flamenco* permissive females. This accumulation takes place near the apical membrane of the somatic follicle cells that surround the oocyte, whereas no derepression occurs in the germline which gives rise to the progeny where integration occurs. This pattern of accumulation is reminiscent of the targeting of enveloped viruses to membrane domains of epithelial cells.

Gypsy ORF 3 is expressed from a spliced messenger RNA that encodes a membrane glycoprotein containing a signal peptide and an endopeptidase cleavage site characteristic of retroviral ENV protein. Direct evidence of Gypsy ENV infectious properties was obtained using a Moloney murine leukemia virus-based retroviral vector pseudotyped by the Gypsy ENV protein. Such particles, produced in the 293GP human cell line, can infect *Drosophila* cells. *Drosophila* cell receptors for Gypsy entry remain to be determined.

Distribution

Southern blot experiments using the Gypsy sequence as a probe have shown that Gypsy-related sequences are widespread in the *Drosophila* genus.

Sequences homologous to Gypsy from *D. subobscura* (Gypsy*Ds*) and *D. virilis* (Gypsy*Dv*) were cloned and entirely sequenced. The sequenced Gypsy*Ds* and Gypsy*Dv* ORF 3s do not have the coding capacity for

Table 1 Insect endogenous retroviruses containing an *env*-like gene.

Element	*Host species*	*env-like spliced RNA*	*ENV protein*	*Infectivity*
Gypsy	*D. melanogaster*	Yes	Yes	Yes
ZAM	*D. melanogaster*	Yes	Yes	?
tom	*D. ananassae*	Yes	Yes	?
TED	*Trichoplusia ni*	?	Yes	?
roo/B104	*D. melanogaster*	Yes	?	?
Tirant	*D. melanogaster*	?	?	?
297	*D. melanogaster*	?	?	?
17.6	*D. melanogaster*	?	?	?
Idefix	*D. melanogaster*	?	?	?
Osvaldo	*D. buzzatii*	?	?	?

functional envelopes because they contain several stop codons generating truncated proteins. The phylogenetic relationship of these three elements is not consistent with the phylogeny of the three host species, suggesting horizontal transfer(s) of the Gypsy elements across species.

All *D. melanogaster* strains contain defective Gypsy proviruses located in pericentromeric heterochromatin. The strains caught in the wild studied so far contain a few additional putatively active Gypsy proviruses (fewer than five) located in euchromatin.

Putative endogenous retroviruses are common in insects. Gypsy is not the only endogenous retrovirus found in insects. Many other envelope-containing insect elements have a similar ORF 3 (**Table 1**) but Gypsy is the only invertebrate retroelement for which infectivity has been shown.

See also: **Endogenous viruses; Host genetic resistance; Replication of viruses; Retroviruses – type D (*Retroviridae*).**

Further Reading

Arhipova IR, Lyubomirskaya NV and Ilyin YV (1995) *Drosophila* retrotransposons. New York: RG Landes, Springer.

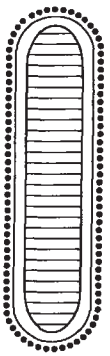
Bucheton A (1995) The relationship between the *flamenco* gene and Gypsy in *Drosophila:* how to tame a retrovirus. *Trends Genet.* 11: 349.

Kim A, Terzian C, Santamaria P *et al* (1994) Retroviruses in invertebrates: the Gypsy retrotransposon is apparently an infectious retrovirus of *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA* 91: 1285.

Pélisson A, Song SU, Prud'homme N *et al* (1994) Gypsy transposition correlates with the production of a retroviral envelope-like protein under the tissue-specific control of the *Drosophila flamenco* gene. *EMBO J.* 13: 4401.

Pélisson A, Teysset L, Chalvet F *et al* (1997) About the origin of retroviruses and the co-evolution of the Gypsy retrovirus with the *Drosophila flamenco* gene. Genetica 100: 29–37.

RHABDOVIRUSES (*RHABDOVIRIDAE*)



Contents

Plant Rhabdoviruses

Andrew O Jackson, Michael Goodin, Ignacio Moreno, Jennifer Johnson and **Diane M Lawrence**, Department of Plant and Microbial Biology, University of California, Berkeley, California, USA

Introduction

Plant viruses have traditionally been included in the Rhabdovirus family based on their distinctive enveloped bacilliform or bullet-shaped particles. These large complex particles can be distinguished readily from the constituents present in uninfected tissue by electron microscopy of extracts or thin sections of infected tissue. Therefore, numerous putative rhabdoviruses have been described in many different plant families. Microscopy of infected cells reveals that plant rhabdoviruses can be distinguished depending on whether the viruses elicit inclusions in the nucleus, bud from the inner nuclear envelope, and accumulate in the perinuclear spaces, or whether they undergo morphogenesis from cytoplasmic membranes and accumulate in the cytoplasm. Thus, the cytopathology of most plant rhabdoviruses differs in several respects from that of the prototype animal rhabdovirus, vesicular stomatitis virus (VSV), which replicates in the cytoplasm and undergoes morphogenesis at the plasma membrane.

Plant rhabdoviruses infect a large number of monocot and dicot plant species (**Table 1**). In some instances, serious disease outbreaks occur on economically important crop plants, some of which result in substantial losses on a recurring basis. The most serious pathogens include maize mosaic virus (MMV), lettuce necrotic yellows virus (LNYV), rice transitory yellowing virus (RTYV) which may be identical to rice yellow stunt virus (RYSV), strawberry crinkle virus (SCV), potato yellow dwarf virus (PYDV), and barley yellow striate mosaic virus (BYSMV). A number of other rhabdoviruses also have considerable disease potential that can be affected by agronomic practices and biological variables of the insect vectors that facilitate their transmission.

Most plant rhabdoviruses are highly dependent on transmission by phytophagous insects, so their prevalence and distribution is influenced to a large extent by the ecology and host preferences of their vectors. Virus–vector interactions are highly specific, and in all cases where known vectors have been carefully examined, they have been shown to support the replication of the plant rhabdoviruses they transmit. Although some rhabdoviruses can be transmitted mechanically by abrasion of leaves, this mode of transmission does not contribute significantly to natural spread in nature due to the labile nature of the virion. Moreover, seed or pollen transmission of plant rhabdoviruses has not been described; thus, aside from vegetative propagation, direct plant to plant transmission is unlikely to be a major factor in the ecology or epidemiology of these pathogens.

Taxonomy and Classification

The International Committee on Taxonomy of Viruses used subcellular distribution patterns to assign plant rhabdoviruses into the *Cytorhabdovirus* and the *Nucleorhabdovirus* genera (**Table 1**). Individual members within these groups have also been shown to be distinct based on host range, vector transmission, serology and nucleic acid hybridization analyses. Presently, eight viruses (BYSMV, broccoli necrotic yellows virus (BNYV), festuca leaf streak virus (FLSV), LNYV, northern cereal mosaic virus (NCMV), sonchus virus (SV), SCV, and wheat American striate mosaic virus (WASMV)) are assigned to the *Cytorhabdovirus* genus. The *Nucleorhabdovirus* genus has seven members (Datura yellow vein virus (DYVV), eggplant mottled dwarf virus (EMDV), MMV, PYDV, RTYV, sonchus yellow net virus (SYNV), and sowthistle yellow vein virus (SYVV)). Of these viruses, SYNV and LNYV have been the most extensively characterized in terms of sequence analysis.

Table 1 List of plant rhabdoviruses and their host and insect specificity

Virus	*Host*	*Vector*	*Virus*	*Host*	*Vector*
Cytorhabdovirus			*Unassigned Plant Rhabdoviruses* (cont.)		
Barley yellow striate mosaic virus (BYSMV)	M	L	Finger millet mosaic virus (FMMV)	M	L
Broccoli necrotic yellows virus (BNYV)	D*	A	Gerbera symptomless virus (GRBSV)	D	
Festuca leaf streak virus (FLSV)	M		Gomphrena virus (GoV)	D*	
Lettuce necrotic yellows virus (LNYV)	D*	A	Holcus lanatus yellowing virus (HLYV)	M	
Northern cereal mosaic virus (NCMV)	M	L	Iris germanica leaf stripe virus (IGLSV)	M	
Sonchus virus (SonV)	D*		Ivy vein clearing virus (IVCV)	D*	
Strawberry crinkle virus (SCV)	D*	A	Laelia red leafspot virus (LRLV)	D*	
Wheat American striate mosaic virus (WASV)	M	L	*Launea arborescens* stunt virus (LASV)	D	
			Lemon scented thyme leaf chlorosis virus (LSCTV)	D	
Nucleorhabdovirus			Lolium ryegrass virus (LoRV)	M	
Datura yellow vein virus (DYVV)	D		Lucerne enation virus (LEV)	D	A
Eggplant mottled dwarf virus (EMDV)	D*		Lupine yellow vein virus (LYVV)	D	
[Pittosporium vein yellowing virus]			*Malva sylvestris* virus (MaSV)	D	
[Tomato vein yellowing virus]			Maize sterile stunt virus (MSSV)	M	L
Maize mosaic virus (MMV)	M	L	Meliotus latent virus (MeLV)	D*	
Potato yellow dwarf virus (PYDV)	D*	L	Melon variegation virus (MVV)	D	
Rice yellow stunt virus (RYSV)	M	L	Oat striate mosaic virus (OSMV)	M	L
[Rice transitory yellowing virus (RTYV)]			Orchid fleck virus (OFV)	D*	
Sonchus yellow net virus (SYNV)	D*	A	Parsley virus (PaV)	D*	
Sowthistle yellow vein virus (SYVV)	D	A	Pelargonium vein clearing virus (PVCV)	D*	
			Pigeon pea proliferation virus (PPPV)	D	
Unassigned Plant Rhabdoviruses			Pineapple chlorotic leaf streak virus (PCLSV)	M	
Atropa belladonna virus (AtBV)	D		Pisum virus (PiV)	D*	
Beet leaf curl virus (BLCV)	D	LW	Plantain mottle virus (PlMV)	D	
Callistephus chinensis chlorosis virus (CCCV)	D		*Ranunculus repens* symptomless virus (RsRSV)	D	
Carnation bacilliform virus (CBV)	D		Raphanus virus (RaV)	D*	
Carrot latent virus (CLV)	D	A	Raspberry vein chlorosis virus (RVCV)	D	A
Cassava symptomless virus (CasSV)	D		Red clover mosaic virus (RCIMV)	D	
Cereal chlorotic mottle virus (CCMV)	M		Sainpaulia leaf necrosis virus (SLNV)	D	
Chrysanthemum frutescens virus (CFV)	D		Sambucus vein clearing virus (SVCV)	D	
Chrysanthemum vein chlorosis virus (CVCV)	D		*Sarracenia purpurea* virus (SPV)	D	
Clover enation virus (ClOEV)	D		Sorghum virus (SV)	M	L
Coffee ringspot virus (CoRSV)	D*	M	Soursop yellow blotch virus (SYBV)	D	
Colocasia bobone disease virus (CBDV)	D	P	*Triticum aestivum* chlorotic spot virus (TaCSV)	M	
Coriander feathery red vein virus (CFRVV)	D*	A	*Vigna sinensis* mosaic virus (VSMV)	D	
Cow parsnip mosaic virus (CPMV)	D*		Winter wheat Russian mosaic virus (WWMV)	M	L
Cynara virus (CyV)	D*		Wheat chlorotic streak virus (WCSV)	M	L
Digitaria striate virus (DSV)	M	L	Wheat rosette stunt virus (WRSV)	M	L
Euonymus fasciation virus (EFV)	D		*Zea mays* virus (ZMV)	M	

Names in brackets are synonomous to those immediately above. Host: D = Dicot, M = Monocot. (*) indicates ability to be mechanically transmitted. Vectors: A = Aphid, L = Leafhopper, LW = Lacewing, M = Mite, P = Planthopper. Blank spaces indicate that no insect vector has been defined.

Most other plant rhabdoviruses have not been investigated in much detail beyond cursory infectivity studies, crude physicochemical analyses of virus particles, and electron microscopic observations of morphogenesis. Consequently, nearly sixty rhabdoviruses await assignment to a genus (**Table 1**). Moreover, sufficient comparative information to determine whether individual descriptions from disparate hosts are due to distant, closely related, or identical viruses is not available from many preliminary descriptions. Hence, more definitive morphological, serological and molecular studies need to

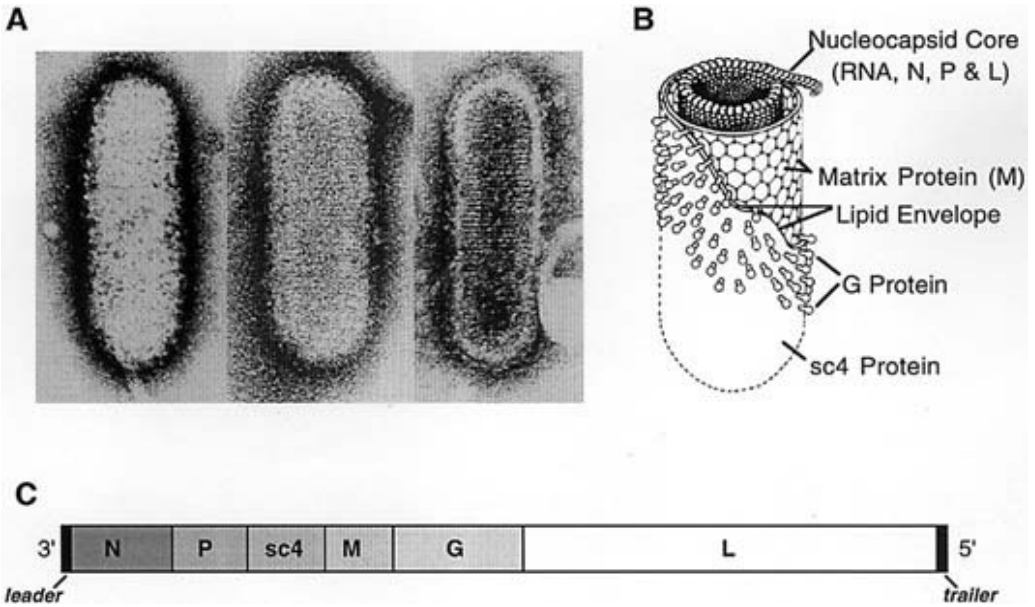


Figure 1 Plant rhabdovirus morphology and depiction of the sonchus yellow net virus genome. (**A**) Electron micrographs of negatively stained particles of SYNV. The panel on the left shows a lightly stained particle illustrating the hexagonal subunit associations at the surface of the virion. The slightly more intense negative stain in the center highlights the surface glycoprotein spikes surrounding the particle. The right panel illustrates a particle with deeper penetration revealing the internal striated nucleocapsid core. (**B**) Depiction of the architecture of the virus particle. The nucleocapsid core is composed of the minus-sense genomic RNA, the nucleocapsid protein (N), the phosphoprotein (P), and the polymerase protein (L). The matrix protein (M) is involved in the attachment of the nucleocapsid core to the envelope. The membrane lipids are host-derived and are interspersed with an orderly array of the glycoprotein (G) spikes. The location of the sc4 protein has not been precisely defined, but it is solubilized by nonionic detergent treatment of virions. (**C**) Depiction of the genome organization of SYNV. The 13 720 nucleotide minus-strand genomic RNA from 3′ to 5′ consists of the leader sequence, the N, P, sc4, M, G and L protein genes and the trailer sequence. The relative sizes of the genes are proportional to the size of the viral RNA. Adapted from Jackson and Wagner (1998).

be carried out to establish reliable criteria for the taxonomic grouping of plant rhabdoviruses, and to more readily verify their roles as causal agents of specific diseases. Additional biological data also need to be acquired with numerous poorly characterized rhabdoviruses whose host range, vector relations and distribution have received only cursory attention. Nevertheless, even though taxonomy assignments and our understanding of the relatedness of the rhabdoviruses are presently rudimentary, modern molecular and cell biological methodology provides great potential for identifying and characterizing unassigned plant rhabdoviruses, and developing additional criteria to better appreciate stages in their evolution.

Virion Morphology and Composition

Plant rhabdoviruses are normally bacilliform after careful fixation, but are bullet-shaped in unfixed preparations (**Fig. 1A**). Estimates of the sizes of virus particles range from 45 to 100 nm in width and 130 to 350 nm in length; however it is very difficult to determine accurately the sizes of the virus particles due to swelling of the fragile virions, shrinking of the nucleocapsid core, and other artifacts. Electron microscopy of particles reveals three distinct layers of varying electron density whose composition has been determined for several viruses by particle disruption, gel electrophoresis and RNA analyses. The outer layer containing 5–10 nm spike-like surface projections is composed of the G protein (**Fig. 1B**). The spikes appear to be arranged as hexamers on the surface and, by analogy with animal rhabdoviruses, G protein trimers may participate in forming the surface lattice. The middle layer consists of a host-derived membrane penetrated by the G protein. The striated inner core, with a periodicity of 4–5 nm, is composed of a helical ribonucleoprotein consisting of the genomic RNA, the nucleocapsid protein (N), the phosphoprotein (P), and the L polymerase protein (**Fig. 1 A, B**). The exact location of the matrix protein (M), has not yet been clearly determined, but it probably participates in coiling of the nucleocapsid and interacts with the G protein to stabilize the particle. A sixth protein (sc4) found in SYNV

particles has no known function, but it can be solubilized from membrane components on treatment with nonionic detergents.

The overall chemical composition (~70% protein, 2% RNA, 20–25% lipid, and a small amount of carbohydrate associated with the G protein) of the plant and animal rhabdoviruses is similar. The minus-sense RNA genomes of plant rhabdoviruses range in size from ~11 to 14 kb based on sedimentation and gel electrophoretic analyses, and are slightly larger than those of most described animal rhabdoviruses. The lipids of plant and animal rhabdoviruses have differences in fatty acid and sterol composition that are related to their respective hosts and sites of morphogenesis. Two nucleorhabdoviruses, SYNV and PYDV, have been examined in the most detail, and the results indicate that a variety of fatty acids, free and esterified sterols are present in purified virus particles. Four sterols predominating in SYNV closely approximate the sterols of the nuclear envelope, whereas those of NCMV, a cytorhabdovirus, are more typical of cytoplasmic membranes.

	I	II	III
Consensus	**AP$_y$UP$_y$UUUU**	**G/C N**	**P$_y$UNNN**
SYNV	**AUUCUUUUU**	**GG**	**UUG A/U A/C**
LNYV	**AUUCUUUU**	**G(N)$_x$**	**CU A/U A/U G/U**
VSV	**ACUUUUUUU**	**G/C U**	**UUGUC**
RV	**ACUUUUUUU**	**C(N)$_x$**	**UUGU A/G**

Figure 2 Alignment of the intergenic regions of selected plant and animal rhabdoviruses. The rhabdovirus consensus sequence is shown at the top followed by the sequences of sonchus yellow net virus (SYNV), lettuce necrotic yellows virus (LNYV), vesicular stomatitis virus (VSV) and rabies virus (RV). The intergenic sequences ('gene-junction' sequences) are separated into three elements: element I constitutes the poly(U) tract at the 3′ end of each gene on the genomic RNA; element II is a short sequence that is not transcribed during mRNA synthesis; element III constitutes the start site for transcription of each mRNA. The bold type in the viral sequences indicates the consensus nucleotides, P_y indicates pyrimidine, $(N)_x$ corresponds to a variable number of nucleotides.

Genomic Structure and Organization

The nucleotide sequence of SYNV RNA and genome mapping studies of LNYV show that both viruses encode six proteins, but otherwise, their genome organization is similar to those of VSV, which encodes five genes. The gene order for SYNV is 3′-leader-*N*-*P*-*sc4*-*M*-*G*-*L*-trailer-5′ (**Fig. 1C**), and the order for LNYV is 3′-leader-*N*-*4a*-*4b*-*M*-*G*-*L*-trailer-5′ (where 4a is thought to represent a phosphoprotein derivative and 4b an undefined extra gene). The coding regions of SYNV and LNYV are flanked by leader sequences at the 3′ end of the minus-sense genomes, and by a noncoding trailer sequence at the 5′ end of the genomes. The leader and trailer sequences of the plant rhabdoviruses have complementary regions, as is the case with other minus-strand viruses, and both sequences are considerably longer than those of animal rhabdoviruses. Another difference is that the transcribed leader RNA of SYNV is polyadenylated, which may represent an adaptation to facilitate its nuclear mode of replication.

The leader and trailer genes of SYNV have little obvious sequence relatedness to those of LNYV or other animal rhabdoviruses. In contrast, the intergenic 'gene-junction' sequences of SYNV and the animal rhabdoviruses are generally highly conserved (**Fig. 2**). These intergenic regions can be grouped into three components constituting a poly(U) tract at the 3′ end of each gene on the genomic template (element I), a short nontranscribed element which separates each gene (element II), and a conserved element located at the beginning of each subsequent gene (element III). The SYNV 'gene junctions' are very similar to the intergenic region following the N gene of the nucleorhabdovirus, RYSV. LNYV also has some similarity to other rhabdoviruses in element I, but elements II and III diverge substantially. Element 1 (AUUCUUUUU) of SYNV is also nearly identical to the analogous regions (AUUCUUUUUU) of Ebola virus, suggesting that some conservation of regulatory regions may extend to the *Filoviridae*. Sequence similarities in this region are also found in paramyxoviruses and in borna disease virus. Thus, intergenic regions of the genome that play an important role in regulating mRNA transcription and replication appear to have been stringently conserved. However, the leader and trailer genes and the genes encoding the proteins appear to have undergone extensive evolution to accommodate diverse host requirements.

Properties of the Encoded Proteins

The structural properties of plant rhabdovirus proteins deduced from nucleotide sequence analyses are presented below in the order of their appearance from the 3′ end of the genome (**Fig. 1C**). Only rudimentary biochemical analyses have been conducted on the proteins and these are mostly confined to SYNV and LNYV. Overall, the plant rhabdovirus proteins appear to have very little sequence relatedness to analogous proteins of animal rhabdoviruses, with the exception of the L protein which has conserved motifs common to those of most rhabdoviruses. A descrip-

tion of these proteins and their probable functions is outlined below.

The nucleocapsid protein (N)

The N protein functions to encapsidate the genomic and antigenomic RNAs and it is a component of the viroplasms and of the polymerase complex that can be isolated from nuclei of SYNV-infected plants. The N protein genes of the nucleorhabdoviruses, SYNV and RYSV, and the cytorhabdovirus, LNYV, have been sequenced. The deduced proteins of these viruses have no extensive sequence relatedness to animal rhabdovirus proteins, although their hydropathy patterns have some similarity. However, the SYNV and RYSV N proteins have limited regions of weak homology that are more closely related to each other than to analogous regions of the N protein of LNYV. The 475 amino acid SYNV N protein contains a nucleoplasmin-like nuclear localization signal close to the carboxy terminus that has some involvement in mediating the nuclear localization of the N protein. A related consensus element is also present in the RYSV N protein, but this element is lacking in the cytorhabdovirus, LNYV. The SYNV, LNYV and RYSV N proteins all contain regions located approximately two-thirds of the way towards the carboxy termini that appear to be weakly conserved with that of VSV.

The phosphoprotein (P)

Sequence information for the P protein and the remaining encoded genes are available only for SYNV. The P gene encodes a 362 amino acid protein with no direct amino acid sequence relatedness to the P proteins of other rhabdoviruses. However, the SYNV P protein appears to have functions similar to those of other rhabdoviruses because it is a component of the viral nucleocapsid core and the nuclear associated replicase complex. The P protein is also capable of forming complexes *in vivo* with the N and L proteins that are analogous to N:P and P:L complexes found in VSV-infected cells that may function in transcription and replicase recycling. The amino terminal half of the SYNV P protein is negatively charged, as is the case with the other rhabdoviruses, but little charge similarity or sequence resemblance is present at the carboxy terminus. The SYNV P protein is phosphorylated *in vivo* at threonine residues and hence differs from the VSV P protein, which is phosphorylated at serine residues. The SYNV P protein accumulates in the nucleus and a basic region approximately 150 amino acids from the amino terminus may have some karyophilic properties.

The sc4 protein

The sc4 protein appears to be associated with the SYNV envelope because nonionic detergent treatments can facilitate its release from virus particles. sc4 does not contain an obvious transmembrane domain or a nuclear localization signal. However, it could be associated with membranes by acylation through attachment of palmitic acid residues found in the viral envelope and thioester (cysteine) or ester (serine or threonine) linkages. Recent studies suggest that sc4 may be phosphorylated *in vivo*. Analysis of the sequence reveals that 16% of the amino acids are serine or threonine residues and that four potential consensus casein kinase II phosphorylation sites are present. The sc4 protein also contains a motif related to an aspartic protease site found in α-amylases and cellular and acid proteases. The function of sc4 has not been elucidated, but it may play a role in aphid transmission or in cell to cell movement. However, irrespective of its function, sc4 appears to be unique to plant rhabdoviruses because its predicted sequence has no similarity to sixth genes encoded by infectious hematopoietic necrosis virus, Sigma virus, Flanders virus, or bovine ephemeral fever virus.

The matrix protein (M)

The 286 amino acid M protein is basic and is thought to function in nucleocapsid coiling and interactions with the G protein, as is the case with other rhabdovirus M proteins. Multiple alignments of the M protein fail to reveal conserved consensus motifs, but short stretches of amino acids display some similarities in composition to the M proteins of other rhabdoviruses. A hydrophobic region of 67 amino acids extending almost to the middle of the protein could be involved in membrane–lipid interactions with the G protein. Recent studies suggest that the SYNV M protein is phosphorylated *in vivo* at both threonine and serine residues.

The glycoprotein (G)

The G protein forms the glycoprotein virion spike. The 632 amino acid sequence deduced for the G protein has no significant homology to G proteins of other rhabdoviruses, but it does contain putative signal sequences, a transmembrane anchor domain, and glycosylation signals. In addition, the SYNV G protein contains a putative nuclear targeting signal near the carboxy-terminus which could be involved in transit to the inner nuclear membrane prior to morphogenesis. Glycosylation inhibitors interfere with G protein *N*-glycosylation and the protein is stable in tunicamycin-treated cells. This treatment blocks SYNV morphogenesis and results in striking

arrays of condensed nucleocapsid cores that fail to bud and accumulate in the nuclei.

The polymerase protein (L)

The SYNV *L* gene encodes a 2116 amino acid protein that is present in low abundance within the nucleocapsid. The L protein is required for polymerase activity, because antibodies raised against a fragment containing the GDNQ (polymerase) motif inhibit transcription. As is the case with other rhabdoviruses, the L protein is positively charged and contains polymerase and RNA binding domains. Alignment of the L protein sequence with polymerases of several other nonsegmented negative-strand RNA viruses reveals conservation within 12 motifs that appear sequentially in the protein. A cluster dendrogram derived from the L protein alignments suggests that SYNV is more closely related to animal rhabdoviruses than to paramyxoviruses, and that animal rhabdoviruses have diverged less from each other than from SYNV.

Polymerase Activity

A viral RNA-dependent RNA polymerase is activated after treatment of LNYV and BNYV cytorhabdovirus virions with mild nonionic detergents. This activity cosediments with the 40–45S loosely coiled nucleocapsid filaments that are released from virions by detergent treatment. The transcribed products are complementary to the genome, as expected of mRNAs. Thus, the described polymerases of these plant rhabdoviruses appear to be similar to the extensively studied polymerase of the animal rhabdovirus prototype, VSV.

In contrast, no appreciable polymerase activity is evident in dissociated preparations of SYNV and other nucleorhabdovirus members. In this regard, the negligible levels of activity are similar to those obtained from rabies virus preparations. However, an active polymerase can be recovered from the nuclei of plants infected with SYNV. The polymerase activity is associated with a nucleoprotein complex consisting of the N, P, and L proteins, and it cosediments with SYNV nucleocapsid cores. The complex can be precipitated in an active form by P protein antibodies, and L protein antibody inhibition experiments show that the L protein is a functional component of the polymerase. Kinetic analysis of transcription products also reveals that the complex is capable of sequentially transcribing a polyadenylated plus-sense leader RNA, and polyadenylated mRNAs corresponding to each of the six SYNV-encoded proteins. Potential replication intermediates consisting of short incomplete minus-strand products homologous to the genomic RNA are also transcribed. These results thus support the hypothesis that polymerases of cytorhabdoviruses are present in an active form in virions and that released cores are capable of initiating primary transcription immediately upon uncoating. Nucleorhabdovirus particles differ by containing an inactive polymerase that appears to require activation by host components early in infection.

Defective Interfering RNAs

Animal rhabdoviruses passaged at high multiplicities of infection commonly accumulate defective-interfering (DI) particles. These DIs are dependent on the wild-type virus for their replication, and their presence results in a substantial decrease in the titer of the helper virus. The RNA molecules associated with DIs are typically internally deleted forms of the wild-type viral genomic RNA that retain the complementary 3′ and 5′ terminal sequences.

Formation of plant rhabdovirus DIs has been observed with PYDV and SYNV. PYDV passaged at high multiplicities of infection developed DIs after 30 successive mechanical transfers. The slowly sedimenting particles appeared not to be infectious in local lesion assays, but their protein composition was similar to those of the parental virus. In addition, the presence of the DIs decreased the amount of PYDV that could be isolated from infected plants. In the case of SYNV DIs, calyx tissues of *Nicotiana edwardsonii* examined 5 months after inoculation were shown to contain a high proportion of particles approximately three quarters as long as those of wild-type SYNV. Purified short particles were not infectious when inoculated alone, but when coinoculated with wild-type virions, short particles predominated upon reisolation. RNA isolated from these short particles was approximately 25% shorter than RNA from complete virions. The DI RNAs were able to hybridize to SYNV cDNA probes, but additional information about their structure has not been reported. From these results, the appearance of plant rhabdovirus DIs appears to be an uncommon occurrence that contrasts with the high frequency of animal rhabdovirus DIs.

Cytopathology and Replication

Unlike the animal rhabdoviruses which replicate and assemble in the cytoplasm, the plant rhabdoviruses vary profoundly in their sites of replication and morphogenesis (**Fig. 3**). The nucleorhabdoviruses, typified by SYNV, replicate in the nucleus, bud in

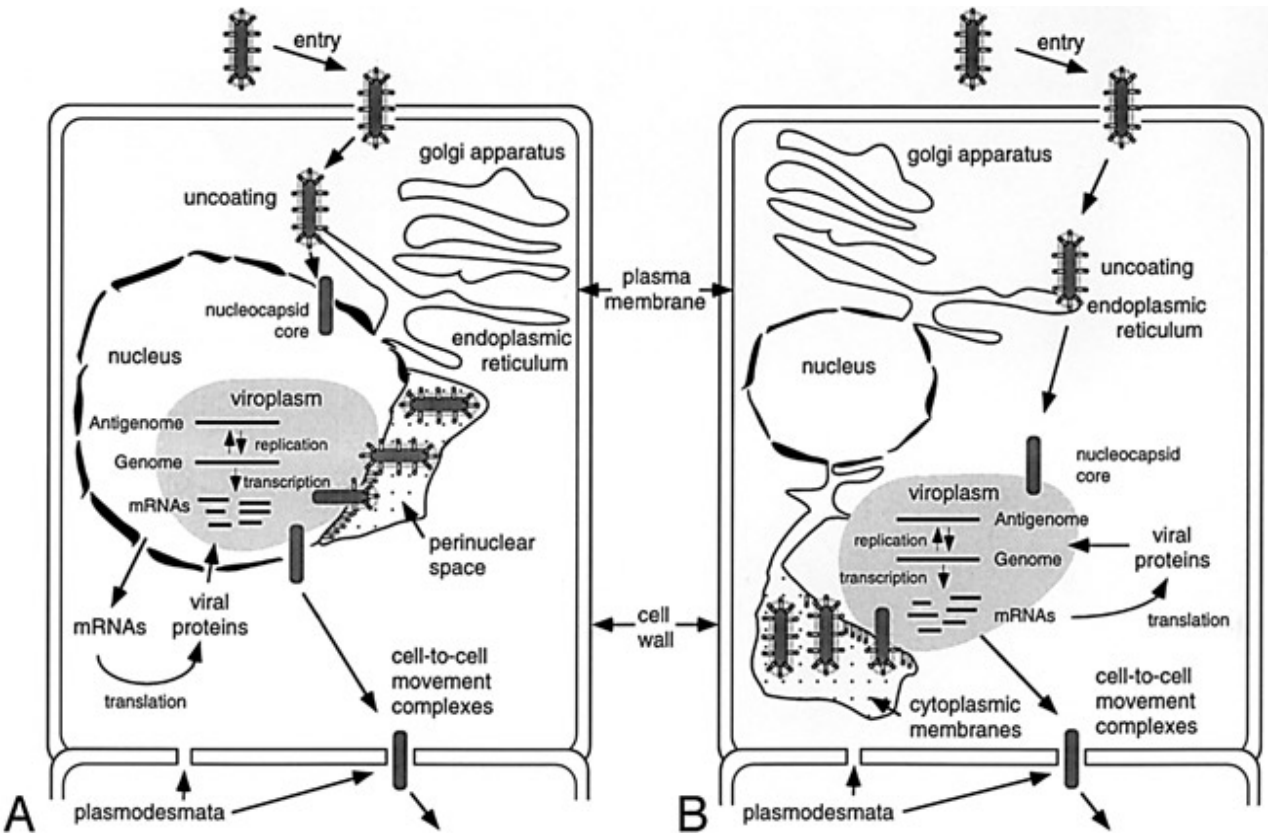


Figure 3 Model for the replication cycle of plant nucleorhabdoviruses (**A**) and cytorhabdoviruses (**B**). See text for details.

association with the inner nuclear envelope, and accumulate in enlarged perinuclear spaces formed between the inner and outer envelopes. Considerable attention has been focused on SYNV, and these studies support the model illustrated in **Fig. 3A.** The early entry and uncoating events are obscure, but based on observations with SYNV-infected protoplasts, it has been hypothesized that during entry into the cell, virus particles associate with the endoplasmic reticulum to release the nucleocapsid cores into the cytoplasm. It is thought that the cores utilize the host nucleocytoplasmic transport machinery to facilitate movement and entry into the nucleus via the nuclear pore complex. During the early stages of infection the virion-associated polymerase probably is activated by host components to produce an active transcriptase that participates in primary transcription. The transcribed mRNAs are transported to the cytoplasm and translated. The newly translated N, P and L polymerase core proteins are transported to the nucleus where they participate in multiple rounds of replication of antigenomic and genomic RNAs and secondary rounds of mRNA transcription. As replication proceeds, the nuclei increase dramatically in volume, and granular electron-dense viroplasms form discrete foci that appear near the periphery of the nuclei. Several recent lines of evidence show that SYNV viroplasms contain the N, P and L proteins, suggesting that the viroplasms are the source of the polymerase activity that can be recovered from nuclei. During the late stages of replication, the matrix protein is thought to participate in coiling of nucleocapsid cores containing the minus-sense RNA genome. These cores then associate with G protein concentrated at sites on the inner nuclear envelope that are in close proximity to the viroplasms. Virus budding apparently requires G protein glycosylation because tunicamycin interrupts budding and causes the appearance of large numbers of the nucleocapsid cores around the periphery of the nucleus. During normal budding, numerous enveloped particles accumulate in perinuclear spaces between the inner and outer nuclear envelope, but in some instances, a few virus particles are present in cytoplasmic vesicles. Since the outer nuclear envelope is continuous with the endoplasmic reticulum and is part of the endomembrane system, this observation is not surprising. However, it does indicate that assignments to the nucleorhabdovirus group need to be based on several different types of ultrastructural evidence. Such evidence should include use of virus specific probes for *in situ* hybridization, immunochemical

localization of nucleocapsid proteins, and high resolution immunoelectron microscopy to probe virus morphogenesis at different stages of infection.

The cytorhabdoviruses appear to replicate in the cytoplasm, bud in association with the endoplasmic reticulum, and accumulate in endoplasmic reticulum-derived vesicles (**Fig. 3B**). Two variations have been proposed based on extensive electron microscopic observations of LNYV and BYSMV cells. During the early stages of LNYV infection, indirect evidence suggests that LNYV may undergo a nuclear phase because the outer nuclear membrane blisters and develops small vesicles that contain some virus particles. However, later in the life cycle masses of thread-like viroplasms appear in the cytoplasm and these are in close proximity to dense networks of the endoplasmic reticulum. These membranes serve as sites for morphogenesis of the majority of virus particles which bud into vesicles that appear to be derived from the proliferated host membranes. A slightly different scenario lacking a nuclear phase has been outlined for BYSMV. In this case, membrane-bound viroplasms appear in the cytoplasm and virus particles are found exclusively in association with cytoplasmic membranes that proliferate in close proximity to the viroplasms. Unfortunately, both of the cytorhabdovirus models have been derived solely from ultrastructural observations, and none of these studies have utilized specific antibodies to individual virus proteins or viral-specific probes for *in situ* hybridization. Clearly more direct cell biological studies must be conducted with such reagents before detailed models of the replication cycles of these viruses can be proposed.

Vector Relationships, Distribution and Evolution

Many members of the rhabdoviruses are transmitted by insects and other arthropods in which they also multiply, so it is possible that members of the rhabdovirus family radiated from a primitive arthropod. This mode of transmission more than any other property, may have been responsible for the widespread occurrence of the rhabdoviruses within the plant and animal kingdoms. Although some plant rhabdoviruses have no known vector, most are transmitted by aphids (*Aphidae*), leafhoppers (*Jassidae*), or planthoppers (*Delphicidae*). Two poorly characterized putative rhabdoviruses, beet leaf curl virus and coffee ringspot virus, have lacebug and mite vectors, respectively, but these viruses need to be examined more rigorously before they can be unambiguously accepted as members of the *Rhabdoviridae*.

Several general patterns indicate that host–vector relationships have profoundly affected plant rhabdovirus distribution. For example, leafhoppers, planthoppers and aphids are prevalent on both monocots and dicots, but rhabdoviruses causing diseases of the *Gramineae* are all transmitted by leafhoppers or planthoppers. Except for PYDV and CBDV, which have leafhopper and planthopper vectors, respectively, the rhabdoviruses infecting dicots are commonly transmitted by aphids. The available evidence indicates that rhabdovirus–vector interactions are highly specific and that generally, a single rhabdovirus is transmitted by closely related species of the same genus. In all cases of insect transmission that have been carefully examined, the rhabdovirus is persistently transmitted in a propagative fashion. Comprehensive transmission trials conducted with PYDV in leafhoppers and SYVV in aphids, as well as less extensive studies with several other leafhopper and aphid transmitted rhabdoviruses, are all consistent with replication in the vector. Indirect evidence for replication is that long latent periods are required before transmission occurs, that virus is often retained throughout the life of the insect, and that transovarial passage can be observed through eggs and nymphs. More persuasive results have been obtained by continued transmission after repeated serial dilution passages from insect to insect. Strain specific infection of tissue culture lines and explants and serological detection of virus in vectors provides additional proof that rhabdoviruses replicate with high specificity in leafhopper and aphid vector cells.

Genetic experiments with PYDV have shown that highly efficient and inefficient leafhopper vectors can be selected. Continuous passage of PYDV by serial injection of insects can also result in isolates that are unable to infect plants. Additional studies have shown that strains that have lost their capacity to be insect transmitted can be recovered after protracted passage in plants. This phenomenon could provide a mechanism for evolution of vectorless rhabdoviruses particularly in cases where infections were established in vegetatively propagated hosts. Rhabdoviruses normally have the capacity to infect a greater range of hosts than the narrow range of species colonized by their vectors, because experimental host ranges usually can be extended considerably by mechanical transmission. One appropriate example is transmission of SCV, which is restricted in nature to cultivated and native strawberry due to feeding preferences of its aphid vector. SCV is very difficult to transmit mechanically from strawberry (**Table 1**). However, alternate solanaceous hosts can be infected by surrogate nonvector aphids injected with extracts

from the strawberry aphid, and the virus can then be mechanically transmitted from these plants. In addition, cowpea protoplast infectivity experiments with the grass rhabdovirus FLSV and with SYNV show that both viruses are able to infect legume protoplasts, but they are unable to infect cowpea plants. These results indicate that plant rhabdoviruses have the ability to infect cells of hosts that are quite distantly related to their native hosts, but that vector feeding and requirements for systemic movement constrain their host specificity.

Plant rhabdoviruses are faced with two major evolutionary challenges of a fundamentally different nature brought about by the necessity to alternately infect plants and insects. In each host, the virus must utilize different entry methods and accommodate distinct cellular and defense mechanisms. Establishment of effective vector relations requires nonpathogenic infections of the insect without substantial interference with longevity, fecundity or normal feeding activities necessary for efficient virus dispersal. Rhabdovirus acquisition by the vector probably necessitates attachment to specific receptors at the surface of cells in the digestive system and active invasion of the reproductive organs, fat bodies and salivary glands. It is highly likely that vector specificity is regulated at the entry stages of infection via receptors on the surface of the host alimentary system. This hypothesis is supported by experiments showing that aphid vector specificity can be extended if the gut barrier is avoided by direct injection of virus. Very different barriers must be circumvented to establish systemic infections of plants. In order to establish a primary infection focus, the cell wall must first be breached by mouthparts of the insect, the virus must be regurgitated into the cell, uncoated and replicated. Then, to establish systemic invasions, the virus must move from cell to cell through very small plasmodesmatal connections, into the phloem cells of the vascular system and throughout the plant. Because the plasmodesmata normally restrict movement of macromolecules, viruses generally move to adjacent cells via mechanisms that increase the permeability of the plasmodesmatal openings. Many plus-strand RNA viruses encode specific nucleic acid binding proteins that function to enlarge plasmodesmata, bind viral RNA and shuttle the genome through the plasmodesmata to neighboring cells. Plant rhabdoviruses face a special challenge at this stage of infection because the approximately 3 nm plasmodesmata are at least an order of magnitude smaller in diameter than virus particles. Therefore, transit of intact viruses would require enormous plasmodesmatal alterations that ought to be easily visible by electron microscopy. Moreover, since the naked genomic RNA of minus-strand viruses is not infectious, the polymerase proteins must accompany the infectious derivative. These constraints probably mandate that rhabdovirus-encoded gene products facilitate enlargement of the plasmodesmata, and that nucleocapsid cores function in cell to cell and vascular movement. These movement activities may well require functions of the sixth genes that have been identified by mapping of SYNV and LNYV.

Epidemiology and Disease Control

Plant rhabdoviruses have been identified in most major crops throughout the world. Although the factors affecting their dispersal have not been investigated extensively, several studies suggest that transmission depends on a delicate balance of interactions involving vector–host plant associations. These include the specificity of the virus–vector relationship, the dependence on insect vectors for local and long distance spread, and possible pathological effects of rhabdovirus infections on the insects. The ecology of host plant and insect vector interactions also has a major role in distribution and spread of plant rhabdoviruses. Therefore, a number of interacting factors, including changes in vector species and weed host populations could affect rhabdovirus ecology and disease cycles. In Berkeley, California, an interesting example resulting in a marked decline of SYVV in natural sowthistle populations has been attributed to the displacement of the aphid vector (*Hyperomyzus lactucae*) with an aggressive invader aphid (*Uroleucon sonchi*) that is not a vector for SYVV. Other obvious components affecting the biology of plant rhabdoviruses and their capacity to cause disease are virus reservoirs in weed hosts or volunteer crop plants that bridge the season between crops, and vectors that survive from one crop generation to the next. An additional element that often is not considered is that synergistic or antagonistic interactions with other viruses may affect viral ecology. One possibility of such interactions comes from a correlation of the presence of bidens mottle virus (BMoV), a potyvirus, in all beggarticks (*Bidens* sp.) harboring SYNV. Potyviruses often act synergistically with a number of viruses, including SYNV; thus it is likely that BMoV may serve to facilitate high levels of SYNV that aid in aphid acquisition.

Several additional interactions also contribute to disease outbreaks. These include populations of viruliferous aphids early in the growing season that can establish infection foci when crops are most susceptible to invasion. After initial infections have been established, rapid distribution of the virus probably is most dependent on a sufficient density

of host plants capable of supporting the vector and transmission of virus to juvenile insects. Concurrently, environmental conditions conducive to short incubation periods after acquisition feeding by vectors, and optimal for rapid disease development in plants after virus transmission will facilitate efficient dissemination of the virus. Consequently, seemingly minor changes in climate, agronomic practices, crop varieties or vector populations may alter virus spread and disease development profoundly, and manipulation of these factors can lead to reductions in yield losses.

For these reasons, rhabdovirus disease control has emphasized a broad range of different strategies. Agronomic or cultural controls applied with some success include elimination of natural weed reservoirs, spraying to reduce vector populations, and production of virus-free vegetative stocks. However, the efficacy of these measures relies on the particular host/virus/vector relationships, and no common method suitable for rhabdovirus disease control has yet been described. In particular, the reported successes indicate that development of effective control measures requires a detailed knowledge of the ecology of the particular virus under consideration, the biology of the host plant and the natural vector reservoirs.

Major factors that can affect rhabdovirus disease incidence are external agents that alter the ecology of host/vector interactions. An interesting anecdotal description of such a situation relates to the epidemiology of LNYV outbreaks in lettuce that occurred in Australia in the early 1960s. These epidemics have been speculated to coincide with the introduction of myxomatosis in Australia to control rabbit populations. The reduced rabbit populations permitted increases in sowthistle plants that constitute a natural reservoir for LNYV and its aphid vector, *Hyperomyzus lactucae*. This combination resulted in disease epidemics due to invasion of newly planted lettuce by large numbers of viruliferous aphids that had increased on viruliferous sowthistle. Fortunately, elimination of weeds for a short distance around fields reduced the entry of vectors into the lettuce crops and provided acceptable disease control. Elimination of rhabdoviruses from vegetatively propagated crops also has considerable potential for disease control. Certification strategies to produce disease-free stock by selection of strawberry propagules free of SCV are proving to be beneficial in California. In other cases, production of virus-free stock, combined with insecticide applications, can provide disease control. Infections of PYDV, which caused serious yield losses before the 1940s in the northeastern United States, appear to have been reduced to a very low frequency as a serendipitous consequence of combinations of insecticides and elimination of the virus from seed potatoes.

Incorporation of disease resistance into crop plants normally provides more effective, durable and economical control than any other measure. Two notable examples of the employment of this strategy exist in rhabdoviruses. The first relates to MMV in Hawaii, where up to 100% losses have been observed in maize lacking disease resistance genes. Useful control is obtained by incorporation of a single gene for tolerance into cultivars, which ameliorates the disease. Another example exists for infections with raspberry veinal chlorosis virus, an unassigned plant rhabdovirus. Raspberries possessing this form of resistance appear to be immune because resistant scions can not be infected by graft inoculation.

The major problem with application of disease resistance is identification of useful genes. Traditionally, such genes have been isolated by screening wild species found near centers of origin of crop plants. However, two major advances in biotechnology provide optimism that resistance can be more widely applied for disease control. Disease resistance genes with specificity to viruses, bacteria and fungi have been cloned from several crops, and some of these genes retain their function when transferred to distantly related species. The evidence also suggests that the recognition motifs of these genes can be engineered to produce novel sources of resistance. A second approach involves engineering synthetic sources of pathogen-mediated resistance by producing transgenic plants expressing portions of the viral genome. Although both strategies have enormous potential for disease control, neither has yet been applied to plant rhabdoviruses. Thus, these approaches represent an important challenge for the future.

See also: **Defective interfering viruses; Fish viruses; Parainfluenza viruses (*Paramyxoviridae*): Animal, Human; Plant virus disease – economic aspects; Rabies virus (*Rhabdoviridae*); Rhabdoviruses (*Rhabdoviridae*): Ungrouped mammalian, bird and fish rhabdoviruses; Vectors: Animal viruses, Plant viruses; Vesicular stomatitus viruses (*Rhabdoviridae*).**

Further Reading

Black LM (1979) Vector cell monolayers and plant viruses. *Adv. Virus Res.* 25: 192.

Francki RIB, Kitajima EW and Peters D (1981) Rhabdoviruses. In: Kurstak E (ed.) *Handbook of Plant Virus*

Infections – Comparative Diagnosis, p. 455. Amsterdam: Elsevier/North Holland.

Jackson AO, Francki RIB and Zuidema D (1987) Biology, structure and replication of plant rhabdoviruses. In: Wagner RR (ed.) *The Rhabdoviruses*, p. 427. New York: Plenum Press.

Jackson AO and Wagner JDO (1998) Procedures for plant rhabdovirus purification, polyribosome isolation, and replicase extraction. In: Foster G and Taylor S (eds) *Plant Virology Protocols: From Virus Isolation to Transgenic Resistance*, vol. 81, p. 77. Totowa, NJ: Humana Press.

Sylvester ES and Richardson J (1989) Aphid-borne rhabdoviruses – relationships with their vectors. *Adv. Vector Dis. Res.* 9: 313.

Ungrouped Mammalian, Bird and Fish Rhabdoviruses

Bishnu P De and **Amiya K Banerjee**, Department of Molecular Biology, The Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, USA

Introduction

The family *Rhabdoviridae* comprises a diverse collection of viruses linked by a common bullet-shaped or bacilliform morphology. They are among the most widely distributed viruses in nature, infecting vertebrates, invertebrates and many plants. A large number are included so far in five genera: *Vesiculovirus, Lyssavirus, Ephemerovirus, Cytorhabdovirus* and *Nucleorhabdovirus*. Many others are virtually uncharacterized and, because of the lack of any serological relatedness to other viruses, they remain ungrouped. This entry deals with those ungrouped rhabdoviruses, including Gossas virus, Klamath virus, Navarro virus, and fish rhabdoviruses. Except for the fish rhabdoviruses, very little is known about these ungrouped viruses. The mammalian and bird rhabdoviruses are grown in most common laboratory cell lines, e.g. BHK-21, Vero, etc., while fish rhabdoviruses are grown in some special cell types such as epithelioma papulosum cyprini (EPC) cells. In common with other rhabdoviruses, mammalian and bird rhabdoviruses readily infect newborn or weanling mice when injected intracerebrally. No human disease is known for any of these viruses.

Gossas Virus

The Gossas virus was originally isolated in 1984 by Bres from the salivary glands of an adult bat (*Tadarida* sp.) caught in Dakar, Senegal. Neither the pathology of infection nor the neutralizing antibody was detected in the bat at that time. However, in laboratory infections antibody can be detected in mice as well as rabbits. The virus did not crossreact antigenically with many other viruses, including Sindbis, Semliki Forest, West Nile or blue tongue virus. Like vesicular stomatitis virus (VSV), the virus grows in BHK-21 and also in Vero cells. Oita rhabdovirus, another ungrouped virus, was isolated from bat by Oya in Japan. No other information is available on this virus.

Klamath Virus

Klamath virus was originally isolated in 1965 by Johnson from a 3-month-old (immature) meadow vole (*Microtus montanus*) collected at Klamath Falls, Oregon. The virus was subsequently detected in Alaska at Dot Lake (red-backed mice, *Clathrionomys rutilus*) and at the University of Alaska (meadow vole, *Microtuseconomus*). The virus is bullet-shaped (167 × 80 nm) in morphology, typical of a rhabdovirus. When inoculated intracerebrally into newborn mice, infectious virus was detected in the lung and brain. The virus grows in chicken embryonated eggs as well as laboratory cell lines. In infected cells, cytoplasm contains the nucleocapsids and matured virions found around the cisternae of endoplasmic reticulum. Antigenic crossreactivity tests using 154 different viruses indicate that Klamath virus is antigenically distinct.

Navarro Virus

The Navarro virus was isolated in 1984 by the Cali Virus Laboratory (Cali, Colombia) from the spleen of an adult wild turkey vulture (*Cathartes aura*) shot in Navarro, Colombia. The known properties of the virus are very similar to the Klamath virus.

Fish Rhabdoviruses

The rhabdoviruses that infect fish are particularly interesting because their hosts live in a wide variety of habitats and include such diverse fish as salmon, trout, cod, carp, pike, perch, etc. These viruses were initially designated as members of either the *Lyssavirus* or *Vesiculovirus* genera on the basis of electrophoretic migration of their proteins. Recently, it has been recognized that these classifications require modification, and the fish rhabdoviruses are now classified as 'unassigned' (formerly known as *Lyssavirus*) and 'vesiculo-like' (formerly known as *Vesiculovirus*) in accordance with the sixth report (1995) of the International Committee on Taxonomy of Viruses (ICTV). The members of the 'unassigned'

Infections – Comparative Diagnosis, p. 455. Amsterdam: Elsevier/North Holland.

Jackson AO, Francki RIB and Zuidema D (1987) Biology, structure and replication of plant rhabdoviruses. In: Wagner RR (ed.) *The Rhabdoviruses*, p. 427. New York: Plenum Press.

Jackson AO and Wagner JDO (1998) Procedures for plant rhabdovirus purification, polyribosome isolation, and replicase extraction. In: Foster G and Taylor S (eds) *Plant Virology Protocols: From Virus Isolation to Transgenic Resistance*, vol. 81, p. 77. Totowa, NJ: Humana Press.

Sylvester ES and Richardson J (1989) Aphid-borne rhabdoviruses – relationships with their vectors. *Adv. Vector Dis. Res.* 9: 313.

Ungrouped Mammalian, Bird and Fish Rhabdoviruses

Bishnu P De and **Amiya K Banerjee**, Department of Molecular Biology, The Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, USA

Introduction

The family *Rhabdoviridae* comprises a diverse collection of viruses linked by a common bullet-shaped or bacilliform morphology. They are among the most widely distributed viruses in nature, infecting vertebrates, invertebrates and many plants. A large number are included so far in five genera: *Vesiculovirus, Lyssavirus, Ephemerovirus, Cytorhabdovirus* and *Nucleorhabdovirus*. Many others are virtually uncharacterized and, because of the lack of any serological relatedness to other viruses, they remain ungrouped. This entry deals with those ungrouped rhabdoviruses, including Gossas virus, Klamath virus, Navarro virus, and fish rhabdoviruses. Except for the fish rhabdoviruses, very little is known about these ungrouped viruses. The mammalian and bird rhabdoviruses are grown in most common laboratory cell lines, e.g. BHK-21, Vero, etc., while fish rhabdoviruses are grown in some special cell types such as epithelioma papulosum cyprini (EPC) cells. In common with other rhabdoviruses, mammalian and bird rhabdoviruses readily infect newborn or weanling mice when injected intracerebrally. No human disease is known for any of these viruses.

Gossas Virus

The Gossas virus was originally isolated in 1984 by Bres from the salivary glands of an adult bat (*Tadarida* sp.) caught in Dakar, Senegal. Neither the pathology of infection nor the neutralizing antibody was detected in the bat at that time. However, in laboratory infections antibody can be detected in mice as well as rabbits. The virus did not crossreact antigenically with many other viruses, including Sindbis, Semliki Forest, West Nile or blue tongue virus. Like vesicular stomatitis virus (VSV), the virus grows in BHK-21 and also in Vero cells. Oita rhabdovirus, another ungrouped virus, was isolated from bat by Oya in Japan. No other information is available on this virus.

Klamath Virus

Klamath virus was originally isolated in 1965 by Johnson from a 3-month-old (immature) meadow vole (*Microtus montanus*) collected at Klamath Falls, Oregon. The virus was subsequently detected in Alaska at Dot Lake (red-backed mice, *Clathrionomys rutilus*) and at the University of Alaska (meadow vole, *Microtuseconomus*). The virus is bullet-shaped (167 × 80 nm) in morphology, typical of a rhabdovirus. When inoculated intracerebrally into newborn mice, infectious virus was detected in the lung and brain. The virus grows in chicken embryonated eggs as well as laboratory cell lines. In infected cells, cytoplasm contains the nucleocapsids and matured virions found around the cisternae of endoplasmic reticulum. Antigenic crossreactivity tests using 154 different viruses indicate that Klamath virus is antigenically distinct.

Navarro Virus

The Navarro virus was isolated in 1984 by the Cali Virus Laboratory (Cali, Colombia) from the spleen of an adult wild turkey vulture (*Cathartes aura*) shot in Navarro, Colombia. The known properties of the virus are very similar to the Klamath virus.

Fish Rhabdoviruses

The rhabdoviruses that infect fish are particularly interesting because their hosts live in a wide variety of habitats and include such diverse fish as salmon, trout, cod, carp, pike, perch, etc. These viruses were initially designated as members of either the *Lyssavirus* or *Vesiculovirus* genera on the basis of electrophoretic migration of their proteins. Recently, it has been recognized that these classifications require modification, and the fish rhabdoviruses are now classified as 'unassigned' (formerly known as *Lyssavirus*) and 'vesiculo-like' (formerly known as *Vesiculovirus*) in accordance with the sixth report (1995) of the International Committee on Taxonomy of Viruses (ICTV). The members of the 'unassigned'

subgroup include infectious hematopoietic necrosis virus (IHNV), viral hemorrhagic septicemia virus (VHSV) and hirame rhabdovirus (HIRRV). The members in the 'vesiculo-like' subgroup include spring viremia of carp virus (SVCV) and pike fry virus (PFV). These viruses may also be classified into two divisions reflecting their host: salmonoid and nonsalmonoid. The IHNV and VHSV are examples of salmonoid fish rhabdovirus, whereas SVCV and PFV belong to the nonsalmonoid group. Because of rapid progression of infection and high mortality, these viruses represent a major threat to aquaculture. Unlike other rhabdoviruses, the fish rhabdoviruses infect and cause disease at characteristically low temperatures (12–15°C), probably due to adaptation to colder aquatic habitats. In the laboratory, these viruses grow in standard cell lines such as BHK-21 and WI-38 and also some poikilothermic cell lines such as FHM, RTG-2 and STE-137. In all cases, the optimum temperature for growth and virus stability is 15–18°C. Like other rhabdoviruses, ultrastructurally they display a bullet-shaped morphology with glycoprotein spikes projecting from the viral envelope. Like VSV, the SVCV and PFV virions have been shown to contain protein kinase activity. Specific characteristics of individual fish rhabdoviruses are described below.

Infectious Hematopoietic Necrosis Virus

The IHNV is enzootic in the sockeye salmon population on the west coast of North America. In recent years, another major host for IHNV was found to be rainbow trout. The infectious hematopoietic necrosis disease was introduced into Japan in 1977 and it appeared in Europe in 1987. The IHNV-infected disease now represents a major threat to aquaculture all over Europe. The virus infection and the disease appear to be cold-dependent, with the characteristic of epizootics at 13°C which disappears at a higher temperature (above 15°C). Two other viruses, namely Oregon sockeye salmon disease virus (OSDV) and Sacramento River Chinook disease virus (SRCDV), are antigenically similar to IHNV and produce diseases with nearly identical symptoms.

Clinical features and pathology

An epizootic of IHNV usually begins with a sudden rise in mortality. Clinical signs are the appearance of dark color, loss of appetite, anemia, exophthalmia, distension of the abdomen with ascites, general viremia and fecal casts. Petechial hemorrhages occur near the base of the fins and on the mesenteries surrounding the viscera. Necrosis of the hematopoietic tissues in the anterior kidney and spleen can be detected by histological examination. With increasing severity of the disease, necrosis is also detected in liver, pancreas, and granular cells in the wall of the alimentary tract.

Fish infected with SRCDV and OSDV do not feed and have symptoms similar to those produced by IHNV. Extensive subcutaneous hemorrhaging occurs, accompanied by the appearance of red blotches on the skin and the gills turning pale. Like IHNV, infection with these viruses also causes necrotic lesions in kidney, pancreas, spleen and adrenal cortex. Virus particles are detected in the interstitial spaces of the infected organs and in some cases in the cytoplasmic vacuoles.

Transmission

The IHNV is transmitted through water, either by feeding on infected carcasses or by exposure to eggs from infected fish. Gills and gastrointestinal tracts are the most probable route of entry of the virus. Transmission also occurs from adult carriers to fry. The virus is readily detected in ovarian or seminal fluid of the infected fish. In the laboratory, defective interfering (DI)-like particles are produced when the cells are infected at higher multiplicity of infection (m.o.i).

Molecular aspects

The morphology and genome size (11 kb) of IHNV is identical to VSV. However, several differences are observed among IHNV and other rhabdoviruses. IHNV specifies six mRNAs rather than five mRNAs as is found in VSV. The five mRNAs encode the viral structural proteins RNA polymerase (L, 225 kDa), envelope glycoprotein (G, 59 kDa), nucleocapsid protein (N, 42 kDa) and two matrix proteins (M1, 26 kDa and M2, 22 kDa. Like P protein of VSV, the M1 protein is phosphorylated and believed to function in transcription, similar to other rhabdoviruses. The sixth mRNA encodes a unique nonstructural protein (NV) that is expressed in infected cells but is not present in purified virions. Recently, the complete nucleotide sequence of the genome of IHNV has been determined. This represents the first complete nucleotide sequence of a fish rhabdovirus genome. The genome organization is 3′-N-M1-M2-G-NV-L-5′. The intergenic region contains the conserved sequence 5′-AGAYAG/C-3′ (antigenomic polarity) which is followed by a stretch of seven adenosine residues. This sequence is present at the end of every sizeable open reading frame found in the IHNV genome and is similar to that found in the intergenic region of VSV.

Viral Hemorrhagic Septicemia Virus

History

VHSV was initially isolated from the rainbow trout (*Oncorhynchus mykis*) in the Egtved region of Jutland (Denmark) in 1950. It represents a major threat to the fish farming industry in continental Europe, causing devastating viral disease in fish. Brown trout (*Salmo trutta*) and brook trout (*Salvelinus fontinalis*) were found to be immune to the disease; however, they could be infected experimentally. Recently, both the geographical distribution and host range of the virus were shown to be wider, as VHSV was isolated in routine control of other species of fish in the USA. The antigenic crossreactivity and the electrophoretic pattern of the viral structural proteins indicate that these viruses are similar to those originating in Europe. The North American isolates, however, were not associated with any specific clinical condition, and no experimental overt infection could be obtained with these strains.

Clinical features and pathology

Infected trout appear black, especially on the head and abdomen, and show exophthalmia of the eyes (sometimes with protruding eyeballs giving a popeye effect due to the hemorrhages in the connective tissues of the eye pit), distended abdomen and severe anemia. The gills appear pale pink or greyish white. Acute hemorrhages are seen at the base of the pectoral fins. At the lateral line they are less frequent or may be entirely absent. In some cases the fish also show neurological and motor disorders, such as spiral swimming at the bottom of the pond, tilted swimming and darting through and out of water. Death occurs within several days.

The most prominent pathological changes are the scattered hemorrhages in skeletal muscles, mouth cavities and sex organs. Histology of the liver and kidney shows necrotic foci with hepatocytes including cytoplasmic vacuoles, karyolysis and pyknosis. Lymphoid and spleen tissues show accumulation of mononucleated and immune erythrocytes.

Immunology and interferon production

Neutralization studies of 76 natural isolates of VHSV from Danish, Norwegian and Swedish rainbow trout showed that 72 of them were essentially identical to the F1 strain, suggesting that the four others may represent different serotypes. However, in cell cultures all were recognized by fluorescein isothiocyanate-conjugated anti-F1 antibody. Diagnosis of the disease is made by immunofluorescent staining of tissue or by direct isolation of the virus from tissue homogenates.

Experimental infection of rainbow trouts with VHSV has been shown to produce interferon that reaches a maximal level (about 2800 units ml^{-1}) at 3 days postinfection. Physicochemical properties of fish interferon have been determined as the molecular mass of about 26 kDa, sedimentation coefficient of 2.5 S and isoelectric point of 4.5–6.2. It has been suggested that the production of interferon may play a role in the antiviral response of the fish at temperatures above 15°C.

Molecular aspects

The VHSV genome, like IHNV, encodes five structural proteins: the nucleocapsid protein N (41 kDa), the polymerase-associated protein M1 (28 kDa) similar to the P protein of VSV, the matrix protein M2 (24 kDa) similar to the M protein in VSV, the glycoprotein G (74 kDa), the polymerase protein L (~150 kDa) and a nonstructural protein NV (14 kDa). By direct sequencing VHSV genome organization was confirmed to be 3′-N-M1(P)-M2(M)-G-NV-L-5′. The NV protein has no significant sequence similarity to the NV protein of IHNV or any other known protein. The predicted sequence of the M1 and M2 proteins are rich in Ser and Thr residues and both are phosphorylated. The RNA polymerase activity of all fish rhabdoviruses, including VHSV, have a lower optimum temperature (15–20°C), as opposed to 30–32°C in VSV. Also, unlike other rhabdoviruses, the RNA polymerase activity of VHSV is stimulated by Mn^{2+} rather than Mg^{2+}.

Spring Viremia of Carp Virus

SVCV belongs to the vesiculo-like genus of the family *Rhabdoviridae*. The infectious dropsy of carp (*Cyprinus carpio*) was originally reported in Europe as early as 1930, but the origin of the disease remained unknown until 1950. Now it is clearly established that SVCV is the causative agent of hemorrhagic swimbladder inflamation and infectious dropsy in common carp. Clinical signs of the virus infection are external and internal hemorrhages, peritonitis and ascites. The symptoms of the disease, however, vary depending on the form of the disease: acute, chronic, asymptomatic or latent. In an overt disease, the central nervous system and peripheral nerves are affected. The fish becomes hyperactive, with the appearance of ulcerative dermal vesicles (carp erythrodermatitis). The kidneys and spleen become enlarged and contain the highest titer of the virus. Peak viremia appears on the 6th day postinfection and again on the 9th and 10 days. Excretion of the virus in

feces and mucus occurs on the 11th day, and finally the fish dies around the 20th day. Since the first isolation of the virus several other fish hosts have been reported, suggesting that SVCV has a wider host range than the carp family. The virus causes significant mortality in both juvenile and adult fish, and therefore it has a large economic impact on the fish farming industry in Europe. In experimental infections, fingerling carp, pike fry and the larvae and carp fry are also susceptible.

Pike Fry (Rhabdo) Virus

The PFV belongs to the vesiculo-like genus of the *Rhabdoviridae* family. It is involved in two diseases of fry of the northern pike (*Esox lucius* L.): a 'head disease' identified by swelling or lumps on the body, or a 'red disease' identified by swelling and reddish color of large areas of the body. These diseases were first seen in the Nieuw-Vennep hatchery in The Netherlands around 1959. Both diseases have a high mortality rate. The hydrocephalus associated with the 'head disease' makes the fish lose equilibrium and swim erratically near the surface of the water. Clinical symptoms are poor growth, hemorrhages in the brain, spinal cord, spleen and pancreas, and degenerative necrotic changes in kidney tubules. The 'red disease' is characterized by pale gills, hemorrhages in trunk and muscle connective tissue, and red swollen areas above the pelvic fins. The virus is detected in the hematopoietic tissues of the kidney. At the molecular level, the PFV is similar to VSV, encoding the structural proteins, N, P, M, G and L.

Hirame Rhabdovirus

The HIRRV is a member of the 'unassigned' genus of the *Rhabdoviridae* family. It was first isolated from Japanese flounder (*Paralichthys olivaceus*) and from ayu fish (*Plecoglossus altivelis*), both of which are valuable cultured fish species in Japan. The clinical signs of HIRRV infection are similar to those caused by SVCV. Crossreactions in serological studies have suggested that HIRRV is related to the well-characterized North American fish pathogen IHNV. The genes of the two matrix proteins (M1 and M2) and the glycoprotein (G) of HIRRV have been sequenced. Sequences of all of the internal gene junctions have also been determined. The matrix protein genes have been shown to share a high amino acid similarity (81.5% for M1 and 86.0% for M2) with the respective genes of IHNV. The G protein shared the highest sequence identity (74.3%) and similarity (83.3%) with the G protein of IHNV.

See also: **Fish viruses; Defective interfering viruses; Rabies virus (*Rhabdoviridae*); Interferons: General features, Therapy of aids and cancer; Vesicular stomatitus viruses (*Rhabdoviridae*).**

Further Reading

Basurco B and Benmansour A (1995) Distant strains of the fish rhabdovirus VHSV maintain a sixth functional cistron which codes for a nonstructural protein of unknown function. *Virology* 212: 741.

Benmansour A, Paubert G, Bernard J and DeKinkelin P (1994) The polymerase-associated protein (M1) and the matrix protein (M2) from a virulent and an avirulent strain of viral hemorrhagic septicemia virus (VHSV), a fish rhabdovirus. *Virology* 198: 602.

Bjorklund HV, Higman KH and Kurath G (1996) The glycoprotein genes and gene junctions of the fish rhabdoviruses spring viremia of carp virus and hirame rhabdovirus: analysis of relationships with other rhabdoviruses. *Virus Res.* 42: 65.

Kurath G, Higman KH and Bjorklund HV (1997) Distribution and variation of NV genes in fish rhabdoviruses. *J. Gen. Virol.* 78: 113.

Pilcher KS and Fryer JL (1980) The viral diseases of fish: a review through 1978. Part I: Diseases of proven viral etiology. *Crit. Rev. Microbiol.* 7: 287.

Schutze H, Enzmann PJ, Kuchling R, Mudt E, Niemann H and Mettenleiter TC (1995) Complete genomic sequence of the fish rhabdovirus infectious hematopoietic necrosis virus. *J. Gen. Virol.* 76: 2519.

Schutze H, Enzmann PJ, Mudt E and Mettenleiter TC (1996) Identification of the non-virion (NV) protein of fish rhabdoviruses viral haemorrhagic septicaemia virus and infectious haematopoietic necrosis virus. *J. Gen. Virol.* 77: 1259.

Wolf KE (1988) Fish viruses and fish viral diseases. Ithaca, NY: Cornell University Press.

RHINOVIRUSES (*PICORNAVIRIDAE*)

Glyn Stanway, Department of Biological Sciences, University of Essex, Colchester, Essex, UK

History

Rhinoviruses are the major cause of the familiar, mild, upper respiratory tract infection known as the common cold and are among the most frequent human pathogens. References to colds are found from earliest times and both Hippocrates and Pliny the Younger discussed possible therapies. Although long suspected, their infectious nature was formally demonstrated in the early 1900s by Kruse, Foster and Dochez, who transmitted colds from donors to recipients using filtered nasal secretions. It was not until the advent of organ and tissue culture that rhinoviruses were first isolated, initially in the laboratories of Pelon and Price. When culture conditions were modified, notably by using temperature and pH similar to those found in the nose, the number of identified rhinovirus serotypes grew rapidly.

Taxonomy and Classification

Viruses designated rhinoviruses have been isolated from humans and cattle. Human rhinoviruses (HRVs) comprise by far the largest group, with over 100 serotypes (HRV-1A, HRV-1B and HRV-2 to HRV-100) and will be the exclusive focus for this discussion. There are three bovine rhinoviruses. All are members of *Picornaviridae* and are classified in the rhinovirus genus. Two viruses isolated from horses, currently named equine rhinoviruses 1 and 2, are also picornaviruses, but sequence analysis shows them to be more similar to members of the aphthovirus genera.

Serotypes are defined in terms of neutralization by antisera and absence of crossreactivity. Several attempts have been made to further divide the large number of HRVs, the most useful being on the basis of receptor tropism. Around 90% of rhinoviruses (the major receptor group) use the membrane protein, intercellular adhesion molecule 1 (ICAM-1) as their cell receptor, whereas most of the rest (the minor group) use members of the low density lipoprotein (LDL) receptor family. A single serotype (HRV-87) apparently recognizes a sialoprotein. Analysis using antivirus agents shows that rhinoviruses can be divided into two classes, but these do not correspond directly to the receptor groups (**Table 1**).

Properties of the Rhinovirus Virion

Electron microscopy reveals the typical picornavirus appearance of spherical, largely featureless particles approximately 30 nm in diameter. The particles are nonenveloped, of icosahedral symmetry and made up of genomic RNA, surrounded by 60 copies each of four capsid proteins, VP1–4. An intermediate in capsid assembly is the protomer, composed of one copy of VP1, VP3 and VP0, a precursor in which VP4 and VP2 are covalently linked. Five protomers, arranged symmetrically about a fivefold axis, give another important intermediate, the pentamer, which will form one of the 12 corners of the icosahedron. Twelve pentamers come together to form the protein coat. Cleavage of VP0 is the final step of assembly and may be related to encapsidation of the nucleic acid and stabilization of the mature particle. One or two

Table 1 Receptor[a] and drug binding[b] grouping of human rhinovirus serotypes

Major receptor group	*Minor receptor group*	*HRV-87 group*
3, **4**, **5**, **6**, 7, **8**, 9, 10, 11, 12, **13**, **14**, 15, 16, **17**, 18, 19, 20, 21, 22, 23, 24, 25, **26**, **27**, 28, **32**, 33, 34, **35**, 36, **37**, 38, 39, 40, 41, **42**, **43**, **45**, 46, **48**, 50, 51, **52**, 53, **54**, 55, 56, 57, 58, 59, 60, 61, 63, 64, 65, 66, 67, 68, **69**, **70**, 71, **72**, 73, 74, 75, 76, 77, 78, **79**, 80, 81, 82, **83**, **84**, 85, **86**, 88, 89, 90, **91**, **92**, **93**, 94, **95**, 96, **97**, 98, **99**, 100	1A, 1B, 2, 29, 30, 31, 44, 47, 49, 62	**87**

[a]The major rhinovirus receptor group are the serotypes which recognize ICAM-1, the minor group recognize the LDL receptor, and HRV-87 binds to a sialoprotein.

[b]Depending on their response to a panel of antivirus agents which bind to a hydrophobic pocket beneath the canyon, rhinoviruses can be divided into two groups, A (serotypes shown in bold type) and B. Structural and sequencing studies suggest that this reflects a fundamental difference between these viruses.

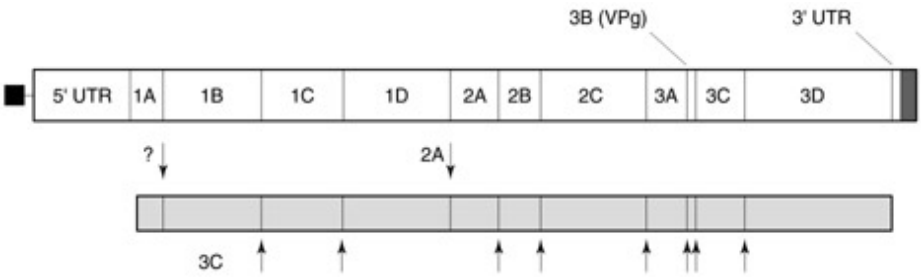


Figure 1 The rhinovirus genome (top) and the polyprotein which it encodes. The single-stranded, positive sense genome has a small protein, VPg, attached to the 5′ terminus (shown as a black box) and is polyadenylated at its 3′ terminus (hatching). Genes are named 1A–3D according to their genomic location. The polyprotein is cleaved by proteases encoded by the 2A and 3C genes and by an unknown activity, at the positions shown by arrows, giving the individual proteins.

copies of VP0 remain uncleaved, but the significance of this is not known.

Our understanding of rhinoviruses has been greatly advanced by work done in the laboratory of Rossmann, where the three-dimensional structures of a number of rhinoviruses have been solved to high resolution. These studies have shown that VP1, VP2 and VP3 are structurally similar, each being composed of an eight-stranded β barrel, and differing mainly in the loops and elaborations which join or project from the β strands making up this core structure. This basic structure for the capsid proteins is seen in all picornaviruses, as is their relative position in the virus particle. At the surface, the area surrounding the fivefold axis of the pentamer is composed primarily of VP1, while VP2 and VP3 are arranged side by side at a greater distance from this axis. VP4 has an extended configuration and is located internally, underneath the other proteins. The pentamer is stabilized by interactions involving the N and C termini of VP1 and VP3, while adjacent pentamers are held together largely by hydrogen bonds between parts of VP2 and VP3. The loops between the β strands of VP1–VP3 are the location of the regions of antigenic importance and they form much of the surface of the virus. The amino acid sequences of these regions are highly variable between serotypes and this is presumably the origin of the many rhinovirus serotypes.

Properties of the Rhinovirus Genome

Rhinovirus genetic information is carried by a single-stranded RNA molecule, the 5′ terminus of which is covalently linked to a small, virus-encoded protein, VPg (**Fig. 1**). The RNA has positive polarity and functions directly as a message, encoding a single polyprotein which is cleaved post-translationally by virus-encoded proteases to give the final products. Several rhinoviruses have been sequenced and they have a genome 7100–7200 nucleotides in length, the shortest known for picornaviruses. This is made up of a 600 nucleotide 5′ untranslated region (UTR), an open reading frame of approximately 2150 codons, a 3′UTR of around 40 nucleotides and a 3′ poly(A) tract. All rhinoviruses have a characteristic nucleotide composition with a preponderance of A and U, particularly in the third position of codons.

Properties of Rhinovirus Proteins

The rhinovirus proteins are numbered 1A(VP4), 1B(VP2), 1C(VP3), 1D(VP1), 2A, 2B, 2C, 3A, 3B, 3C, 3D, according to their location in the initial polyprotein. In addition to these 11 proteins, several precursors, for example 3AB and 3CD, have a significant half-life and are functionally active. The capsid proteins were discussed earlier, and much of our knowledge of the nonstructural proteins is inferred from other picornaviruses. The largest nonstructural proteins, 2C and 3D, together with 3AB, are known to be involved in RNA replication. 2C has amino acid sequence motifs seen in nucleotide binding proteins and, by analogy with other positive-stranded virus proteins, may have a helicase function. 3D has been shown to be the RNA-dependent RNA polymerase and contains motifs (YGDD for instance) which are seen in other polymerases. 2A and 3C are cysteine proteases involved in processing the polyprotein and both are structurally homologous to the trypsin group of proteases. The three-dimensional structure of the HRV-14 3C protease has been solved and this has given insights into the processing mechanism and the additional involvement of 3C, as part of the precursor 3CD, in interacting with the virus RNA during RNA replication. 2A also has other functions, since it is involved in host cell protein synthesis shut off and seems to interact with the 5′UTR to influence translation. The other proteins are not well understood, but 2B may alter host cell

membrane permeability, enhancing virus release, and mutations in this protein have been seen to give changes in cell tropism.

Physical Properties

Rhinoviruses have a buoyant density in CsCl of 1.38–1.42. The characteristic feature which distinguishes them from the similar pathogens, enteroviruses, is their lability below pH 6.0. In contrast, they are relatively thermostable, surviving for days at 20–37°C and this may be an important factor in their spread. As they do not have a lipid envelope, they are resistant to organic solvents such as ether and are unaffected by the detergent sodium deoxycholate. Alcohol/phenol disinfectants are effective virucidal agents.

Replication

Strategy and early events

In common with other viruses, the replication of rhinoviruses requires several steps, including attachment, penetration, uncoating, protein synthesis, RNA replication and assembly. The extent of our knowledge of these steps varies considerably and the study of the first step, attachment, has probably advanced the furthest. Structural analysis by Rossmann has revealed the presence of deep depressions, often termed 'canyons', running at a constant radius around each of the fivefold axes and these contain the sites at which most, if not all, rhinoviruses interact with their cellular receptor. ICAM-1, used by the major receptor group, is an immunoglobulin-like molecule which is sufficiently narrow to penetrate the canyon. The involvement of the canyon has been demonstrated by mutational analysis and by cryoelectron microscopy of HRV-16/ICAM-1 complexes.

The interaction with the receptor, possibly together with exposure to the low pH of endosomes during internalization, is believed to trigger conformational changes. These are centered on the fivefold axis, where a channel through the capsid and the endosomal membrane opens, due to the exposure of hydrophobic domains of VP1 and VP4, allowing the RNA to be released into the cytoplasm.

Translation

Rhinoviruses have a positive sense genome and the first step in macromolecular synthesis is translation, to enable the production of virus-specific proteins. The 5′UTR is very similar to that of enteroviruses, in terms of sequence and predicted secondary structure and translation proceeds in the same manner, i.e. following internal ribosomal entry within the 5′UTR. Similarly, rhinoviruses also shut off host cell protein synthesis, by inactivating the cap binding complex, and their cap-independent, internal ribosome entry allows them to circumvent this inactivation.

Post-translational processing

Synthesis of a single virus polyprotein necessitates cleavage to give the mature proteins and this is brought about by at least two proteolytic activities encoded by the virus itself. $3C^{pro}$ carries out the majority of cleavages, usually at peptide bonds between the dipeptides QG, QS, QA, QT or QM. The amino acids P, A, T or V are often found close to the cleavage site and these, with other features, probably help in its definition. $2A^{pro}$ performs the first cleavage event of processing, liberation of the capsid protein precursor, P1. $2A^{pro}$ usually cleaves between AG, VG or YG residues in rhinoviruses and upstream sequences add specificity to this process. Details of the cleavage of VP0 to VP4 and VP2, which does not occur until the new virus particles are assembled, are not yet fully known.

RNA synthesis

The nature of the genetic material requires that an RNA-dependent RNA polymerase is present and this is virally encoded as no host enzyme exists. Once the first rounds of translation and processing are complete, the synthesized polymerase ($3D^{pol}$), together with other virus products and host proteins, forms a membrane-associated complex. This uses the genomic-sense RNA as a template for producing negative-sense copies which in turn act as templates for genomic-sense RNA synthesis. Some of these act as messages, whereas others are packaged into virus particles. RNA secondary structural elements in both the 5′ and 3′UTR are critical in this process and appear to act as binding sites for host and virus proteins.

Assembly, Release and Cytopathology

Assembly of rhinovirus particles is via the protomers and pentamers already described and takes place in association with membranes. The determinants important in RNA packaging and the details of this process are not known. The gross effect on the cell (the cytopathic effect) probably stems from the inhibition of cellular protein and RNA synthesis, together with the accumulation of virus components. It is also possible that infection triggers apoptosis. Recent evidence suggests that one of the functions of the 2B protein is to affect membrane permeability of the cell, which enhances virus release.

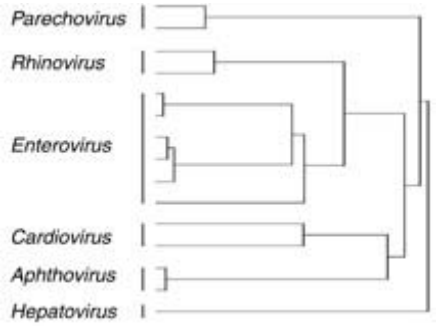


Figure 2 Genetic relationships, based on amino acid identity of the 2C protein, between selected picornaviruses, representing each of the six currently recognized genera. The dendrogram shows that rhinoviruses and enteroviruses are close relatives.

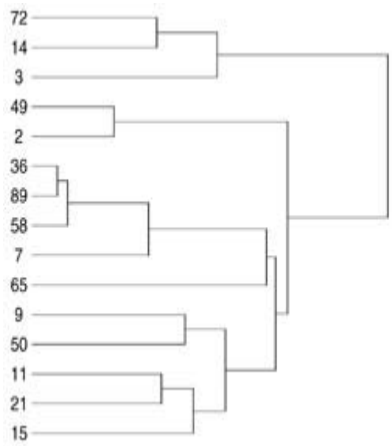


Figure 3 Genetic relationships between a number of HRV serotypes, based on the sequence of part of VP2. The data show that some rhinoviruses, for example HRV-36, HRV-58 and HRV-89 are very closely related. Two distinct molecular groups can be observed and these correlate with those defined on the basis of antivirus drug susceptibility. HRV-3, HRV-14 and HRV-72, which form one molecular group, are all drug group A members, whereas the other serotypes belong to drug group B.

Geographic and Seasonal Distribution

HRVs infect individuals living under all climatic conditions, from arctic to tropical. Infections occur throughout the year, but in temperate countries there is an increased occurrence of HRV colds in September and less markedly in May. These may be correlated with summer/autumn and spring/summer changes in the weather.

Host Range, Virus Propagation and Detection

HRVs exhibit a restricted host range, a major factor probably being the lack of receptors in nonhuman organisms. They cannot be transmitted to any commonly used laboratory animals, and, although some serotypes can infect chimpanzees and gibbons, no symptoms are observed.

Tissue cultures useful for HRV isolation are mainly of human origin and include fetal kidney and tonsil as well as continuous cell lines. Human diploid lung fibroblasts are commonly used for primary isolation, and MRC-5 and HRV-sensitive strains of HeLa (e.g. Ohio-HeLa) give excellent results. Several tissue culture systems need to be used to maximize HRV recovery from clinical samples.

In addition to using virus propagation, routine diagnosis can be performed by polymerase chain reaction (PCR) detection, which is much more rapid and is highly sensitive. PCR employs primers which bind to highly conserved sequences, found in the 5′UTR of all HRVs.

Genetics and Evolution

Rhinoviruses are typical picornaviruses in terms of structure and genome organization. The genetic information is carried on one piece of RNA, precluding reassortment, and the major evolutionary mechanism is the accumulation of point mutations, which occurs at high frequency due to the lack of $3D^{pol}$ proof-reading activity. Recombination has been demonstrated in some picornaviruses and may play a role in rhinovirus evolution, although there is no direct evidence for this. Sequence comparisons with other picornavirus genera shows that rhinoviruses are closely related to enteroviruses and these two genera share features not seen in other picornaviruses, e.g. detailed folding of the 5′UTR and a trypsin-like $2A^{pro}$ (**Fig. 2**). Little is known about rhinovirus evolution on a contemporary time-scale, although antigenic variants can be isolated during an outbreak. Some serotypes, for example HRV-36, HRV-58 and HRV-89, are very closely related at the RNA sequence level, suggesting that they have diverged relatively recently (**Fig. 3**). The existence of intertypes, with antigenic properties intermediate between two serotypes, strengthens the conclusion that some currently known rhinoviruses have a recent common ancestor.

Serological Relationship and Variability

A major factor in the incidence of rhinovirus infection is the large number of serotypes; additionally, several of these can cocirculate within the community. Although there is evidence of an increase in the frequency with which higher numbered and therefore more recently identified serotypes are isolated, many lower-numbered rhinoviruses are still prevalent.

Thus, many serotypes seem to coexist within the human population. The multiplicity of serotypes is in contrast to two other medically important picornaviruses, the polioviruses (three serotypes) and hepatitis A virus (a single serotype). Several serotypes fall into groups on the basis of low-level immunological crossreactivity with hyperimmune serum (HRV-36, HRV-58 and HRV-89; HRV-2 and HRV-49 for example), but it is not known whether this plays any role in protection from heterologous serotypes. These antigenic groups seem to correlate with close molecular relationships (**Fig. 3**).

Epidemiology

Rhinoviruses are a major cause of morbidity and economic loss. They have been implicated in 10–40% of cases of acute respiratory disease, a category accounting for around half of all acute illnesses in the developed world. Thus, although the common cold is less severe than some respiratory diseases, rhinoviruses are responsible for a significant proportion of working days lost in industry, commerce and education.

Rhinovirus infections are most common in young children and babies and the infection rate decreases with age, possibly due to prior exposure to a growing number of serotypes. Estimates of the average incidence of rhinovirus colds have varied, but it is probably at least 0.5 per adult per year. It is agreed that the rate in babies and infants is approximately three times that in adults. Once an individual is infected, the virus often spreads to other members of the family, the home being a major site of transmission. Young children are frequently causes of introduction into the family unit and other young children and the mother the most frequent recipients. The infection rate in mothers possibly reflects greater exposure to infected individuals, although other factors may be involved as it has been shown that the susceptibility to colds varies with the stage of the menstrual cycle. Schools and preschool groups also facilitate rhinovirus transmission.

Transmission and Tissue Tropism

Two routes may be important in rhinovirus spread: direct contact and airborne transmission. People with colds contaminate their hands and environmental surfaces with virus from nasal secretions. The hands of uninfected individuals can then become contaminated by direct contact with the person with a cold or by touching the contaminated surface. The virus can enter the body when the hand is used to rub an eye or pick the nose, common features of human behavior. In experiments which exclude this route, some transmission still occurred, suggesting that contaminated airborne particles or aerosols may also be important. Transmission in the family context correlates with time of exposure to the infected individual, severity of their symptoms and titer of rhinovirus in nasal secretions.

Once introduced into the body, the primary and major site of infection is the epithelial surface of the nasal mucosa. Rhinoviruses thus show a restricted tissue tropism, which may be correlated, among other factors, with their optimum growth temperature (33°C). Clinical manifestations are therefore largely limited to common-cold-like symptoms. Sometimes though, rhinoviruses infect other tissue, particularly the lower respiratory tract, the maxilliary sinus and the middle ear.

Pathogenicity and Clinical Features of Infection

Rhinovirus infections are usually relatively trivial and not life-threatening. Extensive work has been performed on their etiology and pathogenesis using human volunteers. These studies show that a rhinovirus infection can be initiated by less than one $TCID_{50}$ (50% tissue culture infectious dose) of virus, if it is administered to the nasopharynx. Virus shedding can be detected within 24 h and reaches a maximum after 2–3 days, coinciding with the onset of symptoms. Virus titers thereafter fall rapidly but may remain detectable for 3 weeks.

Rhinovirus infection is accompanied typically by the symptoms of a common cold. These vary with the individual and possibly the particular rhinovirus, but usually include nasal discharge and obstruction, often with sneezing, coughing and sore throat. Fever and malaise are less commonly seen than in infections with other respiratory viruses, but gastrointestinal disorders are not uncommon, particularly in children.

Although limited largely to the upper respiratory tract, rhinovirus infections are believed to predispose some individuals to bacterial sinusitis and otitis media. In addition, rhinoviruses can produce serious and debilitating lower respiratory infections, particularly in the elderly, young children and patients with existing disorders, such as cystic fibrosis and bronchopulmonary dysplasia. Up to 40% of exacerbations of chronic bronchitis may be due to rhinovirus infections. Respiratory infections are known to increase the severity and frequency of asthma attacks in susceptible individuals and rhinoviruses are the most important pathogens associated with increased asthma.

Pathology and Histopathology

Physical examination of patients with a rhinovirus infection usually reveals nasal obstruction and discharge, the nasal mucosa being pale and edematous. Elevated levels of bradykinin found locally, possibly contribute to this edema. Neutrophil infiltration is observed in the common cold and this may be caused by rhinoviruses inducing the expression of interleukin 8 (IL-8), a neutrophil chemoattractant. IL-8 may also be involved in asthma exacerbation, which is characterized by rhinovirus-induced enhancement of allergic inflammation and responsiveness to histamine.

The detailed histopathology of rhinovirus infections is not well documented. Nasal mucosa biopsies reveal few histological abnormalities, although shed, virus-containing, columnar epithelial cells can be detected in nasal secretions, suggesting that the epithelial surface of the nasal mucosa is primarily involved. Infection of bovine tracheal organ cultures with bovine rhinovirus leads to the shedding of large numbers of ciliated epithelial cells, leaving a smooth epithelial surface.

Immune Response

Rhinovirus infection stimulates the production of type-specific IgA, IgG and IgM antibodies in up to 90% of individuals. These are detectable within 2–3 weeks in serum and nasal secretions and their levels rise for 5–6 weeks. Most immunoglobulin in nasal secretions is IgA and this is probably a major factor in protection against re-infection (or at least reduction of disease symptoms) by the homotypic rhinovirus. Serum and secretory antibody persist for several years after infection, although their levels decline. As antibody appears late in the infection, it probably plays little part in recovery but it may be involved in final virus clearance. Other mechanisms, including interferon involvement, may therefore be involved in the recovery process. T cell responses which are serotype-crossreactive have been observed, but their contribution to subsequent protection is not known.

Prevention and Control

At present, there is no generally available means of protecting against or treating common colds produced by rhinoviruses. The large number of serotypes apparently precludes conventional vaccines and few attempts have been made to pursue this approach. Most effort has been expended on the development of chemical antivirus agents, but of the many shown to have antirhinovirus activity *in vitro,* none has yet proved clinically useful, although some are currently at the clinical trials stage. An alternative approach is interferon, produced in large amounts by recombinant DNA means. High, intranasal doses, initiated several days before virus challenge, have proved to be effective in preventing illness. However, side effects limit long-term use and as symptoms are only reduced if treatment is commenced before virus infection, interferon has limited applicability. It may prove useful in the family context when it is important to prevent virus spread to specific individuals, e.g. asthmatics and bronchitics. In these cases, it may also be possible to use what we know about the properties of rhinoviruses and their mode of transmission to limit spread. Interrupting transmission by avoiding direct contact with the infected person and by frequent hand-washing is sensible. Furthermore, experiments have been performed in which paper tissues impregnated with virucidal agents (mild acid to exploit rhinovirus lability at low pH) were used for frequent nose-blowing and hand-wiping by infected and uninfected individuals kept together under confined conditions for prolonged periods. The tissues were effective in preventing infection. One semi-empirical approach is the regular topical application of warm, moist air to the upper respiratory tract. Its beneficial effect may be due to the temperature increase in the nose, making it less conductive to rhinovirus replication, although the stimulation of host mechanisms may also be involved. In the absence of specific antivirus agents, proprietary treatments which reduce symptoms are widely used. In addition, both large amounts of vitamin C and zinc acetate tablets have been reported to reduce the severity and duration of colds, although their efficacy have been frequently questioned.

Future Perspectives

The past few years have seen major advances in the study of rhinoviruses, particularly the determination of three-dimensional structures and the identification of receptors. Several nucleotide sequences have been determined, revealing conserved regions which have been exploited by PCR-based systems for rapid detection. These are an improvement on virus isolation procedures for detection and are giving a more complete picture of the role of rhinoviruses in human disease, for instance the recognition of their importance in asthma exacerbation. Rhinovirus serotype diversity means that broadly reactive prophylactic or therapeutic approaches must be devised. The economic and social significance of the common cold continues to stimulate research and our knowledge of the structure of the virus particle, the determinants of antigenicity and cellular receptors, together with

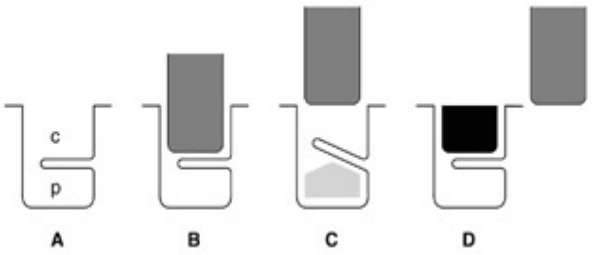


Figure 4 Targets for antirhinovirus therapeutic agents. (**A**) Underneath the canyon (c), which is the site for receptor binding, there is a hydrophobic pocket (p). (**B**) The receptor (gray) docks with the canyon. (**C**) A class of effective antirhinovirus compounds binds strongly to the pocket. This distorts the canyon floor, preventing receptor binding and/or uncoating. (**D**) An alternative approach is to saturate the receptor binding domains with a soluble form of the receptor, thus preventing receptor binding.

other advances, will contribute greatly to the development of rational approaches. It still seems unlikely that a vaccine can be produced, although the ability to pin-point areas of antigenic importance means that it may become possible to construct molecules which can mimic the antigenicity of several serotypes and thus reduce the complexity of the antigenic diversity problem. Even so, several components to the vaccine would be necessary and there may be a reluctance to use such a vaccine, with possible side effects, against what is usually a mild pathogen.

The antirhinovirus drug route may prove more feasible and there are at least three potential targets. Best studied are agents which bind within a hydrophobic pocket located underneath the canyon, blocking virus attachment to cells and/or uncoating (Fig. 4). They have high specificity and efficacy against rhinoviruses *in vitro* and seem to lack toxicity. As the pocket is well conserved, the drugs are potentially broadly reactive and it is possible that one, or a small number, of agents would be effective against all rhinovirus serotypes. Following success with inhibitors of the HIV-1 protease, agents (e.g. peptidyl-aldehydes) which specifically interfere with processing by inhibiting the rhinovirus $3C^{pro}$ are being widely studied. A third approach is to use soluble ICAM-1 to block early virus/cell interactions. However, even such highly specific reagents are likely to suffer from problems which may limit their usefulness. For example, in common with other RNA viruses, rhinoviruses have a high mutation rate and drug-resistant mutants can appear rapidly *in vitro*. Furthermore, maximal benefit from most agents tested is possible only if they are administered before infection. Thus, despite improvements in understanding, rhinovirus infections may continue to be a familiar feature of our lives.

See also: **Antivirals; Interferons: General features, Therapy of aids and cancer; Pathogenesis: Animal viruses; Respiratory viruses; Vaccines and immune response; Viral receptors; Virus structure: Atomic structure, Principles of virus structure; Virus–host cell interactions.**

Further Reading

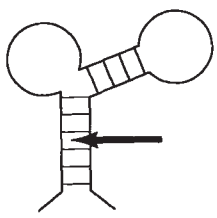
Couch RB (1996) Rhinoviruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 713. Philadelphia: Lippincott-Raven.

Johnson SL (1997) Problems and prospects of developing effective therapy for common cold viruses. *Trends Microbiol.* 5: 58.

Racaniello VR (ed.) (1990) Picornaviruses. *Current Topics in Microbiology and Immunology*, vol. 161. Berlin: Springer-Verlag.

Stott EJ and Garwes DJ (1990) Rhinoviruses, adenoviruses and coronaviruses: their role in repiratory disease. In: Collier LH and Timbury MC (eds) *Topley and Wilson's Principles of Bacteriology, Virology and Immunology*, 8th edn, p. 243. London: Edward Arnold.

RIBOZYMES



Robert H Symons, Department of Plant Science, University of Adelaide, Glen Osmond, South Australia, Australia

History

Ribozymes are catalytic RNA molecules, first identified in the early 1980s. They have the intrinsic ability to break and form covalent bonds in RNA molecules. In many ways they can be compared to the protein enzymes which catalyze cleavage of peptide bonds in other proteins or peptides. However, ribozymes can

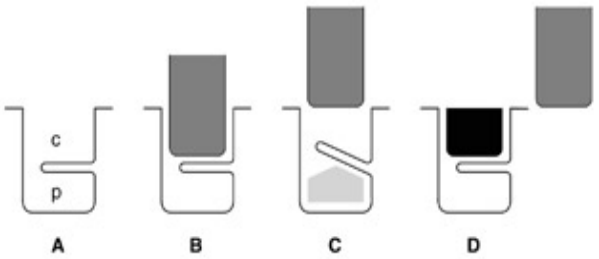


Figure 4 Targets for antirhinovirus therapeutic agents. (**A**) Underneath the canyon (c), which is the site for receptor binding, there is a hydrophobic pocket (p). (**B**) The receptor (gray) docks with the canyon. (**C**) A class of effective antirhinovirus compounds binds strongly to the pocket. This distorts the canyon floor, preventing receptor binding and/or uncoating. (**D**) An alternative approach is to saturate the receptor binding domains with a soluble form of the receptor, thus preventing receptor binding.

other advances, will contribute greatly to the development of rational approaches. It still seems unlikely that a vaccine can be produced, although the ability to pin-point areas of antigenic importance means that it may become possible to construct molecules which can mimic the antigenicity of several serotypes and thus reduce the complexity of the antigenic diversity problem. Even so, several components to the vaccine would be necessary and there may be a reluctance to use such a vaccine, with possible side effects, against what is usually a mild pathogen.

The antirhinovirus drug route may prove more feasible and there are at least three potential targets. Best studied are agents which bind within a hydrophobic pocket located underneath the canyon, blocking virus attachment to cells and/or uncoating (Fig. 4). They have high specificity and efficacy against rhinoviruses *in vitro* and seem to lack toxicity. As the pocket is well conserved, the drugs are potentially broadly reactive and it is possible that one, or a small number, of agents would be effective against all rhinovirus serotypes. Following success with inhibitors of the HIV-1 protease, agents (e.g. peptidyl-aldehydes) which specifically interfere with processing by inhibiting the rhinovirus $3C^{pro}$ are being widely studied. A third approach is to use soluble ICAM-1 to block early virus/cell interactions. However, even such highly specific reagents are likely to suffer from problems which may limit their usefulness. For example, in common with other RNA viruses, rhinoviruses have a high mutation rate and drug-resistant mutants can appear rapidly *in vitro*. Furthermore, maximal benefit from most agents tested is possible only if they are administered before infection. Thus, despite improvements in understanding, rhinovirus infections may continue to be a familiar feature of our lives.

See also: **Antivirals; Interferons: General features, Therapy of aids and cancer; Pathogenesis: Animal viruses; Respiratory viruses; Vaccines and immune response; Viral receptors; Virus structure: Atomic structure, Principles of virus structure; Virus–host cell interactions.**

Further Reading

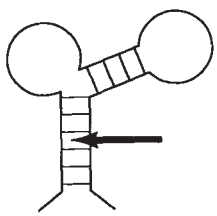
Couch RB (1996) Rhinoviruses. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 713. Philadelphia: Lippincott-Raven.

Johnson SL (1997) Problems and prospects of developing effective therapy for common cold viruses. *Trends Microbiol.* 5: 58.

Racaniello VR (ed.) (1990) Picornaviruses. *Current Topics in Microbiology and Immunology*, vol. 161. Berlin: Springer-Verlag.

Stott EJ and Garwes DJ (1990) Rhinoviruses, adenoviruses and coronaviruses: their role in repiratory disease. In: Collier LH and Timbury MC (eds) *Topley and Wilson's Principles of Bacteriology, Virology and Immunology*, 8th edn, p. 243. London: Edward Arnold.

RIBOZYMES



Robert H Symons, Department of Plant Science, University of Adelaide, Glen Osmond, South Australia, Australia

History

Ribozymes are catalytic RNA molecules, first identified in the early 1980s. They have the intrinsic ability to break and form covalent bonds in RNA molecules. In many ways they can be compared to the protein enzymes which catalyze cleavage of peptide bonds in other proteins or peptides. However, ribozymes can

Table 1 RNAs involved in RNA-catalyzed splicing/cleavage reactions

RNA present in	*Reaction in vivoz*	*End groups on cleaved RNAs*	*Nucleotide cofactor*	*Mechanism*
Ribonuclease P	Processing tRNA precursors	5′-P,3′-OH	No	Hydrolysis
Group I introns	Self-splicing of ribosomal introns	5′-P,3′-OH	Yes	Transesterification
Group II introns	Self-splicing of organelle introns	5′-P,3′-OH	No	Transesterification
Three plant viroids, four circular (virusoids) and four linear satellite RNAs	Cleavage of oligomeric precursors	5′-OH,2′,3′-cyclic phosphate	No	Transesterification
Hepatitis delta virus (HDV) RNA	Cleavage of oligomeric precursors	5′-OH,2′,3′-cyclic phosphate	No	Transesterification
Newt satellite II RNA transcript	Unknown	5′-OH,2′,3′-cyclic phosphate	No	Transesterification
Neurospora mitochondrial plasmid transcript VS RNA	Unknown	5′-OH,2′,3′-cyclic phosphate	No	Transesterification

also be *cis*-acting since the ribozyme component of an RNA molecule can cleave at a specific site in another part of the same molecule. All except one of the naturally occurring ribozymes (**Table 1**) are of the latter type.

In 1983, Altman and his colleagues described the first, and so far the only, truly naturally occurring catalytic ribozyme. The bacterial ribonuclease P is involved in the processing of precursor transfer RNA (tRNA) by specific cleavage of a 5′-terminal sequence (**Fig. 1A**). The enzyme consists of one molecule of protein and one molecule of single-strandard RNA; the isolated RNA can carry out the same processing reaction in *trans* as in the holoenzyme with multiple turnover and without being changed in the reaction.

The term ribozyme was first used by Cech and his colleagues in 1982 to describe the self-splicing activities of an intervening sequence (IVS) of ribosomal RNA precursor sequences in the protozoan *Tetrahymena* (**Fig. 1B**). This is an intramolecular reaction and the ribozyme component only catalyzes a single turnover and is modified during the reaction. It can, therefore, be considered to be acting in a quasicatalytic manner. All introns which self-splice as in **Fig. 1B** are called Group I introns. The molecular aspects of this self-splicing reaction continue to be extensively investigated.

In 1986, three groups reported a new type of intron self-splicing as in **Fig. 1C** and such introns are called Group II introns. They are less common than Group I introns and are found in organellar and bacterial genomes. They exist in fungal mitochondria, plant mitochondria and chloroplasts, algae and bacteria.

Also in 1986 two new ribozymes were identified in small circular plant pathogenic RNAs which were subsequently called the hammerhead and hairpin ribozymes. They both carry out the reaction summarized in **Fig. 1D**. A total of 14 plant pathogenic RNAs have so far been identified which can carry out either the hammerhead or hairpin ribozyme reaction or both the reactions (**Table 1**). Interestingly, only one nonpathogenic RNA has been reported to carry out the hammerhead reaction, the RNA transcripts from satellite DNA of the newt, and none have been reported for the hairpin ribozyme.

The only animal RNA pathogenic RNA known so far to undergo self-cleavage was reported in 1988 for hepatitis delta RNA which is essentially a satellite RNA dependent on hepatitis B virus for its replication. Both plus and minus forms of the approximately 1700 nt circular RNA self-cleave via a pseudoknot structure.

And, finally, a second nonpathogenic RNA was reported in 1990 to undergo the self-cleavage reaction of **Fig. 1D**; this was an 881 nt transcript of a circular mitochondrial DNA plasmid of *Neurospora*. Two-dimensional structural models around the self-cleavage site are different from other known self-cleavage structures.

It is perhaps surprising that all currently known types of self-processing or self-cleaving naturally occurring RNAs in **Table 1** were identified between 1982 and 1990. It remains to be seen whether any new types of reactions will be found from 1998 onwards.

Ribozymes of Non-viral Origin

Since the emphasis in this encyclopedia is on Virology, the nonviral ribozyme systems are only considered briefly here; the newt hammerhead ribozyme system is considered in the following section.

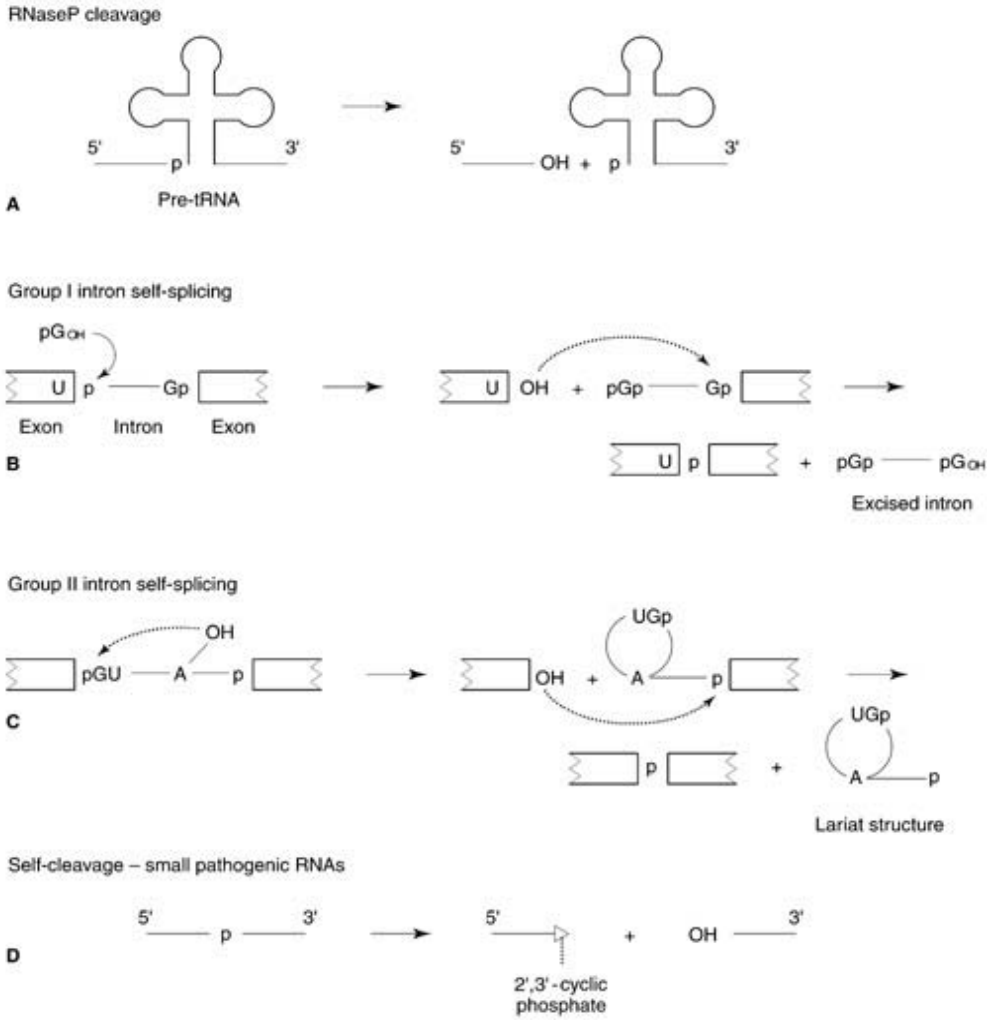


Figure 1 RNA processing reactions that are naturally RNA catalysed. See text and Tables 1 and 2 for further details.

Ribonuclease P (RNase P)

The reaction carried out by this ribozyme is summarized in **Fig. 1A**. RNase P is an endoribonuclease which cleaves precursor tRNAs at their 5′ ends to give the mature 5′ termini of tRNAs (**Fig. 1A**). The best characterized enzymes are those from the eubacteria *Escherichia coli* and *Bacillus subtilis*. The RNase P holoenzyme is composed of one basic protein subunit of approx. M_r 14000 (119 amino acids) and one single-stranded RNA molecule of 377 nucleotides (*E. coli*) or 401 nucleotides (*B. subtilis*). The RNAs make up 90% by weight of these enzymes and are not covalently coupled to the protein component.

The purified RNAs of bacterial RNase P can carry out the same reaction as the holoenzyme but high ionic strength is required in the reaction mixture. This result indicates that, *in vivo*, the catalytic component of RNase P is the RNA moiety.

The nucleotide sequences of the substrate tRNAs are not conserved around the cleavage sites for RNase P; hence there must be common structural features which allow specific recognition. The basic substrate requirements for RNase P activity are shown in **Fig. 2**; a base paired stem, one strand of which contains the eventual 3′ terminal–NCCA of the tRNA and the other strand the cleavage site. Hence, the RNA component of RNase P can act as a true ribozyme in *trans* to specifically cleave pre-tRNAs as well as unrelated RNAs which contain the basic sequence and structural requirements of **Fig. 2**.

Group I introns as ribozymes

The best characterized Group I system is that of the 413 nucleotide intervening sequence (IVS) in the nuclear rRNA precursor from *Tetrahymena thermophila*. The intron can be excised and the two exons

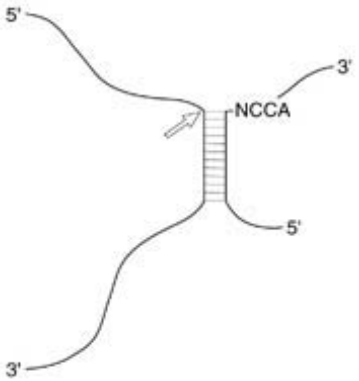


Figure 2 Basic structural and sequence requirements of a substrate for specific cleavage (site indicated by arrow) by RNase P or its RNA.

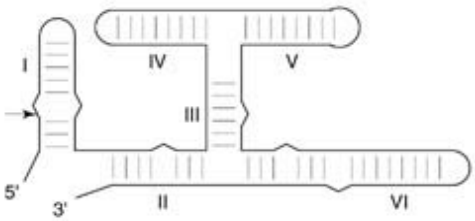


Figure 3 Secondary structure model of part of the 881 nt *Neurospora* VS RNA required for self-cleavage. The minimal sequence required contains 154 nt with only 1 nt 5′ to the self-cleavage site indicated by an arrow.

ligated in the complete absence of protein but there is a requirement for guanosine or a guanosine nucleotide as a cofactor as indicated in **Fig. 1B**. Hence, the IVS catalyzes only its own excision and is modified during the process. The released intron can then undergo further RNA-catalyzed cyclization and cleavage reactions that remove a total of 19 nucleotides from the 5′ end to produce an intron core (L-19 IVS) of 395 nucleotides.

The L-19 IVS RNA can be used *in vitro* in a classical enzymatic fashion in several in *trans* reactions, catalyzing the turnover of more than one substrate molecule and remaining unchanged. One of these reactions is a sequence-specific endonuclease.

Group II introns as ribozymes

Group II introns are found in eubacteria and eubacterial-derived organellar genomes of plants, mitochondria and chloroplasts. They have never been reported in the nucleus of eukaryotes. The overall self-splicing of Group II introns (**Fig. 1C**) involves the initial nucleophilic attack by a 2′-hydroxyl near the 3′

Table 2 Plant and animal pathogenic RNAs with *cis*-ribozyme activity

	Viroid or satellite abbreviation	*Size (nt)*	*Catalytic motif* *Plus RNA*	*Minus RNA*
A. Viroids				
Avocado sunblotch viroid	ASBV	246–251	Hammerhead	Hammerhead
Chrysanthemum chlorotic mottle viroid	CChMV	398–399	Hammerhead	Hammerhead
Peach latent mosaic viroid	PLMV	337–338	Hammerhead	Hammerhead
B. Satellite RNAs				
Sobemoviruses (Virusoids)				
Lucerne transient streak virus	vLTSV	322–324	Hammerhead	Hammerhead
Solanum nodiflorum mottle virus	vSNMV	377	Hammerhead	—
Subterraneum clover mottle virus	vSLMoV	322–388	Hammerhead	—
Velvet tobacco mottle virus	vVTMoV	365–366	Hammerhead	—
Nepoviruses				
Arabis mosaic virus	sARMV	300	Hammerhead	Hairpin
Chicory yellow mottle virus	sCYMV	457	Hammerhead	Hairpin
Tobacco ringspot virus	sTRSV	359–360	Hammerhead	Hairpin
Luteovirus				
Barley yellow dwarf virus	sBYDV	322	Hammerhead	Hammerhead
Hepatitis delta virus				
Hepatitis delta virus RNA	HDV RNA	1860	Pseudoknot	Pseudoknot
Uncharacterized				
Carnation stunt associated viroid-like RNA	CarSV RNA	275	Hammerhead	Hammerhead
Cherry small circular RNA	CSC RNAs	451	Hammerhead	Hammerhead

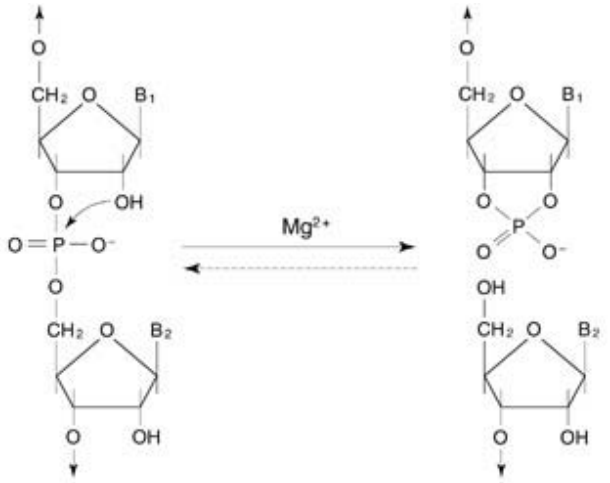


Figure 4 The self-cleavage reaction of RNA catalysed by Mg^{2+} or other divalent cations. This nonhydrolytic, transesterification reaction is theoretically reversible.

end of the intron on the 5′-terminal phosphate of the intron followed by the two steps of **Fig. 1C** to give the ligated exons and the released intron as a lariat structure. The splicing of intron-containing pre-mRNAs follows the same route but it occurs in the nucleus and requires the assistance of the spliceosome complex.

Demonstration of self-splicing of Group II introns *in vitro* requires very unphysiological conditions of high concentrations of salt and of Mg^{2+} and higher than normal temperatures. Only a minority of Group II introns have been shown to have ribozyme activity *in vitro*. Hence, progress in characterizing this ribozyme activity has lagged behind that of the Group I introns.

Neurospora VS RNA can self-cleave

VS RNA is a self-cleaving 881 nt RNA transcript of a mitochondrial DNA plasmid of *Neurospora*. Only 154 nt is required for self-cleavage and a potential two-dimensional structure is shown in **Fig. 3**. As for the hepatitis delta ribozyme (see below), only a single nucleotide 5′ to the cleavage site is required for activity. The RNA can be divided into a two component in *trans* ribozyme system. Even though VS RNA is present at high concentrations in mitochondria, its function is unknown.

Ribozymes of Viral Origin

The plant and animal pathogenic RNAs which have been shown so far to undergo self-cleavage *in vitro* are listed in **Table 2**. In all cases, this self-cleavage activity involves the divalent metal ion-catalyzed nucleophilic attack of the oxygen of the 2′-hydroxyl group at the cleavage site on the phosphate of the internucleotide linkage (**Fig. 4**). The cleavage products contain a 3′ end terminal 2′,3′-cyclic phosphate and a 5′-hydroxyl. The reaction is theoretically reversible.

Experimental evidence, at least for some of the RNAs of **Table 2**, indicates that this self-cleavage reaction is an essential step in their rolling circle replication (**Fig. 5**). Where both the plus RNAs (the dominant form *in vivo*) and the minus RNAs (the minor form) self-cleave, replication follows (**Fig. 5A**). The circular plus strand is copied by a host or viral-coded RNA-dependent RNA polymerase to produce a multimeric minus strand which is processed into unit length minus monomers. These are circularized by a host RNA ligase and the circular molecules copied by the RNA polymerase to give a multimeric plus strand which is cleaved specifically to monomers. These are then circularized to give the progeny circular RNAs, the dominant form found *in vivo*.

Of the RNAs in **Table 1**, only three plant satellite RNAs follow the replication cycle of **Fig. 5B** where the dominant plus form undergoes self-cleavage and the multimeric minus strand is copied directly to give

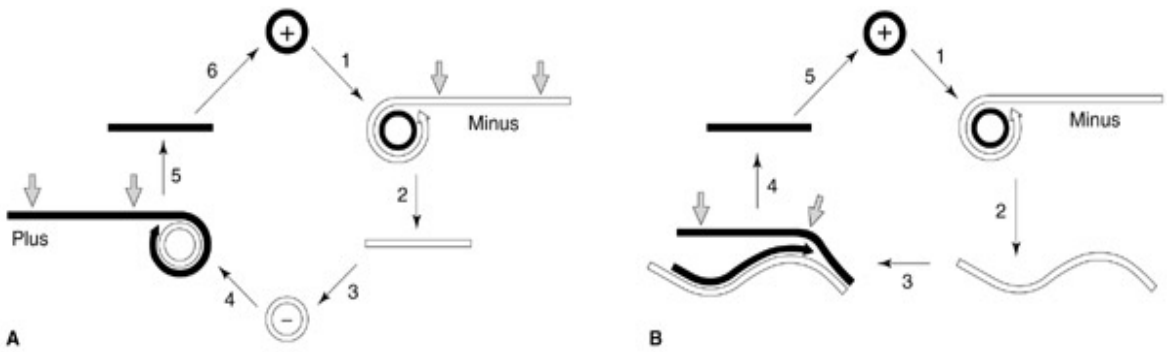


Figure 5 Two routes for the rolling circle replication of small, circular pathogenic RNAs, by an RNA polymerase. (**A**) Six-step pathway where both the multimeric minus and plus linear RNAs self-cleave (open arrows) to give monomers which are ligated *in vivo* to the circular forms. (**B**) Five-step pathway where only the multimeric plus RNA self-cleaves.

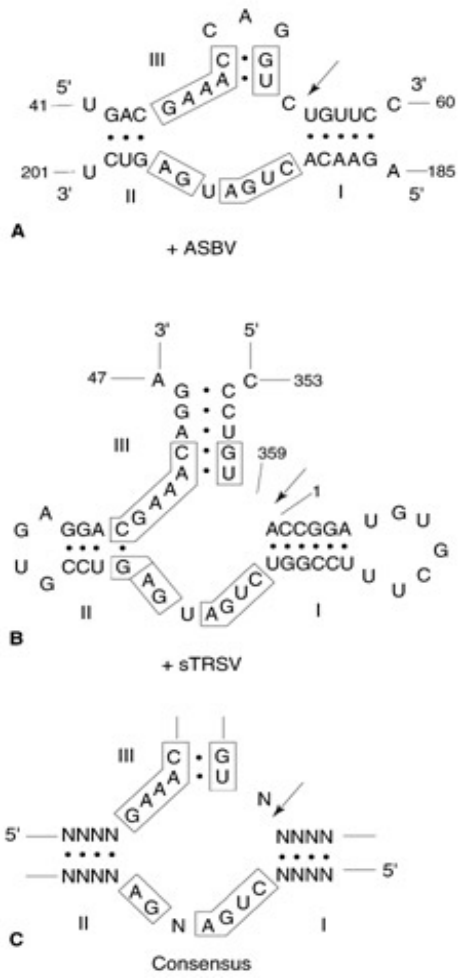


Figure 6 Two-dimensional hammerhead structures in self-cleaving RNAs. (**A**) Hammerhead structure that can form from opposite strands in the 247 nt rod-like structure of plus ASBV. (**B**) Hammerhead structure in the plus strand of the 359 nt satellite RNA of tobacco ringspot virus. (**C**) Consensus hammerhead structure for RNAs in Table 2. Self-cleavage site indicated by arrow. The 13 nucleotides that are highly conserved in all RNAs which cleave by the hammerhead structure are boxed.

the plus strand which self-cleaves to monomers which are then circularized.

Only the three viroids listed in **Table 2**, out of 27 so far identified, undergo self-cleavage *in vitro*. Viroids are single-stranded circular RNAs which vary in size from 246 to 463 nucleotides and infect a wide range of plant species. Many are of agricultural importance. Most of the remaining self-cleaving RNAs in **Table 2** are satellite RNAs in that they are dependent on a helper virus for their replication, including the only animal virus member, the hepatitis delta virus RNA which depends on hepatitis B virus. Two other self-cleaving plant pathogenic RNAs have yet to be characterized at the biological level.

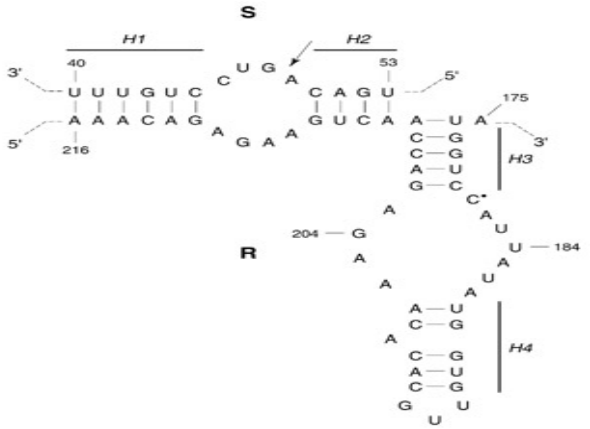


Figure 7 Standard convention for numbering the residues in a hammerhead structure.

So far only three types of two-dimensional structural motifs have been identified at the self-cleavage sites of the 14 pathogenic RNAs of **Table 2**. These are the hammerhead, hairpin and pseudoknot structures.

Hammerhead ribozyme

The hammerhead self-cleavage motif was first recognized in the plus and minus self-cleaving RNAs of avocado sunblotch viroid (ASBV) and of the satellite RNA of lucerne transient streak virus (sLTSV) and the plus form of the satellite RNA of tobacco ringspot virus (sTRSV). The original hammerhead structures predicted around the self-cleavage sites of (+)ASBV and (+)sTRSV together with the highly conserved nucleotides (boxed) in all hammerhead structures are given in **Fig. 6** together with the consensus hammerhead structure for all naturally occurring RNAs. *In vitro*, the RNA sequences in **Fig. 6** are all that are required to ensure efficient self-cleavage in the presence of Mg^{2+}. Hence, such RNAs can be considered as ribozymes since they catalyze a single turnover, intramolecular (*cis*) cleavage reaction.

In addition to the well-studied plant viroids and satellite RNAs in **Table 2**, there are two other naturally occurring circular RNAs where both plus and minus strands self-cleave via the hammerhead structure but which have yet to be characterized in detail at the biological level. One of these is the 275-nt circular RNA from carnation and named carnation small viroid-like RNA (CarSV RNA) which has a dsDNA counterpart in the form of head-to-tail monomers. The other self-cleaving viroid-like RNA is the 451 nt cherry small circular RNA which is associated with a series of double-stranded RNAs of putative viral origin in cherry trees showing the cherry chlorotic rusty spot disease.

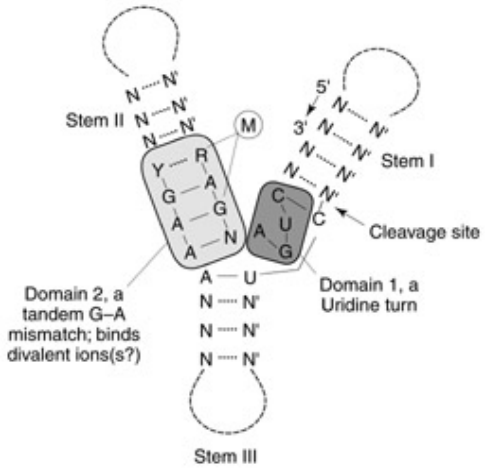


Figure 8 Schematic drawing of the structure of the hammerhead as determined by x-ray crystallography. (Reproduced with permission from McKay (1996).)

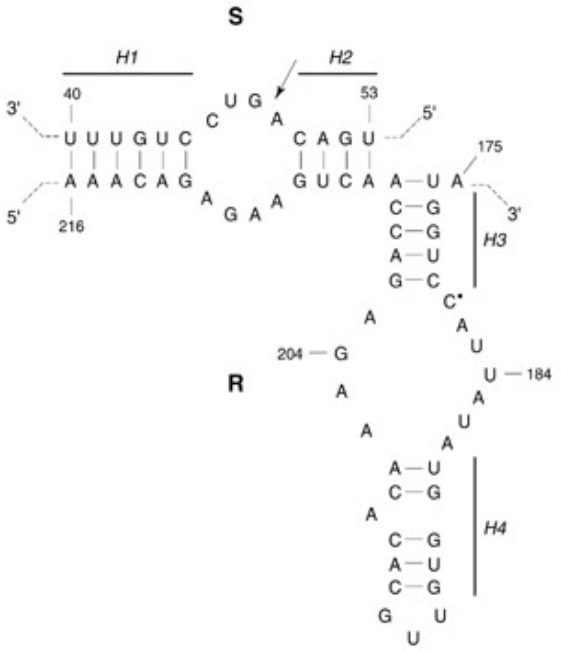


Figure 9 Secondary structure model of the hairpin ribozyme of the minus strand of the satellite RNA of tobacco ringspot virus ((−)sTRSV). The model is divided into a ribozyme component R and a substrate component S which form part of the 359nt sTRSV. The self-cleavage site between G48 and A49 is indicated by an arrow; note that the residue numbering is of the plus strand so that the numbering increases in the 3′ to the 5′ direction. Helices are numbered H1 to H4. UV irradiation of the ribozyme leads to cross-linking between G204 and U184.

In 1987, Epstein and Gall reported that *in vitro*-produced transcripts of tandemly repeated 330bp satellite II DNA of the newt self-cleaved by a hammerhead structure. This is the only non-viral RNA identified so far which self-cleaves via the hammerhead structure; the role of this reaction *in vivo* is unknown.

The small size of the hammerhead structure has led to its extensive characterization and manipulation. To aid in the description of various hammerhead constructs, a standard numbering system was introduced in 1992 (**Fig. 7**) and is now widely used.

Hammerhead reaction in *trans*

The plus and minus hammerhead structures of ASBV as identified in the native rod-like molecules are constituted from sections of the top and bottom RNA strands in the 247 nucleotides viroid. This led Uhlenbeck in 1987 to demonstrate hammerhead self-cleavage in *trans* using a 19nt ribozyme to catalyze cleavage with multiple turnover of a 24nt substrate as well as to cleave sequences embedded in a number of different RNAs. Haseloff and Gerlach in 1988 extended this approach by the design of small riboyzmes for the cleavage of specific sites in native RNA molecules *in vitro*, an approach which is now extensively used *in vitro* and *in vivo*.

Crystal structure of the hammerhead

The first crystal hammerhead structure was reported by McKay in 1994; it consisted of a 34-ribozyme and a 13-mer deoxynucleotide substrate. This was soon followed by Klug in 1995 and 1996 with crystal structures of an all RNA hammerhead. The three-dimensional structures developed from the three approaches are essentially the same (**Fig. 8**). Stem III of the hammerhead forms the stem of a Y-shaped structure where the arms are formed by stems I and II. Stems II and III are nearly collinear.

The hairpin ribozyme

This ribozyme (**Fig. 9**) has it origins in a 359nt linear satellite RNA of tobacco ringspot virus (sTRSV) which is encapsidated within viroids of the helper virus. Circular forms of sTRSV are found *in vivo* in infected plants but these are not encapsidated, in contrast to the encapsidation of the circular viroid-like satellite RNAs or virusoids associated with the Sobemoviruses (**Table 2**). The dominant plus form of sTRSV self-cleaves via the hammerhead structure and the minus form via the hairpin structure. Only three satellite RNA examples of the hairpin ribozyme have been identified, all within the Nepovirus group and having hammerhead self-cleavage in the plus strand.

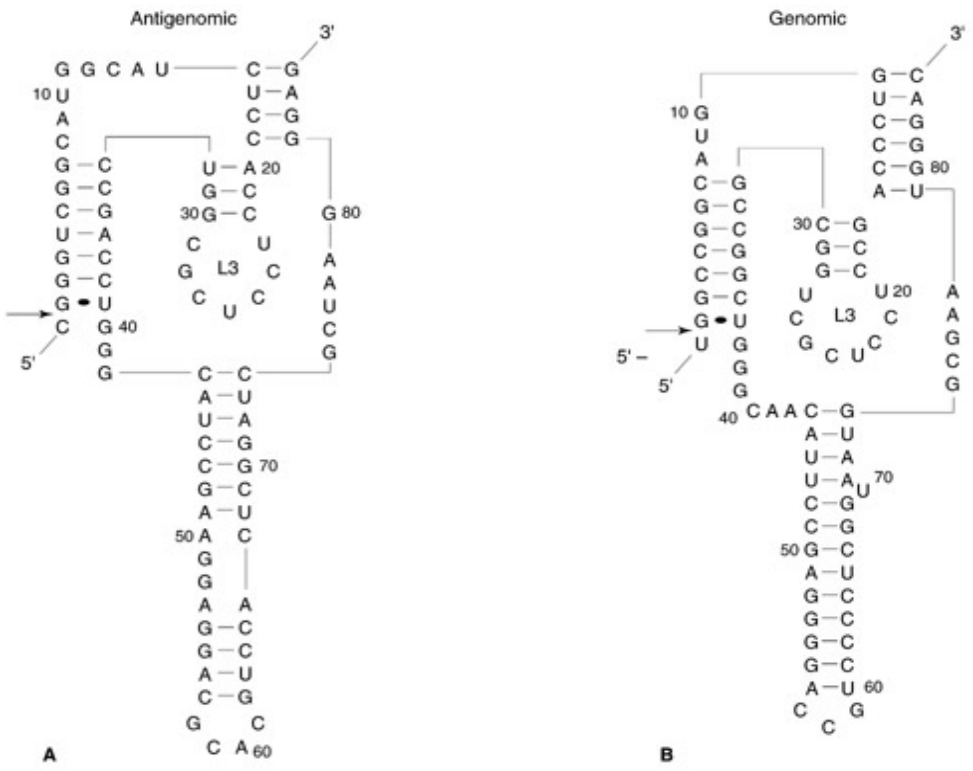


Figure 10 Secondary pseudoknot structures of the (**B**) genomic and (**A**) antigenomic ribozymes of hepatitis delta virus. The self-cleavage site in each ribozyme is indicated by an arrow. Only one nucleotide is needed 5′ to the self-cleavage site for cleavage to occur *in vitro*.

As for the hammerhead sequences in plus and minus ASBV, the nucleotide sequences required for self-cleavage of minus sTRSV are from two well-separated regions of the RNA molecule (**Fig. 9**). This allowed the demonstration of self-cleavage in *trans in vitro* and the manipulation of the hairpin ribozyme to target unrelated RNAs *in vitro* and *in vivo*. This work is at an early stage as compared to the application of the hammerhead ribozyme.

The pseudoknot riboyzme of hepatitis delta virus RNA

Hepatitis delta virus (HDV) is a subviral satellite virus of hepatitis B virus (HBV). The approx. 1680 nt circular genomic RNA is encapsidated in HBV-coded proteins and can fold into an unbranched rod-like structure as can the nonencapsidated complementary antigenomic RNA found *in vivo*. The antigenomic RNA contains an open reading frame coding for a 195 amino acid protein called the delta antigen which is essential for HDV RNA replication. At one end of these molecules there is a viroid-like domain containing the ribozyme domains required for the processing of multimeric intermediates during the rolling circle replication of HDV RNA. There are no known coding regions on the genomic RNA.

The two-dimensional structures of genomic and antigenomic riboyzmes are given in **Fig. 10** and each contain approx. 85 nt. By common usage they are referred to as pseudoknot structures. There is a high conservation of sequence between the two structures. The naturally occurring *cis* ribozyme activity can be converted into in *trans* ribozyme activity by splitting the structures in **Fig. 10** in at least two single-stranded regions to give ribozyme and substrate fragments.

See also: **Hepatitis Delta virus; Luteovirus; Nepoviruses (*Comoviridae*); Satellite RNAs and Satellite viruses; Sobemoviruses; Viroids.**

Further Reading

Been MD and Wickham GS (1997) Self-cleaving ribozymes of hepatitis delta virus RNA. *Eur J Biochem* 247: 741.

Birikh KR, Heaton PA and Eckstein F (1997) The structure, function and application of the hammerhead ribozyme. *Eur J Biochem* 241: 1.

Cech TR and Herschlag D (1996) Group I ribozymes:

substrate recognition, catalytic strategies, and comparative mechanistic analysis. *Nucleic Acids Mol. Biol.* 10: 1.
Hampel A (1998) The hairpin ribozyme: discovery, two-dimensional model, and development of gene therapy. *Prog. Nucleic Acid Res. Mol. Biol.* 58: 1–39.
McKay DB (1996) Three-dimensional structure of the hammerhead ribozyme. *Nucleic Acids Mol. Biol.* 10: 161.
Nolan JM and Pace NR (1996) Structural analysis of the bacterial RNase P. *Nucleic Acids Mol. Biol.* 10: 109.
Pyle AM (1996) Catalytic reaction mechanisms and structural features of Group II intron ribozymes. *Nucleic Acids Mol. Biol.* 10: 75.
Scott WG and Klug A (1996) Ribozymes: structure and mechanism in RNA catalysis. *Trends Biochem. Sci.* 21: 220.

RINDERPEST AND DISTEMPER VIRUSES (*PARAMYXOVIRIDAE*)

Tom Barrett, Institute for Animal Health, Pirbright Laboratory, Pirbright, Surrey, UK

History

Rinderpest virus

Rinderpest (or cattle plague) is one of the oldest known plagues of domestic livestock, with recognizable descriptions dating back to the fourth century AD. It is of ancient Asiatic origin but in more recent times devastating plagues of rinderpest swept across Europe in the eighteenth and nineteenth centuries. In 1711 the disease entered Europe through Venice and had spread as far as Britain by 1714. The economic effect of the subsequent European plagues was so drastic that it led to the establishment of veterinary schools to deal specifically with the problems of animal health, the first being the veterinary school at Lyon in France in 1762. A vigorous slaughter and quarantine policy succeeded in controlling the disease and by the beginning of the twentieth century Europe was free of rinderpest. Subsequently there have been periodic introductions through importation of live infected animals; the last serious outbreak in domestic cattle occurred in Belgium in 1920 and was caused by infected zebu cattle in transit from India to Brazil mixing with local cattle at the docks. Following the 1920 outbreak in Europe, the Office International des Epizooties (OIE) was set up in Paris to deal with matters concerning animal health in relation to international trade. The last known case reported in Europe was in an imported zoo animal in Rome in 1949. Isolated cases of rinderpest have occurred in Brazil (1920) and Australia (1924), again in association with importation of live infected cattle.

In 1889 a catastrophic outbreak of rinderpest occurred in Africa and was caused by the importation of infected cattle from India to feed Italian soldiers engaged in a military campaign in Abyssinia (Ethiopia). The subsequent panzootic spread to nearly all parts of the continent, reaching South Africa by 1897. Over 90% of domestic cattle, along with other highly susceptible wild animals such as buffalo (*Syncerus caffer*), eland (*Taurotragus* spp.), kudu (*Tragelaphus imberbis*), giraffe (*Giraffa* spp.) and wildebeest (*Connochaetes* spp.), were wiped out (**Table 1**). At the time most transport relied on oxen and, as the South African railway system had not been fully established, the economic consequences were devastating. In Kenya the Masai tribe suffered greatly and many people starved. Descriptions at the time stated that the East African Plains were so littered with dead carcasses that the vultures were unable to clear the carrion. Previously rinderpest was only seen in Africa in Egypt and parts of Senegal, where it was periodically introduced from Europe or the Middle East.

A similar plague in small ruminants, peste des petits ruminants, was first described in West Africa in 1942 by Gargadennec and Lalanne. This disease is

Table 1 Susceptibility of wildlife to rinderpest

Susceptibility	*Animals*
Very high	Buffalo, eland, kudu, warthog
High	Giraffe, bushbuck, bushpig, sitatunga, Uganda cob, bongo, wildebeest
Moderate	Reedbuck, topi, gemsbok, blesbok, bontbok, oribi, impala, springbok
Low	Waterbuck, dukier, orynx, Grant's gazelle, dikdik, hartebeest
Very low	Thomson's gazelle, hippopotamus, gerenuk

substrate recognition, catalytic strategies, and comparative mechanistic analysis. *Nucleic Acids Mol. Biol.* 10: 1.
Hampel A (1998) The hairpin ribozyme: discovery, two-dimensional model, and development of gene therapy. *Prog. Nucleic Acid Res. Mol. Biol.* 58: 1–39.
McKay DB (1996) Three-dimensional structure of the hammerhead ribozyme. *Nucleic Acids Mol. Biol.* 10: 161.
Nolan JM and Pace NR (1996) Structural analysis of the bacterial RNase P. *Nucleic Acids Mol. Biol.* 10: 109.
Pyle AM (1996) Catalytic reaction mechanisms and structural features of Group II intron ribozymes. *Nucleic Acids Mol. Biol.* 10: 75.
Scott WG and Klug A (1996) Ribozymes: structure and mechanism in RNA catalysis. *Trends Biochem. Sci.* 21: 220.

RINDERPEST AND DISTEMPER VIRUSES (*PARAMYXOVIRIDAE*)

Tom Barrett, Institute for Animal Health, Pirbright Laboratory, Pirbright, Surrey, UK

History

Rinderpest virus

Rinderpest (or cattle plague) is one of the oldest known plagues of domestic livestock, with recognizable descriptions dating back to the fourth century AD. It is of ancient Asiatic origin but in more recent times devastating plagues of rinderpest swept across Europe in the eighteenth and nineteenth centuries. In 1711 the disease entered Europe through Venice and had spread as far as Britain by 1714. The economic effect of the subsequent European plagues was so drastic that it led to the establishment of veterinary schools to deal specifically with the problems of animal health, the first being the veterinary school at Lyon in France in 1762. A vigorous slaughter and quarantine policy succeeded in controlling the disease and by the beginning of the twentieth century Europe was free of rinderpest. Subsequently there have been periodic introductions through importation of live infected animals; the last serious outbreak in domestic cattle occurred in Belgium in 1920 and was caused by infected zebu cattle in transit from India to Brazil mixing with local cattle at the docks. Following the 1920 outbreak in Europe, the Office International des Epizooties (OIE) was set up in Paris to deal with matters concerning animal health in relation to international trade. The last known case reported in Europe was in an imported zoo animal in Rome in 1949. Isolated cases of rinderpest have occurred in Brazil (1920) and Australia (1924), again in association with importation of live infected cattle.

In 1889 a catastrophic outbreak of rinderpest occurred in Africa and was caused by the importation of infected cattle from India to feed Italian soldiers engaged in a military campaign in Abyssinia (Ethiopia). The subsequent panzootic spread to nearly all parts of the continent, reaching South Africa by 1897. Over 90% of domestic cattle, along with other highly susceptible wild animals such as buffalo (*Syncerus caffer*), eland (*Taurotragus* spp.), kudu (*Tragelaphus imberbis*), giraffe (*Giraffa* spp.) and wildebeest (*Connochaetes* spp.), were wiped out (**Table 1**). At the time most transport relied on oxen and, as the South African railway system had not been fully established, the economic consequences were devastating. In Kenya the Masai tribe suffered greatly and many people starved. Descriptions at the time stated that the East African Plains were so littered with dead carcasses that the vultures were unable to clear the carrion. Previously rinderpest was only seen in Africa in Egypt and parts of Senegal, where it was periodically introduced from Europe or the Middle East.

A similar plague in small ruminants, peste des petits ruminants, was first described in West Africa in 1942 by Gargadennec and Lalanne. This disease is

Table 1 Susceptibility of wildlife to rinderpest

Susceptibility	*Animals*
Very high	Buffalo, eland, kudu, warthog
High	Giraffe, bushbuck, bushpig, sitatunga, Uganda cob, bongo, wildebeest
Moderate	Reedbuck, topi, gemsbok, blesbok, bontbok, oribi, impala, springbok
Low	Waterbuck, dukier, orynx, Grant's gazelle, dikdik, hartebeest
Very low	Thomson's gazelle, hippopotamus, gerenuk

also caused by a virus and is known as kata in West Africa. At first it was thought to be a variant of rinderpest virus adapted to grow in sheep and goats; however, it was subsequently shown to be an immunologically distinct virus with a separate epizootiology in areas where both viruses were enzootic. A disease of small ruminants, which was almost certainly caused by peste des petits ruminants virus, was first described in Senegal in 1871.

Canine distemper virus

Canine distemper virus is also a disease with a long history. Edward Jenner studied its neurological symptoms but it was Carré in 1906 who first showed that it was caused by a virus. In French the virus is known as 'la maladie de Carré'. In the summer of 1988 a large number of harbour seals (*Phoca vitulina*) died in the Baltic Sea and on the North Sea coasts of Northern Europe with clinical signs very similar to canine distemper in dogs. The epizootic was eventually shown to have been caused by a virus, at first thought to be canine distemper. Monoclonal antibody analyses and nucleic acid hybridization showed that it was a new virus distinct from canine distemper and it is now named phocid distemper virus. A disease with similar clinical signs caused mass mortality in Siberian seals (*Phoca sibirica*) in Lake Baikal in the winter of 1987. There was no obvious link between the virus outbreaks in the two seal populations and subsequent work showed that, in contrast to the European situation, the Russian epizootic was caused by a virus indistinguishable from canine distemper. The source of virus in this outbreak is thought to have been lakeside dogs which were suffering from canine distemper at that time. So, in addition to a wide range of land mammals, canine distemper virus can also infect aquatic carnivores.

In 1990 large numbers of striped dolphins (*Stenella coeruleoalba*) in the Mediterranean Sea were found dying from a virus infection which was referred to as the dolphin morbillivirus. Two years previously a virus had been isolated from porpoises (*Phocoena phocoena*) which was then found to be very closely related to the new dolphin virus. They were shown to be genetically distinct from other morbilliviruses, including phocid distemper virus. Serological evidence of infection with this virus was subsequently found in many other cetacean species in the Atlantic Ocean and the term cetacean morbillivirus has been suggested as a suitable name.

Taxonomy and Classification

Rinderpest was first shown to be a filtrable agent in 1902 and, along with the other viruses described above, is classified in the *Morbillivirus* genus (from the latin *morbus* meaning disease) within the *Paramyxoviridae* family. Antigenically the animal morbilliviruses are closely related to human measles virus, the type virus of the genus. Morbilliviruses are large enveloped viruses with a negative-strand RNA genome of about 16 kb. The virus particles are pleomorphic with an average diameter of around 200–300 nm. Measles virus is the only member of the group which has been shown to haemagglutinate red blood cells reproducibly. Unlike other members of the *Paramyxoviridae*, neuraminidase activity is generally not found in morbilliviruses; however, a highly substrate-specific neuraminidase activity has recently been demonstrated in rinderpest and peste des petits ruminants viruses.

Geographic Distribution

Rinderpest is enzootic on the Indian subcontinent and in parts of the Middle East and Eastern Africa. Sporadic outbreaks occur in countries bordering the enzootic regions. Peste des petits ruminants is enzootic in parts of West Africa but in the past few years it has spread across a broad belt of sub-Saharan Africa and eastwards through the Middle East and to southern Asia as far as Bangladesh (**Fig. 1**). It was thought until recently that India was free from peste des petits ruminants virus and that the morbillivirus-like disease prevalent in small ruminants was caused by rinderpest virus. However, in 1988 its presence in southern India was confirmed using specific cDNA hybridization probes.

Canine distemper has a worldwide distribution but is not found in very hot, arid regions. The development of an attenuated vaccine for canine distemper virus in the 1950s greatly reduced the incidence of disease in domestic dogs. However, many wild-life species are susceptible to the disease and can act as reservoirs of infection.

The origin of the European seal morbillivirus is unknown but it appears to be enzootic in Arctic waters, as sera collected from Greenland seals dating back to the early 1980s have been shown to be positive for morbillivirus antibodies. Arctic harp seals (*Phoca groenlandica*) show a high seropositivity and, unlike seals in European waters, are present in sufficient numbers to maintain the disease. The morbilliviruses isolated from porpoises and dolphins were probably transmitted by contact with another cetacean species in which the virus is enzootic. The most likely candidate in this case is the pilot whale (*Globicephalus melas*), which is gregarious and present in sufficient numbers to maintain the virus.

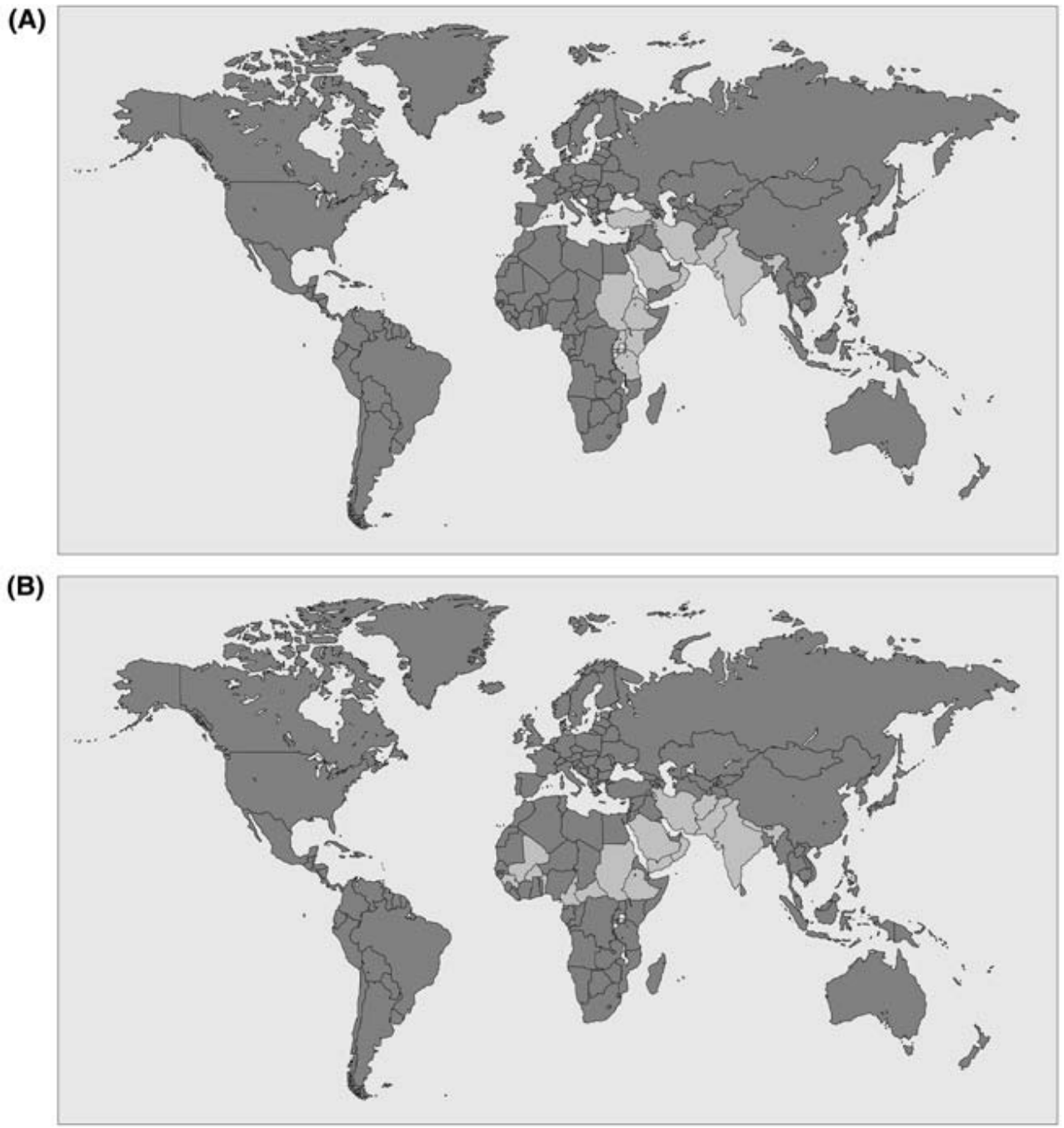


Figure 1 The present (1999) distribution of **(A)** rinderpest and **(B)** peste des petits ruminants viruses. Countries reporting disease since 1990 are in lighter shading.

Host Range and Virus Propagation

All species of the order *Artiodactyla* can be infected with rinderpest virus but some species are more susceptible to disease than others. In the case of cattle, Asian breeds are more resistant than European, while the opposite is the case in pigs. In the case of domestic ruminants, peste des petits ruminants virus only causes clinical disease in sheep and goats – goats being particularly susceptible. Peste des petits ruminants virus can also infect wild ruminants, as was illustrated by an outbreak of peste des petits ruminants virus in a zoo in the United Arab Emirates, but its full host range is unknown. In that outbreak

gazelle (*Gazella dama*), ibex (*Capra ibex nubiana*) and gemsbok (*Oryx gazella*) were involved. Some large ruminants and pigs can be infected subclinically but they are dead-end hosts and do not transmit the disease.

Canine distemper causes disease in all *Canidae* (dog, wolf, fox), *Mustelidae* (ferret, weasle, mink), *Procyonidae* (racoon, panda) and collared peccaries (*Tayassu tajacu*). Canine distemper can probably infect all carnivore species but in certain cases, e.g. some *Felidae* (cats), the infection may be subclinical. Until recently it was thought that canine distemper could not cause disease in large cats such as tigers, lions and panthers; however, in 1991–1992 canine distemper was found to have been responsible for the deaths of large cats in several zoos in the USA. Shortly afterwards a distemper outbreak had a devastating effect on the population of lions in the Serengeti in Africa. The virus responsible for infection in large cats was most closely related to that circulating in local wild carnivores, e.g. racoons in the USA and hyaenas (*Hyaena crocuta*) in Africa, and not a new strain adapted specifically to felids. The outbreak of canine distemper virus in Siberian seals has extended its range to aquatic carnivores.

Phocid distemper virus is known to infect several species of seal, ranging from the harp seals and ringed seals (*Phoca hispida*) of the North Atlantic to the grey (*Halichoerus grypus*) and harbor seals which have a more southerly distribution. During the 1988 European epizootic, although few grey seals succumbed to the infection, many were found to have developed morbillivirus-specific antibodies. This indicated a difference in susceptibility to disease as harbor seals died in large numbers. Phocid distemper virus accidentally infected mink in Denmark during the 1988 epizootic and so it can also cause disease in terrestrial carnivores.

The morbilliviruses can be isolated in a variety of cell types. Primary bovine kidney cells are usually used to isolate field strains of rinderpest virus and primary lamb kidney cells for peste des petits ruminants virus. More recently a marmoset lymphoblastoid cell line (B95a) and a *Theileria parva*-transformed bovine lymphocyte cell line have been reported to be suitable for rinderpest virus isolation. Virus can be best isolated from tissues such as mucosal lesions, lymph nodes or by cocultivation of washed buffy coat with susceptible cells such as bovine kidney or B95a cells. Cytopathic effects are usually evident between 3 and 12 days after infection, whereas control cells treated with antirinderpest antiserum should remain healthy.

Canine distemper virus is usually isolated by cocultivation of lymphocytes from infected animals with mitogen-stimulated canine or ferret lymphocytes and can then be adapted to grow in MDCK or Vero cells. Lung tissue is also a good source of virus for canine distemper and phocid distemper virus isolation. Primary seal kidney cells were used initially to isolate phocid distemper virus but the virus can also be adapted to grow in Vero cells. Typical cytopathic effects such as cell elongation, cell rounding, the formation of stellate cells and syncytia can be observed between 3 and 12 days postinfection. Several blind passages may be necessary before cytopathic changes are observed in the cells.

Properties of the Virion and Genome

The rinderpest virus particle is made up of a lipid envelope derived from the host cell, six virus structural proteins and a genome consisting of a single strand of negative-sense RNA. Two other virus-specific proteins (C and V) found in infected cells, but not so far in virus particles, are termed nonstructural proteins. All morbilliviruses are identical in genetic configuration. In the case of rinderpest, the virion RNA consists of a short 3′ leader RNA (55 nucleotides) followed by the coding regions of the six structural protein genes and ending in a short 5′ trailer RNA (37 nucleotides). There are semiconserved start–stop sequence motifs at the start and end of each gene, and between these is a trinucleotide untranscribed intergenic sequence (usually GAA). Measles, rinderpest and canine distemper viruses have been completely sequenced; measles virion RNA is 15 894 nucleotides long, rinderpest 15 882 nucleotides long and and canine distemper virion RNA slightly shorter, at 15 690 nucleotides.

The two virus encoded glycoproteins, the haemagglutinin (H protein) and fusion proteins (F protein), are embedded in a lipid membrane which is derived from the host cell during budding and this forms the virus envelope. A nonglycosylated matrix protein (M protein) interacts both with the cytoplasmic domains of the envelope glycoproteins and with the nucleocapsids formed within the host cell during genome replication. The M protein is thought to be essential for virus morphogenesis and budding. The newly synthesized virion RNA is surrounded and protected in the cytoplasm by the nucleocapsid (N protein) protein which, in association with the virus polymerase (L protein) and phosphoprotein (P protein), forms the ribonucleoprotein complex (**Fig. 2**).

Replication

The two nonstructural proteins found in infected cells are derived from messenger RNAs transcribed from

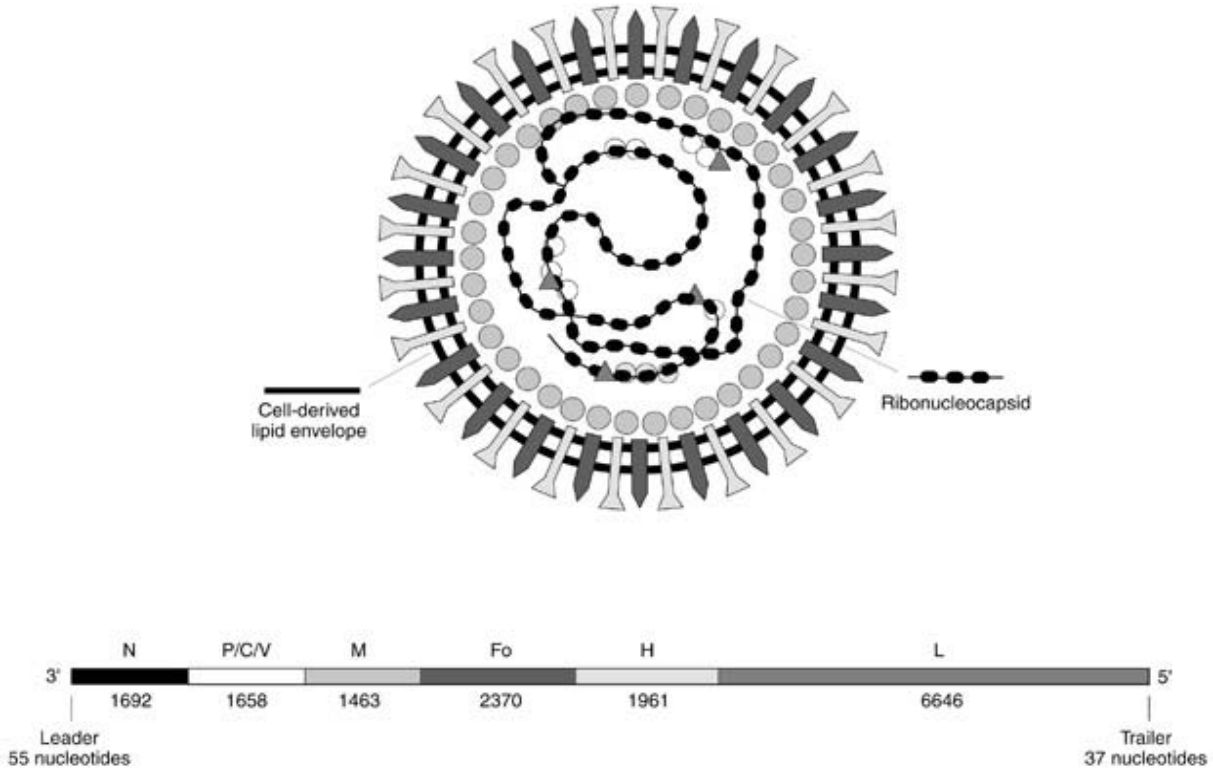


Figure 2 A typical morbillivirus showing the outer envelope with the two projecting surface glycoproteins and the inner helical nucleocapsid containing the virion RNA. The matrix protein is shown underneath the virus envelope. The arrangement of the genes along the virion RNA is shown below.

the P gene. The C nonstructural protein is translated from an alternate reading frame in the phosphoprotein mRNA beginning at the second AUG codon. The V protein is translated from an mRNA which is not an exact copy of the P gene sequence but from one which has an extra G residue inserted about halfway along the gene. This process is often referred to as 'editing' but is more correctly called alternative transcription and is a property of the virus polymerase, as it does not occur in other artificial systems. A conserved sequence motif is found in the region where the insertion occurs. Addition of an extra G occurs in about 50% of the mRNAs transcribed from the P gene in the case of measles and rinderpest viruses and there is evidence that it occurs in all other morbilliviruses. Translation of this mRNA produces a chimeric protein consisting of the N-terminus of the P protein with a new C-terminus, rich in cysteine residues, derived from RNA sequence in the third reading frame. This mRNA is also capable of translating the C protein as its coding region is located in front of the editing sequence position. The functions of these proteins are unknown but they most likely play a part in the control of transcription and replication of the genome RNA.

Evolution

Rinderpest virus

Monoclonal antibody and sequence analyses indicate that rinderpest virus is most closely related to measles virus, and phocid distemper virus to canine distemper virus. The nucleic acid homology between the N genes of phocid and canine distemper viruses is only about 77%. The two viruses are, therefore, almost as different as rinderpest and measles (70% homology) and must have had a long period during which they diverged from a common ancestor. The new dolphin and porpoise morbilliviruses are very closely related to each other, being different strains of the same virus, and are now often referred to as the cetacean morbillivirus. They are antigenically more related to rinderpest and peste des petits ruminants viruses than to phocid and canine distemper viruses but they fall into a distinct lineage group when their sequences are compared with other morbilliviruses (**Fig. 3**).

The exact evolutionary relationship between the different morbilliviruses is unknown but rinderpest has been proposed as the 'archevirus' of the group. Monoclonal antibody reactivities support this conclusion as rinderpest reacts with a broader range of

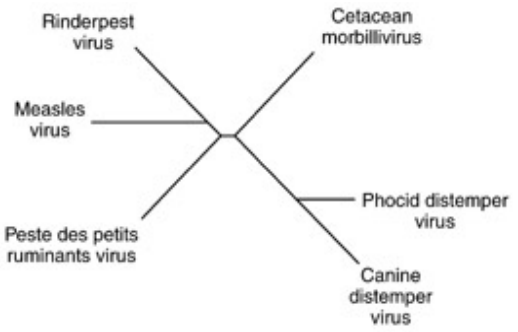


Figure 3 A computer-generated tree showing the phylogenetic relationships between the different morbilliviruses. The tree was generated using the PHYLIP programs to analyze the complete N gene coding region.

monoclonals produced against other morbilliviruses. The fragility of the virus particle and the lifelong immunity following infection (see Prevention and Control) dictate that morbilliviruses have a constant supply of susceptible hosts for their survival. Populations of 300 000 or more are required to maintain measles virus. Animal populations of the required size would most likely have been large herds of ruminants before humans formed sufficiently large settled groups. When cattle were domesticated they could have passed a morbillivirus infection to humans which eventually evolved into measles virus.

Canine distemper virus

Canine distemper virus infects a wide range of carnivores and is maintained by a virus reservoir in a large variety of wild carnivore species. This virus, unlike rinderpest virus, can in rare cases maintain a persistent infection from which the disease can reactivate as old dog distemper. It is conceivable, though less likely, that canine distemper is the progenitor of the group and that predators passed the disease to other nonpredator species, which then evolved into the distinct morbilliviruses. Little is known about the evolution of the morbilliviruses isolated from aquatic mammals. It is possible that seals contracted canine distemper from dogs or other carnivores with which they came into contact while hauling up on land or ice flows to breed. Their relatively close genetic relationship supports this suggestion. In the case of cetaceans the situation is much less clear. They do not come on land to breed and there are not many opportunities for contact with terrestrial mammals. This, and the great genetic distance between the cetacean and other morbilliviruses, indicates that the virus must have been present in cetaceans for a very long time. Pilot whales have been suggested as the most likely species in which the virus is naturally enzootic, as they are present in relatively large numbers and serological studies have shown a high prevalence of morbillivirus-specific antibodies in this species.

Serologic Relationships and Variability

All viruses in the group are antigenically related – the matrix, fusion and nucleocapsid proteins being the most highly conserved across the group. In fact, immunity induced to the F protein may be responsible for the strong crossprotection seen after vaccination with heterologous virus. Rinderpest vaccine is routinely used to vaccinate against peste des petits ruminants virus, and inactivated canine distemper virus vaccine has been shown to protect seals against infection with phocid distemper virus. It has also been shown experimentally that measles virus can protect dogs against distemper and cattle against rinderpest, and that distemper virus can protect humans against measles. This has no epidemiological significance as the viruses do not naturally crossinfect. The H protein, responsible for attachment to the host cell receptor, is the least conserved and least crossreactive morbillivirus protein. In immunoprecipitation reactions only the H protein fails to crossprecipitate with heterologous antisera, although some one-way precipitations are seen, e.g. measles antiserum will precipitate canine distemper H but not vice versa. Strain variations within each virus group can be readily demonstrated using monoclonal antibodies but these variations do not result in different serotypes for each virus.

Epizootiology

Rinderpest virus

Traditionally, rinderpest outbreaks follow wars and civil disturbance where there is unrestricted movement of people and troops with live food animals which can carry the virus. Recent outbreaks in Lebanon, the Middle East and Sri Lanka follow this pattern. The outbreak in Sri Lanka in 1987 was seen after a 40 year span free from the disease and the likely source was live goats brought from India with the troops and traded locally. More recently rinderpest reappeared in Turkey as a consequence of the Gulf war.

Rinderpest and peste des petits ruminants viruses are normally introduced into an area by importation of live infected animals from an enzootic area. Transmission by infected meat is very rare and considered to be a low risk. The most dangerous sources of virus are subclinically infected animals. Subclinically infected pigs act as a source of virus for

cattle. Sheep, goats and possibly other small ruminants can be infected with rinderpest virus and pass the infection to cattle. Experimentally this has been shown to occur with Asian strains of rinderpest virus which readily infect small ruminants. In contrast, African strains of rinderpest do not appear to productively infect sheep and goats. Another factor which may be important in the maintenance of rinderpest is the presence of strains which cause mild or subclinical infections in some enzootic areas. The incubation period for these viruses can be up to 15 days and this, along with their low transmission rates, means that they can persist unnoticed for many years in cattle populations. It is possible that the disease may flare up clinically when animals are put under stress, such as when they are moved to markets. In Africa the situation is also complicated by the presence of large numbers of susceptible wildlife species which can help spread the virus in an uncontrolled manner. Wild ruminants vary greatly in their response to rinderpest infection with species such as buffalo, kudu, eland and warthog (*Phacochoerus aethiopicus*) being highly susceptible, and others such as the hippopotamus (*Hippopotamus amphibius*) and Thompson's gazelle (*Gazella thomsoni*) highly resistant. The epizootiology of rinderpest virus on the two continents is therefore quite different. In Asia there is one known lineage of the virus, while in Africa two distinct virus lineages coexist (**Fig. 4**).

The role of wildlife species in maintaining the disease is unclear. There is no good evidence that wild ruminants act as a reservoir of infection for domestic animals but they may be important in helping to spread disease once an outbreak occurs. To date the evidence suggests that domestic animals are generally the source of infection for wildlife species. The virus was successfully eradicated from South Africa and Tanzania, despite the presence of considerable numbers of wild animals. In fact some highly susceptible wild animals may act as sentinels for the disease, as illustrated by the recent severe outbreak of rinderpest virus in lesser kudu, eland and buffalo in Kenya. The epizootiology of peste des petits ruminants virus and the role wildlife plays in its maintenance has not been studied in any detail.

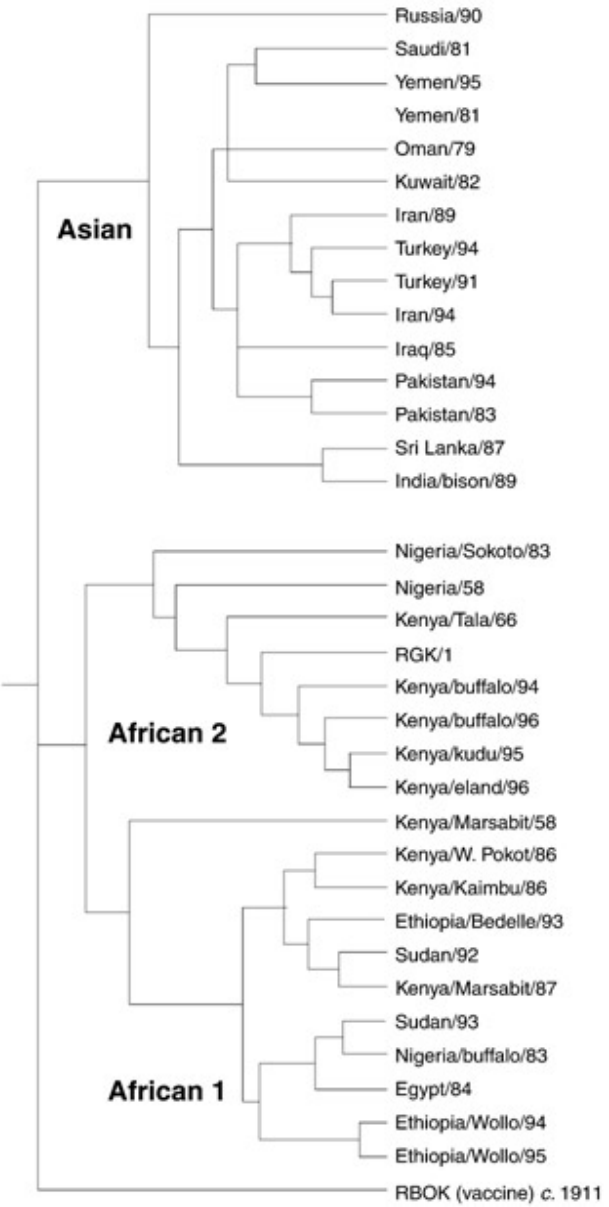


Figure 4 A computer-generated tree showing the relationship between African and Asian lineages of rinderpest virus. The tree was generated with the PHYLIP programs using partial sequence data from the F protein gene.

Canine distemper virus

Canine distemper virus is enzootic in wild carnivores and it remains a problem in poor urban areas where there are many stray dogs and vaccination is not carried out rigorously. The virus is also an important factor in the ecology of wild animal populations. The last free-living population of black-footed ferrets in Wyoming was almost wiped out by a canine distemper infection. The recent canine distemper outbreak in free-living lions in the Serengeti and in captive lions, tigers and panthers in zoos in the USA indicate that it must now be considered a threat to these species. As noted previously, canine distemper and a closely related phocid distemper virus have recently been isolated from several species of pinniped. Such a serious disease could have a devastating effect on small populations of rare sea mammals, such as that of the monk seals (*Monachus monachus*) in

the Mediterranean Sea and the Atlantic Ocean or in the Siberian seals in Lake Baikal. It is important to monitor these species for signs of infection and possibly vaccinate those taken into seal sanctuaries with safe subunit vaccines. The epizootiology of phocid distemper virus is poorly understood. Analysis of historic sera from Canadian harp seals and arctic ringed seals indicated that they were infected with a morbillivirus several years before the appearance of phocid distemper along the coasts of northern Europe. It has been suggested that climatic changes caused the harp seals to migrate further south, so passing the infection to other seal species which lacked herd immunity to the virus and as a result suffered a severe epizootic.

Transmission and Tissue Tropism

Morbillivirus transmission is by direct contact with secretions or excretions of infected animals. The morbilliviruses are highly contagious: all discharges can carry the virus. However, since the virus is extremely sensitive to environmental factors, such as heat, sunlight and chemical inactivation, it requires close contact with an infected animal for successful transmission. It is therefore relatively easy to control by regulating animal movements, in conjunction with a strict quarantine and slaughter policy where necessary. Even without the availability of vaccines rinderpest was successfully controlled and eliminated from Europe by these means.

The morbilliviruses are highly lymphotropic and cause a transient immunosuppression in infected animals. The more virulent strains of the virus also have a strong tropism for epithelial tissues and this helps the spread of the disease by contact, as high titres of virus are then excreted. Mild strains do not replicate so readily in epithelial surfaces and so are more difficult to transmit by contact. With the exception of rinderpest and peste des petits ruminants, morbilliviruses are known to infect brain cells, where they can set up persistent, eventually fatal, infections.

Pathogenicity

Although there is only one serotype of each virus, differences between isolates can be shown using monoclonal antibodies, and strains also vary in their ability to cause disease in infected animals. In the case of rinderpest, extreme variation in pathogenicity has been reported, ranging from the mild strains currently circulating in eastern Africa to highly virulent strains, such as those found in the Middle East, which can cause 90–100% mortality in susceptible hosts.

Variation in the severity of clinical disease and neurotropism have also been reported with different strains of canine distemper, which are known as 'biotypes'. Very little is known about the pathogenicity of the marine morbilliviruses. Different hosts are known to have different susceptibilities, as evidenced by the greater number of deaths in harbor seals relative to gray seals during the European seal epizootic in 1988. Likewise, pilot whales show a high level of seropositivity to cetacean morbillivirus but only dolphins and porpoises were severely affected during the epizootic in the Mediterranean Sea in 1990–1991. However, because of the immunosuppressive nature of morbilliviruses, secondary bacterial and concurrent parasitic infections can greatly influence the outcome of the disease and it is sometimes difficult to assess the contribution of virus infection. Nothing is yet known concerning the molecular basis of pathogenicity in morbilliviruses.

Clinical Features of Infection

Rinderpest virus

Typical rinderpest is an acute febrile disease, with mortality reaching close to 100% with some of the most virulent strains of rinderpest virus, such as the Saudi/81 strain. The course of the disease is divided into five stages: after a short (1) incubation period of 3–5 days, the (2) prodromal phase is seen in which there is a rapid rise in temperature. This is followed by the (3) mucosal phase in which severe mouth lesions are seen and there is a copious nasal and ocular mucopurulent discharge. The affected animals become depressed and anorexic and on postmortem examination many lesions are seen throughout the digestive and lymphatic system. This is followed by the (4) diarrheal phase where there is severe bloody diarrhea, the animal is prostrated and dies from dehydration and weakness. In nonfatal cases there follows the (5) convalescent phase which may take many weeks and during which pregnant animals may abort. Some strains, such as those prevalent in Eastern Africa, are generally mild and there may be no clinical signs in many cases, even though the animals seroconvert. With the mild strains the incubation period can extend up to 15 days.

Clinically peste des petits ruminants virus infection closely resembles rinderpest. There is an incubation period of 4–5 days, then a rise in temperature followed by nasal and ocular discharges, which can be very severe. Mucosal lesions and severe diarrhea then appear and the animals, in fatal cases, die of dehydration within 6 days of the onset of fever. Bronchopneumonia is a frequent complication.

Canine distemper virus

The incubation period for canine distemper virus is usually about 1 week. There are often two temperature peaks, the second corresponding with other signs such as nasal discharge, conjunctivitis and anorexia. As in the case of rinderpest, respiratory and gastrointestinal lesions follow and severe leukopenia is also seen. In some cases central nervous system (CNS) signs, such as convulsions and seizures, are seen as part of the clinical disease, or they may follow a subclinical infection. Recovered dogs frequently show persistent nervous 'ticks' or involuntary movements of one or more legs. Old dog encephalitis is a rare disease thought to be caused by canine distemper virus persistence in the brain and nervous tissue. Seals infected with phocid distemper virus show clinical signs, including CNS lesions, reminiscent of canine distemper infection in dogs, and this gave the first clue to the nature of the etiological agent.

Pathology and Histopathology

Severe morbillivirus infections are accompanied by a marked leukopenia, leading to a deficiency in the immune system. This often results in the activation of latent or other concurrent infections. In addition, secondary bacterial infections are common. These factors may complicate both the clinical and pathomorphological findings. In the case of rinderpest, leukopenia is most marked during the erosive mucosal phase. Histologically the virus shows a tropism for lymphoid and epithelial cells. All lymphoid organs are affected, with the greatest damage occurring in the mesenteric lymph nodes and gut-associated lymphoid tissue; severe destruction of the B and T cell areas is seen. Intracytoplasmic and intranuclear eosinophilic inclusion bodies are commonly found in the cells of morbillivirus infected animals.

Epithelial tissues of the upper respiratory, urogenital and alimentary tracts are also infected by the highly pathogenic, and highly contagious, strains of the virus. In acute rinderpest and peste des petits ruminants infections there is extensive erosive inflammation of mucosal surfaces of the digestive and upper respiratory tracts, with cellular necrosis and the formation of syncytia. Mild strains induce less extensive mucosal lesions and this may account for the reduced ability of these strains to transmit by contact. In the cecum, colon and rectum of animals infected with rinderpest and peste des petits ruminants viruses, so-called zebra or tiger stripes are commonly found. The stripes are caused by greatly distended capillaries packed with erythrocytes. Similar pathological changes are seen in infections with the other animal morbilliviruses. Dehydration in acute morbillivirus infections, the result of profuse diarrhea, causes changes in hematology and blood chemistry. There is an apparent increase in erythrocyte count and the packed cell volume increases by 40–65%. At death the blood is dark, thick and slow to coagulate. In acute canine distemper, phocid distemper and peste des petits ruminants virus infections a serous inflammation of intestinal and respiratory surfaces, associated with interstitial pneumonia, occurs. A nonsuppurative encephalitis is also seen.

Immune Response

There is a strong cell-mediated component in the response to morbillivirus infection. In the case of measles virus, individuals with a genetic defect leading to agammaglobulinemia can quite easily overcome measles infection but immunosuppressed individuals are at extreme risk from the disease. Animals with any detectable neutralizing antibody are considered immune to rinderpest and immunity following morbillivirus infection is lifelong. Either the F or H surface glycoprotein can confer immunity to disease, as poxvirus recombinant vaccines, expressing either the H or F proteins of rinderpest virus, are protective. Similar observations were made in animal model systems in the case of measles virus. In addition, it was found that vaccinia recombinants expressing the N protein could protect mice from lethal intracerebral challenge with measles virus, whereas a similar recombinant expressing the N gene of rinderpest virus gave no protection. In contrast, purified H and F antigens are not protective unless presented as immune stimulating complexes in association with Quil A (ISCOM vaccines). ISCOM vaccines are known to stimulate cytotoxic T cell responses in animals.

Prevention and Control

Morbilliviruses are extremely fragile; they are sensitive to sunlight, high temperature, low and high pH and chemicals which can destroy their outer lipid-containing envelope. Outbreaks of these viruses are therefore easily controlled by proper quarantine and hygienic measures. There is only one serotype of each virus and, once recovered from the infection, the animal is immune for life. As there is no evidence for a persistent or carrier state in the recovered animals, vaccination is a very effective means of controlling these diseases.

Diagnosis

Rapid and accurate differential diagnosis is the key to success in controlling a morbillivirus outbreak. Identification of the morbilliviruses is generally based on the species in which the disease is seen, e.g. measles in humans, rinderpest in cattle, canine distemper in dogs, etc. However, this is not always accurate as the viruses can sometimes infect unusual hosts and the diseases share clinical signs with other virus infections, so diagnosis can not be based solely on the host and clinical signs. For example, rinderpest and bovine virus diarrhea are often confused, and peste des petits ruminants virus can be mistaken for pasteurellosis or other microbial pleuropneumonias. Laboratory diagnosis, based on either virus isolation or specific antigen detection, is essential to confirm the presence of these diseases. A general morbillivirus-specific antigen detection test, the agar gel immunodiffusion (AGID) test, is currently used in field situations in Africa and Asia but it cannot distinguish between the viruses of rinderpest and peste des petits ruminants. Differential neutralization tests can be used to classify virus isolates but these are time consuming. However, rapid competitive ELISA systems using morbillivirus species-specific monoclonal antibodies, which can distinguish rinderpest and peste des petits ruminants viruses, have been developed for virus antigen and antibody detection. Rapid penside tests for the detection of rinderpest and peste des petits ruminants viruses have been developed based on latex bead technology and specific monoclonal antibodies. These new techniques are now being introduced into countries where these diseases are endemic. The most recent advance has been the introduction of the polymerase chain reaction for specific morbillivirus diagnosis (**Fig. 5**). This technique, in combination with sequence analysis of the DNA produce, has enabled a much more precise analysis of the epidemiology of these viruses in the field.

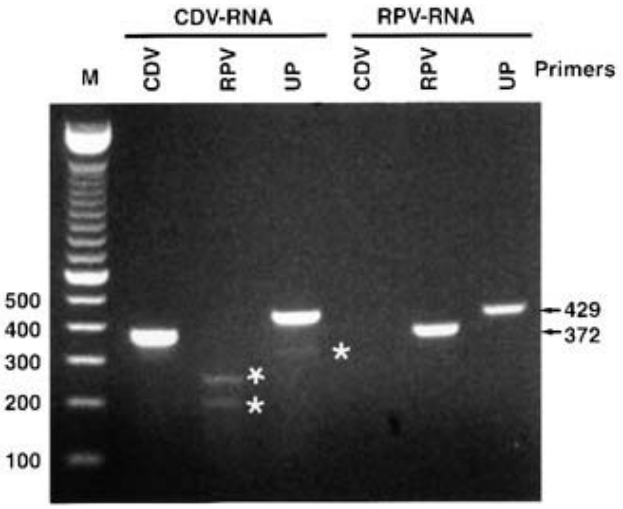


Figure 5 Example of a differential RT/PCR test to distinguish rinderpest (RPV) and canine distemper virus (CDV). Both a universal primer set (based on P gene sequences) and specific primer sets (based on F gene sequences) were used. Nonspecific amplification products are indicated by asterisks.

Vaccination

During the 1930s attenuated rinderpest vaccines were developed by passage of the virus in nonnatural hosts, e.g. rabbit and embryonated eggs (lapinized/avianized) or goats (caprinized). The Japanese lapinized/avianized vaccine was used extensively to control the disease in Asia. In India and Africa the caprinized virus was used; however, this virus was not completely attenuated and it may have been responsible for the circulation of rinderpest in small ruminants in India. In the early 1960s the Plowright tissue culture attenuated vaccine was introduced; it is safe and relatively easy to produce, with no clinical signs following vaccination in domestic animals. In addition, the virus does not replicate at epithelial surfaces and cannot be transmitted by contact. Immunity following vaccination is complete and lifelong. The vaccine is, however, very heat labile and establishment of an effective cold-chain and follow-up seromonitoring to determine the level of herd immunity are essential prerequisites for a successful vaccination campaign. Improvements in freeze-drying techniques have greatly increased the stability of the vaccine in the dry form but it is still very labile when reconstituted and must be used within a very short period.

In the 1960s an internationally funded rinderpest eradication campaign (Joint Programme 15 or JP 15) was carried out in Africa, using the tissue culture attenuated vaccine, and almost succeeded in clearing the disease from Africa. However, political instability, lack of funds to continue vaccination and disease surveillance and the existence of mild strains of the disease resulted in a resurgence in the 1980s. Quite often countries are reluctant to report cases of rinderpest or peste des petits ruminants virus infection because of the repercussions for trade in live animals and this makes the task of controlling outbreaks more difficult. An internationally funded vaccination campaign has been underway in Africa, the Pan African Rinderpest Campaign (PARC), since the mid-1980s and has successfully contained rinderpest virus to eastern Africa. Currently a Global Rinderpest Eradication Programme (GREP) has been established by the United Nations Food and Agricul-

tural Organization in an attempt to eradicate the disease early in the twenty-first century.

Generally rinderpest vaccine is used to control the spread of peste des petits ruminants virus but a homologous vaccine has now been developed and is being field tested in West Africa. The role that wildlife species play in maintaining rinderpest and peste des petits ruminants viruses needs to be more clearly understood before it will be possible to be certain that vaccination of domestic animals alone can eliminate these diseases.

Canine distemper vaccines are not attenuated for all species, and in some, such as the lesser panda (*Ailurus fulgens*), they cause quite severe disease. There are two widely used vaccines for canine distemper virus: the Onderstepoort strain was attenuated by growth in avian cells and the Rockborn strain in canine tissue culture cells. Immunity lasts for several years following vaccination with either vaccine. A less effective ISCOM subunit vaccine (see Immune Response) must be used for nondomestic species, particularly in the case of valuable zoo animals. Since there is a large wildlife reservoir of canine distemper virus and the vaccines are not attenuated for many wild animal species, it may be impossible to eradicate this virus disease.

Future Perspectives

Much research has gone into the development of poxvirus recombinants expressing morbillivirus antigens in an effort to obtain more heat-stable vaccines, which would make them easier to use in hot climates. They have proved to be effective vaccines in short-term protection studies; however, the duration of immunity and the level of protection these vaccines give in comparison with the conventional tissue culture vaccines needs to be established. The most exciting new development in the field of morbillivirus research has been the ability to rescue live virus from DNA copies of their genomes. This technology will enable us to study the molecular mechanisms that underlie pathogenic variation in the morbilliviruses. Identification of attenuating mutations will help in the design and production of safer vaccines. Another great advance will be the ability to introduce genetic markers into the viruses, which will enable vaccinated animals to be distinguished serologically from animals which have been naturally infected. A genetically marked vaccine will be a very valuable tool for GREP in its aim to eradicate rinderpest virus by the end of the first decade of the twenty-first century.

See also: **Parainfluenza viruses (*Paramyxoviridae*): Animal; Measles virus (*Paramyxoviridae*); Bovine diarrhea virus and Border disease virus (*Flaviviridae*); Immune response: Cell mediated immune response, General features; Pathogenesis: Animal viruses.**

Further Reading

Anderson J, Barrett T and Scott GR (1996) *Manual on the Diagnosis of Rinderpest,* 2nd edn. Rome: Food and Agriculture Organization.

Appel M (1987) Canine distemper virus. In: Appel MJ (ed.) *Virus Infections of Carnivores,* p. 133. Amsterdam: Elsevier.

Barrett T, Blixenkrone-Moller M, DiGuardo G *et al* (1995) Morbilliviruses in aquatic mammals: report on round table discussion. *Vet. Microbiol.* 44: 261.

Losos G (1986) Peste des petits ruminants. In: *Infectious Tropical Diseases of Domestic Animals,* p. 549. Harlow: Longman.

Plowright W (1982) The effects of rinderpest and rinderpest control on wildlife in Africa. In: Edwards MA and McDonnell U (eds) *Animal Disease in Relation to Animal Conservation,* p. 1. Symposia of the Zoological Society of London, no. 50. London: Academic Press.

Scott GR (1985) Rinderpest in the 1980s. In: Pandey R (ed.) *Infections and Immunity in Farm Animals,* p. 145. Progress in Veterinary Microbiology and Immunology, no 1. Basel: Karger.

ROSS RIVER VIRUS AND BARMAH FOREST VIRUS (*TOGAVIRIDAE*)

Lynn Dalgarno and **Ian D Marshall**, Division of Biochemistry and Molecular Biology, School of Life Sciences, The Australian National University, Canberra, Australia

History

The first reports of a disease probably caused by Ross River virus (RRV) appeared in 1928, describing epidemics of transient arthritis and rash in two Murrumbidgee River towns on the semiarid inland plains of New South Wales (NSW), south-eastern Australia. During World War II, epidemics of arthritis with rash were described in troops serving in the tropical regions of Australia and on islands to the immediate north. Most of these outbreaks were differentially diagnosed against a background of endemic dengue fever, and published reports allowed the description of a syndrome adequate for clinical diagnosis of the disease, at least during epidemics. Several attempts failed to isolate or define the nature of the causative agent. These wartime reports provided the terms 'epidemic polyarthritis' and 'epidemic polyarthritis with rash' to characterize the disease; although these are still widely used, the noncommittal 'Ross River virus disease' is gaining currency. As fever is usually absent or unremarkable, 'Ross River fever' is inappropriate.

The first large, adequately documented community epidemic occurred in the Murray Valley of south-eastern Australia in 1956, but again two groups of investigators failed to isolate the causative agent. However, following consideration of the epidemiology and nature of the disease, and subsequent serological testing of convalescent sera, it was concluded that a mosquito-borne Group A arbovirus was the most likely candidate, and for several years the Malaysian alphavirus Bebaru was used as a surrogate diagnostic and survey antigen. Eventually, in 1963, the specific alphavirus was isolated from a pool of *Aedes vigilax* mosquitoes collected during dengue investigations near the Ross River at Townsville, coastal north Queensland. The first human isolate was in 1971 from the serum of a mildly febrile 7-year-old aboriginal boy, but, as is usual before puberty, characteristic signs and symptoms did not develop. The final incrimination of RRV did not occur until 1979, when the virus was introduced to Fiji, presumably by a viremic tourist from Australia; RRV was isolated in newborn mice inoculated with the acute stage serum of the indicator case. Numerous isolations were subsequently made during the ensuing series of virgin soil epidemics which, over the next 2 years, extended across the south Pacific from New Caledonia to the Cook Islands. RRV has since been isolated from patients in Australia but only from sera incidentally taken before onset of symptoms (see below).

Over recent years it has become apparent that a significant number of clinically well-defined cases of viral polyarthritis with rash are due to Barmah Forest virus (BFV), which was first isolated from mosquitoes in 1974 (see below). In 1988 BFV was shown to be the causative agent of a disease that is currently regarded as being clinically indistinguishable from that due to RRV infection. Although RRV and BFV are both alphaviruses, BFV has a number of features which are atypical. RRV and BFV are antigenically quite distinct. There have been several epidemics where both viruses circulated concurrently and the causative virus of individual cases was distinguished serologically.

Taxonomy and Classification

RRV and BFV are mosquito-borne arboviruses belonging to the genus *Alphavirus* of the *Togaviridae* family. RRV is a subtype of Getah virus in the Semliki Forest virus (SFV) serological complex. BFV is the sole member of a seventh alphavirus complex.

Properties of the Virion

Purified RRV virions have two glycosylated envelope proteins: E1, the hemagglutinin (molecular mass, 52 kDa) and E2, the neutralizing antigen (49 kDa). The nucleocapsid protein, C, is 32 kDa. RRV is sensitive to chloroform, ether, detergents, ultraviolet irradiation and low pH. The infectivity titre of wild-type RRV is virtually unaffected by incubation in cell growth medium at 50°C for 45 min. Under the same conditions, a mutant of RRV T48 with a deletion of seven amino acids (residues 55–61) in the E2 glycoprotein is thermolabile, showing a three log unit loss of infectivity.

Cryoelectron microscopy and image reconstruction have provided a detailed structural picture of RRV,

the first for an alphavirus. The nucleocapsid (approximately 40 nm in diameter) possesses icosahedral symmetry with a triangulation number (*T*) of 4 and is surrounded by a lipid bilayer (4.8 nm thick). The core structure, at a radius below 17 nm, is composed of both the genomic RNA and the basic, N-terminal domain of the nucleocapsid protein. The surface of the virion is largely protein. There are 240 heterodimers of E1 and E2 assembled into 80 trimeric spikes on the surface. The E1 protein forms the core of the trimeric spike and E2 is found largely on the outer surface. The trimers separate immediately above the lipid bilayer to form a propeller-like, tripartite head; at the end of each heterodimer E1 and E2 appear to separate. Fab fragments from neutralizing monoclonal antibodies directed against E2 determinants bind to one tip of the heterodimer. The spike has a hollow base which may have a role in fusion mediated by an E1 homotrimer. The heterodimers form one-to-one associations with nucleocapsid monomers across the lipid bilayer; this contact is due to the association of the 'cytoplasmic domain' of E2 and the ordered, C-terminal domain of the nucleocapsid protein.

Properties of Genome

The prototype RRV (T48) genome is a single-stranded, positive-sense RNA molecule of 11851 nucleotides excluding the poly(A) tail. The 5′ two-thirds of the RNA encodes the nonstructural proteins (NSPs); the 3′ one-third is expressed as a subgenomic (26S) mRNA which is transcribed from full length negative strands. In the genome, a 5′ noncoding region of 78 nucleotides is followed by an open reading frame (ORF) of 7440 nucleotides which is interrupted after 5586 nucleotides by a UGA termination codon. By analogy with Sindbis virus (SIN), the 5′ two-thirds of the genome encodes two polyproteins. One is NSP1-2-3 (1862 amino acids), and the second is produced by read-through of the 'leaky' UGA codon to generate NSP1-2-3-4. Four in-phase stop codons, three of which are in a region corresponding to the 5′ noncoding sequence of the 26S subgenomic RNA, ensure termination of NSP translation. The 3′ one-third of the genomic RNA has an ORF of 3762 nucleotides which encodes the polyprotein C-E3-E2-6K-E1. The length of the 3′ noncoding region can vary between isolates. For the prototype the length is 524 nucleotides; four closely related sequence blocks, 48–58 nucleotides in length, are found in the 3′ noncoding region of RRV (T48). Deletions, insertions, sequence rearrangements and single nucleotide substitutions are commonly observed in the 3′ noncoding region of different isolates of RRV.

The RRV genome has three regions which are strongly conserved between alphaviruses, including BFV. These are (1) a tract of 23 nucleotides next to the 3′ poly(A) tail; (2) 21 nucleotides at the 3′ terminus of the *NSP4* gene; and (3) 50 nucleotides near the 5′ end of the *NSP1* gene. The genome also contains a moderately conserved sequence element close to its 5′ end. All four sequence elements appear to have roles in the regulation of viral RNA replication. The 23 nucleotide tract is believed to be a promoter for negative strand synthesis; the complement of the 21 nucleotide element may be recognized, in the negative strand, by the 26S RNA transcriptase.

The prototype BFV (BH2193) genome is 11 488 nucleotides long, excluding the poly(A) tail. The 5′ and 3′ noncoding regions are 62 and 445 nucleotides in length respectively. The genome encodes an NSP of 2411 amino acids and a structural polyprotein of 1239 amino acids. The 3′ noncoding region has two unrelated sequence blocks of around 50 nucleotides which are each repeated once. One of these is unrelated to sequences in other sequenced alphaviruses; the other is closely similar to a repeat in the 3′ noncoding region of RRV and Getah RNAs. Thus recombination in the 3′ noncoding region of the RNA of common ancestor viruses may be part of the evolutionary history of these three alphaviruses. SIN, the fourth Australian alphavirus, shows no homology in its 3′ noncoding region with those of RRV/BFV/Getah.

Virus Proteins

Sequence data predict that for RRV, C, E3, E2, the '6K' protein and E1 are 270, 64, 422, 60 and 438 amino acid residues in length respectively, assuming no post-translational trimming. The RRV polyprotein is 75 and 48% homologous with the SFV and SIN polyproteins respectively. The capsid protein is highly basic in its N-terminal half, consistent with a role in interacting with genomic RNA. The N-terminal ten amino acids of E3 are hydrophobic and presumably form part of a signal sequence for the insertion of p61, the E2 precursor, into the host endoplasmic reticulum. Three neutralization epitopes on RRV E2, which together make up a significant antigenic site, have been mapped to residues 216 (epitope *a*), 232 and 234 (epitope *b1*) and 246, 248 and 251 (epitope *b2*). These epitopes are flanked in the primary sequence by asparagine-linked glycosylation sites at residues 200 and 262. This site is important in early virus-cell interactions and in virulence, as judged by biological studies on RRV mutants selected during epidemics (see below), selected during passage in cell culture or mice, or generated using infectious RNA derived from

cDNA clones. The existence of a '6K' protein has not been reported in RRV-infected cells. The hemagglutinin, E1, has an uncharged tract (residues 80–96) which is highly conserved between alphaviruses and may be involved in fusion with cell membranes during virus entry. Comparative sequence data predict that the genomic RNA encodes two polyproteins (NSP1-2-3, the major species; NSP1-2-3-4, a minor species) which are processed to four NSPs of 533, 798, 531 and 611 residues respectively.

Sequence data predict that for BFV (BH2193), C, E3, E2, 6K and E1 are 253, 68, 421, 58 and 439 residues in length. For the NSPs the corresponding figures are 533, 798, 470 and 610. BFV E2 appears to be unique among alphavirus proteins in its relatively low observed molecular mass (43 kDa) and in the absence of *N*-linked glycosylation sites; the molecular mass of BFV E1, which has two glycosylation sites, is high (56 kDa) relative to other alphaviruses.

Replication and Virus Assembly

In cultured vertebrate cells RRV infection is cytopathic. The latent period is 3–5 h; maximum extracellular virus titers (10^8 Vero PFU ml^{-1}) and levels of viral RNA synthesis are at 8–10 h, at which time the shutdown of host cell protein synthesis is virtually complete. Virus-specific RNAs formed in BHK and *Ae. albopictus* cells include RF, RI, 45S, 26S and small amounts of 38S and 33S RNA (conformational variants of 45S and 26S RNAs respectively). Seven major virus-specific polypeptides are detected in vertebrate cells: p127, p95, p61 (E2 precursor), p52 (E1), p49 (E2), p32 (capsid protein) and E3. In Vero cells RRV is commonly found in small cytoplasmic vesicles; 'type 1 cytopathic vacuoles' are observed. There is a pronounced accumulation of nucleocapsids, particularly late in infection. In vertebrate cells BFV replication appears to involve analogous steps to those seen for RRV and other alphaviruses.

In cultured *Ae. albopictus* cells at 28°C RRV generates a non-cytopathic, persistent infection with peak titers (2×10^7 Vero PFU ml^{-1}) at 2–3 days. At 12 and 48 h after infection, 85 and 5% of cells respectively assay as 'infective centres'. Viral protein synthesis is sustained over the period 10–24 h after infection but is quantitatively less than in vertebrate cells; no p95 is observed. There is no shutdown of host protein synthesis, and cell division rate is unaffected by infection. Virus matures within large electron-dense, cytoplasmic inclusions and at the cell membrane. Free nucleocapsids are infrequent. When titers decrease during the later stages of infection, inclusions are transformed into microvesiculated vacuoles which may result from fusion with lysosomal vesicles.

Geographic and Seasonal Distribution

An extensive serological survey of neutralizing antibodies in humans has established that before 1979 RRV occurred only in Australia, the islands of New Guinea, New Britain and the Solomons, with activity in these islands decreasing from west to east. Chikungunya virus was the predominant alphavirus west and north of New Guinea through to south-east Asia. No alphavirus activity was detected in island groups further to the east. Unfortunately the pathogenicity of BFV was not appreciated at that time, and evidence for BFV activity outside Australia has not been sought.

RRV and epidemic polyarthritis occur in every state of Australia. Limited surveys in New Guinea indicate that activity is confined to the lowlands and deep valleys of the central mountain ranges. The south Pacific island epidemics which started in 1979 petered out early in 1981, and there is no evidence that the virus has become enzootic in the region.

RRV infection occurs throughout the year in tropical and subtropical northern regions of Australia, with the highest incidence during the wet season, December to April. In temperate southern regions outbreaks occur mainly in late summer and autumn. Major RRV epidemics occasionally occur in years of flood in the Murray-Darling basin and persist from spring through to the fall. The epidemics are particularly severe in irrigation districts. Frequent localized outbreaks occur in coastal regions of eastern and southern Australia, including Tasmania. Cases have occurred on the fringes of most major cities during periods of increased coastal activity. Brisbane and Perth have experienced infrequent but significant suburban outbreaks.

The prototype BFV strain was isolated from *Cx annulirostris* mosquitoes trapped in February 1974 at Barmah Forest on the Murray River, north-eastern Victoria. It was independently isolated from mosquitoes collected at about the same time near Charleville, southern Queensland. In 1975 it was isolated from *Culicoides* spp collected near Darwin in the Northern Territory. BFV continued to be isolated sporadically in the eastern States, both inland and in coastal regions, but it was not until 1989 that it was recorded in Western Australia (in the far north). BFV distribution appears to coincide with that of RRV at least in eastern and northern Australia. In Western Australia more than 85% of the population is concentrated in the coastal districts of the south-west corner of the state. In and to the south of Perth, RRV is endemic in

the area, with an average of 154 cases annually. In the early spring and through the summer of 1992–1993 RRV appeared to be completely replaced by BFV; 18 BFV isolates were obtained from mosquito pools and there were 22 serologically confirmed BFV polyarthritis cases, the first indication of the virus in the region. The explanation for the apparent replacement of RRV by BFV during this episode is not known. No RRV was isolated from >70 000 mosquitoes tested during 1993, but since then, as in other States, both viruses have been active.

Host Range and Virus Propagation

In the primary cycle the vertebrate host range of RRV is effectively limited to placental and marsupial mammals. There is a lack of specificity in mosquito vector species; the virus has been recovered from 11 species encompassing five genera. Horses are commonly infected, sometimes resulting in lameness and constitutional or nervous disturbances of varying severity; RRV is suspected as a cause of equine death, but proof is lacking. No other domestic or native animal is known to show signs, but naturally acquired antibodies are found in most mammals, and virus has been recovered from macropods and from a horse. Antibodies are rarely found in birds, and, in the laboratory, viremia has been demonstrated only in recently-hatched chickens. It has been concluded that birds are not involved in the primary or amplification cycles for RRV. Viremia has been readily produced in a range of small and large adult marsupial species and in the small native rodent, *Pseudomys novaehollandiae*.

The host range of BFV is not known but limited serological investigations indicate that marsupials are more likely to be the primary hosts than are birds.

Newborn and weanling outbred mice have been the most commonly used experimental host. Many cell lines are susceptible to RRV and are used to prepare stocks, and in virus assay by plaque formation or induction of cytopathic effects. The C6/36 line of *Ae. albopictus* mosquito cells is the most sensitive available cell line, and is now the most common means of recovering RRV from patients and field material. Infection of mosquito cell monolayers cannot usually be visualized by cytopathic effect so they are commonly used together with immunofluorescent detection methods or in conjunction with Vero or BHK cells.

Genetics and Evolution

RRV genetic types and subtypes have been demonstrated from *Hae*III restriction digest profiles of cDNA to virion RNA and by sequencing genomic RNA. The examination of 14 isolates of RRV led to the identification of three genetic types with an estimated 1.5–5% nucleotide sequence divergence between types. RRV is not a bird virus and the relative immobility of mammalian hosts may allow the emergence of geographic variants adapted to local hosts. Based on pathogenicity for outbred infant mice there are a number of variants of RRV with degrees of mouse virulence. The prototype T48, from north Queensland, kills all infant mice in 5–6 days; a Nelson Bay (central coastal NSW) isolate kills 0–25% of infant mice in 10–12 days. There is no evidence for an association between a particular RRV genetic type and vector species.

Sequence studies on the genomes of RRV (T48) (genetic type I), and a strain from Nelson Bay (type III), showed 284 nucleotide differences, equivalent to 2.4% nucleotide sequence divergence. Most of the differences are 'silent'. There are 36 amino acid differences in the NSPs and 12 in the structural proteins, five of which are in E2. The distribution of these differences correlates with the location of nonconserved regions in the proteins of alphaviruses more generally.

Under conditions of natural selection in 'virgin soil' outbreaks the RRV genome is remarkably stable. During the first 10 months of the RRV epidemic in Pacific island communities (1979–1981), involving thousands of infections of humans, domestic animals and mosquitoes, changes in the E2 gene from the sequence seen in the earliest isolate obtained during the outbreak (the April 1979 Fijian strain) were confined to a single nucleotide which altered residue 219 (in the region of an antigenic site; see above). This mutation was first detected in a strain from an American Samoan patient infected in August 1979; there was no further change in the E2 gene in a strain from a Cook Islands patient infected in February 1980. No changes were observed in the 3′ noncoding region of the genome during the entire outbreak.

For BFV, the nucleotide sequence of the E2 gene from 12 isolates (1974–1995) differed by up to 1.7% in pairwise comparisons. The 3′ noncoding region can vary markedly between isolates, as is seen with RRV. BFV (BH2193) pathogenesis in infant mice is inconsistent even within a litter of day-old outbred mice; some will die within 24 h of peripheral inoculation, with high concentrations of virus generated in brain and muscle. Other mice will gradually develop signs and die 6–7 days after inoculation. No evidence has been obtained for differences in pathogenicity for infant mice between isolates.

In comparisons between the sequences of the BFV, RRV and SFV structural and nonstructural polypro-

teins, the percentage amino acid identity is relatively uniform along their lengths. There is therefore no evidence that BFV results from recombination between ancestral viruses in the coding region of the genome. Phylogenetic trees derived from sequence comparisons show that BFV, RRV and SFV arise from a separate evolutionary branch to that giving rise to SIN and the equine encephalitis viruses. RRV and SFV are more closely related to each other than either is to BFV.

Serological Relationships and Variability

RRV shares group- and genus-specific antigens with other alphaviruses. Based on antigenic relationships determined by hemagglutination inhibition (HAI), complement fixation tests and plaque reduction neutralization tests, RRV is in the SFV complex, and is a subtype of Getah virus. BFV shows little crossreaction in complement fixation tests or neutralization tests with RRV, SFV and other alphaviruses, although BFV is clearly related to RRV and SFV at the nucleotide and amino acid sequence levels.

Differences between the surface antigens of RRV geographic variants have been demonstrated with kinetic HAI and kinetic complement fixation tests. Homologous and heterologous virus/polyclonal antibody kinetic tests with RRV strains collected over a period of 13 years in north Queensland indicated antigenic identity. Similarly, strains collected over 3 years at Nelson Bay, NSW were antigenically identical by kinetic tests. However, heterologous kinetic tests between Queensland and Nelson Bay viruses and antibodies gave no crossreaction in HAI and significantly reduced crossreaction in complement fixation tests. The control crossreaction tests using standard incubation times suggested that all virus strains were antigenically identical.

Epidemiology

In Australia, the most important RRV vectors in coastal regions are brackish-water mosquitoes breeding in mangrove or melaleuca swamps. In these habitats *Ae. vigiliax* is the dominant vector in tropical and subtropical coastal regions, but in cooler southerly coastal regions such habitats are shared with or dominated by *Ae. camptorhynchus*. In the inland regions of Australia the major vector is the summer-breeding *Cx. annulirostris*. BFV has been isolated from a range of mosquito species similar to that for RRV in tropical and temperate ecosystems on the Australian mainland.

In Australia, RRV persists in a mammalian wildlife–mosquito primary cycle, probably augmented by a low-level transovarial cycle in *Aedes* mosquitoes. Year-round sporadic cases and the initiation of epidemics are presumably due to mosquitoes that have been infected in the primary cycle. It has now been demonstrated that there is a symptomless prelude of viremia prior to the onset of disease manifestations in Australian patients so that, as in the Pacific islands, a direct man–mosquito–man cycle can develop and accelerate into an epidemic.

Clinical Features and Infection

There are three major manifestations of RRV disease: rheumatic, rash and constitutional. Arthralgia usually develops very rapidly; the most common signs are pain on movement, tenderness and slight swelling. Wrists are most frequently involved, followed closely by knees, ankles and fingers. Elbows, toes and tarsal joints are also commonly affected. Pain is often more intense than indicated by observed signs. Rash occurs in about two-thirds of patients, but is rarely the sole manifestation of the disease. Most commonly it appears as erythematous macules and papules 1–5 mm across, distributed sparsely to thickly on trunk and limbs, and less frequently on face and scalp. Appearance on palms, soles and digital webs is characteristic, particularly when there is no rash elsewhere. Scattered purpura may be found, usually on the feet and lower legs. Except for hyperesthesia of the palms there is usually no discomfort due to the rash, and it resolves within 7–10 days. Pyrexia, one of the most common of the constitutional effects of other virus infections, is usually absent or slight. Myalgia is common, and carpal tunnel syndrome can be induced or exacerbated. Fatigue is the most consistently apparent constitutional effect and seems to be independent of other manifestations. Clinical signs and symptoms of RRV are rarely detected in infected prepubertal children but there is a normal antibody response.

Although diagnostic criteria of 'chronic fatigue syndrome' are difficult to define it seems that, in Australia, RRV infection might now be the most common precursor of the syndrome. In these cases fatigue is usually accompanied by persistent intermittent arthralgia. Such complications are uncommon in patients under the age of 30 years.

Although BFV causes disease in humans with a similar set of signs and symptoms to that caused by RRV, the possibility that BFV is responsible for milder symptoms than RRV has been suggested. Chronic fatigue has not been reported following BFV infections.

Pathogenicity

Differences in the pathogenicity of RRV strains involved in various outbreaks can be inferred from differences in the duration and severity of signs and symptoms, which can range from subclinical to a so-called chronic form. In reports of discrete epidemics there are differences in the incidence, severity and persistence of rash; in the presence or absence of muscle pain; in the average duration of incapacity; in the occurrence of relatively rare signs such as buccal and palatal enanthems; and in the correspondence of disease onset with viremia or antibody production.

By comparison with earlier unsuccessful attempts at virus isolation during outbreaks in Australia, virus isolation from humans was readily accomplished during the Pacific island outbreaks. In successfully isolating virus, investigators used mice, Vero or mosquito cell monolayers or *Toxorhynchites amboinensis* mosquitoes. Differences in the disease profile were also apparent. The incubation period (days from infection to onset of symptoms) was 2–3 days in the Pacific islands, compared with 9–11 days in Australia. The Pacific island patients were viremic for up to 7 days and HAI antibodies were usually detected by day 10; in some cases a brief remission of symptoms occurred after viremia, followed by relapse as antibodies developed. The onset of symptoms in Australian patients usually coincides with antibody production, so the level and duration of the symptomless viremia has not been determined.

Considering the apparent dearth of potential primary cycle mammals on these Pacific islands, it is probable that only an RRV strain which induces a relatively prolonged high level viremia in humans could sustain a 'virgin soil' epidemic for 2 years.

Studies on the molecular basis of RRV virulence for mice have demonstrated the involvement in virulence of a number of genetic determinants including E2, the NSPs and E1. The introduction of the E2 gene from the mouse-attenuated Nelson Bay RRV strain into a cDNA clone of RRV (T48) attenuates mouse virulence. Chimeric viruses have been constructed in which the 5′ and 3′ noncoding regions of the RRV and SIN genomes have been exchanged. Virus containing heterologous 5′ noncoding regions show host-dependent defects in replication; exchange of the 3′ noncoding regions gives rise to virus that grows surprisingly well.

Pathology and Histopathology

In the early stages of RRV infection in humans, cells in the synovial fluid and joint effusions are predominantly mononuclear and remarkable for the proportion of mitotic figures and highly vacuolated and phagocytic macrophages. In later effusions macrophages appear less activated and small lymphocytes predominate. RRV antigen has been detected by immunofluorescence on the surface of 20–30% of the larger cells in synovial fluid during the first few days after onset of symptoms, but attempts to isolate virus have failed. There is no erosion nor permanent damage to joints. In a minority of cases relapses occur over a year or more; these gradually decline in incidence and intensity.

The histology of the rash is variable. The dermis shows a chiefly perivascular mononuclear cell infiltrate, vasodilatation and varying degrees of edema. Diffuse to dense erythrocyte extravasation is quite common. Histologically detectable changes in the epidermis are present in about half the cases, although rarely recognized macroscopically. It is not clear whether rash is due to the direct action of virus or is the result of immunological processes.

Signs and symptoms of epidemic polyarthritis can be confused with those of rubella infection. There is no evidence that RRV is teratogenic, so, if termination is being considered in first trimester pregnant women, it is important to differentially diagnose cases of polyarthritis with rash.

The pathology and histopathology of BFV infection has not been explored.

Immune Response

The detection of RRV and BFV antibodies is now routinely performed by ELISA, although their first appearance can usually be detected several days earlier by standard alphavirus HAI tests. ELISA can also be used in antibody class capture assays to detect IgM, but precautions must be taken to avoid false positives due to the presence of rheumatoid factor, which is not causally present in epidemic polyarthritis. Specific IgM often persists for many months in RRV infection so is not a reliable indicator of recent infection; as with many other arbovirus infections, a rising titer of IgG antibodies from acute to convalescent stages is a more reliable diagnostic tool. Signs and symptoms can persist in the presence of antibodies. It is likely that infection bestows immunity to all genetic types of RRV.

In the laboratory, RRV titers in mice and in macrophage cultures are readily enhanced by low levels of specific antibody. Whether waning levels of antibody can exacerbate a second infection in humans is unknown but should be explored when considering the development of vaccines.

Prevention and Control

As with other zoonoses involving wildlife as vertebrate hosts, particularly those that are vectored by insects, there can be no prospect of eradicating RRV or BFV. A degree of control can be achieved by reducing the interaction of vectors and humans through education, and at the community level by carrying out mosquito abatement programmes appropriate to the district. At the personal level the avoidance of mosquito attack can be achieved by screening windows and doors, remaining indoors during periods of maximum vector mosquito activity, and by the use of efficient repellents.

Vaccination against a disease which is not life-threatening and is without permanent sequelae can only be administered on a request basis. RRV-infected children rarely express signs and symptoms, which, superficially, augurs well for the development of a live virus childhood vaccine. However, little is known about persistence or the nature of immunity after subclinical or frank RRV infection, nor whether the wide range of individual responses to infection, including long-term relapses, is related in any way to the prior immune status of the patient. Before developing candidate vaccines, prospective studies should be carried out to assess the duration of effective immunity following natural infection.

See also: **Chikungunya, O'nyong nyong and Mayaro viruses (*Togaviridae*); Epidemiology of viral diseases; Equine encephalitis viruses (*Togaviridae*); Immune response: General features, Cell mediated immune response; Pathogenesis: Animal viruses; Replication of viruses; Sindbis and Semliki Forest viruses (*Togaviridae*); Vectors: Animal viruses; Zoonoses.**

Further Reading

Faragher SG, Meek ADJ, Rice CM and Dalgarno L (1988) Genome sequences of a mouse-avirulent and a mouse-virulent strain of Ross River virus. *Virology* 163: 509.

Fraser JRE (1986) Epidemic polyarthritis and Ross River virus disease. *Clin. Rheum Dis* 12: 369.

Lee E, Stocks C, Lobigs P *et al* (1997) Nucleotide sequence of the Barmah Forest virus genome. *Virology* 227: 509.

Marshall ID and Miles JAR (1984) Ross River virus and epidemic polyarthritis. In: Harris KF (ed.) *Current Topics in Vector Research*, p. 31. New York: Praeger.

Strauss JH and Strauss EG (1994) The alphaviruses: gene expression, replication, and evolution. *Microbiol. Rev.* 58: 491.

ROTAVIRUSES (*REOVIRIDAE*)

Contents

General Features

Robert D Shaw, State University of New York at Stony Brook, Northport VA Medical Center, Northport, New York, USA

Harry B Greenberg, Division of Gastroenterology, Stanford University Medical School, Stanford, California, USA

History

Diarrhea was long recognized worldwide as a leading cause of morbidity and mortality, but the search for important etiologic agents of human disease was not fruitful until the early 1970s. Rotavirus was known as a mouse diarrheal pathogen in the 1950s and as a simian and bovine pathogen in the 1960s, but it was not until 1972 that a virus was positively implicated as a cause of human gastroenteritis when Kapikian and coworkers used immune electron microscopy to identify Norwalk virus in diarrheal stools. The following year, investigators in several locations identified rotaviruses in intestinal biopsies and diarrheal stools of children. Rotaviruses are now identified as the leading cause of severe dehydrating gastroenteritis in infants and children. Efficient *in vitro* cultivation of many human rotavirus strains in the early 1980s, followed by the successful cloning of the rotavirus genome and expression of individual rotavirus proteins in recombinant systems, has provided a detailed knowledge of rotavirus structure and function, as well as many aspects of rotavirus serology, immunity and pathogenesis.

Prevention and Control

As with other zoonoses involving wildlife as vertebrate hosts, particularly those that are vectored by insects, there can be no prospect of eradicating RRV or BFV. A degree of control can be achieved by reducing the interaction of vectors and humans through education, and at the community level by carrying out mosquito abatement programmes appropriate to the district. At the personal level the avoidance of mosquito attack can be achieved by screening windows and doors, remaining indoors during periods of maximum vector mosquito activity, and by the use of efficient repellents.

Vaccination against a disease which is not life-threatening and is without permanent sequelae can only be administered on a request basis. RRV-infected children rarely express signs and symptoms, which, superficially, augurs well for the development of a live virus childhood vaccine. However, little is known about persistence or the nature of immunity after subclinical or frank RRV infection, nor whether the wide range of individual responses to infection, including long-term relapses, is related in any way to the prior immune status of the patient. Before developing candidate vaccines, prospective studies should be carried out to assess the duration of effective immunity following natural infection.

See also: **Chikungunya, O'nyong nyong and Mayaro viruses (*Togaviridae*); Epidemiology of viral diseases; Equine encephalitis viruses (*Togaviridae*); Immune response: General features, Cell mediated immune response; Pathogenesis: Animal viruses; Replication of viruses; Sindbis and Semliki Forest viruses (*Togaviridae*); Vectors: Animal viruses; Zoonoses.**

Further Reading

Faragher SG, Meek ADJ, Rice CM and Dalgarno L (1988) Genome sequences of a mouse-avirulent and a mouse-virulent strain of Ross River virus. *Virology* 163: 509.

Fraser JRE (1986) Epidemic polyarthritis and Ross River virus disease. *Clin. Rheum Dis* 12: 369.

Lee E, Stocks C, Lobigs P *et al* (1997) Nucleotide sequence of the Barmah Forest virus genome. *Virology* 227: 509.

Marshall ID and Miles JAR (1984) Ross River virus and epidemic polyarthritis. In: Harris KF (ed.) *Current Topics in Vector Research*, p. 31. New York: Praeger.

Strauss JH and Strauss EG (1994) The alphaviruses: gene expression, replication, and evolution. *Microbiol. Rev.* 58: 491.

ROTAVIRUSES (*REOVIRIDAE*)

Contents

General Features

Robert D Shaw, State University of New York at Stony Brook, Northport VA Medical Center, Northport, New York, USA

Harry B Greenberg, Division of Gastroenterology, Stanford University Medical School, Stanford, California, USA

History

Diarrhea was long recognized worldwide as a leading cause of morbidity and mortality, but the search for important etiologic agents of human disease was not fruitful until the early 1970s. Rotavirus was known as a mouse diarrheal pathogen in the 1950s and as a simian and bovine pathogen in the 1960s, but it was not until 1972 that a virus was positively implicated as a cause of human gastroenteritis when Kapikian and coworkers used immune electron microscopy to identify Norwalk virus in diarrheal stools. The following year, investigators in several locations identified rotaviruses in intestinal biopsies and diarrheal stools of children. Rotaviruses are now identified as the leading cause of severe dehydrating gastroenteritis in infants and children. Efficient *in vitro* cultivation of many human rotavirus strains in the early 1980s, followed by the successful cloning of the rotavirus genome and expression of individual rotavirus proteins in recombinant systems, has provided a detailed knowledge of rotavirus structure and function, as well as many aspects of rotavirus serology, immunity and pathogenesis.

Classification

The family *Reoviridae* contains the *Rotavirus* genus and eight other genera. Rotaviruses share several common features that form the basis of the classification. Viral particles are approximately 102 nm in diameter (including surface spikes) and consist of two protein capsids surrounding a central protein core that contains the genome (designated as a 'triple-layered' particle). The extracellular icosahedral particles are not enveloped by a lipid membrane. Ten monocistronic and one bicistronic genomic segments form the organization of double-stranded RNA. The negatively stained electron microscopic appearance of the complete rotavirus particle is responsible for the name *rotavirus*, which derives from the Latin word *rota*, meaning wheel. The outer capsid appears as a sharply defined rim, to which spokes appear to radiate from a large central hub. Recent advance in cryo-electron microscopy with computer-enhanced reconstructed images has provided a more detailed view of rotavirus structure (**Fig. 1**). Notable features include the presence of spikes on the outer capsid that extend over 10 nm and pores that penetrate from the virion surface into the viral genome. These channels may permit importation of metabolites required for viral RNA transcription and export of the nascent RNA transcripts for subsequent viral replication processes.

Geographic and Seasonal Distribution

Rotaviruses are ubiquitous among humans and many animal species throughout the world, and are usually important causes of gastroenteritis wherever they occur. Human rotavirus illness predominantly occurs in the cooler months in developed countries, peaking in January and February, while it is an unusual cause of gastroenteritis in the summer months. Rotavirus infections in the USA occur in a wave, starting in the Southwest in November and spreading on to New England and the Canadian Maritime provinces in March. This seasonality does not occur in tropical climates (10° latitude from the equator) where rotavirus infections occur throughout the year.

Host Range and Virus Propagation

Rotaviruses are recovered from diarrheal stools shed by a multitude of animal species. As an indication of the breadth of the host range of group A rotaviruses, infection in species other than human occurs in simian, equine, porcine, canine, feline, lapine, murine, bovine, ovine and avian, although rotavirus is not an important cause of disease in all of these species. Among laboratory animals, group A rotaviruses do not infect guinea pigs or rats, although group B rotaviruses infect the latter. Animal strains, even those with serotypes indistinguishable from human strains, rarely infect humans. Recently, a few human rotavirus isolates have been described that are probably derived from feline, canine, bovine or porcine rotaviruses. The potential of animal rotavirus reservoirs as a source of genetic diversity for the evolution of new human strains is unknown.

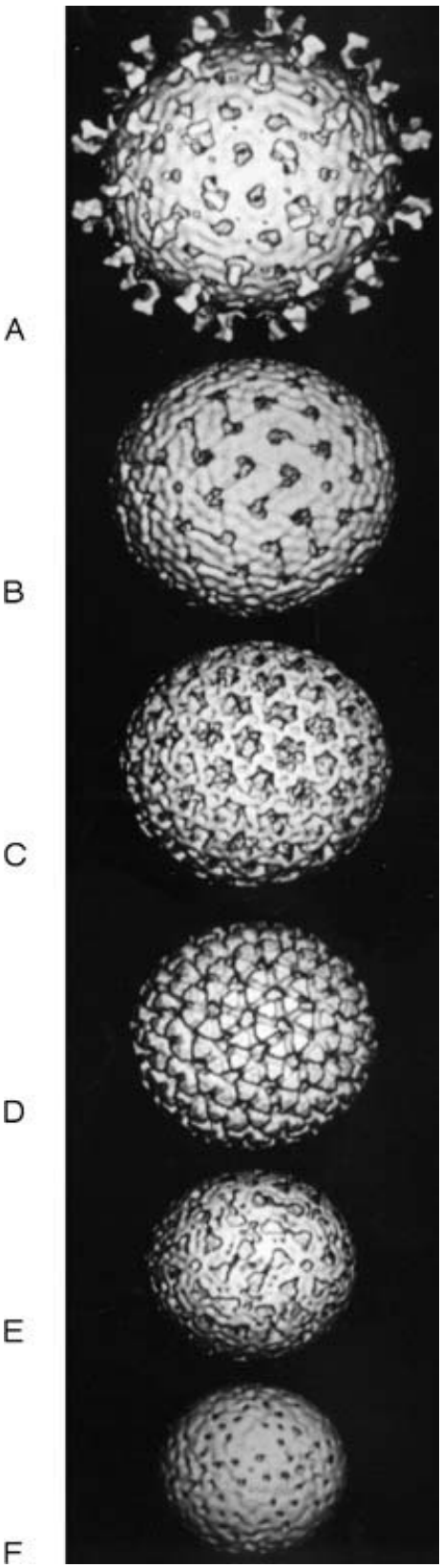
Rotaviruses were first propagated *in vitro* in 1963, when Mahlerbe and coworkers reported the isolation of simian SA11 from a vervet monkey kidney cell culture. Cultivation of human rotaviruses was not successful until the discovery that trypsin exposure, which cleaves amino acid bonds at three closely spaced sites of the VP4 protein, dramatically enhances virus infectivity in culture. Group B and C viruses are not generally cultivatable, with the exception of several porcine and bovine group C strains. Group A rotaviruses are usually cultivatable in simian kidney cell lines in the presence of trypsin. The most efficiently cultured animal rotaviruses typically yield 10^7–$10^{8.5}$ plaque-forming units (PFU) per milliliter in tissue culture systems. Human viruses tend to produce one to two orders of magnitude fewer PFU in tissue culture, although they are often shed in diarrheic stools in quantities of 10^9–10^{10} particles per milliliter.

Genetics

The double-stranded RNA (dsRNA) segments of the rotavirus genome have masses between 2×10^5 and 2.2×10^6 Da. The genes distribute by size into four classes that produce a characteristic pattern (the 'electropherotype') when separated by polyacrylamide gel electrophoresis. Electropherotype classification of rotaviruses was particularly important before *in vitro* cultivation of human rotaviruses. The wide variability of electropherotypes and the observation that serotypes and electropherotypes do not correlate well has decreased the importance of this technique in classification. However, some epidemiologic studies continue to use electropherotypes for detection and characterization of rotavirus, and it is still a useful technique for detection of non-group A rotaviruses.

Rotavirus RNA lacks 3′-terminal polyadenylated sequences and contains 5′-capped structures. Both ends of each segment contain short highly conserved regions of approximately eight nucleotides. These highly conserved sequences may be of importance in transcription, replication and assortment of the virus genome. These features are also characteristic of other *Reoviridae*.

The RNA itself is not infectious; rotaviruses contain within the double-layered particle an endogenous RNA-dependent RNA polymerase that tran-



scribes the gene segments into mRNA. Transcripts are full-length positive strands from which negative-strand synthesis occurs following the formation of replicase particles in the cytoplasm. RNA transcripts can be identified 3 h postinfection. The proteins and structural requirements of RNA replication are not fully understood. Reassortment of gene segments occurs at high frequency during mixed infection with two or more rotavirus strains, although, unlike influenza, there is no evidence that this is a mechanism for generation of serotypic diversity in nature.

Gene-coding assignments for group A rotavirus genes have been clearly established as follows. Genes 5, 7, 8, 10 and 11 code for nonstructural proteins, which were originally designated by the prefix 'NS' followed by the molecular mass, but are presently known as 'NSP' 1–5. The gene product of genes 5 (nonstructural protein NSP1) seems to have a role in the early stages of virus assembly. The gene 7 product (NSP3) has been detected in infected cells in association with complexes containing replicase activity and has specific RNA-binding function, but little else is known. The gene 8 product (NSP2) is associated with replicase activity but has a poorly defined function. NSP4, coded by gene segment 10, is a membrane-associated glycoprotein which plays an important role in viral assembly, in which the protein serves as a receptor for VP6 in the endoplasmic reticulum. In addition, NSP4 has been implicated in diarrheal pathogenesis (see below). NSP5, coded by gene segment 11 is a phosphoprotein of unknown function coded by gene segment 11. Genes 1–4, 6 and 9 code for structural proteins VP1–4, 6 and 7, respectively (in most group A viruses). VP4 and VP7 are the two

Figure 1 Three-dimensional structure of rhesus rotavirus by cryoelectron microscopy and icosahedral image reconstructure (Yeager *et al* (1990) *J. Cell Biol.* 110: 2133). The surface-shaped representations were obtained by truncating the three-dimensional maps with spherical envelopes to reveal the internal structure. The six structures from top to bottom with their corresponding diameters (in parenthesis) are as follows: (**A**) The virion outer capsid surface displays 60 spikes attributed to the VP4 hemagglutinin (102 nm). (**B**) The smoothly-rippled outer capsid surface attributed primarily to VP7, is perforated by 132 aqueous holes (79 nm). (**C**) The space between the outer and inner capsids forms an open aqueous network that may provide pathways for the diffusion of ions and small regulatory molecules as well as the extrusion of RNA (72 nm). (**D**) The inner capsid has a 'bristled' outer surface composed of 260 trimeric columns, attributed to VP6 trimers (66 nm). (**E**) The VP6 trimers merge with a smooth, inner capsid shell which is perforated by holes in register with those in the outer capsid (58 nm). (**F**) A third protein shell (referred to as the 'core') is thought to be formed by VP1, VP2 and VP3 and encapsidates the dsRNA segmented genome (53 nm).

surface proteins of the virion while VP6 is the major constituent of the double-layered particle.

The electropherotypes of non-group A rotaviruses differ from group A viruses primarily in gene segments 7, 8 and 9. The organization of the non-group A 7–9 genome segments lacks the tight triplet formation in polyacrylamide gel electrophoresis that characterizes the group A electropherotype. Non-group A rotaviruses are morphologically identical to group A strains but they are antigenically distinct and they do not crosshybridize in Northern blot analyses, even under conditions of low stringency. There appear to be at least five (B–F) non-group A rotavirus types. Groups B and C have been identified in humans as well as in animals. Sequence data are now available for several non-group A rotaviruses and this has allowed some gene coding assignments to be made. The development of serologic reagents from expressed viral proteins will extend the understanding of the epidemiology and importance of non-group A viruses.

Evolution

Little is known about the evolution of rotaviruses. While strong similarities in genomic sequences exist among some human and animal strains, there remains little evidence as to the origin of any particular strain. High rates of mutation exist in RNA genomes, and the capacity of the segmented genome to reassort during mixed infections provides theoretical opportunities for rotaviruses to mutate and evolve rapidly. However, during multiple passages in cell culture without the presence of obvious selection pressure, rotaviruses do not appear to change appreciably.

Serologic Relationships and Variability

Six of the viral proteins are structural, but only three have played important roles in the classification of group A rotaviruses by virtue of antigenic or functional properties. Non-group A rotaviruses have not been widely cultivated *in vitro* and far less is presently known about the structure and serologic classification of these viruses. VP6, the major structural protein of the group A secondary capsid, bears most of the common group antigens as well as the subgroup antigens. Rotaviruses that cause most human disease are classified as group A strains but occasionally group B and C also infect humans (see below, Epidemiology). Animal viruses have been classified into groups A–F. Human and animal group A rotaviruses are subdivided into subgroups (I and II) on the basis of serologic reactivity with subgroup-specific monoclonal antibodies directed at VP6. All group A rotaviruses share antigenic determinants on VP6, and some shared epitopes appear to be present on group C rotaviruses as well.

The glycoprotein VP7, the major neutralization protein of the outer capsid constituting 20% of the viral mass, induces serotype-specific neutralizing antibodies. VP4 is the other outer capsid protein, which is only 2% of the viral mass. It is the viral hemagglutinin, an important determinant of virulence, and is the cell attachment protein. VP4 is also responsible for inducing neutralizing antibodies. The contribution of VP4-specific neutralizing antibodies seems to be less important than VP7-specific neutralizing antibodies in hyperimmune sera used in determination of viral serotypes, but evidence delineating the relative roles of these antibodies in natural infections and following vaccination in many species is conflicting. A combined role of VP7- and VP4-specific immunity in the serologic classification of rotaviruses (such as the influenza binary system using hemagglutinin and neuraminidase antigens) has been adopted. In this system the VP7 serotype is classified as a G (glycoprotein) type and the VP4 type as a P (protease) type.

At least 14 group A G types have been identified, 10 of which have been isolated from humans (G1–4 are the major human pathogens worldwide; 8, 9 and 12 have been occasionally recovered from humans; and porcine G5 and bovine G6 and 10 have been recovered from children with diarrhea). Initially, serotyping required tissue culture growth of the viruses in the presence of defined serotype-specific sera, but serotype-specific monoclonal antibodies as well as serotype-specific nucleic acid probes are now readily available and have dramatically reduced the time and expense of the serotyping procedure. A total of 18 P types have been defined by amino acid sequence homologies, including seven in humans (P4, 6, 8, 9, 10, 11 and HCR3). The number of serotypes is steadily increasing as larger numbers of isolates are tested. In some studies, 70–80% of human isolates are either G1P8 or G2P4. Although it is assumed that the serotype classification of rotavirus strains is important in determining immunity to these viruses, the relationship of serotype to protective immunity is not entirely clear. For instance, VP6, the subgroup determinant, has recently been shown to elicit antibodies that mediate intracellular viral neutralization. A VP6 DNA vaccine has also resulted in protection from infection in a murine model, and particles containing only VP2 and VP6 have induced protective immunity in mice and rabbits.

Epidemiology

Group A rotaviruses are the principal cause of severe gastroenteritis in infants and young children through-

out the world, accounting for 12–71% (median 34%) of all diarrheal episodes requiring hospitalization in children under the age of 2 years. In developing countries, the annual toll includes roughly 18 million cases of severe diarrhea and nearly a million deaths. In the USA, over 60 000 children are hospitalized, and about 50 deaths are annually attributed to rotavirus infection.

Infants in the first 2–3 months of life seem to be relatively protected from severe rotavirus disease, probably because of residual maternal immunity mediated by transplacental antibodies. Severe disease is most common among children of 6 months to 2 years of age. Rotavirus infections occur beyond 3 years of age and into adult life but they are typically mild or asymptomatic. However, group A rotavirus may occasionally cause severe diarrhea in immunologically competent adults. Epidemics are known to occur in institutional settings, especially among the elderly in nursing homes, and occasional cases may be fatal.

The epidemiology of rotavirus infections and viral shedding can be monitored by serologic assays or by electrophoretic patterns of viral RNA (electropherotype) obtained from fecal specimens. Electropherotype studies have demonstrated that several genomic patterns may coexist within a community, but these patterns do not necessarily correlate with serotypic classifications. Several serotypes may also coexist within a community, but each season is usually dominated by a single serotype that may vary from year to year. Group A serotypes G1–4 cause most human disease and appear to be equally virulent. These serotypes have been identified in developed and underdeveloped settings and have been in circulation for at least 30 years.

Transmission and Tissue Tropism

Rotaviruses are transmitted by the fecal–oral route, as has been conclusively demonstrated by transmission of illness in human volunteers by oral inoculation of fecal filtrates. Transmission by the respiratory route has been considered but the evidence to support this route is weak. Rapid appearance of antibodies to rotaviruses is noted by 3 years of age in all areas of the world regardless of hygiene. Viral shedding is not always associated with symptoms, and asymptomatic infection occurs frequently in newborn nurseries and daycare centers. The virus is quite stable on environmental surfaces for prolonged periods. These factors complicate efforts to control hospital outbreaks, which are not always successful even if patients are carefully monitored for virus excretion and appropriate control measures are followed.

Rotaviruses replicate within and are primarily shed from mature small intestine epithelial cells located at the villous tips. Recent evidence of rotavirus infection of the liver in a small number of immunocompromised children has demonstrated that infection of other tissues is possible in some rare cases.

Pathogenicity

The genetic correlates of rotavirus pathogenicity have not yet been completely determined. Host range restrictions limit crossinfection between species in most cases. Animal strains have been used as human vaccine candidates as they possess antigenic similarity with human viruses but do not cause disease, except when given in very large doses. Animal rotavirus vaccine candidates appear to replicate in humans at a low level and stimulate local and systemic immunity. All serotypes of rotavirus seem to be equally virulent, although some neonatal strains have been associated with asymptomatic infection. Other studies, however, have indicated that it is the newborn host rather than the rotavirus strain that determines the avirulent phenotype in the newborn.

Clinical Features of Infection

Rotavirus gastroenteritis is seen most commonly in children between the ages of 3 months and 2 years. Asymptomatic infection is common in infants less than 2 months old or individuals older than 2 years, although episodic severe disease in adults may result from group A rotavirus infection. Group B rotavirus infection causes epidemics in older children and adults in China.

In some animal species, illness is strictly limited to the very young. Age-related restriction of rotavirus illness appears to be due, at least in part, to nonimmune mechanisms. The incubation period in humans and most animals appears to be 24–72 h. Malnutrition may increase the severity of the symptoms. In addition to the symptoms listed in **Table 1**, those related to severe volume depletion, such as lethargy, irritability, confusion, and eventually vascular collapse and death, can be seen.

Pathology and Histopathology

The pathologic lesion resulting from rotavirus infection varies somewhat depending on the species and age in question. For instance, porcine rotavirus causes a particularly large amount of cellular damage, whereas murine infection may be characterized by much more selective destruction. Infection of the very young of most species will characteristically produce more cell destruction than infection in adults. Blunt-

Table 1 Clinical features of rotavirus gastroenteritis

Symptom	*Frequency (%)*
Diarrhea	98
Diarrhea >10 times daily	28
Vomiting	87
Vomiting >5 times daily	51
Fever	84
Abdominal pain	18
Blood in stool	1
Hospitalization	39

Adapted from Uhnoo *et al* (1986) *Arch. Dis. Child.* 61: 732–738. British Medical Association, Tavistock Square, London, WC1H 9JR, with permission.

ing of intestinal villi and vacuolation of enterocytes may be seen within hours after infection, prior to the presence of detectable viral antigen. Also seen are mononuclear cell infiltration of the lamina propria, distended endoplasmic reticulum, mitochondrial swelling and denuded microvilli. Viral particles may be seen within columnar epithelial cells, goblet cells, phagocytic cells and M cells in the small intestinal mucosa (the colon is generally spared). Production of viral antigen in the intestine peaks around 48–72 h postinfection in most species. Large amounts of viral proteins accumulate in the cytoplasm (viroplasm), which may appear swollen and vacuolated. However, some damaged cells may be seen without detectable viral antigen present. Intestinal cellular morphology returns to normal in about 7 days, although much of the damage is repaired as quickly as 3 days after infection.

The mechanism of virus-induced diarrhea is not clear. Lytic infection is not prominent in intestinal cell lines, nor is the histologic damage in the host clearly related to diarrhea. Toxin-like effects have been suggested, as exogenous administration of the rotavirus NSP4 protein has been reported to induce diarrhea in mice, and inactivated rotaviruses that do not cause tissue damage have also been demonstrated to cause diarrhea in mice. NSP4 has been reported to stimulate chloride secretion, but the role of this effect in disease is as yet unclear. Water absorption by the small intestine is impaired, but can be corrected by the administration of glucose–salt solutions. Abnormal motility may contribute to rotavirus-induced diarrhea. Also, carbohydrate malabsorption and secondary osmotic diarrhea may occur. An integrated understanding of the roles of these many factors in the pathogenesis of diarrhea has not yet been achieved.

Group B and C rotaviruses also cause small intestinal lesions in several animal species as well as in humans. Villous blunting is seen in various small intestinal regions. Syncytia including up to 20 enterocytes are seen during group B infection, a finding not observed in group A infections.

Immune Response

The antibody-based immune response to rotavirus infection has been studied in many animals as well as in humans. Serum and mucosal antibodies are detected, beginning several days after primary infection. Cytotoxic T cells have been identified in the intestinal mucosa of mice undergoing experimental rotavirus infection. The rapid resolution of rotavirus diarrhea during an acute infection occurs somewhat before the immune response is fully developed, so at least some of the factors responsible for resolution of the illness may be nonimmune. Immune factors are most likely to have substantial roles in the prevention of subsequent infections, although it is still unclear precisely which factors determine susceptibility to rotavirus infection.

Genetic studies using specific viral reassortants and passive transfer studies using monoclonal antibodies directed at specific rotavirus proteins have demonstrated that antibodies directed at either VP4 or VP7 (but not other rotavirus proteins) can neutralize virus and protect susceptible hosts. However, the bulk of antibodies elicited by rotavirus infection are directed at the major structural protein of the inner capsid VP6 (which constitutes 51% of the viral mass). While antibodies to VP6 may not neutralize virus in the intestinal lumen or in tissue cultures, VP6-specific IgA antibodies may prevent viral replication intracellularly during the process of transcytosis through intestinal epithelial cells. Protective efficacy against infection of IgG antibodies raised in mice following systemic vaccination (including adjuvant) with recombinant proteins assembled into virus-like particles has also been recently shown, despite the absence in the particle of VP4 or VP7. In addition, murine protection appears to be conferred by DNA vaccination with the gene that codes for VP6. Thus, several mechanisms and antigens appear to be capable of mediating protection from infection in animal models. The utility of these mechanisms in vaccine strategies or their role in response to natural infection remains to be determined.

The locations of the amino acid-defined regions of VP4 and VP7 that elicit neutralizing antibodies have been mapped. One large and complex conformationally determined neutralization domain exists on VP7, and at least two domains are found on VP4 (one on

each of the two fragments resulting from trypsin cleavage of VP4, which are referred to as VP5* and VP8*). Most neutralizing antibodies elicited by VP7 are serotype-specific, although at least one epitope is heterotypic and binds antibodies that are broadly crossreactive. VP4 serotypic diversity is still not well understood. There are at least two important neutralization regions on VP4, one on either side of the site of trypsin cleavage (cleavage of this site enhances growth in tissue culture; see above, Host Range and Virus Propagation). The N-terminal fragment, VP8*, contains neutralization sites that are limited to particular strains. The C-terminal fragment VP5* has a domain that is crossreactive among several human strains, and a similarly crossreactive region is shared among several animal strains.

Primary rotavirus infection induces neutralizing antibodies to VP7 and VP4. There is conflicting evidence, in various animal models and in humans, concerning the relative importance of these two groups of antibodies in the establishment of protection against subsequent infections. Prevailing opinion at the present time could be simplified to state that a primary human infection results in predominantly serotype-specific immunity, although heterotypic immunity is frequently detected at lower levels. Individuals gradually establish broader immunity with reinfections, although whether this is due to accumulated diversity of homotypic responses to serial VP7 exposures or a gradual increase in the immune response to the major heterotypic regions on VP4 or VP7, or even VP6, remains unknown.

The complexities of the intestinal immune environment have impaired the development of a complete understanding of the immune response to rotavirus infection and vaccination. The immunological environment of the intestinal mucosa is relatively difficult to monitor, either by serum or intestinal fluid measurements. Animal studies have confirmed that most of the specific antirotavirus antibodies generated in response to infection are IgA, which is secreted predominantly by lymphocytes in the small intestinal lamina propria. It has long been inferred from animal studies that replication of the virus in the intestinal tract was a prerequisite for the development of substantial local immunity. While infectious rotavirus administered directly into the intestinal tract has been demonstrated to be a powerful mucosal antigenic stimulus to specific antibody formation, it is not yet clear that replication is vital; or, if it is, the mechanism by which replication enhances the response is not determined. Furthermore, rotavirus-specific cytotoxic lymphocytes have been identified in the intestinal mucosa following parenteral administration of killed rotavirus. In immunodeficient murine model studies, passive transfer of immune cytotoxic T cells has been shown to prevent acute rotavirus infection and resolve ongoing rotavirus infection.

The mechanisms of rotavirus antigen processing and presentation in the mucosal immune compartment are largely unexplored. Rotaviruses, like many other particulate antigens, including reoviruses, are known to bind to and be internalized by M cells overlying intestinal lymphoid aggregates, at least in a porcine model. The importance of this route of contact with immune cells, as opposed to penetration of virus directly into the lamina propria or presentation of viral antigens by major histocompatibility complex (MHC) class I- or II-bearing enterocytes, is unknown.

Prevention and Control of Rotavirus

Two avenues leading to prevention and control of rotavirus disease are vaccination and oral rehydration therapy. Treatment with oral rehydration solutions containing glucose and electrolytes is highly effective for ameliorating the consequences of rotavirus infection, but there are serious logistical, cultural and educational difficulties limiting the distribution of this treatment resource into underdeveloped areas. Recent evaluations of this approach in the USA suggest that it is underutilized even in a developed setting.

Breast feeding has been advocated as an inexpensive and effective means of rotavirus disease suppression, as breast milk is effective in the reduction of morbidity and mortality caused by bacterial gastroenteritis. However, despite the presence of antirotavirus antibodies in breast milk, there is little evidence that breast feeding can protect against rotavirus infection or serious rotavirus disease.

Vaccination strategies that have been tested have utilized the host range restrictions of animal rotaviruses in a 'Jennerian' approach to disease prevention. For example, simian and bovine rotaviruses have been used as naturally attenuated vaccine strains in children. Field trials demonstrated these vaccines to be safe and immunogenic, and efficacy against severe disease was high in developed countries. Efficacy in underdeveloped countries has been more difficult to demonstrate, although at least one large study showed substantial efficacy. Animal rotaviruses have induced both homotypic and heterotypic protection in some studies, but in other circumstances these vaccines have failed to protect in both developed and less-developed settings. Overall, these findings have been considered encouraging by most authorities, and live attenuated 'Jennerian' vaccines with significant protection against severe disease may soon be

commercially available. However, further investigation may reveal an optimal vaccine strategy that will routinely provide reproducible protective efficacy in all parts of the world.

Future Perspectives

Vaccine strategies currently under consideration are varied. Both nonpathogenic neonatal rotavirus strains and multivalent collections of reassortant rotaviruses that contain human gene 4 segments are presently undergoing intensive testing. One example of the latter has now been approved by the FDA for use in the USA. Vaccines made from synthetic viral proteins or particles administered systemically or enterally are planned or under investigation, but the ability of these constructs to stimulate protective immunity in humans is unknown.

A better understanding of the mechanisms of viral antigen processing and presentation, the determinants of the magnitude and specificity of the antibody and cytotoxic T cell responses and a more precise determination of the mechanisms of naturally-occurring protective immunity may permit a more efficient and effective design for synthetic vaccine products that will elicit sufficiently broad protective immunity.

See also: **Enteric viruses; Norwalk and related viruses (*Caliciviridae*); Pathogenesis: Animal viruses, Plant viruses; Rotaviruses (*Reoviridae*): Molecular biology; Vaccines and immune response.**

Further Reading

Ball JM, Tian P, Zeng CQ, Morris AP and Estes MK (1996) Age-dependent diarrhea induced by a rotaviral nonstructural glycoprotein [see comments]. *Science* 272 (5258): 101.

Blacklow N and Greenberg H (1991) Viral gastroenteritis. *N. Engl. J. Med.* 325: 252.

Burke B and Desselberger U (1996) Rotavirus pathogenicity. *Virology* 218: 299.

Iqbal N and Shaw R (1997) Rotaviruses. In: D. Richman, R. Whitley and F. Hayden (eds) *Clinical Virology*, p. 765. New York: Churchill Livingstone.

Midthun K and Kapikian AZ (1996) Rotavirus vaccines: an overview. *Clin. Microbiol. Rev.* 9: 423.

Offit PA (1996) Host factors associated with protection against rotavirus disease: the skies are clearing. *J. Infect. Dis.* 174: S59.

Patton JT (1994) Rotavirus replication. [Review]. *Curr. Top. Microbiol. Immunol.* 185: 107.

Perez-Schael I, Guntinas MJ, Perez M *et al* (1997) Efficacy of the rhesus rotavirus-based quadrivalent vaccine in infants and young children in Venezuela [see comments]. *N. Engl. J. Med.* 337: 1181.

Ramig RF (1997) Genetics of the rotaviruses. *Annu. Rev. Microbiol.* 51: 225.

Molecular Biology

Mary K Estes, Division of Molecular Virology, Baylor College of Medicine, Houston, USA

Properties of the Virion

Rotaviruses are members of the *Reoviridae* and are characterized by a genome of double-stranded (ds) RNA and a nonenveloped icosahedral structure (**Figure 1** also see Plate 31 for color). The rotaviruses were named (from the Latin *rota*, meaning wheel) based on the distinctive morphologic appearance of particles visualized by negative-stain electron microscopy. Virus particles resemble a wheel, with short spokes and a well-defined rim. The virion contains six structural proteins (VP1, VP2, VP3, VP4, VP6 and VP7) that make up a triple-layered protein shell. In the interior of particles, the genomic dsRNA is highly ordered, with about 25% of the genome making up a dodecahedral structure (**Figure 2**). Surrounding the genome is the innermost shell that is composed of VP2. VP2 shells also are called single-layered particles, and two internal proteins, VP1 and VP3, interact with the inner surface of VP2 at the fivefold axes where they are visualized as flower-shaped structures in three-dimensional reconstructions of virus-like particles containing proteins 1/2/3/6. VP1 is the viral transcriptase and VP3 is the guanylyltransferase. The addition of VP6 to single-layered particles results in structures called double-layered particles. These particles possess an active transcriptase activity and mRNAs are extruded from particles at the fivefold axes. The outer protein shell has a rippled surface composed of trimers of a glycoprotein, VP7; dimeric spikes composed of VP4 emanate through the VP7 surface and the base of the VP4 spikes interacts with the inner capsid protein VP6.

Three-dimensional reconstructions of rotavirus particles using images of particles embedded in vitreous ice have provided the most detailed description of particle structure (**Figures 1** and **2** see Plates 31 and 32 for color). The outer shell has a diameter of 76.5 nm, the second shell is 70.5 nm and the inner core shell is 50 nm in diameter. The two outer icosahedral layers have a $T = 13\,l$ symmetry, and the inner shell is composed of 120 molecules of VP2 arranged with a $T = 1$ symmetry. The most distinctive feature of the outer shell is the presence of 60 spikes, at least 10 nm

commercially available. However, further investigation may reveal an optimal vaccine strategy that will routinely provide reproducible protective efficacy in all parts of the world.

Future Perspectives

Vaccine strategies currently under consideration are varied. Both nonpathogenic neonatal rotavirus strains and multivalent collections of reassortant rotaviruses that contain human gene 4 segments are presently undergoing intensive testing. One example of the latter has now been approved by the FDA for use in the USA. Vaccines made from synthetic viral proteins or particles administered systemically or enterally are planned or under investigation, but the ability of these constructs to stimulate protective immunity in humans is unknown.

A better understanding of the mechanisms of viral antigen processing and presentation, the determinants of the magnitude and specificity of the antibody and cytotoxic T cell responses and a more precise determination of the mechanisms of naturally-occurring protective immunity may permit a more efficient and effective design for synthetic vaccine products that will elicit sufficiently broad protective immunity.

See also: **Enteric viruses; Norwalk and related viruses (*Caliciviridae*); Pathogenesis: Animal viruses, Plant viruses; Rotaviruses (*Reoviridae*): Molecular biology; Vaccines and immune response.**

Further Reading

Ball JM, Tian P, Zeng CQ, Morris AP and Estes MK (1996) Age-dependent diarrhea induced by a rotaviral nonstructural glycoprotein [see comments]. *Science* 272 (5258): 101.

Blacklow N and Greenberg H (1991) Viral gastroenteritis. *N. Engl. J. Med.* 325: 252.

Burke B and Desselberger U (1996) Rotavirus pathogenicity. *Virology* 218: 299.

Iqbal N and Shaw R (1997) Rotaviruses. In: D. Richman, R. Whitley and F. Hayden (eds) *Clinical Virology*, p. 765. New York: Churchill Livingstone.

Midthun K and Kapikian AZ (1996) Rotavirus vaccines: an overview. *Clin. Microbiol. Rev.* 9: 423.

Offit PA (1996) Host factors associated with protection against rotavirus disease: the skies are clearing. *J. Infect. Dis.* 174: S59.

Patton JT (1994) Rotavirus replication. [Review]. *Curr. Top. Microbiol. Immunol.* 185: 107.

Perez-Schael I, Guntinas MJ, Perez M *et al* (1997) Efficacy of the rhesus rotavirus-based quadrivalent vaccine in infants and young children in Venezuela [see comments]. *N. Engl. J. Med.* 337: 1181.

Ramig RF (1997) Genetics of the rotaviruses. *Annu. Rev. Microbiol.* 51: 225.

Molecular Biology

Mary K Estes, Division of Molecular Virology, Baylor College of Medicine, Houston, USA

Properties of the Virion

Rotaviruses are members of the *Reoviridae* and are characterized by a genome of double-stranded (ds) RNA and a nonenveloped icosahedral structure (**Figure 1** also see Plate 31 for color). The rotaviruses were named (from the Latin *rota*, meaning wheel) based on the distinctive morphologic appearance of particles visualized by negative-stain electron microscopy. Virus particles resemble a wheel, with short spokes and a well-defined rim. The virion contains six structural proteins (VP1, VP2, VP3, VP4, VP6 and VP7) that make up a triple-layered protein shell. In the interior of particles, the genomic dsRNA is highly ordered, with about 25% of the genome making up a dodecahedral structure (**Figure 2**). Surrounding the genome is the innermost shell that is composed of VP2. VP2 shells also are called single-layered particles, and two internal proteins, VP1 and VP3, interact with the inner surface of VP2 at the fivefold axes where they are visualized as flower-shaped structures in three-dimensional reconstructions of virus-like particles containing proteins 1/2/3/6. VP1 is the viral transcriptase and VP3 is the guanylyltransferase. The addition of VP6 to single-layered particles results in structures called double-layered particles. These particles possess an active transcriptase activity and mRNAs are extruded from particles at the fivefold axes. The outer protein shell has a rippled surface composed of trimers of a glycoprotein, VP7; dimeric spikes composed of VP4 emanate through the VP7 surface and the base of the VP4 spikes interacts with the inner capsid protein VP6.

Three-dimensional reconstructions of rotavirus particles using images of particles embedded in vitreous ice have provided the most detailed description of particle structure (**Figures 1** and **2** see Plates 31 and 32 for color). The outer shell has a diameter of 76.5 nm, the second shell is 70.5 nm and the inner core shell is 50 nm in diameter. The two outer icosahedral layers have a $T = 13\,l$ symmetry, and the inner shell is composed of 120 molecules of VP2 arranged with a $T = 1$ symmetry. The most distinctive feature of the outer shell is the presence of 60 spikes, at least 10 nm

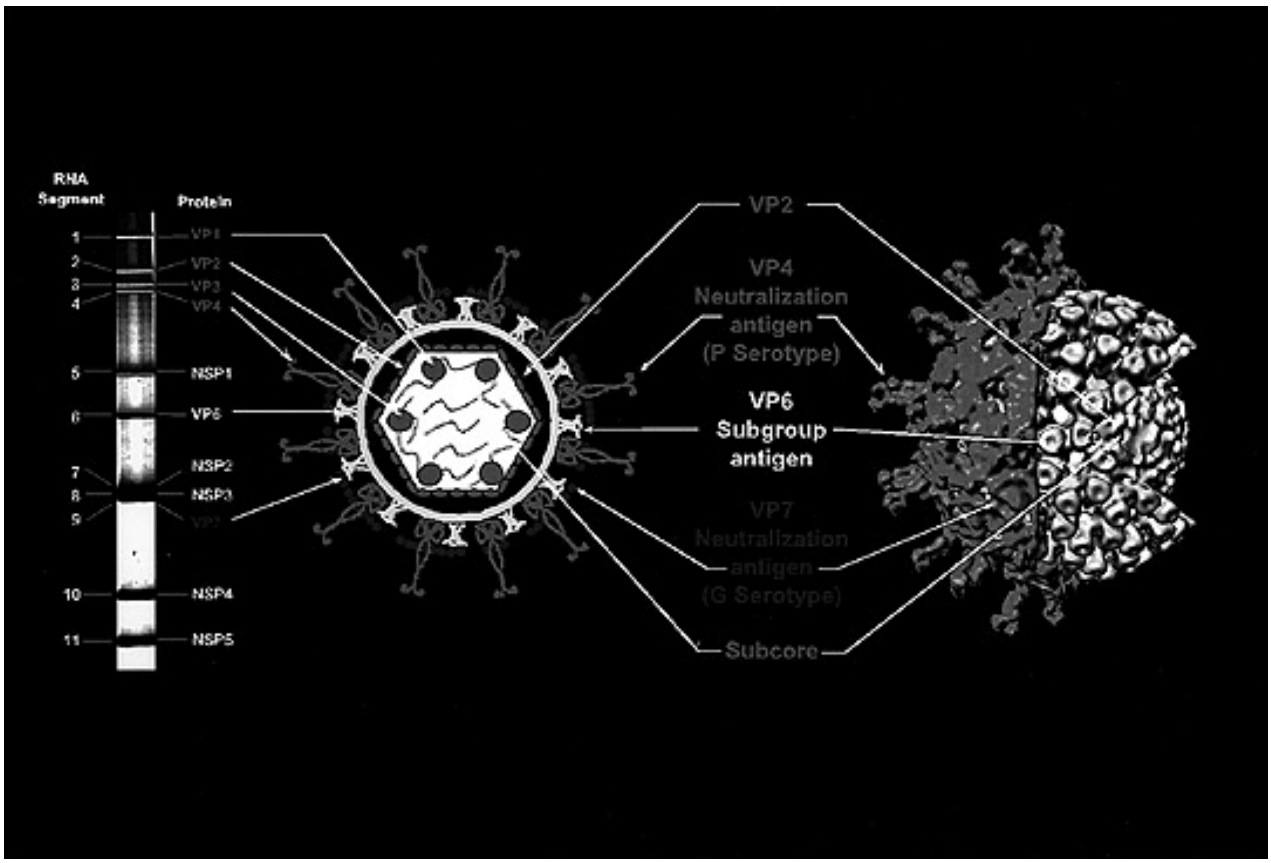


Figure 1 Rotavirus genes, proteins and structure. The left panel shows the RNA segments and the gene coding assignments for the simian rotavirus SA11. See **Table 1** for details on the genes and proteins. The location of the viral structural proteins in the different shells of the virus particles is shown in the schematic in the center. The major structural proteins that make up the outer shell of particles (VP4 and VP7), the intermediate shell (VP6), the inner shell (VP2) and the subcore region composed of ordered viral RNA are illustrated in the ~25 Å three-dimensional structure of particles shown in the right panel. (This reconstruction was kindly provided by B.V.V. Prasad.) **(For color references see Color Plate 28.)**

(100 Å) in length, that extend from the surface of particles. These spikes are made up of dimers of VP4, the protein product of genome segment 4. The spikes are situated at the edge of a subset of three types of 132 channels that lead from the viral surface to the center of the virion. The channels are involved in importing the metabolites for RNA transcription and exporting nascent RNA transcripts for subsequent virus replication processes. The channels at the fivefold axes are the conduits for the export of mRNA that first interacts with the enzyme complexes at the inner surface of these axes. Cleavage of VP4 is associated with enhanced viral infectivity. This proteolytic cleavage results in the appearance of two products, VP5* and VP8*, that both remain associated with virions. Cleavage of VP4 is thought to be important in viral penetration into cells. The VP6 shell has a bristle-like structure composed of trimers of VP6. These trimers have a central indentation and 132 channels lie in register with the channels in the outer capsid.

Properties of the Genome

The sequences of the genome of several rotaviruses (the simian rotavirus SA11, bovine rotavirus RF, and human rotavirus K8 strains) have been determined completely. The following summary of information about the genome is based on data known primarily for the group A rotaviruses, of which SA11 is the prototype strain. The viral genome consists of 11 RNA segments that range in size from 667 (segment 11) to 3302 (segment 1) base pairs, with the total genome containing 18 556 base pairs (**Table 1**). The RNA segments are thought to be encapsidated in association with protein molecules. Each RNA segment encodes one protein, with the exception of gene 11 which codes for two proteins.

Each genomic RNA segment contains a methylated cap 5′ sequence $m^7GpppG^{(m)}GPy$ followed by a 5′ nontranslated sequence, an open reading frame coding for the protein product, another set of noncoding sequences, and ending with a 3′-terminal

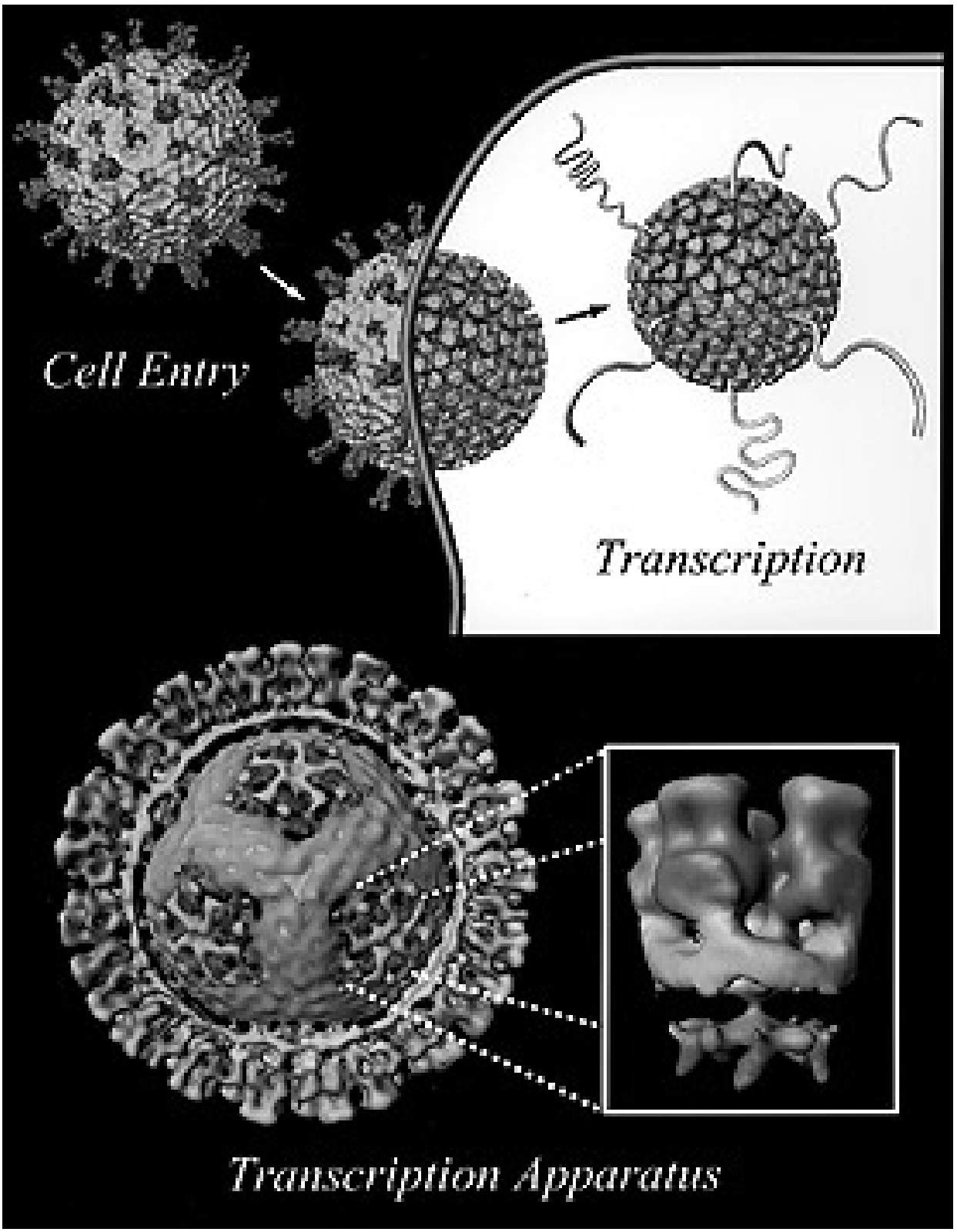


Figure 2 Rotavirus transcription. The top panel illustrates the early changes in rotavirus structure that lead to transcription and release of the mRNA molecules from the double-layered particles. The bottom panel shows the internal structure of rotavirus double-layered particles that are surrounded by trimers of the intermediate shell protein VP6 (blue). The double-stranded RNA genome is organized as a dodecahedral shell inside the particles and the minor internal proteins VP1 and VP3 are organized in a flower-shaped structure at the fivefold axes. The right-hand insert shows a side view of this structure highlighting VP6 (blue), VP2 (green) and the complex of VP1 and VP3 (orange). The newly made transcripts are extruded from these complexes at the fivefold axes (see top panel). (Figure kindly provided by J. Lawton.) (**For color references see Color Plate 29.**)

Table 1 Rotavirus genome RNA segments and protein products[a]

Segment	Number of base pairs	Number of noncoding sequences[b] 5′	3′	Protein product[c]	Nascent polypeptide mol.wt (no. of amino acids)[d]	Mature protein modified	No. molecules per virion[e]	ts mutant group[f]	Remarks
1	3302	18	17	VP1	125 005 (1088)		12	C	Core protein, slightly basic, polymerase
2	2690	16	28	VP2	102 431 (880)	Cleaved	120	F	Single-layered particle RNA binding, leucine zipper
3	2591	49	34	VP3	98 120 (835)		12	B	Core protein, basic, guanylyltransferase
4	2362	9	22	VP4	86 782 (776)	Cleaved VP5*(529)[g] VP8*(247)[g]	120	A	Surface spike protein, dimer, hemagglutinin, protease-enhanced infectivity, neutralization antigen, cell attachment protein, virulence, putative fusion region
5	1611	30	93	NSP1	58 654 (491)			NA	Slightly basic, zinc fingers
6	1356	23	139	VP6	44 816 (397)	Myristilated	780	G	Inner capsid protein, trimer, hydrophobic subgroup antigen
7	1104	25	131	NSP3	34 600 (315)			NA	Slightly acidic, RNA binding, oligomer
8	1059	46	59	NSP2	36 700 (317)			E	Basic, role in RNA replication?
9	1062	48 135	33 33	VP7(1) VP7(2)	37 368 (326) 33 919 (297)	Cleaved signal sequence, *N*-linked high mannose glycosylation and trimming[h]	780	NA	Surface glycoprotein, rough ER integral membrane glycoprotein, neutralization antigen, 2 hydrophobic NH_2-terminal regions, Ca^{2+}-binding
10	751	41	182	NSP4	20 290 (175)	Uncleaved signal sequence *N*-linked high mannose glycosylation and trimming		NA	Nonstructural rough ER transmembrane glycoprotein, 2 hydrophobic NH_2-terminal regions, role in morphogenesis, putative Ca^{2+}-binding site, enterotoxin
11	667	20	49	NSP5	21 725 (198)	Phosphorylated, *O*-linked glycosylation		NA	Nonstructural, slightly basic; serine–threonine-rich, RNA binding, protein kinase
				NSP6	21 000	Phosphorylated		NA	Nonstructural phosphoprotein

[a] For the Group A/Si/SA11 strain. Modified from Estes (1996) and Mattion *et al.* (1994). [b] Number of 5′ noncoding sequences is up to the first AUG; number of 3′ noncoding sequences does not include the termination codon. [c] Determined by biochemical and genetic approaches. The size (in thousands) of the primary translation product is given for the nonstructural (NSP) proteins. [d] Molecular weights are calculated from the deduced amino acid sequences from nucleotide sequence data. The molecular weights are calculated from the largest potential open reading frame. [e] The calculated numbers of molecules of VP2, VP4, VP6 and VP7 are made from predicted structural analyses of individual particles using electron cryomicroscopy. [f] NA indicates none assigned. [g] There are two trypsin cleavage sites in SA11 4fM VP4 at amino acid 241 and 247. The indicated mature products are those based on use of only the preferred second cleavage site. [h] Mature cleaved VP7 contains 276 amino acids.

cytidine. A poly(A) tract is not found in the genomic RNAs and viral RNA transcripts are not polyadenylated. The 5′- and 3′ ends of each genome segment contain terminal consensus sequences of 7–10 nucleotides (nt). These consensus sequences are present in each RNA segment as part of the noncoding sequences. The 5′- and 3′-terminal consensus sequences are unrelated, implying they have functional differences. The terminal consensus sequences are assumed to be important *cis*-acting signals, which presumably include, at the 3′ ends, the viral promoters. The termini are also thought to contain sequences important in packaging and in the regulation of rotavirus gene expression at the levels of transcription, replication and translation. Both the 5′ and 3′ termini have been shown to be important for *in vitro* replication of the genome and they likely interact during translation of the viral mRNAs.

Properties of the Proteins

Descriptive properties of the six structural and six nonstructural proteins are quite well characterized (**Table 1**). Biochemical and antigenic information about the structural proteins is quite extensive, but molecular mechanisms of how these proteins function remain unknown. The two outer capsid proteins (VP4 and VP7) are the proteins studied in most detail because of their expected important roles in virus replication and in the induction of protective immunity. Indeed, VP4 and VP7 each have been shown to induce antibodies with neutralizing activity, and they function to mediate early events (attachment and penetration) in the replication cycle. Virus serotypes are defined based on antigenic properties of VP4 and VP7. VP7 serotypes (also called G for glycoprotein types) now are easily characterized and classified using monoclonal antibodies (MAbs). VP4 types (also called P for protease types) are still being characterized and VP4 typing MAbs are not yet available. VP4 and VP7 also interact with one another in still undefined ways, and these interactions can affect specific biologic and antigenic properties of virions. VP6 is the most immunogenic protein based on the ease of detection of antibodies to this protein following infection, and the most sensitive diagnostic assays are based on detection of this protein or antibodies to it. The minor subcore proteins, VP1 and VP3, are part of the RNA-dependent RNA polymerase activity associated with double-shelled particles. VP1 functions as the polymerase in association with VP3, which is the guanylyltransferase responsible for capping the newly made transcripts. VP2 is an RNA-binding protein that can self-assemble into single-layered particles. It is unclear if VP2 has an active role in the transcription process. VP2 is needed for replication of genomic RNA, and it may be one of the key proteins responsible for the packaging of the nascent RNA segments into newly forming particles in infected cells. VP6 is needed for transcriptase activity. However, it is not known whether VP6 simply functions as a structural component required to keep VP1, VP2, VP3 and the RNA segments in the proper conformation to permit transcription or if VP6 participates actively in the transcription process. Based on electron cryomicrograph studies, it is estimated that particles contain 120 molecules of VP2, 120 molecules of VP4, and 780 molecules of each VP6 and VP7.

The nonstructural proteins, found only in infected cells and not in mature virions, function in the steps of genome replication, mRNA translation and virion assembly. However, the precise role of only two of the nonstructural proteins (NSP3 and NSP4) in these processes is currently clear. Several of the nonstructural proteins involved in RNA replication may function as complexes. The nonstructural protein NSP3 binds specifically to four nucleotides at the 3′ end of viral RNAs and initially was suggested to be involved in RNA encapsidation. While this remains a possibility, recently NSP3 was found to interact with eIF4GI, a human eukaryotic initiation factor, and this interaction has been confirmed in rotavirus-infected cells by coimmunoprecipitation. In addition, the amount of poly(A) binding protein (PABP) present in eIF4F complexes decreases during rotavirus infection, and PABP is removed from eIF4F complexes after incubation with NSP3. These results indicate a physical link between the 5′ and the 3′ ends of mRNA is necessary for efficient translation of viral mRNAs and strongly support a closed loop model for the initiation of translation. These results also suggest that NSP3, by taking the place of PABP on EIF4GI, is responsible for the shut-off of cellular protein synthesis. Thus, NSP3 is involved in a new kind of viral mRNA translational regulation.

The nonstructural glycoprotein NSP4 is a unique protein that functions in the unusual rotavirus morphogenesis pathway that includes the transport of subviral particles across the membrane of the endoplasmic reticulum membrane, and acquisition and subsequent loss of a transient envelope on particles. This process culminates in the assembly of the outer capsid glycoprotein on to particles in the lumen of the ER. NSP4 has been found to have other properties including modulating intracellular calcium levels and chloride secretion. These activities relate to NSP4 functioning as an enterotoxin; NSP4 represents the first viral enterotoxin to be described.

Physical Properties of the Virions

The three forms of particles (triple-layered, double-layered and single-layered) are easily distinguished by electron microscopy and these also can be separated easily by centrifugation in gradients of cesium chloride (CsCl). The physical property of virions used most often to purify virus particles is virion density. In CsCl gradients, virions are relatively stable and viral infectivity is stable to extremes of pH (3.5–9.0). Virion stability is strain-dependent and some strains, particularly human virus strains, may be much less stable than viruses isolated from animals. Studies with reassortants suggest that virion stability during purification and storage at 4°C is determined by a particular gene 4 or its encoded protein VP4, and by specific interactions of VP4 with VP7. The outer capsid proteins can be removed by treatment of virions with chelating agents, such as 10 mM EDTA, or with ethanol, and this results in inactivation of infectivity. VP7 is a calcium-binding protein. In some cases, virus infectivity is less stable when virions contain cleaved VP4. In addition, the VP4 spikes can be removed by treatment of virions at pH 11.2 with ammonium hydroxide; removal of VP4 also may occur during other physical or chemical manipulations such as treatment with organic solvents. The ease of removing the VP4 spikes suggests that most virus preparations are quite heterogeneous and they probably contain many noninfectious particles (that lack a full complement of VP4 spikes). This heterogeneity of virus preparations may explain why early estimates of the approximate percent of each protein in particles determined by biochemical methods did not agree with the calculations made from electron cryomicrographs.

Replication

General features of the replication of rotaviruses are that infectious triple-shelled rotavirions bind to a host cell receptor and virions enter cells by still poorly characterized mechanisms. The outer shell of the virion apparently is disrupted or removed as part of the virus entry process and this allows activation of the RNA-dependent RNA polymerase (transcriptase) activity that is associated with the particles that contain VP1, VP2, VP3 and VP6. The virion-associated transcriptase synthesizes viral messenger RNAs (mRNAs) that are end-to-end transcripts of each of the 11 genome segments. These mRNAs are capped by the virion-associated capping enzyme. The mRNAs subsequently are translated, giving rise to all the viral proteins. The accumulation of viral proteins in the cytoplasm results in the formation of large perinuclear electron dense inclusions, termed viroplasms, that are thought to be the sites of genome replication and the assembly of progeny subviral particles. Subviral particles that contain a subset of newly made structural proteins (VP1, VP2, VP3, VP6) and of nonstructural proteins and mRNAs are apparently formed. Some subviral particles contain an associated RNA polymerase (replicase) activity that uses the mRNAs as templates for the synthesis of minus-strand RNA resulting in the formation of dsRNA. It has been proposed that the replicase particles mature into double-layered particles, and some of these nascent double-layered particles will function as transcriptase particles and synthesize additional mRNAs, thus leading to an amplification of the level of RNA replication in the cell. Other newly-formed double-layered particles associate with regions of the endoplasmic reticulum (ER) containing the nonstructural protein NSP4 and bud into the endoplasmic reticulum. During this process, the particles acquire a membrane that is subsequently lost, while VP4 and VP7 condense around the particles producing the outer shell of protein found on mature virus particles. VP4 probably associates with NSP4 and VP6 prior to the budding of particles into the ER.

Stages of the Replication Cycle

Adsorption, penetration and uncoating

The initial stages of virus replication have been examined by biochemical and morphologic (electron microscopic) procedures and they are not yet completely understood. Only triple-layered particles attach to cells when monitored by electron microscopy or by infectivity assays. Virus attachment is thought to occur via VP4 but VP7 may be involved in this process as well. Binding to cells does not require cleaved VP4 or glycosylated VP7.

The identity of the cellular receptor for rotaviruses appears to be different for different virus strains and cells. Some virus strains bind to cells in a sialic acid (SA)-dependent fashion. A study of the binding of radiolabeled simian rotavirus SA11 (a SA-dependent virus) to monkey kidney (MA104) cells found approximately 13 000 receptor units per cell. Binding is sodium-dependent, pH-insensitive between 5.5 and 8, and is dependent on sialic acid residues in the membrane. Virus binds but is not internalized at 4°C. The SA-dependent porcine rotavirus OSU has been shown to bind to ganglioside receptors NeuGcGM3 and NeuAcGM3 on porcine enterocytes. The SA-independent receptor has not yet been identified.

Many rotavirus strains contain a hemagglutinin, as demonstrated by their ability to bind to red blood cells. The hemagglutination activity has been mapped to VP8*, the N-terminal cleavage fragment of VP4. Studies of virus binding to red blood cells were the first to show that neuraminidase treatment reduces virus binding, indicating a role of SA in virus attachment. SA-containing compounds such as fetuin and mucin also inhibit virus binding to cells. These results add rotaviruses to an increasing number of viruses (such as reoviruses and influenza) that require SA for binding to cells. However, these studies have not determined whether virus binds directly to SA or whether SA maintains the configuration of the binding site without directly interacting with the virus. Binding to SA is not essential for rotavirus infectivity, as most human and animal rotaviruses infect cells in a SA-independent manner.

After binding to susceptible cells occurs, virus is internalized. Rotavirus infectivity is enhanced by proteolysis and this effect is not due to increased efficiency of virus attachment to host cells, but to facilitation of the virus internalization (penetration) step. Internalization will not take place at 0–4°C, indicating that this step requires active cellular processes. All virus is internalized by 60–90 min after binding. The mechanism of internalization (penetration) into cells remains unclear and controversial.

Both morphologic and biochemical approaches have been used to investigate the mode of entry of rotaviruses into cells. Current data indicate that trypsin-treated and nontrypsin-treated virus enter cells by different mechanisms. Nontrypsin-treated virus is thought to enter cells by receptor-mediated endocytosis, while trypsin-activated rotavirus is thought to enter cells more quickly by inducing permeability alterations in the plasma membrane which result in direct penetration of the cell membrane. Either pathway is essential for initiating penetration of the cell membrane but not for further steps in virus infectivity, as double-layered particles that lack the outer capsid proteins are infectious if they are delivered into the cytoplasm of cells by using a facilitator such as lipofectamine.

Other viruses that initiate infection by mechanisms involving receptor-mediated endocytosis often depend on the acidification of endosomes for partial uncoating or entry into the cell. The acidification of endosomes is not important for the entry of rotaviruses into cells. Unlike other viral systems, including reoviruses, lysosomotropic agents (ammonium chloride, chloroquine, methylamine, amantadine) or endocytosis inhibitors (dansylcadaverine and cytochalasin D or the vaculoar proton-ATPase inhibitor bafilomycin A1) have little inhibitory effect on rota virus replication as measured by RNA synthesis, polypeptide synthesis or virus yields. Energy inhibitors (sodium azide and dinitrophenol) have a minimal effect on rotavirus infection suggesting that rotaviruses do not use endocytosis to enter cells. However, it remains possible that these inhibitors (and the conditions tested) specifically affect the cell processes (if any) required for rotaviruses to enter cells.

It seems clear that the passage of rotaviruses from endocytic vesicles to the cytoplasm does not occur by a pH-dependent fusion mechanism, but this does not prove that rotaviruses are not taken up by endocytosis. The most direct support for the idea that rotaviruses enter the cell through direct penetration of the plasma membrane is the demonstration that trypsin-treated triple-layered rotavirus (but not nontrypsinized virus or double-layered particles) causes release of a fluorophore encapsulated within liposomes. A putative fusion region in VP5* has been identified that has sequence homology with Sindbis virus, and it has been suggested that this region might mediate virus penetration into cells. This remains to be demonstrated. It also remains to be determined if the interaction of cleaved VP4 with lipids occurs only at the plasma membrane or if this might occur in the endosome, or during virus morphogenesis (see below). It is possible that more than one mechanism, including endocytosis and direct passage, is operative for rotaviruses, as has been proposed for poliovirus and reoviruses. Recently, internalization of rotavirus or virus-like particles composed of proteins 2/4/6/7, but not of proteins 2/6/7 or 2/6, were shown to be able to induce a rapid and transient coentry of α-sarcin, a toxin that inhibits translation. Studies of the entry of this toxin may be useful to reveal the route used by rotaviruses to traverse the cell membrane and initiate productive infection.

Transcription and replication

The synthesis of viral transcripts is mediated by a viral RNA-dependent RNA polymerase (transcriptase) which has a number of enzymatic activities. The transcriptase is a component of the virion and properties of this enzyme (or enzyme complex) have been inferred by studying the characteristics of products from *in vitro* transcription reactions. Rotavirus particles presumably contain the same enzymatic activities found in reoviruses, including transcriptase, nucleotide phosphohydrolase, guanyltransferase and two methylases. These activities are inferred because rotavirus transcripts made *in vitro* in the presence of *S*-adenosyl methionine possess a methylated 5′-terminal cap structure, m^7GpppGm, and transcription is inhibited by pyrophosphate. Particles also

contain a poly(A) polymerase activity whose precise function remains unknown; it has been postulated to be responsible for the synthesis of oligo(A) molecules.

The virus-associated transcriptase is latent in triple-layered particles and can be activated *in vitro* by treatment with a chelating agent or by heat shock treatment. Such treatments result in removal of the outer capsid proteins with conversion of triple-layered particles to double-layered particles. In infected cells, triple-layered particles have been shown to be uncoated to double-layered particles, and transcription in cells occurs from such particles (**Figure 2**). Transcription is asymmetric and all transcripts are full-length (+) strands made off the (−) dsRNA strand. The intracellular site of transcription is unknown.

Activation of transcriptase activity is a process that is not well understood. 'Activation' may be a misnomer, since in reoviruses it has been suggested this process does not actually modify the enzyme complex but instead releases the templates from structural constraints, allowing them to move past the transcriptase catalytic site. Rotavirus transcription requires a hydrolyzable form of ATP and studies with analogues that inhibit transcription suggest that ATP is required in reactions other than polymerization. ATP may be used for initiation or elongation of RNA molecules, as has been described for vesicular stomatitis virus or vaccinia virus RNA polymerases. It remains unclear if distinct polypeptides in the transcriptively active particles perform distinct functions or if the inner core polypeptides function as an enzymatic complex, but the location of VP1 and VP3 as a structural complex at the fivefold axes favor the latter hypothesis. VP1 has been crosslinked with a photoreactable nucleotide analogue, indicating that VP1 is a component of the transcriptase. VP3 in virus particles and expressed alone in insect cells can bind GTP, suggesting this protein is the guanyltransferase. Whether VP3 also possesses transcriptase activity alone or in association with VP1 remains unclear. Similarly, the role of VP2 in the transcription process is unclear.

The synthesis of plus- and minus-strand RNA has been studied in SA11-infected cells and in a cell-free system. Optimization of an electrophoretic system that allows separation of the plus and minus strands of rotavirus RNAs based on the complementary strands migrating at different rates in acid urea agarose gels facilitated these studies. Analysis of the kinetics of RNA synthesis in infected cells showed that plus- and minus-strand RNAs are detected initially at 3 hours postinfection, in agreement with other studies that looked at the time of incorporation of [^{3}H]uridine into rotavirus RNA. After 3 hours, the level of transcription increases until 9–12 hours, at which time the levels of plus-strand RNAs are maximal. The ratio of plus- to minus-strand RNA synthesis changes during infection and the maximal level of minus-strand RNA synthesis is seen several hours prior to the peak of plus-strand RNA synthesis.

The delay in obtaining maximal plus-strand RNA synthesis has been hypothesized to be due to a requirement for the accumulation of stoichiometric amounts of a protein (e.g. VP6) necessary for the assembly of transcriptase particles. Both newly synthesized and pre-existing plus-strand RNA can act as templates for minus-strand RNA synthesis throughout infection, an unexpected result based on earlier studies with reoviruses. The observation that the level of RNA replication does not increase continually in conjunction with the increasing levels of plus-strand RNA suggests that RNA replication is regulated by factors other than the level of plus-strand RNAs in the infected cell.

The synthesis of dsRNA also has been analyzed using a cell-free system to study the replication of rotavirus RNA. The components of this system include: (1) open core particles prepared from purified double-layered particles or virus-like particles composed of VP1/2/3/6; (2) exogenously added viral mRNA or a synthetic transcript; and (3) salts and nucleoside triphosphates. The *in vitro* replication system does not require the nonstructural proteins and specifically replicates rotavirus templates. The synthesis of dsRNA *in vitro* is an asymmetrical process in which a nuclease-sensitive positive-strand RNA acts as template for the synthesis of negative-strand RNA. After its synthesis, dsRNA remains associated with subviral particles, suggesting free dsRNA is not found in cells.

This *in vitro* system supports the initiation of negative-strand RNA using exogenous viral positive-strand RNA as template. The conversion of exogenous mRNA to dsRNA by subviral particles provides a method of studying (1) the specificity of viral proteins in recognition and replication of rotavirus mRNAs, and (2) the effect of adding exogenous synthetic RNAs containing specific mutations on replication. Finally, the possibility exists that nascent replicated dsRNA can be assembled into these viral particles in this *in vitro* replication system. Unfortunately to date, the efficiency of the system has not been adequate to achieve this goal. Together these results suggest that a cell-free system to support rotavirus RNA replication, transcription and the assembly of subviral particles can be established. This system should be useful to help define the defects in rotavirus mutants and to study the RNA sequences and proteins involved in virus replication and assembly. The role

of individual proteins and specific protein complexes in RNA replication and viral morphogenesis will probably not be solved until they are studied *in vitro* with pure species of native rotavirus proteins and viral RNAs.

The sites and precise details of RNA replication remain unclear. However, electron-dense viroplasms are probably the sites of synthesis of the single-shelled particles that contain RNA. This conclusion is based on the localization of several of the viral proteins (VP2, NSP2, NSP5) to viroplasms and of VP4 and VP6 to the space between the periphery of the viroplasm and the outside of the ER, and on the observation that particles emerging from these viroplasms often seem to directly bud into the ER that contains VP7 and NSP4.

Assembly

The distinctive feature of rotavirus morphogenesis is that subviral particles, which assemble in cytoplasmic viroplasms, bud through the membrane of the ER and maturing particles are transiently enveloped. This is one of the more interesting aspects of rotavirus replication differing from virus members of other genera in the *Reoviridae* family. The envelope acquired in this process appears to be lost as particles move toward the interior of the ER, and it is replaced by a thin layer of protein which ultimately comprises the outer capsid of mature virions.

The sites of synthesis or localization of the viral proteins have been examined by ultrastructural immunocytochemistry using polyclonal monospecific or monoclonal antibodies and by studying the distribution of proteins by immunofluorescence or by subcellular fractionation. Taken together, the morphologic and biochemical data are consistent with rapidly assembling double-layered particles serving as an intermediate stage in the formation of triple-layered virions. Most of the rotavirus structural proteins and all of the nonstructural proteins are synthesized on the free ribosomes, although the nascent proteins on free ribosomes have not been analyzed. Instead, this conclusion has been drawn based on the absence of signal sequences that would indicate targeting to the ER and lack of protection to digestion in *in vitro* protease protection studies. In contrast, the glycoproteins VP7 and NSP4 are synthesized on ribosomes associated with the membrane of the ER and they are cotranslationally inserted into the ER membrane due to signal sequences at their N-termini. The glycoprotein NSP4 is a homotetramer oriented with the C-terminus on the cytoplasmic side of the ER membrane. This cytoplasmic domain of NSP4 acts as a receptor to bind to VP6 on the outer surface of nascent double-layered particles. This binding is thought to be the first step that initiates the membrane budding event.

VP7 is detected in the ER of SA11-infected cells in two pools. One pool is found only on intact particles and is detected only by a neutralizing monoclonal antibody. The second pool of VP7 is unassembled, it remains associated with the ER membrane, and it is detected by a polyclonal antibody made to denatured VP7. A kinetic study of the assembly of VP7 and of other structural proteins into particles has shown the incorporation of the inner capsid proteins into double-layered particles occurs rapidly, while VP4 and VP7 appear in mature triple-layered particles with a lag time of 10–15 minutes. Kinetic analyses of the processing of the oligosaccharides on the two pools of VP7 have shown the virus-associated VP7 oligosaccharides have a 15 minute lag compared with that of the membrane-associated form, suggesting that the latter is the precursor to virion VP7. This lag appears to represent the time required for virus budding and outer capsid assembly. NSP4, VP7 and VP4 also can form hetero-oligomers that are not associated with any known subviral particle; these hetero-oligomers are thought to be present at sites on the ER membrane where maturation to triple-layered particles begins. The proteins of the outer shell apparently are assembled on to the double-layered particles either during the budding process or once the particles reach the ER lumen.

Rotavirus maturation is a calcium-dependent process, based on the observation that virus yields are decreased when produced in cells maintained in calcium-depleted medium. Viruses produced in the absence of calcium were found to be exclusively double-layered, and budding of virus particles into the ER was not observed. Among the viral proteins, reduced levels of VP7 were observed, and subsequent studies showed that such reduced levels were due to the preferential degradation, and not to the impaired synthesis, of VP7. An interesting finding of these studies is that unglycosylated (but not mature) VP7 made in the presence of tunicamycin is relatively stable in a calcium-free environment. It is possible calcium stabilizes or modulates folding or compartmentalization of the newly glycosylated VP7 for subsequent assembly into particles. Alternatively, calcium deprivation may destabilize the ER or ER proteins required for the stable association of glycosylated VP7 with the membrane.

Virus release

Electron microscopy studies of infected tissue culture cells have shown the infectious cycle ends when

progeny virus is released by host cell lysis. Extensive cytolysis during infection and drastic alterations in the permeability of the plasma membrane of infected cells resulting in the release of cellular and viral proteins have been demonstrated. In spite of cell lysis, most double-layered and many triple-layered particles remain associated with the cellular debris, suggesting these particles interact with structures within cells. Interactions with cell membranes and the cell cytoskeleton have been suggested to occur and these may play a role in movement of the viral proteins or particles within the cell. Whether the cytoskeleton provides a means of transport of viral proteins and particles to discrete sites in the cell for assembly or acts as a stabilizing element at the assembly site and in the newly budded virions or if particles are simply trapped by the cytoskeleton remains to be determined. It also is possible that virus may not be released from infected enterocytes because of cytopathic effect and cell lysis. Instead, virus-infected enterocytes may merely be sloughed intact into the intestinal lumen. This possibility has been suggested by studies of rotavirus replication in polarized human intestinal epithelial cells. These cells are infected in a symmetric manner and cell functions are shut off before the development of cytopathic effect and extensive virus release. Recent studies in polarized epithelial cells have suggested that rotaviruses are released from cells by a novel vesicular transport that does not result in extensive cytopathic effects.

Future Perspectives

Future basic research is expected to define the functions of each of the nonstructural proteins in the replication cycle and to understand the mechanisms of RNA replication and genome packaging. This may lead to the ability to use reverse genetics to probe in great detail the functions of any gene and to construct virions with desired properties. Knowledge of the three-dimensional structure of these complex virions is awaited for further understanding of the interactions between the outer capsid proteins and between the proteins in each of the capsid shells.

See also: **Rotaviruses (*Reoviridae*): General features; Reoviruses (*Reoviridae*): Molecular biology; Influenza viruses (*Orthomyxoviridae*): General features.**

Further Reading

Chen D, Zeng Q-Y, Wentz M *et al* (1994) Template-dependent *in vitro* replication of rotavirus RNA. *J. Virol.* 68, 7030.

Estes MK (1996) Rotaviruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn. Philadelphia: Lippincott-Raven.

Lawton JA, Estes MK and Prasad BVV (1997) Three-dimensional visualization of mRNA release from actively transcribing rotavirus particles. *Nat. Struct. Biol.* 4: 118.

Mattion NM, Cohen J and Estes MK (1994) The rotavirus proteins. In: Kapikian AZ (ed.) *Virus Infections of the Gastrointestinal Tract*, 2nd edn, p. 169. New York: Dekker.

Patton JT (1995) Structure and function of the rotavirus RNA-binding proteins. *J. Gen. Virol.* 76: 2633.

Prasad BVV and Estes MK (1997) Molecular basis of rotavirus replication: structure–function correlations. In: Chiu W, Burnett RM and Garcea R (eds) *Structural Biology of Viruses*, p. 239. Oxford: Oxford University Press.

Prasad BVV, Rothnagel R, Zeng CQ-Y *et al* (1996) Visualization of the ordered genomic RNA and the transcription complex in rotavirus by three-dimensional electron cryomicroscopy. *Nature* 382: 471.

RUBELLA VIRUS (*TOGAVIRIDAE*)

Teryl K Frey, Department of Biology, Georgia State University, Atlanta, Georgia, USA

Jerry S Wolinsky, Department of Neurology, The University of Texas Health Science, Center at Houston, Houston, Texas, USA

History

Acute rubella virus infection causes a generally benign disease known as rubella or German measles that is usually acquired during childhood. The virus is endemic worldwide and causes epidemics at irregular intervals. First described by German physicians in the eighteenth century as distinct from scarlet fever and

progeny virus is released by host cell lysis. Extensive cytolysis during infection and drastic alterations in the permeability of the plasma membrane of infected cells resulting in the release of cellular and viral proteins have been demonstrated. In spite of cell lysis, most double-layered and many triple-layered particles remain associated with the cellular debris, suggesting these particles interact with structures within cells. Interactions with cell membranes and the cell cytoskeleton have been suggested to occur and these may play a role in movement of the viral proteins or particles within the cell. Whether the cytoskeleton provides a means of transport of viral proteins and particles to discrete sites in the cell for assembly or acts as a stabilizing element at the assembly site and in the newly budded virions or if particles are simply trapped by the cytoskeleton remains to be determined. It also is possible that virus may not be released from infected enterocytes because of cytopathic effect and cell lysis. Instead, virus-infected enterocytes may merely be sloughed intact into the intestinal lumen. This possibility has been suggested by studies of rotavirus replication in polarized human intestinal epithelial cells. These cells are infected in a symmetric manner and cell functions are shut off before the development of cytopathic effect and extensive virus release. Recent studies in polarized epithelial cells have suggested that rotaviruses are released from cells by a novel vesicular transport that does not result in extensive cytopathic effects.

Future Perspectives

Future basic research is expected to define the functions of each of the nonstructural proteins in the replication cycle and to understand the mechanisms of RNA replication and genome packaging. This may lead to the ability to use reverse genetics to probe in great detail the functions of any gene and to construct virions with desired properties. Knowledge of the three-dimensional structure of these complex virions is awaited for further understanding of the interactions between the outer capsid proteins and between the proteins in each of the capsid shells.

See also: **Rotaviruses (*Reoviridae*): General features; Reoviruses (*Reoviridae*): Molecular biology; Influenza viruses (*Orthomyxoviridae*): General features.**

Further Reading

Chen D, Zeng Q-Y, Wentz M *et al* (1994) Template-dependent *in vitro* replication of rotavirus RNA. *J. Virol.* 68, 7030.

Estes MK (1996) Rotaviruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn. Philadelphia: Lippincott-Raven.

Lawton JA, Estes MK and Prasad BVV (1997) Three-dimensional visualization of mRNA release from actively transcribing rotavirus particles. *Nat. Struct. Biol.* 4: 118.

Mattion NM, Cohen J and Estes MK (1994) The rotavirus proteins. In: Kapikian AZ (ed.) *Virus Infections of the Gastrointestinal Tract*, 2nd edn, p. 169. New York: Dekker.

Patton JT (1995) Structure and function of the rotavirus RNA-binding proteins. *J. Gen. Virol.* 76: 2633.

Prasad BVV and Estes MK (1997) Molecular basis of rotavirus replication: structure–function correlations. In: Chiu W, Burnett RM and Garcea R (eds) *Structural Biology of Viruses*, p. 239. Oxford: Oxford University Press.

Prasad BVV, Rothnagel R, Zeng CQ-Y *et al* (1996) Visualization of the ordered genomic RNA and the transcription complex in rotavirus by three-dimensional electron cryomicroscopy. *Nature* 382: 471.

RUBELLA VIRUS (*TOGAVIRIDAE*)

Teryl K Frey, Department of Biology, Georgia State University, Atlanta, Georgia, USA

Jerry S Wolinsky, Department of Neurology, The University of Texas Health Science, Center at Houston, Houston, Texas, USA

History

Acute rubella virus infection causes a generally benign disease known as rubella or German measles that is usually acquired during childhood. The virus is endemic worldwide and causes epidemics at irregular intervals. First described by German physicians in the eighteenth century as distinct from scarlet fever and

rubeola (measles), rubella only gained international acceptance as a unique disease in the late 1800s. Experimental transmission studies confirmed the viral nature of the disease in 1938. Three years later, Norman Gregg reported an epidemic of congenital cataracts often in association with cardiac malformations as a consequence of gestational rubella; this observation established rubella virus as a major teratogen. Rubella virus was not isolated in cell culture until 1962. The last major epidemic of rubella to impact the United States occurred two years later and the resulting load of 20 000 congenital rubella syndrome (CRS) cases led to rapid development of live-attenuated vaccines which were placed in use in vaccination programs in the US, Canada, Japan, and several European countries by 1969. These programs have been successful in greatly reducing the incidence of both rubella and CRS in these countries. However, since rubella virus is endemic in the rest of the world, it remains a challenge to countries in which vaccination programs are in place. Rubella virus is also of interest due to unique properties in its replication cycle that distinguish its closest relatives and its association with immune-mediated human disease.

Classification

Rubella virus is a member of the *Togaviridae* family and the only member of the genus *Rubivirus*. The other *Togavirus* genus, *Alphavirus*, contains 26 members all of which are arthropod-borne. In common with other togaviruses, the rubella virion consists of a single stranded, plus-sense genomic RNA contained within a quasispherical core or capsid which is surrounded by a lipid envelope. The capsid is composed of multiple copies of a single virus-specified protein, C, and embedded in the envelope are two types of glycoproteins, E1 and E2. Unlike other togaviruses, rubella virus has no invertebrate vector and the only known natural reservoir for rubella virus are humans. Further, rubella virus is serologically distinct from alphaviruses and shares no significant homology at either the nucleotide or amino acid level. Specific features of the genomic organization of rubella virus also distinguish it from the alphaviruses as discussed below.

Host Range and Virus Propagation

Rubella virus has no known natural host other than humans. It has been reported that nonhuman primates and a variety of laboratory animals can be infected with rubella virus, however the extent of viral replication is inconsistent among these reports and no reliable animal model exists for the study of rubella virus pathogenesis. Rubella virus replicates in a number of laboratory cell culture lines, however in most of these no cytopathic effect (CPE) is routinely observed. Primary African green monkey kidney cells (AGMK) were used for the initial isolation of rubella virus and subsequently were recommended as the standard cells for routine isolation of the virus from clinical specimens. Within several days, AGMK cells inoculated with positive clinical specimens exhibit interference with ECHO-11 virus. This interference assay is the classical standard assay for detection of rubella virus, however currently used detection assays generally employ immunofluorescence or polymerase chain reaction for detection of the virus. Continuous cell lines routinely used to propagate rubella virus include Vero (AGMK), RK-13 (rabbit kidney), and BHK-21 (baby hamster kidney). The highest yields of cell-free virus are obtained in Vero cells and these cells also exhibit CPE when infected with laboratory-adapted strains of the virus. In all cell lines in which rubella virus replicates, persistent infections are readily established and maintained. The noncytocidal nature of rubella virus predisposes it to persistent infection. Additionally, the virus persists in spite of the interferon response it induces in most of these cell lines. Finally, persistent rubella virus infection in cell culture cannot be cured by the inclusion of neutralizing antibodies in the culture medium because virus budding occurs at intracytoplasmic locations. Thus, rubella virus is highly adapted for persisting infection both at the cellular level and in the presence of a humoral response.

Properties of the Virion

In cross-section, rubella virions are 60–70 nm spherical particles composed of a 30 nm electron-dense core separated by an electron-lucent zone from the lipid envelope. In negative-stained preparations, virions exhibit a marked degree of pleomorphism. In the best preparations, a 6–8 nm fringe formed by glycoprotein spikes can be observed around the envelope surface, however more commonly protrusions from the enveloped surface are not observable. The virion has a density of 1.18–1.19 g ml^{-1} while isolated capsids have a density of 1.44 g ml^{-1}. The capsid is formed by multiple copies of the C protein (~34 kDa) which is present as disulfide-linked homodimers. Based on the alphavirus model, it is presumed that the rubella virus capsid has icosahedral symmetry, however its three-dimensional structure has never been solved.

The virion spikes are formed by two virion glycoproteins, E1 and E2. E1 has a molecular weight of 59 kDa whereas E2 is a heterogeneous species

ranging from 44 to 50 kDa due to differential glycosylation. Both E1 and E2 are class I membrane proteins with hydrophobic transmembrane sequences near the COOH-terminus and both contain covalently attached fatty acids. Both E1 and E2 contain N-linked glycans and E2 additionally contains O-linked glycans. In the virion, E1 and E2 appear to be primarily in the form of a heterodimer which is easily disrupted by routine preparation techniques. Disulfide-bonded E1–E2 heterodimers and E1–E1 homodimers are also detected. The higher order architecture of the virion spikes is entirely unclear. E1 is more exposed on the virion surface than is E2 and contains both the viral hemagglutinin and receptor site. E1 is also immunodominant in terms of the humoral response, although in most individuals antibodies are induced against all three of the virion proteins.

Rubella virions are stable at physiological pH values and can be frozen for years without loss of infectivity at temperatures below −20°C. Infectivity decays with a half-life of 5–20 min at 56°C, 1–2 h at 37°C, and more than a week at 4°C. The virus can be lyophilized and the vaccine is stored and distributed in lyophilized form. Rubella virions are susceptible to most commonly used inactivating agents, such as formaldehyde, UV light and lipid solvents.

Genomic Organization

The rubella virus genomic RNA is 9762 nucleotides in length and contains a 5′ terminal cap structure and a 3′ terminal poly(A) tract. A distinctive feature of the genomic RNA is that it contains 30% guanine residues and 39% cytosine residues, the highest G+C content of all RNA viruses. The genome contains two long, non-overlapping open-reading frames (ORFs) (**Fig. 1**). The 5′ proximal ORF encodes a 2116 amino acid product that is proteolytically cleaved into two products of 150 kDa (P150) and 90 kDa (P90), which are at the N- and C-termini of the ORF, respectively. The cleavage is mediated by a papain-like cysteine protease located at the C-terminus of P150. P150 and P90 function in virus RNA replication. By computer-assisted comparisons with other viruses, P150 contains a domain predicted to have methyltransferase activity whereas P90 contains both a helicase domain and an RNA-dependent RNA polymerase domain. The 3′ proximal ORF encodes a 1063 amino acid product that is proteolytically processed into the virion proteins by a cell protease, signal endopeptidase. The order of the virion protein genes within the ORF is 5′ C-E2-E1 3′. The SP-ORF is translated from a subgenomic RNA that is synthesized in infected cells. The start site for the subgenomic RNA is in between the ORFs and the subgenomic RNA contains the sequences from the start site through the 3′ end of the genome. An infectious clone for rubella virus has been developed. An infectious clone is a cDNA copy of the viral RNA contained in a plasmid in which it is placed adjacent to an RNA polymerase promoter. Since rubella virus has a plus-sense genome, *in vitro* transcripts from the plasmid will initiate virus replication following transfection into susceptible cells. The infectious clone allows for site-directed mutagenesis studies of the rubella virus genome.

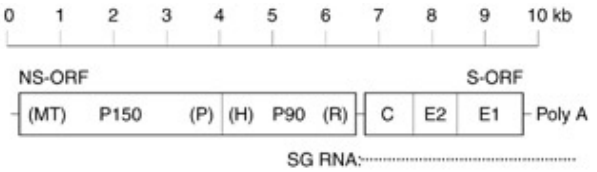


Figure 1 Coding strategy of the rubella virus genome. Shown is a schematic representation of the rubella virus genomic RNA with untranslated regions represented as solid black lines and coding regions (ORFs) as open boxes (NS-ORF, nonstructural protein ORF; S-ORF, structural protein ORF). Within each ORF, the coding sequences for the proteins processed from the translation product of the ORF are delineated and additionally within the NS-ORF, the locations of motifs associated with the following activities are indicated: methyl transferase (MT), protease (P), helicase (H) and replicase (R). The sequences encompassed by the subgenomic RNA (SG RNA) are also shown. The scale at the top of the diagram is in kilobases.

Intracellular Replication Cycle

The receptor for rubella virus on the surface of susceptible cells has not been identified. Following attachment to the receptor, the virus is taken into the cell by receptor-mediated endocytosis. Compared with other enveloped viruses, the time required for internalization following attachment is prolonged, taking as long as 8 h. In the reduced pH environment of the endocytic vesicle, fusion between viral envelope and the vesicular membrane occurs, releasing the capsid and genomic RNA into the cytoplasm of the cell. How release of the RNA from the capsid is accomplished is not known, however there is evidence to indicate that the capsid adheres to the membrane of the endocytic vesicle following fusion and subsequently dissociates.

The genomic RNA is translated to produce the nonstructural protein precursor which is cleaved into P150 and P90 (**Fig. 2**). These proteins then use the genomic RNA as a template for synthesis of a genome-length, minus-sense RNA. Host cell proteins may be involved in the replication process and it has been shown that a specific cell protein, calreticulin,

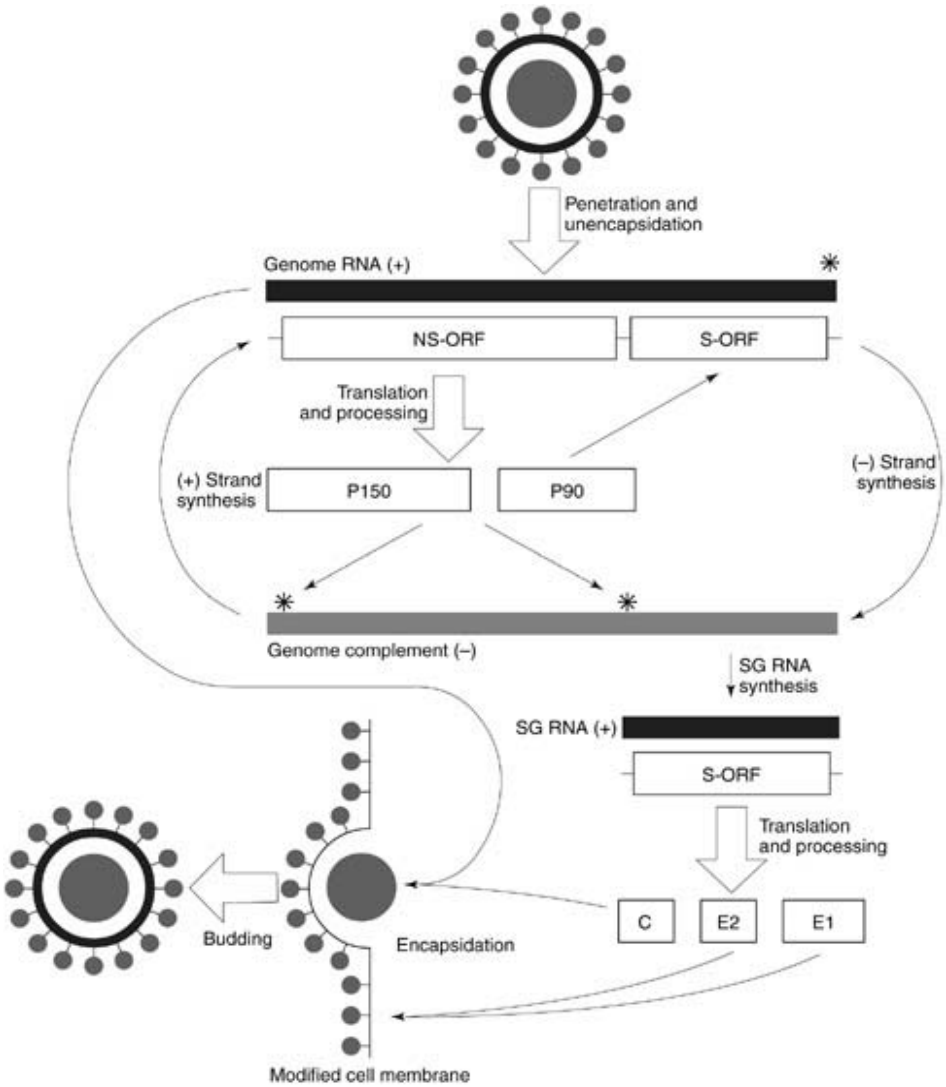


Figure 2 Replication strategy of rubella virus. The plus-sense genome and subgenomic RNA are represented by solid black lines; beneath each, the ORFs that they contain are shown as open boxes. The minus-sense genome RNA complement, represented as a hatched line, is used solely as a template for the two plus-sense RNA species. Putative *cis*-acting sequences on each RNA that are recognized by the virus replicase to initiate synthesis of complementary RNAs are marked with an asterisk. The general functions of the virus proteins are indicated by arrows (e.g. P150 and P90 functioning as the replicase by interacting with *cis*-acting sequences on the viral RNA species and synthesizing complementary strands).

binds to a stem-loop structure near the 3′ end of the genomic RNA. In so doing, it may facilitate recognition of the 3′ end by the virus replicase. The genome-length, minus-sense RNA is then used as the template for synthesis of both the genomic RNA and the subgenomic RNA. Synthesis of the subgenomic RNA is initiated by internal recognition of sequences on the genome-length, minus-sense RNA template. RNA synthesis is asymmetric in infected cells in that more of both of the plus-sense species than the minus-sense RNA is produced. In alphavirus-infected cells, the activity of the nonstructural (NS) protease (which cleaves the nonstructural precursor into four proteins rather than two proteins) is important in regulating plus- and minus-sense RNA synthesis. It has not been determined whether the rubella virus NS protease plays a similar role. Rubella virus RNA synthesis occurs in cytopathic vacuoles of lysosomal origin which occur in infected cells.

In the structural protein ORF, E2 and E1 are immediately preceded by hydrophobic signal sequences that function to direct translation of secreted and membrane-associated proteins into the lumen of the endoplasmic reticulum. Therefore, following translation of the C sequences within the ORF, the E2 signal sequence mediates association of the

translation complex with the ER. C-E2 cleavage is mediated by signal endopeptidase, or signalase, which functions in the lumen of the ER to remove signal sequences from secreted and membrane-associated proteins (unlike alphaviruses, rubella C protein does not have autocatalytic protease activity). Following cleavage, the E2 signal sequence remains associated with C. Similarly, the E1 signal sequence maintains the association of the translational complex with the ER, signalase mediates the E2–E1 cleavage, and the E1 signals sequence remains attached to E2. Heterodimerization of E2 and E1 occurs in the lumen of the endoplasmic reticulum soon after synthesis. The three-dimensional folding of E1 appears to be a complicated process that requires intramolecular disulfide-bond formation by all 20 Cys residues in the ectodomain of the protein. Both E1 and E2 acquire high-mannose glycans in the ER; E1 contains three potential glycosylation sites and E2 contains four and all appear to be occupied. The site of O-glycosylation of the E2 is not known. E1 appears to contain an ER retention signal that is only over-ridden once conformational folding is complete after which the E1–E2 heterodimer migrates to the Golgi. In the Golgi, the N-glycans of both E2 and E1 are modified to complex form, although modification is not complete and the extent of modification is heterogeneous on both proteins. Modifications of the O-glycans on E2 also occur and account for the size heterogeneity of E2. E2 contains a Golgi retention signal, indicating that the Golgi is the preferred site of viral budding in infected cells. However, late in infection E1 and E2 migrate to the cell surface and budding also occurs at this site.

Rubella virus capsid morphogenesis occurs in association with cell membranes. The association of rubella virus capsid protein with membranes is probably mediated by the E2 signal sequence which is retained at the COOH terminus of C. In fact, C may associate with the E2–E1 heterodimer and migrate as a passenger on the cytoplasmic side of vesicles transporting E2–E1 from the ER to the Golgi and among the Golgi stacks. Unlike the alphaviruses with which capsids accumulate in infected cells, rubella virus capsids only become visible in association with deformed, thickened membranes that appear to be in the early process of budding. A putative encapsidation signal has been localized near the 5′ terminus of the genomic RNA. Early in infection, the Golgi is the preferred site of viral budding, but late in infection budding occurs from the plasma membrane as well. Interestingly, in cells in which the complete SP-ORF is expressed in the absence of genomic RNA, virus-like particles form which have the same morphology and isopycnic density as do virions. It would be anticipated that these particles contain nonviral RNAs, however specific RNA contained within these particles have not been demonstrated.

Rubella virus replicates in the cytoplasm of the infected cell. Whether any of the virus proteins migrate to the nucleus during infection is under investigation; preliminary evidence indicates that P90 binds to the p105Rb tumor suppressor protein. Rubella virus infection does not appear to inhibit cell macromolecular synthesis in any grossly detectable manner, however perturbations of specific macromolecular products and induction of specific genes may occur. Microscopically, rubella virus-infected cells appear similar to uninfected cells, however rearrangements of cellular cytoskeletal elements and organelles such as mitochondria have been reported. Rubella virus inhibits growth in primary human cell cultures in part due to an inhibition of mitosis, however the virus has no reproducible effect on the growth of stable cell lines. In those cell lines which exhibit CPE (Vero and RK-13 cells), cell death is due to apoptosis.

Genetics and Evolution

The rubella virus genome appears to be relatively stable. Currently, the genomes of three independent strains of rubella virus have been sequenced in their entirety and all three are identical in terms of the size of the genome as well as the coding and noncoding regions within the genome. More extensive phylogenetic analysis has focused on the *E1* gene. Isolates from Europe, North America, and Japan vary by up to 4% and form a single genotype. A second genotype that varies from the first by 7–10% occurs in Asia. Although the natural variation at the nucleotide level is not as great as encountered with other RNA viruses, the evolution rate of rubella virus has been measured as 0.1% per year which is fairly typical of RNA viruses. A unique feature of rubella virus evolution is that changes to G and C are selected for, indicating an adaptive advantage of the high G+C content of the genome.

Since their are no known animal relatives of rubella virus, the origin of the virus prior to its introduction into the human population is unknown. Rubella virus and the alphaviruses share no nucleotide homology except for short stretches at the 5′ end of the genome and at the subgenomic promoter site. Thus these two genera are only distantly related. Rubella virus and the alphaviruses both belong to the 'alphavirus-like superfamily' of plus-sense RNA viruses which includes a large number of plant viruses as well as human hepatitis E virus, which is currently classified as a calicivirus. Within this superfamily, computer-assisted phylogenetic analysis of the nonstructural

proteins indicates that rubella virus is more closely related to HEV and beet necrotic yellow vein virus than to the alphaviruses. This dissimilarity is borne out by differences in order of motifs in the NS-ORF. Thus, it is hypothesized that the evolution of the genera of the *Togavirus* family may have been more complicated than simple divergence from a common ancestor and probably involved recombination between progenitors of the current alphaviruses, rubella virus, HEV and possibly plant viruses.

Serologic Relationships and Variability

Rubella virus is monotypic and immunological characterization of diverse strains has only revealed subtle antigenic differences. These reside on C and E2 as E1 has been found to be antigenically identical using a variety of assays. The recent discovery of the second Asian genotype raised the possibility of greater antigenic variation, however the two genotypes only vary by 1–3% in predicted amino acid sequence of E1 and none of the recognized monoclonal epitopes contain major changes. Additionally, immune human serum neutralizes viruses from both genotypes with similar kinetics. As might be anticipated by the lack of serologic crossreaction with the alphaviruses, there is no important homology between rubella virus and sequenced alphaviruses in the subgenomic region that specifies the structural polypeptides of the virion.

Epidemiology

Rubella virus is endemic worldwide. In temperate zones, seasonal peaks occur in the spring and before widespread vaccine use, rubella epidemics occurred at 5–9-year intervals. There is considerable geographic variation in rubella attack rates in different age groups. In developed, temperate zone countries, peak infection rates occur in 5–9-year-old children. However, in much of Africa, the highest infection rates occur in children under 5, and 80% of all children are immune by 10 years of age. In contrast, in island and rural tropical populations the incidence of rubella is low, with high percentages of susceptible women of childbearing age. Much of this regional variation is explained by population densities, socioeconomic factors, and levels of medical sophistication. An additional factor in rubella epidemiology is that rubella virus is not as transmissible as is measles virus and thus even during epidemics, susceptibles are missed. Thus, infection of adolescents and young adults in any population is not uncommon.

Needless to say, vaccination programs have considerably altered the epidemiology of rubella in countries in which they are employed. Vaccination strategies and their effect on the incidence of rubella are discussed below.

Transmission and Tissue Tropism

Rubella virus is transmitted between individuals by aerosolation. Congenitally infected infants shed virus for three to six months following birth and are a source of transmission. Although vaccine virus can be recovered from vaccinees, transmission of vaccine virus to susceptible individuals has not been observed.

The epithelium of the buccal mucosa provides the initial site for rubella virus replication after infection and the mucosa of the upper respiratory tract and nasopharyngeal lymphoid tissue serve as portals of virus entry. The virus is then spread by local lymphatics which seed regional lymph nodes where further virus replication occurs. After an incubation period of 7–9 days, virus appears in the blood. The secondary sites of replication which account for the maintenance of viremia have not been identified, however infection of mononuclear cells contributes. Viremia ceases with the onset of detectable rubella-specific antibody shortly after the rash appears 2–3 weeks postinfection. Through the viremia, virus is seeded into the nasopharynx where it is shed by aerosolation. Patients are most infectious immediately preceding and during the rash; virus generally disappears from nasopharyngeal secretions within four days of appearance of the rash.

Reinfection with rubella virus does occur and is more frequent among vaccinees than naturally infected individuals due to lower antibody levels. Reinfection usually proceeds without viremia, clinical illness or virus shedding, however, reinfection with clinical illness has been reported. There are a small number of cases in which rubella virus reinfection of pregnant women with well-documented immunity has resulted in CRS.

During pregnancy, placental tissues are very susceptible to infection. Placental infection results in scattered foci of necrotic syncytiotrophoblast and cytotrophoblast cells and damage to vascular endothelium. Following placental infection, virus can spread to the fetus but this does not always occur and rubella virus is more often recovered from placental tissue than from fetal products of conception. Once fetal infection occurs, virus spreads throughout the fetus and almost any organ may be infected. *In vitro* cell cultures derived from infected fetuses are persistently infected with rubella virus. Severe fetal damage is only associated with infection during the first trimester of pregnancy. This is due to a combination of an apparent decline in the efficiency of placental transfer after the first trimester and a reduction in the

ability of the virus to inflict fetal damage after this time of gestational development.

Clinical Features of Infection

Rubella acquired in childhood or early adulthood is usually mild, however symptoms in adults tend to be more severe than in children. It is estimated that up to 50% of rubella infections are clinically inapparent. Symptomatic rubella encompasses combinations of maculopapular rash, lymphadenopathy, low-grade fever, conjunctivitis, sore throat and arthralgia. The rash is the most prominent and earliest feature and appears following an incubation period of 16–20 days. The rash begins as distinct pink maculopapules on the face that then spread over the trunk and distally onto the extremities. The maculopapules coalesce and the rash rapidly fades over several days. An associated posterior cervical and suboccipital lymphadenopathy is also characteristic. Fever is typically low grade. The entire clinical syndrome usually resolves in a few days. Infrequently occurring complications include thrombocytopenia and post-infectious encephalitis. Acute polyarthralgia and arthritis following natural rubella virus infections of adults are common and occur more frequently and with greater severity in women than in men. Joint involvement is usually transient, resolving within one to several weeks, however chronic arthritis persisting or recurring over several years has been reported. The most common symptoms of rubella, lymphadenopathy, erythematous rash, and low-grade fever, are nonspecific and easily confused with similar illnesses caused by other common viral and nonviral pathogens or drug-induced eruptions. Therefore, a definitive diagnosis of rubella requires confirmation by virus isolation or, more commonly, by serology.

Fetal infection with rubella virus has dire consequences for fetal development. The rate of CRS following maternal infection is highest early in pregnancy; 50%, 25% and 10% during the first, second, and third months, respectively. CRS is rare following maternal infection after week 16 of gestation. The clinical manifestations of CRS apparent at birth vary widely, most frequently including thrombocytopenia purpura ('blueberry muffin syndrome'), intrauterine growth retardation, congenital heart disease (patent ductus arteriosus or pulmonary artery or valvular stenosis), psychomotor retardation, eye defects (cataract, glaucoma, retinopathy), suspected or confirmed hearing loss and hepatomegaly and/or splenomegaly. Less frequent features include adenopathy, bony radiolucencies, hepatitis usually with jaundice, and hemolytic anemia. Nearly 80% of CRS children show some type of neural involvement, particularly neurosensory hearing loss.

Most clinical manifestations of congenital rubella are evident at or shortly following birth and some are transient. However, recognition of retinopathy, hearing loss and mental retardation may be delayed for several years in some cases. Progressive consequences of congenital rubella have become increasingly appreciated as CRS children from the 1964 epidemic have been followed longitudinally. These predominantly involve endocrine dysfunction (diabetes mellitus, which ultimately affects 40% of CRS patients, and thyroid dysfunction). A rare, fatal neurodegenerative disease, progressive rubella panencephalitis (PRP), was also described in CRS patients that bears superficial resemblance to subacute sclerosing panencephalitis associated with measles virus. Subsequently, PRP cases were also reported in individuals who were infected postnatally.

Pathogenesis, Pathology and Histopathology

There is limited information on the pathogenesis of uncomplicated rubella because of the benign nature of the illness. With respect to the complications that can accompany acute rubella, the postinfectious encephalitis is thought to be autoimmune in nature since rubella virus cannot be isolated from cerebrospinal fluid or the brain at autopsy. Interestingly, however, extensive inflammation and demyelination are not observed. In a few cases of rubella arthritis, the presence of rubella virus in synovial fluid and/or cells has been demonstrated and therefore it is assumed that virus persistence is involved. However, considering the age and sex factors in the incidence of arthritis, it seems likely that immunopathological mechanisms also play a role. No predisposition for development of arthritis following rubella, immunological or otherwise, has been identified.

Following fetal infection, virus can be isolated from practically every organ of abortuses or infants who die soon after birth. However, only 1 in 10^3 to 1 in 10^5 cells are infected and it is not known how such a low infection rate leads to the profound birth defects exhibited in CRS. Affected organs are routinely small for gestational age and contain reduced numbers of cells. Considering the inhibitory effect of rubella virus on primary cells, it is thought that virus infection early in organogenesis inhibits cell division leading to both retardation and alteration in organ development. Virus persistence continues after birth as evidenced by shedding which generally ceases within six months of age. Whether virus persistence continues beyond cessation of shedding and plays a role

in the delayed and progressive manifestations of CRS is not known.

Histologically, affected organs from CRS show a limited number of well-recognized malformations with noninflammatory histopathology predominating. Particularly apparent are vascular lesions and focal destruction in tissue bordering these lesions. These lesions are likely to be due to virus replication in the vascular endothelium and the damage to neighboring tissue may play a role in the pathogenesis of CRS. The neuropathology of CRS is of interest not only because of the defects manifest shortly after birth, but also because some CRS patients develop schizophrenia-like symptoms later in life. CRS brains are generally free of gross morphological malformations with a common tendency towards microcephaly. Vascular damage, leptomeningitis, decreased numbers of oligodendroglial cells, and alteration of white matter are observed. Recently, magnetic resonance imaging of a group of CRS adults with schizophrenia-like symptoms revealed specifically reduced cortical gray matter and enlargement of the ventricles, which were not previously observed aspects of CRS-induced neuropathology. Interestingly, the comparative finding that non-CRS schizophrenia patients exhibit a pattern of brain dysmorphy similar to CRS patients with schizophrenia-like symptoms supports the hypothesis that the pathogenesis of schizophrenia is developmental in nature (there is some evidence for a viral trigger to schizophrenia).

Immune Response

The earliest detectable serological response to rubella virus infection is the presence of immunoglobulin (Ig)M antibodies at the time of onset of the rash. Since these antibodies generally wane within a month or two, serodiagnostic testing for the presence of IgM is the primary means for diagnosis of acute rubella virus infection currently employed. In the succeeding weeks, antirubella virus antibodies appear in all immunoglobulin classes. The dominant early and persistent IgG response is in the IgG_1 subclass and antibodies of this class persist indefinitely after natural infection in healthy individuals. Immunoprecipitation studies disclose that the majority of the antibody response is directed to the E1 glycoprotein, with proportionally lesser amounts of the response directed at E2 or C. Although neutralizing and complement fixing antibodies are induced as well, the classical assay for the presence of antirubella virus antibodies was hemagglutination inhibition (HAI) and the current standard titer recognized for immunity of 10 IU ml^{-1} is based on a reciprocal HAI titer of roughly 1:8. Because of the importance of serodiagnostic testing for rubella, a worldwide commercial market for rubella tests exists and a number of companies offer such kits, most of which are based on latex agglutination or enyzme immunoassay.

Rubella virus-specific cellular immune responses are measurable within one to two weeks of onset of rubella. These decline over several years but persist at low levels indefinitely following natural rubella. MHC-restricted CD4+ epitopes have been mapped to all three of the virus structural proteins, however CD8+ epitopes have thus far only been mapped to the C protein.

Following fetal infection, the fetus produces IgM antibody, detectable at 18–20 weeks of gestation, and maternal IgG antibody crosses the placenta. Both types of antibody exhibit virus neutralizing activity *in vitro*, however neither is sufficient to resolve virus infection during gestation. As discussed above, the intracellular maturation of virus probably shields it from antibody. After birth, the presence of IgM or a lack of decline of IgG titer are both considered diagnostic of fetal infection. CRS infants exhibit impairment in the cellular immune response to rubella virus to varying degrees and it is thus a deficiency in this arm of the immune response that allows the virus to persist. Considering this deficiency in CRS infants, it is curious that detectable virus persistence ends relatively shortly after birth. The means by which virus persistence is cleared under these conditions is not understood.

Prevention and Control of Rubella

As discussed above, live attenuated vaccines were developed and placed in use by 1970. The vaccine used in most countries, with two exceptions, is the RA 27/3 vaccine. This vaccine was developed by multiple passaging of a virus isolate from an explant culture of a fetal human kidney in WI-38 human fetal diploid lung cells. Several of the passages were done at 30°C and limiting dilutions were used at some of the passages. Production of the vaccine is done in both WI-38 and MRC-5 diploid human cell cultures. In Japan, five attenuated vaccine strains were developed and are currently in use. Additionally, at least one Chinese vaccine strain is currently in use in China.

Rubella attenuated vaccines cause subclinical infection with transient viremia in susceptible recipients. However, transmission of vaccine virus has not been reported. The RA27/3 vaccine strain produces seroconversion in greater than 95% of recipients. Vaccine-induced titers are lower than those induced by natural infection but appear to last indefinitely. The rubella vaccine is generally administered to children in trivalent form with the measles and

mumps attenuated vaccines. Additional testing has shown that the recently licensed varicella vaccine can be combined with these vaccines in a tetravalent vaccines with no detectable interference between the component vaccine viruses.

In general, the rubella vaccines have been among the most successful in terms of induction of immunity with an absence of side effects. However, two issues have arisen concerning rubella vaccination. The first is that the vaccine virus can cross the placenta and infect the fetus. However, in a registry kept in the US between 1971 and 1988 of over 300 deliveries to women inadvertently vaccinated within three months of conception or during the first trimester of pregnancy, no congenital abnormalities were reported. Nevertheless, vaccination during pregnancy is contraindicated and is deferred until post-partum. Second, is the occurrence of arthralgia and arthritis following vaccination. Joint complications are nonexistent in children with the currently used rubella vaccines, however transient arthralgia and arthritis is reasonably common among adult female vaccinees. There have also been reports of chronic arthritis and related neurological involvement following vaccination of adult women. Although these complications are consistent with complications that can accompany natural rubella in adult females, recent studies have shown that the incidence of such vaccine-related complications is rare and cannot be statistically differentiated from the incidence of similar symptoms in control, unvaccinated populations.

Since the inception of rubella vaccination, the US has employed a strategy of universal vaccination at 15 months of age augmented with vaccination of seronegative 'at-risk' individuals (women planning pregnancy, health care workers) which was successful in bringing the incidence of rubella and CRS to record low levels by 1988. However, a resurgence occurred between 1989 and 1991 among foci of unvaccinated individuals concentrated primarily on college campuses, among Amish communities in the Northeast, and among the Hispanic population in the Southwest. Since most of the infected individuals were of adolescent age or older, the ratio CRS to rubella cases was higher than in unvaccinated populations and over 50 CRS cases occurred despite the fact that only 3000 rubella cases were reported. Since the resurgence, a second vaccine at age 5–10 has been included in the vaccination program as well as more strict enforcement of vaccine requirements for school admission. The incidence of rubella and CRS has subsequently dropped to minuscule levels. Most of these cases are thought to be imported and no rubella was reported during a two month period in 1996, possibly indicating a break in endemic transmission.

In Japan and Europe, a strategy of vaccination of adolescent girls was initially adopted since it was felt that natural immunity was more robust than vaccine-induced immunity and thus it was considered desirable that as many individuals as possible should contract natural rubella. Predictably, rubella virus continued to circulate and thus postadolescent women who managed to break through without a rubella titer were infected and CRS was not eliminated. Therefore, most of these countries adopted the universal strategy in the late 1980s. Aggressive implementation of the universal policy in the UK and Scandinavia has brought rubella down to very low levels; however, in many countries with rubella vaccination programs, vaccination is not comprehensively pursued and thus both rubella and CRS still occur.

Rubella vaccination is practiced in only a few countries outside of the US, Canada, Europe and Japan. In underdeveloped countries, this is primarily because of the expense of the vaccine, the nature of national public health infrastructures, and the general mildness of the disease in comparison to those caused by life-threatening pathogens. However, rubella exacts a societal load in every country in which it is endemic. Because rubella virus is exclusively a human virus and excellent vaccines for use against it exist, it is potentially eradicable and recently attention has been focused on the possibility of elimination or eradication efforts for two major reasons. First, because the rubella and measles vaccines are administered together in most childhood vaccination programs, it would be efficient to include the rubella vaccine in currently ongoing worldwide measles elimination efforts. In addition to controlling two diseases with one effort, measles surveillance requires diagnosis of rubella because of the similarity of symptomology and thus inclusion of rubella vaccine would also concomitantly reduce surveillance costs by reducing rubella cases. Secondly, rubella elimination is potentially of great benefit to the countries which maintain expensive comprehensive vaccination and control programs. In these countries, most rubella outbreaks are due to importation and thus a substantial reduction or elimination of rubella would allow easing of control efforts as well as eventual discontinuation of vaccination. Therefore, a concerted worldwide effort on rubella control appears to be forthcoming.

Future

Because of its association with a diverse group of clinical diseases, rubella virus will remain a fascinating pathogen. As an example, the incidence of diabetes in CRS patients is the best statistically direct

association between a specific human virus and a specific autoimmune disease. The mechanism of viral involvement in each of these diseases is not fully understood. Unfortunately, our present understanding of disease mechanisms in the rubella virus-related syndromes is hindered by the current lack of a suitable animal model system that fully mimics the infection seen in humans and development of an animal model is a research priority. Virologically, rubella virus is taxonomically unique and appears to have evolved as a recombinational hybrid of other distantly related viruses. Thus, investigation of its molecular biology will likely reveal novel replication strategies and yield insight into virus evolution. The biggest challenge concerning rubella virus, however, will be potential forthcoming elimination efforts which could well consume the next quarter to half century.

See also: **Defective interfering viruses; Diagnostic techniques: Detection of viral antigens, nucleic acids and specific antibodies; Sindbis and Semliki Forest viruses (*Togaviridae*); Nervous system viruses; Epidemiology of viral diseases; Immune response: Cell mediated immune response, General features; Persistent viral infection; Vaccines and immune response.**

Further Reading

Frey TK (1994) Molecular biology of rubella virus. *Adv. Virus Res.* 44: 69.

Frey TK (1997) Neurological aspects of rubella virus infection. *Intervirology* 40: 167.

Gillam S (1994) The Jeanne Mannery Fisher memorial lecture 1994: Molecular biology of rubella virus structural proteins. *Biochem. Cell. Biol.* 72: 349.

Plotkin SA (1994) Rubella vaccine. In: Plotkin SA and Mortimer Jr EA (eds) *Vaccines*, 2nd Edn, p. 303. Philadelphia: WB Saunders.

Pugachev KV, Abernathy ES and Frey TK (1997) Improvement of the specific infectivity of the rubella virus infectious clone: determinants of cytopathogenicity induced by RUB map to the nonstructural proteins. *J. Virol.* 71: 562.

Wolinsky JS (1996) Rubella virus. In: Fields BN, Knipe DM, Howley PM *et al.* (eds) *Virology* 3rd edn., p. 899. Philadelphia: Lippincott-Raven Publishers.

Russian Spring Summer Encephalitis *see* **Encephalitis Viruses**

S

SALMONELLA PHAGE P22 (*PODOVIRIDAE*)

Anthony R Poteete, Department of Molecular Genetics and Microbiology, University of Massachusetts Medical Center, Worcester, Massachusetts, USA

Introduction

P22 is a temperate bacteriophage that mediates generalized transduction of its host, *Salmonella typhimurium*. This capability forms the basis of the first claim of P22 to distinction: the discovery of transduction described by Zinder and Lederberg in 1952 involved the transfer of *Salmonella* genes by P22. P22 continues to the present day to be an indispensable tool of *Salmonella* genetics. It is an unassigned species in the family *Podoviridae*.

The molecular genetics of P22 itself have been studied intensively. Research with P22 has contributed to our understanding of a number of biological processes, including replication, recombination, and regulation; protein folding and assembly; and viral morphogenesis and evolution.

Virion Structure

The P22 virion can be described as an icosahedral head, with a diameter of approximately 570 Å, attached at one vertex to a short tail/baseplate structure, from which a single, slender fiber extends (radially with respect to the head) (**Fig. 1**). The virion is roughly half protein and half DNA by weight.

P22 virions contain nine protein species. The major one is the coat protein, which is present in about 400 copies arranged in an icosahedral ($T = 7$ laevo) shell. One minor structural polypeptide, the portal protein, forms a ring-shaped dodecamer around the place in the head where the tail/baseplate attaches. Another, described as the tailspike protein, forms trimers (**Fig. 2**); six trimers attach to the tail to form the baseplate. The remaining six proteins of the capsid have structurally undefined roles in forming the tail and in DNA injection.

The single P22 chromosome is a linear double-stranded DNA molecule of approximately 43 400 bp. Its precise structure varies from one virion to another, due to the nature of the viral assembly process. P22 DNA is packaged from a large DNA molecule, called a concatemer, consisting of multiple tandem repetitions of the phage genome. Packaging into a preformed head structure, called a prohead, starts at a specific sequence, *pac*, and proceeds unidirectionally until a headful of DNA has been taken up. At that point, wherever it occurs in the phage DNA sequence, packaging into the first prohead terminates, and packaging into a second prohead initiates. This sequential packaging can continue for many headfuls. The process, in the case of wild-type P22, results in

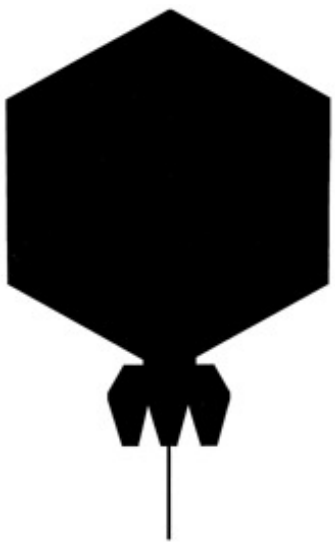


Figure 1 Schematic drawing representing the appearance of P22 virions in electron micrographs of negatively stained preparations. The capsid is icosahedral, but can appear hexagonal in outline. The baseplate is hexagonally symmetric. Adapted from Casjens S and Hendrix R (1988) Control Mechanisms is dsDNA backeriophage assembly. In: Calendar R (ed.) *The Bacteriophages*, vol. 2. New York: Plenum

from P_{ant}, encodes antirepressor, which induces lytic growth of any P22-related prophage that happens to be resident in the infected cell.

The P22 genes that are expressed as a result of gene *24* protein action include ones that promote homologous recombination, DNA replication, and late transcriptional regulation. The late transcriptional regulatory gene, *23*, encodes a protein that antiterminates transcription from the promoter P_{LATE}. The late transcript thus extended is over 20 000 bp in length, and includes all of the genes required for phage assembly and host cell lysis.

Replication of P22 DNA requires the action of two phage-encoded proteins, whose function is to recruit host replisomes to the phage replication origin. The key product of replication is the linear concatemer that serves as a substrate for sequential headful DNA packaging. This concatemer is generated from a circular P22 chromosome by a rolling circle mechanism. The circular form of the P22 chromosome is formed by homologous recombination between the repetitious ends of the linear DNA molecule injected by the phage.

Assembly of P22 virions proceeds by an ordered pathway that starts with the formation of proheads. Coat protein aggregates into a roughly spherical shell around a core of scaffolding protein. Four minor proteins, including the portal protein, also participate in this process. Proheads of normal appearance in electron micrographs can assemble in the absence of any of these minor proteins; however, they cannot mature into infectious phage. During the DNA packaging process, the scaffolding protein is ejected intact; it subsequently participates, catalytically, in the formation of new proheads. The coat protein shell also expands and rearranges. Addition of three minor proteins is required for the formation of a stable head with a short tail, to which trimers of the tailspike protein attach as a final step.

Packaging of DNA by the prohead requires the action of two proteins which are not incorporated into the final structure, one to bring the prohead to the pac site, and the other to pump DNA inside. P22 mutants, called HT, direct the synthesis of altered versions of the first of these, and reduce the specificity of *pac* recognition. HT mutants exhibit elevated frequencies of transduction of bacterial genes, and are especially useful for *Salmonella* genetics.

Lysis of the infected cell results from the action of two proteins. One of the proteins, called a holin, inserts into the cytoplasmic membrane, creating channels that permit the passage of small proteins. The second protein, a lysozyme, thereby gains access to the cell wall peptidoglycan, which it hydrolyzes to effect disruption of the host cell.

Lysogeny

Like other temperate phages, P22 can follow either of two pathways following infection of a sensitive host cell: it can grow lytically, or form a lysogen. Which of these pathways is favored depends on a variety of factors, including the multiplicity of infection and the nutritional status of the cell. The key element in the choice between pathways is a transcriptional regulatory protein, the product of gene *c1*, which is transcribed as a result of antitermination by gene *24* protein. Regulation of c1 protein synthesis and stability is complex and only partially understood. When present at sufficient levels, c1 protein stimulates transcription from the promoter P_{RE}. The c2 protein, encoded in the P_{RE} transcript, is then synthesized, and acts by binding to operators overlapping P_L and P_R, repressing transcription; at the same time, it turns on its own transcription from the otherwise silent promoter P_{RM}. An additional transcriptional repressor, the product of the *mnt* gene, is required for maintenance of the lysogenic state; the function of this protein is to turn off transcription of antirepressor.

Establishment of stable lysogeny requires, in addition to transcriptional repression, integration of the phage DNA into the bacterial chromosome to form a prophage. This function is carried out by integrase, a protein that promotes reciprocal recombination between specific sequences called attachment sites in the circular phage and bacterial chromosomes.

In the lysogenic state, a number of P22 genes are expressed. In addition to the c2 and Mnt repressors, these include *sieA*, *sieB* and *a1*. The functions of the latter genes all involve keeping out infecting phages: the product of *sieA* interferes with DNA injection by P22 and related phages; *sieB* causes the lytic cycle of certain other *Salmonella* phages (not P22 itself) to abort at an early stage; and *a1* alters the structure of the lysogen's O-antigen, interfering with adsorption by P22 and related phages.

The lysogenic state is homeostatic. The c2 protein turns on its own gene, and turns off most of the other phage genes; spontaneous induction is a rare event. On the other hand, treatment of lysogens with agents that damage DNA results in efficient induction. The mechanism of this induction is based on the host cell's normal reaction to such agents. A signal, perhaps single-stranded DNA, generated as a consequence of DNA damage, activates RecA protein, which in turn promotes proteolysis of LexA protein, a cellular repressor that controls transcription of the global SOS regulon. Some phage repressors, including c2 protein, are homologues of LexA protein; they are similarly cleaved in response to activated RecA

protein. With repressor thus rendered inactive, transcription from P_L and P_R initiates. The resulting lytic growth requires the reversal of integration–excision, promoted by the combined action of the phage-encoded integrase and excisionase, but is otherwise similar to lytic growth following infection.

Research Directions

Current research with P22 is focused on the molecular mechanisms of selected aspects of the phage's biology. Some of this research is comparative. For example, P22 frequently serves as a system in which particular mechanisms of λ gene regulation can be tested for generality. P22 has also found use as a component of certain specialized cloning systems. In addition, a few P22-encoded proteins serve as model systems for the study of protein folding and stability, of DNA binding, and of transcriptional regulation.

Antirepressor

P22 departs from the familiar pattern of lambdoid phage transcriptional regulation by possession of an antirepressor (the protein product of gene *ant*). Embedded in the late operon of P22, *ant* is subject to regulation by its own set of three repressors: two proteins, the products of genes *arc* and *mnt*, and one antisense RNA, the *sar* transcript. All of these have been extensively characterized.

The *ant* operon has been employed in the construction of a specialized cloning system. In it, a DNA sequence thought to function in gene regulation is installed in P22 in place of the operator that normally controls *ant* expression. The resulting hybrid phage will invariably kill a bacterium that can not control its synthesis of antirepressor; it can be used to select among bacterial clones for those which express a protein that can bind specifically to the DNA site in question. A closely related P22 strain provides a direct selection for bacteria expressing a protein that can bind specifically to a site in RNA. The phage is constructed so that the RNA site question overlaps the *ant* ribosome binding site.

DNA packaging

The molecular basis of the limited sequence specificity of one of the DNA packaging proteins (the product of gene *3*) has been the subject of recent investigation. This research has led to understanding of the sequence determinants of *pac* activity, as well as a picture of what constitutes a *pac*-like site. The DNA packaging apparatus of P22 has been used in a cloning system in which it is combined with the ends of the transposon-phage Mu. The resulting replicon, when inserted into the host cell's chromosome (essentially anywhere), becomes a locked-in prophage, unable to excise. However, upon induction, it packages the host chromosome, one headful at a time; sequences located near and to one side of the insertion are packaged with particularly high efficiency.

Homologous recombination

P22 depends on genetic recombination to circularize its chromosome following infection. This recombination must take place at high efficiency within the 1600 bp sequence repeated at the ends of the linear DNA injected by the phage. The recombination system of the bacterial host is inefficient in this process. P22 encodes proteins that work in conjunction with the host system to increase its efficiency. One of these proteins, Abc, binds to the host cell's RecBCD nuclease/helicase and modulates its nuclease activity. Another, Erf, is a single-stranded DNA-binding protein that accelerates association of complementary strands. A third protein, Arf, is a general recombination enhancer of unknown mechanism. Though the phage and host proteins work together, the combined system contains functional redundancies, and can operate at nearly normal efficiency in the absence of any of the known host functions.

Like P22, phage λ encodes a homologous recombination system, called Red, that can function in the absence of the known host recombination proteins, but that works most efficiently in their presence. Both phages encode strand exchange-promoting proteins (Erf and Redβ, respectively) analogous to RecA. However, the two phages use different strategies for interactions with RecBCD. Whereas P22 modifies RecBCD and uses it in promoting phage recombination, λ completely nullifies RecBCD and elaborates its own exonuclease. The two phage recombination systems share no apparent sequence homology, but are functionally interchangeable. The functional significance of such an efficient recombination system for λ is not obvious, as it is in the case of P22, as the λ chromosome circularizes by an entirely different mechanism. The explanation may reside in the idea of the lambdoid phages as mosaics of interchangeable genetic modules; efficient recombination systems are required to retain the capability of headful packaging in the group.

Tailspike

The P22 tailspike protein has proven to be an interesting object for studies of protein structure, folding and assembly, and aggregation. The mature form of the protein is a trimer of identical 666 amino acid residue subunits. The most prominent feature of the monomer is a large β-helix, consisting of 13

helical turns of parallel β strands. In the trimer, the helical coils pack into a parallel bundle. Distally, the three polypeptides intertwine to create an extremely stable bundle of β sheets.

Folding and assembly of the tailspike occur in concert: monomers partially fold, assemble into a structure called a protrimer, then complete folding together. Folding and trimerization of the tailspike polypeptide are slow *in vivo* under certain physiological conditions, and the various forms of the tailspike exhibit different solubilities and electrophoretic mobilities. It has thus been possible to dissect the tailspike folding and assembly pathway *in vivo* as well as *in vitro*.

The mature tailspike trimer is an unusually stable protein. However, its folding/assembly process is unusually temperature sensitive. At 40°C, most of the subunits fail to fold correctly, and end up in insoluble aggregates. Investigators have characterized mutant proteins that exhibit increased and decreased tendencies to aggregate.

P22 in novel settings

P22 does not form plaques on *Escherichia coli*, but only because it cannot adsorb. If *E. coli* is given the capability of synthesizing the *S. typhimurium* O-antigen, it will support lytic growth, lysogeny and generalized transduction by P22. Plasmid-borne *rfb* and *rfc* genes from *S. typhimurium* are sufficient for this purpose. This observation suggests the possibility of using P22 as a generalized transducing phage in other, less genetically characterized Gram-negative bacteria.

Early in this century, a key aim of bacteriophage researchers was to use these viruses as antibacterial therapeutic agents. This line of investigation was discontinued due to technical difficulties and the advent of antibiotics. However, the recent proliferation of antibiotic resistant strains has revived interest in this area. Recently, investigators have described the isolation of mutants of phages λ and P22 that have extended lifetimes in the murine circulatory system, and can abort experimental infections.

See also: **Coliphage lambda (*Siphoviridae*); Lysogeny and prophage; Phage Homologous Recombination.**

Further Reading

Campbell A and Botstein D (1983) Evolution of the lambdoid phages. In: Hendrix RW, Roberts JW, Stahl FW and Weisberg RA (eds) *Lambda II*. New York: Cold Spring Harbor Laboratory.

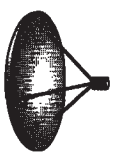
Casjens S and Hendrix R (1988) Control mechanisms in dsDNA bacteriophage assembly. In: Calendar R (ed.) *The Bacteriophages*, vol. 2, New York: Plenum.

King J, Haase-Pettingell C, Robinson AS, Speed M and Mitraki A (1996) Thermolabile folding intermediates: inclusion body precursors and chaperonin substrates. *FASEB J* 10: 57.

Poteete AR (1988) Bacteriophage P22. In: Calendar R (ed.) *The Bacteriophages*. vol. 2, New York: Plenum Press.

Susskind MM and Botstein D (1978) Molecular genetics of bacteriophage P22. *Microbiol. Rev.* 42: 385.

SATELLITE RNAs AND SATELLITE VIRUSES



ME Taliansky and **PF Palukaitis**, Virology Department, Scottish Crop Research Institute, Invergowrie, Dundee, UK

Introduction

Satellites are a heterologous collection of subviral agents which comprise nucleic acid molecules that depend for their productive multiplication (replication) on a helper virus, but which contain sequences substantially distinct from those of the genomes of either their helper virus or their hosts. Satellites are thus distinct from either defective (D) or defective interfering (DI) RNAs, because the latter are wholly derived from their helper virus genomes. However, there are some hybrid RNAs between satellites and parts of the viral genome, such as the chimeric molecules formed from part of a satellite RNA associated with turnip crinkle virus (TCV) and part of a DI RNA formed from the virus genome. Satellites may encode their own coat protein (CP) (satellite viruses), or they may rely on the helper virus for encapsidation as well as replication (the satellite RNAs or satellite DNA). Satellites are not needed

helical turns of parallel β strands. In the trimer, the helical coils pack into a parallel bundle. Distally, the three polypeptides intertwine to create an extremely stable bundle of β sheets.

Folding and assembly of the tailspike occur in concert: monomers partially fold, assemble into a structure called a protrimer, then complete folding together. Folding and trimerization of the tailspike polypeptide are slow *in vivo* under certain physiological conditions, and the various forms of the tailspike exhibit different solubilities and electrophoretic mobilities. It has thus been possible to dissect the tailspike folding and assembly pathway *in vivo* as well as *in vitro*.

The mature tailspike trimer is an unusually stable protein. However, its folding/assembly process is unusually temperature sensitive. At 40°C, most of the subunits fail to fold correctly, and end up in insoluble aggregates. Investigators have characterized mutant proteins that exhibit increased and decreased tendencies to aggregate.

P22 in novel settings

P22 does not form plaques on *Escherichia coli*, but only because it cannot adsorb. If *E. coli* is given the capability of synthesizing the *S. typhimurium* O-antigen, it will support lytic growth, lysogeny and generalized transduction by P22. Plasmid-borne *rfb* and *rfc* genes from *S. typhimurium* are sufficient for this purpose. This observation suggests the possibility of using P22 as a generalized transducing phage in other, less genetically characterized Gram-negative bacteria.

Early in this century, a key aim of bacteriophage researchers was to use these viruses as antibacterial therapeutic agents. This line of investigation was discontinued due to technical difficulties and the advent of antibiotics. However, the recent proliferation of antibiotic resistant strains has revived interest in this area. Recently, investigators have described the isolation of mutants of phages λ and P22 that have extended lifetimes in the murine circulatory system, and can abort experimental infections.

See also: **Coliphage lambda (*Siphoviridae*); Lysogeny and prophage; Phage Homologous Recombination.**

Further Reading

Campbell A and Botstein D (1983) Evolution of the lambdoid phages. In: Hendrix RW, Roberts JW, Stahl FW and Weisberg RA (eds) *Lambda II*. New York: Cold Spring Harbor Laboratory.

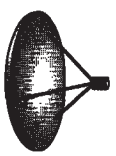
Casjens S and Hendrix R (1988) Control mechanisms in dsDNA bacteriophage assembly. In: Calendar R (ed.) *The Bacteriophages*, vol. 2, New York: Plenum.

King J, Haase-Pettingell C, Robinson AS, Speed M and Mitraki A (1996) Thermolabile folding intermediates: inclusion body precursors and chaperonin substrates. *FASEB J* 10: 57.

Poteete AR (1988) Bacteriophage P22. In: Calendar R (ed.) *The Bacteriophages*. vol. 2, New York: Plenum Press.

Susskind MM and Botstein D (1978) Molecular genetics of bacteriophage P22. *Microbiol. Rev.* 42: 385.

SATELLITE RNAs AND SATELLITE VIRUSES



ME Taliansky and **PF Palukaitis**, Virology Department, Scottish Crop Research Institute, Invergowrie, Dundee, UK

Introduction

Satellites are a heterologous collection of subviral agents which comprise nucleic acid molecules that depend for their productive multiplication (replication) on a helper virus, but which contain sequences substantially distinct from those of the genomes of either their helper virus or their hosts. Satellites are thus distinct from either defective (D) or defective interfering (DI) RNAs, because the latter are wholly derived from their helper virus genomes. However, there are some hybrid RNAs between satellites and parts of the viral genome, such as the chimeric molecules formed from part of a satellite RNA associated with turnip crinkle virus (TCV) and part of a DI RNA formed from the virus genome. Satellites may encode their own coat protein (CP) (satellite viruses), or they may rely on the helper virus for encapsidation as well as replication (the satellite RNAs or satellite DNA). Satellites are not needed

Table 1 Satellite viruses

Helper virus/satellite virus	*Particle size (nm)*	*CP (NW)*	*Size of satellite RNA (nt)*
Subgroup 1			
Tobacco necrosis necrovirus (TNV)/Satellite TNV (STNV)	17	~21 600	1239 620[a]
Panicum mosaic sobemovirus (PMV)/Satellite PMV (SPMV)	17	~17 000	826
St. Augustine decline sobemovirus (SADV)/Satellite SADV (SSADV)	17	~17 000	24
Tobacco mosaic tobamovirus (TMV)/Satellite TMV (STMV)	17	~17 500	1059
Maize white line mosaic virus (MWLMV)/Satellite MWLMV (SMWLMV)	17	23 961	1168
Subgroup 2			
Chronic bee-paralysis virus (CPV)/CPV associated satellite virus (CPVA)	17	NR[b]	~1100 (three species)

[a] Satellite RNA of STNV.
[b] NR, not reported.

by helper viruses for their accumulation. However, some RNA molecules which are not required by the helper virus for experimental infection, appear to be essential for the natural life cycle of the virus, by contributing to vector transmissibility. Such examples are the RNAs associated with groundnut rosette virus (GRV) and beet necrotic yellow vein virus (BNYVV), which have been described and are referred to as satellite-like RNAs. A related group of subviral agents is that containing RNAs that resemble satellites but which depend on a helper virus only for encapsidation rather than for replication [e.g. hepatitis delta virus (HDV) and beet western yellows virus (BWYV) ST9-associated RNA].

History

The term satellite virus was adopted by Kassanis to describe the small 17 nm diameter particles associated with tobacco necrosis virus (TNV), in 1962. It was serologically unrelated to TNV and hence encoded its own CP. In 1969, a satellite was found associated with tobacco ringspot virus (TRSV), which was encapsidated by the helper virus, and the term 'satellite' was broadened to include satellite RNAs. In recent years, a large number of other satellite viruses and satellite nucleic acids associated with different groups of viruses have been described. Most known satellites consist of single-stranded (ss) RNA, with ssRNA plant viruses as helpers. However, satellite ssDNAs and double-stranded (ds) RNAs have also been described.

Classification

Satellites do not constitute a homogeneous taxonomic group. They represent quite different disparate groups of subviral agents associated with plant, animal, protozoan and fungal viruses containing different types of genetic material such as ssRNA, dsRNA or ssDNA. It seems very unlikely that such different groups of satellites might be evolutionarily related. There are also no taxonomic correlations between the viruses that support satellites. Satellitism would appear to have arisen independently a number of times during the evolution of viruses. Thus, classification of satellites is based largely on features of the genetic material of the satellites. The nature of the helper virus and helper virus host range are important secondary characters. All known satellites appear to be included into the following categories.

Satellite viruses

- Subgroup I. Satellite tobacco necrosis virus (STNV)
- Subgroup II. Chronic bee paralysis associated satellite virus (CPVA)

Satellite nucleic acids

- ss satellite DNA
- ds satellite RNA
- ss satellite RNA
  - Subgroup I. Large ss satellite RNA with messenger properties
  - Subgroup II. Small linear ss satellite RNA
  - Subgroup III. Circular ss satellite RNA

As mentioned above, satellite-like RNAs and subviral agents dependent on a helper virus only for encapsidation may also be regarded as satellite-related agents. A complete list of satellites and satellite-related agents is presented in **Tables 1–3**.

Table 2 Satellite nucleic acids

Helper virus/satellite nucleic acid	*Size of satellite nucleic acid (encoded protein MW)*
ss sat-DNA (circular)	
Tomato leaf curl virus (TLCV)/TLCV sat-DNA	682 nt (NR)[a]
ds sat-RNAs (linear)	
L-A ds RNA virus of *Saccharomyces cerevisiae*/M sat-RNA	~1000–1800 nt (NR)
Trichomonas vaginalis virus (TVV)/TVV sat-RNA	~500 nt, ~700 nt, ~1700 nt (NR)
ss sat-RNAs	
Large sat-RNAs with messenger properties	
Nepovirus	
Arabis mosaic virus (ArMV)/ArMV large sat-RNA	1104 nt (~39 kDa)
Chicory yellow mottle virus (CYMV)/CYMV large sat-RNA	1145 nt (~39 kDa)
Grapevine Bulgarian latent virus (GBLV)/GBLV sat-RNA	~1500 nt (NR)
Grapevine fanleaf virus (GFLV)/GFLV sat-RNA	1114 nt (~37 kDa)
Myrobalan latent ringspot virus (MLRV)/MLRV sat-RNA	~1400 nt (~45 kDa)
Strawberry latent ringspct virus (SLRV)/SLRV sat-RNA	1118 nt (~36 kDa)
Tomato black ring virus (TBRV)/TBRV sat-RNA	1372–1375 nt (~48 kDa)
Potexvirus	
Bamboo mosaic virus (BaMV)/BaMV sat-RNA	836 nt (~20 kDa)
Small linear ss sat-RNAs	
Carmovirus	
Turnip crinkle virus (TCV)/TCV sat-RNA	194 nt, 230 nt, 356 nt
Cucumovirus	
Cucumber mosaic virus (CMV)/CMV sat-RNA	333–405 nt
Peanut stunt virus (PSV)/PSV sat-RNA	393 nt
Robinia mosaic virus (RbMV)/RbMV sat-RNA	~390 nt
Umbravirus	
Pea enation mosaic virus (PEMV)/PEMV sat-RNA	717 nt
Necrovirus	
Tobacco necrosis virus (TNV)/TNV small sat-RNA	620 nt
Nepovirus	
Chicory yellow mottle virus (CYMV)/CYMV small sat-RNA	457 nt
Tombusvirus	
Artichoke mottled crinkle virus (AMCV)/AMCV sat-RNA	~700 nt
Cymbidium ringspot virus (CymRSV)/CymRSV sat-RNA	621 nt
Carnation Italian ringspot virus (CIRV)/CIRV sat-RNA	~700 nt
Pelargonium leaf curl virus (PLCV)/PLCV sat-RNA	~700 nt
Petunia asteroid mosaic virus (PAMV)/PAMV sat-RNA	~700 nt
Tomato bushy stunt virus (TBSV)/TBSV sat-RNA	612 nt, 822 nt
Circular ss sat-RNAs	
Polerovirus	
Barley yellow dwarf virus (BYDV-RPV)/BYDV-RPV sat-RNA	322 nt
Nepovirus	
Arabis mosaic virus (ArMV)/ArMV small sat-RNA	~300 nt
Tobacco ringspot virus (TRSV)/TRSV sat-RNA	359 nt
Sobemovirus	
Lucerne transient streak virus (LTSV)/LTSV sat-RNA	324 nt
Rice yellow mottle virus (RYMV)/RYMV sat-RNA	~210 nt
Solanum nodiflorum mottle virus (SNMV)/SNMV sat-RNA	377 nt
Subterranean clover mottle virus (SCMV)/SCMV sat-RNA	332 nt, 388 nt
Velvet tobacco mottle virus (VTMoV)/VTMoV sat-RNA	365 nt–366 nt

[a] NR, not reported.

Table 3 Related subviral agents

Helper virus/satellite-like RNA/subviral agent	*Size of satellite-like RNA/subviral agent (MW of encoded protein)*
Satellite-like ssRNAs (dependent on a helper virus for replication and encapsidation, but essential for vector transmission of a helper)	
Umbravirus	
Groundnut rosette virus (GRV)/GRV sat-RNA	895–903 nt
Benyvirus	
Beet necrotic yellow vein virus (BNYVV)/	
BNYVV RNA3	1774 nt (~25 kDa)
BNYVV RNA4	1467 nt (~31 kDa)
BNYVV RNA5	1342–1347 nt (~26 kDa)
Agents dependent on a helper virus for encapsidation but not for replication	
Hepadnavirus	
Hepatitis B virus (HBV)/	
Hepatitis delta virus (HDV)	1679 nt (~22 kDa; δAg-S) (δAgS+19 aa; δAg-L)
Luteovirus	
Beet western yellows virus (BWYV)/BWYV-associated RNA ST9	2843 nt (~85 kDa) + several others

General Properties of Satellites and their Effects on Helper Virus and Host Plants

The satellite viruses

Satellite viruses occur as nucleoprotein particles that are morphologically and serologically distinct from their helper viruses. No serological relationships were found between different satellite viruses except between satellite panicum mosaic virus (SPMV) and satellite St Augustine decline virus (SSADV), the helper viruses of which, PMV and SADV, respectively, are closely related. All known satellite viruses including CPVA contain ssRNAs between 800 and 1200 nucleotides (nt). Particles of all known satellite viruses are isometric and are 17 nm in diameter. Whereas helper viruses of most satellite viruses also have isometric particles, the helper virus of satellite tobacco mosaic virus (STMV) has rod-shaped particles.

Subgroup I The most studied of satellite viruses are plant virus satellites belonging to subgroup I. The three-dimensional structures of STNV, STMV and SPMV have been determined at high resolution by x-ray crystallography. The shell of all these satellite viruses is composed of 60 protein subunits. Most satellite virus RNAs are presumably monocistronic. The CP is the sole product of *in vitro* translation of the STNV, satellite maize white line mosaic virus (SMWLMV) and SPMV RNAs, although the last contains several additional open reading frames (ORFs) which are untranslatable *in vitro*. However, in the case of STMV, in addition to the CP, another product of about 6.8 kDa is produced during *in vitro* translation, although it remains unclear if this product is functionally active.

Although the STNV CP is not related to that of the helper virus, TNV, both particles bind specifically to the surface of zoospores of the fungal vector *Olpidium brassicae*. The replication of STNV inhibits that of TNV and the extent of the inhibition depends on the particular combination of strains of STNV and TNV. A second satellite RNA of about 620 nt has been detected in STNV particles. It is distinct from STNV RNA, in that it has no messenger RNA activity, but still depends on TNV for replication. Therefore this RNA may be considered as a satellite of TNV for replication, and a satellite of STNV for encapsidation (**Table 1**).

The effect of the multiplication of SPMV on the accumulation of its helper, PMV, is unknown, but it is known that the presence of SPMV alters the mild mosaic symptoms induced by PMV in maize to severe mosaic and necrosis. On the other hand, no differences in symptomatology have been observed between isolates of MWLMV or TMV containing or lacking satellite viruses.

Subgroup II Less is known about satellite viruses belonging to the subgroup II (**Table 1**). RNA of CPVA satellite virus consists of three species, each about 1.1 kb, which are distinct from CPV helper RNA, although some T1 oligonucleotides appear in common between CPV and CPVA RNA. The satellite virus interferes with CPV replication.

Satellite nucleic acids

ss Satellite DNAs This category comprises tomato leaf curl virus (TLCV) satellite DNA with a circular ssDNA genome, which does not encode a satellite CP (**Table 2**). The 682 nt DNA contains no ORFs and shows little sequence similarity to the helper virus genome, limited to two short motifs present in two separate stem–loop structures. One of these motifs is universal for all geminiviruses, and the other motif is identical to a replication-associated protein binding site in TLCV. The satellite DNA is strictly dependent on the helper virus replication-associated protein and is encapsidated by TLCV CP. Replication of TLCV satellite DNA is also supported by other taxonomically distinct geminiviruses, including African cassava mosaic virus and beet curly top virus.

ds Satellite RNAs These RNAs have been found in association with viruses of the family *Totiviridae*. The dsRNA range from 0.5 kb to 1.8 kb and are encapsidated by the helper virus CP. These particles often also contain a positive-sense ssRNA copy of the dsRNA. The presence of satellites in helper virus cultures can affect markedly the virulence of the helper virus infection. This category comprises the M satellites of L-A dsRNA virus of *Saccharomyces cerevisiae* and satellites of *Trichomonas vaginalis* virus (TVV) (**Table 2**).

Some strains of *S. cerevisiae* secrete a protein toxin that is lethal to other strains, but not to the toxin-secreting strain, which is said to be immune or resistant. The protein responsible both for the killer phenotype and for immunity to the toxin (in the form of the prototoxin) is encoded by M_1, a dsRNA satellite of the major yeast dsRNA virus L-A. Several other satellite dsRNAs of L-A, each encoding a toxin-immunity system, have been described and are called M_2, M_3, M_{28}, etc. The family of these satellites comprises dsRNAs, varying from ca. 1.0 kb to 1.8 kb. These RNAs completely depend on the helper virus (L-A) encoded proteins Gag and Gag-Pol for encapsidation and replication. In addition, a strict dependence of M_1 satellite multiplication on host chromosomal genes has also been described. M_1 needs 30 so-called *MAK* (maintenance of killer) genes, of which only three are required by the helper virus L-A. The M_1 satellite represses the copy number of the L-A helper virus.

Another series of dsRNA satellites is associated with a virus infected with a sexually transmitted protozoan, *T. vaginalis* (TVV). Three different dsRNA satellites of TVV have been found, containing approximately 500 bp, 700 bp and 1700 bp. All these RNAs are synthesized conditionally and are present in only some *T. vaginalis* isolates harboring the virus, which seems to replicate and encapsidate them.

ss Satellite RNAs This is the most numerous group of known satellites. The ss satellite RNAs range in size from just under 200 nt to approximately 1500 nt. The larger satellite RNAs appear to contain functional ORFs, although as yet no precise function has been assigned to any of their gene products. Small ss satellite RNAs do not appear to encode any functional ORFs, but tend to be highly structured. Small ss satellite RNAs may be either linear or circular. Despite their small size and the usual absence of any potential products, these ss satellite RNAs may have a dramatic effect on the symptoms induced by their helper virus, ranging from amelioration to severe exacerbation. These symptom effects vary with the helper virus, host plant, and satellite.

Subgroup I: Large ss Satellite RNAs with Messenger RNA Properties The most studied of this type of satellite RNAs is that of the nepoviruses (**Table 2**). These satellite RNAs, ranging in size from approximately 1100 to 1500 nt, resemble the genomic RNA of their helper viruses in that they have a 3′-terminal poly(A) sequence and a 5′-terminal genome-linked protein (VPg). The VPgs of satellite RNAs of tomato black ring virus (TBRV), arabis mosaic virus (ArMV) and grapevine fanleaf virus (GFLV) are indistinguishable from those attached to the helper virus genome RNA and therefore must be encoded by the helper virus genome. These satellites encode a nonstructural protein with M_r ranging from 36 to 48 kDa, which at least in some cases, such as in TBRV, GFLV and ArMV satellites, has been shown to be essential for satellite RNA replication. Comparison of amino acid sequences of proteins encoded by different satellite RNAs of nepoviruses revealed several domains: the terminal regions are strongly basic, most notably the N-terminal region, and the central region contains both basic and acidic residues. The satellite-encoded proteins are also relatively rich in cysteine and histidine residues, mostly in the 3′-terminal halves. It has been suggested that these proteins may be involved in adapting the helper virus replicase to the satellite RNA. The most characteristic biological property of these RNAs is that the symptoms induced by infection with the helper virus are only slightly or not at all affected by the presence of the satellite RNA. TBRV, strawberry latent ringspot virus (SLRV), chicory yellow mottle virus (CYMV) and GFLV satellite RNAs induce no changes in the symptoms induced by the helper virus. However, in the case of the large ArMV satellite RNA, the satellite RNA was found to exacerbate symptoms in three

species and ameliorate symptoms in ten species out of 42 plant species tested. The large satellite RNAs of nepoviruses have little or no effect on the accumulation of their helper viruses, even in plants in which satellite RNA enhanced or ameliorated the symptoms of ArMV infection.

Another large ssRNA satellite which may be included in this category is associated with the potexvirus bamboo mosaic virus (BaMV). This RNA is a linear molecule of 836 nt [excluding the poly(A) tail] and like other satellite RNAs is encapsidated by the CP of its helper virus. However, it differs from all other known satellites in that the RNA is encapsidated into rod-shaped particles. The BaMV satellite RNA contains an ORF for a protein of 183 amino acids (20 kDa). This protein shares 46% sequence identity with the CP amino acid sequence of SPMV (see above). The ORF for the BaMV satellite RNA appears to be translated *in vitro* and *in vivo*. However, the encoded protein is not essential for satellite RNA replication. The presence of the BaMV satellite RNA caused a reduction of BaMV genomic RNA accumulation, in the range 65–85%.

Subgroup II: small linear ssRNAs This subgroup comprises the satellites with genomes less than 0.8 kb (**Table 2**). No circular molecules are present in infected cells. Some of these satellites contain potential ORFs, but the evidence indicates that there are no *in vivo* functions for these ORFs. Although small satellite RNAs of some cucumber mosaic virus (CMV) strains have been shown to direct the synthesis of protein products *in vitro*, these ORFs are not conserved among different isolates. Moreover, neither mutagenesis of the 5′-proximal initiation codon in a biologically active cDNA clone nor a frameshift mutation of the ORFs altered the biological activity of CMV satellite RNAs. Other small, linear, ssRNA satellites also do not appear to encode functional proteins, and hence the biological functions of these satellite RNAs must rely on the nucleotide sequence and the corresponding secondary structure. The small satellite RNAs have highly ordered secondary structures. The small size and high degree of secondary structure are probably responsible for the high stability and survivability of satellites both *in vitro* and *in vivo*. The latter may in turn account for the highly infectious nature of many satellite RNAs.

Small RNA satellites can dramatically alter the symptoms induced by the helper virus. The alterations can be either an attenuation or an exacerbation of the virus-induced symptoms. Some satellite RNAs of CMV can ameliorate the symptoms induced by the helper virus on one host and intensify them on another host. The symptom modulation can also be affected by the particular strain of helper virus. Thus, a three-factor interaction involving the particular satellite, the strain of helper virus, and the species of host determines the type of host response. In most cases, satellites attenuate the symptoms. Exacerbation of symptoms is much rarer. Attenuation of symptoms is usually accompanied by a reduction in the virus titer. This has led to the suggestion that the competition for the replicase between the satellite and helper virus genomes results in a reduction in the concentration of the helper virus elicitor of host pathogenesis. However, it has also been shown that the chimeric satellite RNA of TCV may inhibit movement of the virus helper (rather than replication), thus reducing its accumulation and symptom expression; the CP of TCV is involved in this type of interaction. However, in some other satellite virus: helper combinations [for example CMV satellite RNA and the helper tomato aspermy virus (TAV)], a reduction in pathogenicity was not accompanied by a reduction in the titer of the helper virus. Thus, more than one mechanism may be responsible for satellite RNA-mediated symptom attenuation.

A few satellites exacerbate the symptoms induced by the helper virus. Of these, the best characterized are the satellite RNAs of CMV. Examples of symptom intensification include chlorosis on tobacco (and pepper), chlorosis on tomato, and necrosis on tomato. As in the case of satellite-mediated symptom attenuation, there might be different mechanisms for symptom intensification. One of them may be based on the interaction of the satellite RNA with a helper virus-encoded product. Chlorosis and some forms of necrosis involve interaction between specific regions (pathogenicity domains) of CMV satellite RNA and a factor(s) derived from CMV RNA 2, i.e. either the encoded 2a or 2b proteins or RNA 2 itself. In addition, whereas interactions between certain satellite RNAs and either RNA 2 or the 2a protein induce severe pathogenic responses in some host species, on other plant species the same combinations attenuate symptoms. The host component(s) involved in the above interactions is (are) clearly a crucial factor. Another possible mechanism of symptom exacerbation seems to involve direct interaction between the pathogenicity domain in the negative strand of the CMV satellite RNA with some helper component(s) without essential contribution from a helper virus.

Subgroup III: small circular ss satellite RNAs This subgroup comprises satellites with genomes that contain about 350 nt and occur as circular as well as linear molecules (**Table 2**). Replication of some has been shown to involve self-cleavage of linear, multi-

meric, progeny molecules by an RNA-catalyzed reaction. The helper viruses of these satellite RNAs come from three different genera. The sobemoviruses specifically encapsidate the circular satellites (these satellites have been referred to as virusoids in some of the literature because of their structural resemblance to viroids and the erroneous, premature conclusion that they were essential for the replication of the helper virus). Members of the other two virus genera (*Nepovirus* and *Polerovirus*) encapsidate the linear form of the satellite RNA. However, the circular forms, which are essential for rolling circle replication (see below), can be found in infected tissues. As with small, linear satellite RNAs, circular satellites do not display messenger RNA activity, although some short ORFs are detected in the genomes of some of these circular satellites. The effect on symptoms induced by helper viruses may vary from attenuation to exacerbation, as in the case of small, linear, satellite RNAs. Satellite RNAs of TRSV generally reduce the titer of the helper virus as well as the severity of TRSV-induced symptoms. The satellite RNA of barley yellow dwarf virus (BYDV)-RPV (Rho-palosiphum padi virus) also reduces the accumulation of the helper virus and attenuates symptoms. By contrast, circular satellite RNAs of sobemoviruses, such as velvet tobacco mottle virus (VTMoV) sat-RNA, greatly enhance the severity of symptoms induced by the helper virus.

Satellite related agents

Satellite-like ssRNAs Two examples have been reported of RNA molecules which have many similarities to satellites and are not required for mechanical transmission/infection of the helper virus; however, in contrast to the true satellites these RNAs are required for the natural infection of the helper virus (**Table 3**). One of these, GRV (an umbravirus) ss satellite-like RNA, contains 895–903 nt. This RNA relies on GRV for its replication, but it is needed in addition to groundnut rosette assistor virus (a luteovirus) for aphid transmission of GRV. Therefore, it is essential for the survival of GRV in nature and is thus regarded as a satellite-like RNA. This RNA shares no significant sequence similarity with the GRV genomic RNA and does not code any functional proteins. It is the satellite-like RNA that is largely responsible for the symptoms of groundnut rosette. The GRV satellite contains two nontranslatable elements involved in symptom induction. Symptoms produced by different GRV satellites are independent of the helper GRV isolate, and indeed indistinguishable symptoms are produced when a different virus, pea enation mosaic virus (PEMV) is substituted for GRV as a helper virus. Thus it seems that this RNA may induce symptoms itself without an essential contribution from the GRV helper. Normally, satellite-like RNA isolates of GRV do not affect the accumulation of GRV genomic and subgenomic RNAs in infected plants, but a few, so-called mild GRV satellites have been identified that drastically diminish the replication of the helper GRV in infected or transgenic plants. Sequence alignment revealed striking similarities between GRV satellite-like RNAs and a true satellite RNA of PEMV, belonging to the subgroup II of small, ss, linear, satellite RNAs.

Although beet necrotic yellow vein virus (BNYVV) (a benyvirus) has a bipartite RNA genome, field isolates of BNYVV have two or three additional satellite-like RNAs of which RNA 3 (1774 nt) and RNA 4 (1467 nt) are essential for spread in root tissues and transmission by the fungal vector of the virus, respectively (**Table 3**). However RNA 5 (1342–1347 nt) of BNYVV may be a true satellite RNA, since it is not essential for spread of the virus in nature. The satellite-like RNA 3 has been also implicated in symptom expression (rhizomania). The 25 kDa protein encoded by the longest ORF of the RNA 3 is probably needed for symptom production. Another ORF, ORF N, which is translationally silent on full-length RNA 3 but is translationally activated by a long, internal deletion, may also contribute to symptom production in some hosts. RNA 4 also contains a long ORF encoding a potential 31 kDa protein, although it is not clear if it is essential for the functional activity of RNA 4. RNA 5 can intensify the severity of symptoms induced by BNYVV, and a 26 kDa protein encoded by this RNA may be important for the expression of symptoms.

Other satellite related agents *Hepatitis Delta Virus (HDV)* HDV is a subviral, human pathogen that propagates only in the presence of its helper. With this helper, HDV can replicate most efficiently in hepatocytes and can greatly increase the severity of liver damage caused by a hepatitis B virus (HBV) infection. The replication of HDV genome takes place in the nuclei of infected cells. The host RNA polymerase II (that usually directs DNA-dependent RNA synthesis) is probably responsible for this RNA-directed transcriptional event in infected cells, which is independent of the hepadnavirus helper. Therefore, by definition, HDV is not a satellite. However, HDV is dependent on the envelope proteins of the helper virus required for the assembly and release of infectious virions. The HDV genome is a single-stranded, circular, 1679 nt RNA that folds into an unbranched rod-like structure in which 70% of its

nucleotides are paired. This genomic RNA exists both within virions and in infected nuclei as a ribonucleoprotein (RNP), which contains the only protein encoded by HDV, the delta antigen. Two forms of this antigen are observed during infection. The small delta antigen (δAg-S), a 22 kDa nuclear phosphoprotein, is synthesized early and is required for replication to occur. Later in infection, a specific RNA-editing event leads to the mutation of the δAg-S termination codon and the synthesis of the large antigen, δAg-L, which contains an additional 19 amino acids at its C-terminus. δAg-L acts as a potent inhibitor of HDV genome replication and in combination with δAg-S promotes the assembly of the HDV RNP into HBV envelope particles.

Beet western yellows virus (BWYV)-associated RNA The ST9 strain of BWYV encapsidates not only the 5.6 kb genomic RNA that is typical of luteoviruses, but also a ss, linear 2843 nt-associated RNA (ST9aRNA), which has a distinct nucleotide sequence. The ST9aRNA was postulated to be a satellite RNA. However, this RNA has been shown to be able to replicate without a helper virus. Thus, ST9aRNA is an infectious, subviral agent of plants which depends on its associated virus, BWYV (ST9 strain), for encapsidation but not for replication. Plants infected with BWYV containing the ST9aRNA exhibit a more severe symptom phenotype and contain ~10-fold more virions than do plants infected with BWYV containing no ST9aRNA. The ST9aRNA contains three large ORFs, which, at least *in vitro*, can be translated to yield several products, the largest of which is ~85 kDa, derived from various combinations of readthrough of the three ORFs. The deduced amino acid sequence of two regions of an 85 kDa protein contain significant homology with the RNA-dependent RNA polymerase of carmoviruses.

Structure and Replication of Satellite RNA

Information on secondary structure and replication mechanisms has been obtained mostly for ss satellite RNAs. These small satellite RNAs have highly ordered secondary structures with base pairing up to 73%. The small size and high degree of secondary structure are probably responsible for the high stability, survivability and infectivity of many satellites. As mentioned above, small satellite RNAs may strongly modify symptoms induced by their helper viruses. The sequences involved in pathogenicity are located in discrete small domains. For example, in the case of the CMV satellite RNAs, distinct RNA elements, present in the positive or negative strands, may program chlorosis induction in tobacco and tomato or necrosis in tomato.

RNAs of some satellites can form a tRNA-like domain similar to some plant virus RNAs. For example, the 3′ end of the STMV RNA folds into a tRNA-like structure similar to that in TMV RNA. Accordingly, functional assays have shown that STMV RNA can be aminoacylated *in vitro* with histidine as is the case for TMV. The biological implications of these observations remains unknown.

Satellites are dependent on their helper viruses for replication. The helper virus in turn is dependent on the host plant to supply some components necessary for replication, and thus a complex three-way interaction between satellite, helper virus and plant host is required for satellite replication. The specificity of satellite replication occurs at the level of both the helper virus and the host plant species. With the satellite RNAs of TRSV and CMV, the apparent level of satellite replication varies widely with the strain of helper virus. In addition, related viruses may or may not replicate the same satellite RNAs. For example, most satellite RNA isolates of CMV are replicated efficiently by the related cucumovirus, TAV, but not by another cucumovirus, peanut stunt virus (PSV). Moreover, PSV satellite RNA replication is not supported by CMV. In addition to the helper virus specificity, the host plant often plays a significant role in the efficiency of satellite replication. The small satellite RNA of ArMV replicates very efficiently in *Chenopodium quinoa*. By contrast, the large satellite RNA of another nepovirus, TBRV, replicates poorly in *C. quinoa*, but replicates very efficiently in *Nicotiana clevelandii*. Replication of satellite RNAs has been presumed to occur using the replicase of the helper virus. However, satellite RNA replication does not always involve the same mechanism as replication of the viral RNAs, and it seems likely that other factors which are specific for satellite replication are involved. For large satellite RNAs associated with nepoviruses, the satellite RNA-encoded proteins may function as such factors interacting and modifying the helper virus replication complex. In the case of noncoding, linear, satellite RNAs like the CMV or TRSV satellites, such factor(s) might be host component(s).

Circular, ss, RNA satellites are replicated by a rolling circle mechanism. The replication cycle involves the copying of the encapsidated circular plus (+) strand by an RNA-dependent RNA polymerase to yield a longer than unit length minus (−) strand, which in most cases can self-cleave to yield monomeric products. (For satellite RNAs present in virions as linear molecules this stage is preceded by a ligation

of (+) linear molecules to yield a circular (+) strand.) Then, these (−) RNA monomers are circularized and copied to produce a multimeric, linear (+) strand, which self-cleaves to form linear (+) monomers, which then self-ligate to form circular satellite RNAs. For those multimeric (−) RNAs which are not processed (e.g. satellites of some sobemoviruses), the multimeric (−) RNA is copied to give a multimeric (+) strand which then undergoes cleavage to monomers. The (+) RNA monomers are either circularized and then encapsidated in the circular form (as in the case of sobemovirus satellite RNAs), or packaged as a linear form [as in the case of the BYDV-RPV satellite RNA (a luteovirus), and the small nepovirus satellite RNAs]. Replication of HDV RNA also occurs via a rolling circle mechanism and self-cleaving reactions. HDV is the only animal pathogenic RNA which replicates via a rolling circle mechanism.

There are two types of ribozyme structure found in satellite RNAs possessing self-cleavage activity: the hammerhead and the hairpin ribozymes. Hammerhead structures have been identified in both (+) and (−) RNA strands of lucerne transient streak virus (LTSV) (a sobemovirus) and BYDV-RPV (a luteovirus), as well as in the (+) strands of small satellite RNAs of nepoviruses. Hairpin structures have been found in (−) strands of small nepoviruses. The HDV RNA also contains self-cleaving sequences. However, these appear different from hammerhead and hairpin ribozymes.

Sequence Variation, Evolution, Origins

Some sequence variants of plant satellite viruses (in particular STMV, STNV and SSADV) as well as plant satellite RNAs (predominantly satellite RNAs of CMV and TRSV) have been described. The STNV and CMV satellite RNAs exhibit differences in biological properties, often in a particular host. The large number of CMV satellite RNA isolates sequenced has been used to establish the evolutionary relationships between different groups of CMV satellite RNAs and to demonstrate the presence of structural constraints on satellite RNA evolution. In the case of CMV satellite RNAs, the actual evolution of satellite RNAs in the greenhouse as well as in the field has been observed, as a function of passage with different helper virus strain.

Nothing is known about the origin of satellite RNAs. Limited sequence similarity was observed between one satellite RNA of CMV and chloroplast RNA sequences, and between a satellite RNA of CMV and the potato spindle tuber viroid.

Strategies for Virus Control

The ability of satellite to attenuate the disease symptoms induced by their helper viruses has led to the suggestion that satellite may be useful as biological control agents of pathogenic molecules. Two approaches to test the viral control potential of satellite have been used. In the first approach, the application of mild strains of CMV containing satellite RNA to greenhouse and field crops has been evaluated. In several cases, CMV containing satellite RNA are able to protect plants to various extents against infection by more virulent strains. In the second approach, transgenic plants expressing CMV satellite RNA, TRSV satellite RNA, or GRV satellite-like RNA sequences were found to be either resistant or tolerant to virus infection. Moreover, plants expressing CMV satellite RNA sequences are also resistant to potato spindle tuber viroid, with which CMV satellite RNA shares some similarity.

Further Reading

Colmer CW and Howel SH (1992) Role of satellite RNA in the expression of symptoms caused by plant viruses. *Annu. Rev. Phytopathol.* 30: 419.

Roossinck MJ, Sleat D and Palukaitis P (1992) Satellite RNAs of plant viruses: structures and biological effects. *Microbiol. Rev.* 56: 265.

Symons RH (1997) Plant pathogenic RNAs and RNA catalysis. *Nucleic Acids Res.* 25: 2683.

S13m Bacteriophage *see* **Coliphage ϕX174 and Related Phages (*Microviridae*)**

San Miguel Sea Lion Virus *see* **Caliciviruses**

Scrapie *see* **Prions**

Semliki Forest Virus *see* **Sindbis and Semliki Forest Viruses**

SENDAI VIRUS (*PARAMYXOVIRIDAE*)

Morio Homma, Department of Home Economics, Kobe Women's University, Kobe, Japan

Masato Tashiro, Department of Viral Diseases and Vaccine Control, National Institute of Infectious Diseases, Tokyo, Japan

History

Sendai virus was first discovered in 1952 by Kuroya, Ishida and Shiratori at Tohoku University Hospital, Sendai, Japan, during an epidemic of fatal pneumonitis among newborn babies. A new virus with hemagglutinating activity antigenically different from known influenza viruses was recovered from mice inoculated with the autopsied lung tissues. The virus was originally named newborn pneumonitis virus, type Sendai. However, the question soon arose as to the causative agent of human disease because this virus was reported to be widely spread among laboratory rodents and pigs in Japan at that time. Since then, Sendai virus has been shown to prevail worldwide and to cause enzootic or epizootic infections in mice and rats. Sendai virus was designated hemagglutinating virus of Japan (HVJ) in 1955 by the Society of Japanese Virologists. Later, Sendai virus was shown to be related to human parainfluenza virus type 1, isolated by Chanock in 1955 and named hemadsorption virus type 2 (HA2). As Sendai virus causes a respiratory infection in mice, this virus–animal system has been widely investigated in pathogenesis and immunology as a suitable model of respiratory viral infections. Okada's finding of cell fusion by Sendai virus in 1958 has developed a new field of cell biology, e.g. the production of hybrid cells such as heterokaryons and hybridomas. Proteolytic activation of the fusion glycoprotein was first described for Sendai virus by Homma and Ohuchi in 1973. Additionally, most of the information on the molecular biology of paramyxoviruses has been obtained from studies on Sendai virus, the prototype of the family *Paramyxoviridae*. Recently, a reverse genetics technology to recover infectious viral particles from a cDNA copy of the Sendai virus genome was developed by Kato, by which any mutant viruses could be obtained.

Taxonomy and Classification

Sendai virus belongs to the genus *Respirovirus* in the subfamily *Paramyxovirinae* of the family *Paramyxoviridae* of RNA viruses. Based on serological relationships, Sendai virus is assigned to a murine subtype of human parainfluenza virus type 1, the classification being supported by phylogenetical analyses of the genome nucleotide sequences among paramyxoviruses.

Properties of Virion and Genome

The virions are pleomorphic in size and shape. They are roughly spherical and 150–250 nm in diameter but larger particles are common. The particles consist of a nucleocapsid with helical symmetry (18 nm in width, 1 μm in length), which is enclosed by a lipid envelope derived from host cell plasma membrane. The nucleocapsid is composed of a nonsegmented, linear, negative-stranded genomic RNA (15 384 nucleotides) containing six genes, covalently linked in tandem, and associated proteins NP, P and L (**Fig. 1**). The envelope contains protein M beneath the inner layer, and two spike-like projections, composed of tetramers of glycoprotein HN and trimers of glycoprotein F, respectively. They penetrate the lipid bilayer beyond the outer surface to make the fuzzy appearance of the envelope surface when viewed by electron microscopy. The NP protein (58 kDa), bound directly to the genomic RNA, is the major structural component of the nucleocapsid. Proteins P (72 kDa) and L (255 kDa) act in concert as RNA polymerase complexes with transcriptase and replicase activities. The P–NP complex functions to encapsidate the RNA genome. The C and V proteins, nonstructural proteins and possible minor virion components encoded by overlapping reading frames within the P gene region, are not essential for viral replication but

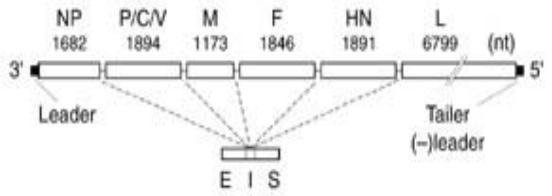


Figure 1 Sendai virus genome RNA. The extragenetic 3′-terminal leader and the complementary 5′-tailer regions are shown by bold lines. The conserved transcription regulatory sequences (E, end; I, intergenic; S, start) are located at the gene boundaries.

appear to regulate mRNA transcription and genome replication. The M protein (40 kDa) has a dual affinity for the NP protein and the cytoplasmic domain of the glycoproteins and plays an important role in the virus assembly process. The larger glycoprotein HN (68 kDa) has receptor binding, hemagglutinating and neuraminidase activities, while the smaller glycoprotein F (63 kDa), when proteolytically cleaved into disulfide-linked F_1 and F_2 subunits, has membrane fusing and hemolytic activities and mediates virus entry through envelope fusion.

Replication

In common with other paramyxoviruses, virus replication takes place exclusively in the cytoplasm and does not require DNA synthesis of host cells. A one-step replication cycle takes about 12–15 h and one infected cell usually yields thousands of progeny viral particles.

Virus Entry

The HN glycoprotein mediates attachment of viral particle to the sialic acid-containing receptors on the carbohydrate side chains of the glycoproteins or glycolipids of host cell surface. After adsorption, the F (F_1 + F_2) glycoprotein mediates, at neutral pH, an envelope fusion between the viral envelope and the plasma membrane, by which the nucleocapsid is introduced into the cytoplasm to initiate the primary transcription catalyzed by the virion-associated RNA polymerase complexes.

Characterization of Transcription

Transcription of the genome RNA to synthesize mRNA occurs in the cytoplasm, for which host factors, e.g. tubulin, are required. The genomic RNA consists of the leader sequence at the 3′ end and the related tailer sequence at the 5′ end. There is a tandem set of six genes ordered NP, P/C/V, M, F, HN and L, and bound by the consensus transcriptional regulatory sequences for the poly(A) initiation site (E), intergenic (I) and a gene start sequence (S). The P–L polymerase complexes associated with the nucleocapsid catalyze the primary transcription to synthesize monocistronic mRNA species, with adding poly(A) tail at the 3′ end, corresponding to each polypeptide. Several polycistronic mRNAs may also be synthesized by reading through the stop signals at the intergenic sequence. The P/C/V gene encodes the P mRNA of the complementary copy of the genomic RNA, from which the P protein is translated. The C protein, a small basic protein, is also translated from a (+1) open reading frame in the 5′ end region of the P mRNA by internal translational initiation at another site. In addition to the P gene transcript, a second mRNA species is synthesized by RNA editing, with insertion of an additional G residue not coded by the genome at the specific editing site, resulting in a frameshift downwards. The second mRNA encodes the V protein, a hybrid protein with the N-terminus half of the P protein and a new reading frame downwards for a cysteine-rich C-terminus region. The secondary transcription is catalyzed by the RNA polymerase complexes of the viral proteins newly synthesized in infected cells.

Characterization of Translation

Each monocystronic mRNA translates a corresponding polypeptide. With the P mRNA, however, four nonstructural proteins (C, C′, Y_1, and Y_2) are synthesized from reading frames overlapping the P gene using different initiation codons by ribosomal scanning. The glycoproteins HN and F are synthesized on membrane-bound polysomes and subjected to post-translational processing.

Post-translational Processing

The F protein, a type I membrane protein, is translated and inserted into the membrane of the rough endoplasmic reticulum by the signal peptide at the N-terminus, which is removed after insertion. On the other hand, the HN is a type II membrane protein anchored to the lipid bilayer at a hydrophobic region in the N-terminus. Both proteins are glycosylated and during transport to the smooth endoplasmic reticulum the carbohydrate side-chains are processed to mature forms. The HN polypeptide forms homotetramers, a pair of disulfide-linked dimers, while the F polypeptide forms homotrimers by noncovalent bonds.

This precursor form of F glycoprotein is biologically inactive, and gains the fusion activity by proteolytic cleavage into the disulfide-linked subunits, F_1 and F_2 (**Fig. 2**). The cleavage site of the F protein,

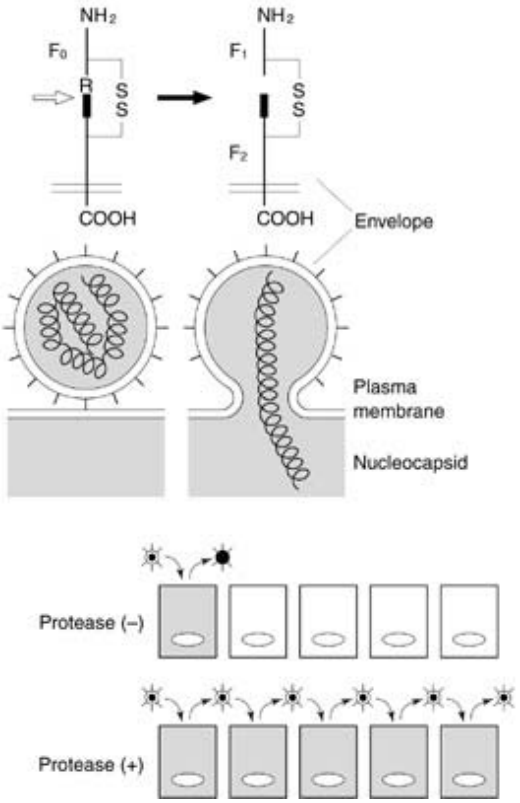


Figure 2 Proteolytic activation of Sendai virus F glycoprotein and infectivity. Upper panel: Sendai virus infects target cells through envelope fusion between viral envelope and plasma membrane. Sendai virus possessing the precursor F_0 is noninfectious. Proteolytic cleavage of F_0 by an activating protease at residue 116-arginine (indicated by open arrow) into disulfide-linked subunits, F_1 and F_2, results in the exposure of hydrophobic fusion-inducing domain (shown by bold lines), which is required for membrane fusion activity and infectivity. Lower panel: Sendai virus can undergo multiple cycles of replication and show pathogenicity in tissues where suitable activating protease(s) is present. Open circles indicate activated (infectious) virus particles and closed circle indicates nonactive (noninfectious) particle.

consisting of a single arginine residue, is not cleavable by ubiquitous cellular proteases present in the trans-Golgi region that cleave preferentially dibasic or multibasic motifs. Accordingly, Sendai virus produced in most tissue culture cells possesses uncleaved F protein and remains nonactive, lacking cell fusion and hemolytic activities and infectivity. These activities are restored by *in vitro* treatment of the virus with trypsin, blood clotting factor Xa in the chorioallantoic fluid of chicken eggs, and tryptase Clara, a serine protease secreted by Clara cells of rat bronchial epithelia. By contrast, progeny virus is recovered in the activated form in several host cells or tissues, such as the chorioallantoic membrane of chick embryos, primary monkey kidney cells and mouse lungs. In these cases, the cleavage of F protein takes place extracellularly by secreted host proteases which cleave the single arginine residue of the F protein.

Strategy of RNA Replication

Replication of the genome RNA is mediated by polymerase complexes composed of the gene products of the primary transcripts, P, L and probably some of the nonstructural proteins. The NP proteins assembled with the genomic RNA also interact with the replication complexes. The leader sequence at the 3′ end and the tailer sequence at the 5′ end of the genomic RNA are required for genome replication. The C protein appears to regulate the switch between mRNA transcription and genome replication. Genome replication provides templates for secondary transcription and for further amplification of the genome, and finally supplies the mature progeny genome for incorporation into virus particles. The entire nucleotide sequence of the negative-stranded genomic RNA is copied complementarily to the antigenomic positive-stranded RNA that serves as an intermediate template for the synthesis of progeny genome RNA. Subgenomic RNA species are frequently synthesized and incorporated into viral particles producing defective-interfering (DI) particles.

Assembly and Release

The progeny genome RNA is combined with the NP protein to form a helical structure to which the P and L proteins are incorporated to form the assembled nucleocapsid. The glycoproteins F and HN are transported through the Golgi apparatus to the cytoplasmic membrane, where they replace host membrane proteins. In polarized epithelial cells, the glycoproteins are transported to the apical membrane domain, where virus assembly occurs. The M protein accumulates beneath the cytoplasmic domain of the glycoproteins and binds the glycoprotein tails with the nucleocapsid. Viral particles are then assembled by budding with incorporation of the lipid bilayer of the host plasma membrane to form the viral envelope. Cellular actin, which is incorporated into the envelope, plays a role in the budding process. The neuraminidase residing on the HN glycoprotein facilitates the release of viral particles from infected cells by destroying cellular receptors.

Cytopathology

Sendai virus usually produces a lytic-type cytopathic effect, which is associated with disruption of the cytoskeletal systems likely caused by the M protein. Apoptotic cytolysis is also reported with Sendai virus-

infected cells. Since the F protein is not activated in ordinary tissue culture cells, syncytial formation mediated by 'fusion from within' is not observed, unless trypsin is added to the culture medium. Inhibition of cellular RNA or protein synthesis is not remarkable. Sendai virus can set up a variety of persistent infections in tissue culture cells without killing infected cells.

Cell Fusion

Sendai virus causes cell fusion and produces multinuclear giant cells. The F protein is directly responsible for the fusion activity for which proteolytic activation is required. Coincidence of the HN glycoprotein is specifically required for inducing cell fusion. Cell fusion falls into two categories: 'fusion from without (FFWO)' and 'fusion from within (FFWI)'. FFWO occurs in cells within a few hours after infection at high multiplicity of virus infection and does not require virus replication. FFWO has been used for producing hybrid cells, such as heterokaryons and hybridomas, and for introducing macromolecules into cells. On the other hand, FFWI occurs late in the replication cycle and is mediated by, in concert with the HN glycoprotein, the F protein synthesized in infected cells and expressed at the cell surface in the activated form.

Geographic Distribution

Retrospective serological surveillance revealed that Sendai virus initially appeared in Japan in the early 1950s and became prevalent worldwide by 1970 as a most common contaminant in laboratory rodent colonies under conventional conditions.

Host Range and Virus Propagation

Mice and rats are natural hosts of Sendai virus, while other rodents, including hamsters, guinea pigs and rabbits, and also pigs can be infected. Suckling and weanling mice are highly susceptible to the respiratory infections. Susceptibility to experimental infections varies considerably among mouse strains, probably due to differences in immunological responses. Sendai virus can grow efficiently in the chorioallantoic cavity of embryonated chicken eggs and replicates productively in a broad spectrum of tissue culture cell lines as well. However, exogenous trypsin is usually needed for proteolytic activation of the F glycoprotein to support multiple cycles of replication in tissue culture cells. Primary monkey kidney cells support multiplication of the virus without exogenous proteases. Sendai virus isolated from mice and passaged only in mouse lungs or monkey kidney cells retains original pathogenicity in mice, whereas it becomes less pathogenic, due to a mutation in the C gene, when passaged in chick embryos. Sendai virus replication is detected by hemagglutination of chicken erythrocytes and the virus is identified by the hemagglutination inhibition test.

Serological Relationship and Variability

The Sendai virus core soluble (S) antigen, mainly composed of the NP protein, exhibits a serological relationship to, but distinguishable from, human parainfluenza virus type 1. Envelope components, HN, F and M proteins, share antigenicity with their human counterparts among the members of the *Paramyxoviridae*, as determined by analyses of the genome nucleotide sequences. Sendai virus is antigenically homogeneous, i.e. there are no subtypes.

Epidemiology

Sendai virus is a most frequent contaminant maintained persistently in conventional colonies of laboratory mice and rats, while no evidence for the infection in wild rodents has been obtained. Both acute enzootic and epizootic infections occur in laboratory rodents.

Curiously, in the 1950s, Sendai virus was prevalent nationwide among pigs in Japan, but by 1961 the virus had disappeared from pigs. Infection of Sendai virus in pigs has not been reported in other countries.

Transmission and Tissue Tropism

Virus replication in the respiratory tract of mice reaches a peak 4–6 days after infection, declines gradually, and virus shedding terminates within 2 weeks. However, the virus may be recovered for up to 6 weeks after infection. In suckling mice, the virus persists longer. Virus transmission occurs either by direct contact with infected mice or by airborne route. Vertical transmission is suggested to occur from pregnant mice to fetus, resulting in stillbirths or a variety of malformations in offspring.

Sendai virus is exclusively pneumotropic in weanling and adult mice, the target tissues being restricted to the epithelial cells of the upper respiratory tract, trachea, bronchi and bronchioles. Neither subepithelial invasion from the surface mucosa nor spread to systemic organs via viremia occurs. Alveolar cells are not infected under usual conditions. Infection of alveolar and peritoneal macrophages results in abortive infections. In newborn and suckling mice, the virus may spread from the nasal epithelium to the brain via the olfactory route. Intracranial inoculation

of the mice causes infections in the ependyma and meninges.

Pathogenicity

Pneumotropism of Sendai virus in mice cannot be explained by cellular receptors in the lungs, as receptors for the virus are also present in other organs not permissive for Sendai virus infection. Instead, pneumotropism has been shown to be primarily determined by host protease-mediated activation of the F glycoprotein. Activated virus with the cleaved F ($F_1 + F_2$) protein will infect the respiratory mucosa and replicate in multiple cycles, as the F protein of progeny virus undergoes cleavage activation in the bronchial lumen by tryptase Clara, an arginine-specific serine protease secreted by Clara cells of the bronchial epithelium. Upon Sendai virus infection, secretion of tryptase Clara is stimulated, whereas that of pulmonary surfactant, an inhibitor of the protease also secreted by Clara cells, is reduced, thereby producing a condition in the bronchial lumen preferable for activation of progeny virus. As a result, infected cells are increased in number and lung lesions are extended. In contrast, infection by a protease-activation mutant, whose F protein is not cleavable by trypsin and tryptase Clara but by chymotrypsin, terminates after a single cycle of replication in the lung, because the progeny virus remains noninfectious. Various organs of mice other than lungs, which lack the protease(s) required for the activation of wild-type F protein, have a potential capacity to support replication of wild-type virus but only for a single cycle. On the other hand, a pantropic mutant, whose F protein is cleavable by ubiquitous host proteases distributed in various organs, causes a systemic infection in mice. These results, together with similar observations on the virulence of Newcastle disease virus and avian influenza viruses, indicate that organ tropism and pathogenicity of Sendai virus are primarily determined by the presence of activating proteases for the F protein in target tissues.

The mode of virus budding at the primary target of infection is considered an additional determinant for organ tropism. The budding site of wild-type Sendai virus in the bronchial epithelial cells is restricted to the apical membrane domain, whereas the pantropic mutant buds bidirectionally at the apical and basolateral domains, due to amino acid exchanges in the M protein. This may explain why infection by wild-type virus remains localized in the surface epithelium of the respiratory tract, whereas the pantropic mutant readily invades subepithelial tissues to gain access to the spread to distant organs via viremia.

A deletion mutant in the V protein replicates less efficiently in mouse lungs, causing a slighter lung lesion, although it is highly cytopathic in tissue culture cells. The results suggest a regulatory role of the V protein in the pulmonary pathogenicity.

Clinical Features of Infection

With enzootic infections the virus usually produces a subclinical infection in mice and rats. Experimental infections as well as epizootic infections cause symptoms characteristic of acute respiratory infection. Moderate fever, ruffled furs and nasal discharge are common signs of the upper respiratory infection, appearing 2–3 days after exposure. Dyspnea, cough, anorexia and loss of body weight, which usually appear on the sixth or seventh day after infection, will indicate progression to bronchopneumonia. With weanling mice, retardation of body weight gain reflects the severity of the disease. In the second week of infection, infected animals die of bronchopneumonia or begin to recover from the infection. In newborn or 1- to 2-day-old suckling mice, intracranial inoculation causes meningitis and ependymitis, resulting in hydrocephalus.

Pathology and Histopathology

Major pathology of Sendai virus infection in mice is mild rhinitis, moderate tracheitis and severe bronchopneumonia. Immunohistological studies reveal that target tissue of the virus is confined to the epithelial mucosa of the upper respiratory tract, trachea, bronchi and bronchioles. Subepithelial tissues, alveolar epithelium and infiltrating cells are not usually involved. Macroscopically the lungs become swollen and hyperemic in a few days after intranasal infection, and lung consolidation, looking like the liver or spleen, begins to appear around day 7. Light microscopic studies reveal infected epithelial cells to be swollen and pyknotic with destruction of cilia within day 1. Submucosal edema and hyperemia occur with a peribronchial infiltration by neutrophils and mononuclear cells. For several days such infected cells increase progressively in number, meanwhile becoming necrotic and desquamated. The cellular infiltration progresses for 2 weeks, with massive edema and bleeding in the interstitium and alveolar spaces. Resolution of the lesion, if it occurs, begins about day 10 and proceeds rapidly, but complete resolution takes more than a month. When 1- to 2-day-old mice are infected intracranially, meninges, ependyma, choroid plexus and labyrinth are involved with respective inflammations.

Immune Response

Recovery from Sendai virus infections involves both humoral and cellular immunities. Envelope glycoproteins, HN and F, are mainly responsible for the humoral response and internal proteins, NP and M, are also of importance as target antigens for the cellular responses. Virus is cleared mainly by CD8+ cytotoxic T lymphocytes (CTLs) in a class I-restricted manner, and therefore, in nude mice, virus infection persists for more than 2 months. CTL response in mice depends on the *H-2* haplotype. Macrophages, natural killer cells and interferons contribute to the virus clearance in concert with the T lymphocytes. Lung consolidation is mainly caused by CD4+ T cells primed by the internal proteins.

Mucosal IgA antibodies to the HN are primarily responsible for resistance to infection. Serum IgG antibodies are less effective for preventing replication of initially infecting virus, but can minimize further replications and lung lesions. The role of cellular immunity in protection is controversial.

The HN molecule contains at least four antigenic epitopes. Antibodies against HN inhibit hemagglutinating and neuraminidase activities, and neutralize infectivity. Antibodies to the F protein, with at least four antigenic sites, inhibit fusion and hemolytic activities, and can also prevent infection, specifically cell-to-cell infection. When mice are infected, considerable titers of serum antibodies become detectable. Since the HN, F, NP and M proteins share antigenic determinants with other members of paramyxoviruses, most closely to human parainfluenza virus type 1, heterotypic antibody responses may occur when Sendai virus-primed animals are immunized or boosted with a related virus, or vice versa.

Sendai virus is a strong inducer of interferons in tissue culture cells and in mice.

Prevention and Control

Control of Sendai virus infection is practically important for breeding and maintenance of laboratory animals and for the performance of animal experiments. Contamination with the virus will interfere with experimental data, specifically in immune responses and lung histology, and often interrupts animal experimentation by causing epizootic acute infections, with or without devastating animal death. A drop in breeding efficiency as a result of infection can be critical for maintenance of animal strains.

Virus-free animals are produced by cesarean birth and can be maintained under specific pathogen-free conditions isolated from conventional colonies. Once Sendai virus-free colonies are established, frequent serological surveillance and quarantine of contaminated colonies are needed for maintenance of laboratory animals.

Inactive vaccines prepared from egg-grown virus are commercially available, but are presently far from being in general use. Experimental live vaccines of protease-activation mutants, temperature-sensitive mutants or defective-interfering particles with considerable efficacy have been developed. Recombinant vaccinia viruses with genes encoding Sendai virus proteins or oligopeptides corresponding to the epitopes responsible for immune protection have been shown to induce protection in mice.

See also: **Defective interfering viruses; Genetics of animal viruses; Parainfluenza viruses (*Paramyxoviridae*): Animal, Human; Immune response: Cell mediated immune response, General features.**

Further Reading

Brownstein DG (1986) Sendai virus. In: Bhatt PN, Jacoby RO, Morse HC III and New A (eds) *Viral and Mycoplasmal Infections of Laboratory Rodents*, p. 37. New York: Academic Press.

Collins PL, Chanoock RM and McIntosh K (1996) Parainfluenza viruses. In: Fields BN, Knipe DM and Howley PM (eds) *Fields Virology*, 3rd edn, p. 1205. Philadelphia: Lippincott-Raven.

Ishida N and Homma M (1978) Sendai virus. *Adv. Virus Res.* 23: 349.

Lamb A and Kolakofsky D (1996) Paramyxoviridae: the viruses and their replication. In: Fields BN, Knipe DM and Howley PM (eds) *Fields Virology*, 3rd edn, p. 1177. Philadelphia: Lippincott-Raven.

Okada Y (1988) Sendai virus-mediated cell fusion. *Curr. Top. Memb. Trans.* 32: 297.

Tashiro M and Rott R (1996) The role of proteolytic cleavage of viral glycoproteins in the pathogenesis of influenza virus infections. *Semin. Virol.* 7: 237.

SEQUIVIRUSES (*SEQUIVIRIDAE*)

MA Mayo and **AF Murant**, Scottish Crop Research Institute, Invergowrie, Dundee, UK

History

The genus name *Sequivirus* was adopted in 1995 for the former 'parsnip yellow fleck virus group'. The eponymous member (PYFV) was first described in 1968 and two serotypes have been recognized, both infecting plants in the family Umbelliferae: parsnip serotype, found in parsnip, celery and *Heracleum sphondylium*; and anthriscus serotype, found in carrot and *Anthriscus sylvestris*. Both serotypes depend on a helper virus, anthriscus yellows virus (a tentative member of the genus *Waikavirus* of the family *Sequiviridae*) for transmission by aphids in a semipersistent manner. The only other species in the genus *Sequivirus* is dandelion yellow mosaic virus (DYMV) which was first isolated from diseased *Taraxacum officinale* (Compositae), but causes a severe necrotic disease in lettuce (*Lactuca sativa:* Compositae). DYMV has been relatively little studied but it is aphid-transmitted and the limited data available suggest a semipersistent mode of transmission. There is no evidence to show if a helper virus is involved.

Taxonomy and Classification

Viruses in the genus *Sequivirus* have isometric particles of *c.* 30 nm diameter and monopartite, positive-sense, single-stranded (ss) RNA genomes of about 10 kb. The RNA-dependent RNA polymerase encoded by PYFV RNA is 'picorna-like'. The genus is classified, together with the genus *Waikavirus* (type member, rice tungro spherical virus), in the family *Sequiviridae*. Viruses in both genera have genomes that encode a large polyprotein from which the mature virus proteins are cleaved by protease action. The viruses possess three coat proteins whose genes are located near the 5′ ends of the genomes, properties which are shared with picornaviruses. Sequiviruses have sometimes been referred to as 'plant picornaviruses'.

Properties of the Virion

Although PYFV may have an infectivity dilution end-point of up to 10^{-5} in sap of *Spinacia oleracea* (spinach), the virus concentration is often very low in extracts of glasshouse-grown plants. Best virus yields have been obtained by growing infected *S. oleracea* plants at 15°C with light at 10 000 lux for 8 h per day, and collecting systemically infected leaves 19 days after inoculation of the plants. Yields of about 5–20 mg of viral nucleoprotein per kilogram of leaf material are obtained by clarifying leaf extracts with diethyl ether or butan-1-ol followed by differential centrifugation.

Particle preparations of PYFV contain 30 nm diameter isometric particles which sediment either as 'top component' (apparently empty protein shells) or 'bottom component' (infective nucleoprotein particles). The top component particles are relatively fragile. Top and bottom components of an isolate of the parsnip serotype, separated by sedimentation in sucrose density gradients or by isopyknic banding in CsCl or Cs_2SO_4 solutions, respectively had sedimentation coefficients ($s_{20,w}$) of 60 S and 152 S, buoyant densities in CsCl of 1.29 and 1.49 g ml^{-1}, and A_{260}:A_{280} ratios of 0.8 and 1.7. Top and bottom component particles of PYFV contain three major protein species of M_r ($\times 10^{-3}$) 31, 26 and 22.5 (parsnip serotype) or 31, 26 and 24 (anthriscus serotype). Bottom component particles of PYFV each contain one ssRNA molecule of *c.* 10 kb.

The infectivity dilution end-point of DYMV in sap of *Chenopodium amaranticolor* or *C. quinoa* may be up to 10^{-5}. Purification involves clarification of phosphate-buffered extracts of leaf by adding butan-1-ol to a concentration of 8% (v/v), followed by differential centrifugation; however, the yields are low. The nucleoprotein particles are isometric, *c.* 30 nm in diameter, and have an $s_{20,w}$ of 159 S, A_{260}:A_{280} of 1.67 and a buoyant density in Cs_2SO_4 of 1.42 g ml^{-1}. DYMV particles have three protein species, of M_r ($\times 10^{-3}$) 32, 29.5 and 27, and one ssRNA molecule of *c.* 10 kb.

Properties of the Genome

Most of the 9871 nucleotide ssRNA genome of PYFV comprises one large open reading frame that encodes a polyprotein of 3027 amino acids. Mature coat proteins, such as the virion proteins, are produced from the primary polyprotein translation product by proteolysis. **Figure 1** illustrates the position of this open reading frame in PYFV RNA. The genome RNA does not have a 3′-terminal poly(A) sequence and is thought to have a 5′-terminal genome-linked protein.

Figure 1 also shows the polyprotein translation product of the PYFV genome RNA and indicates

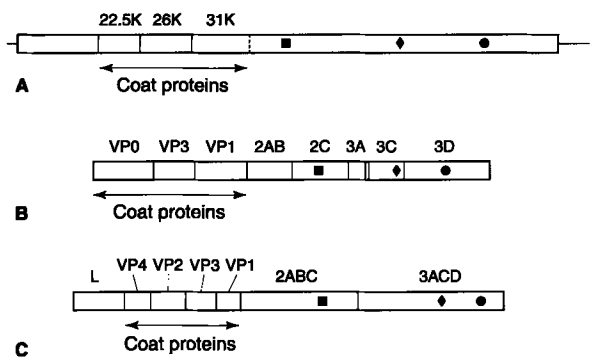


Figure 1 Genome organization of (**A**) PYFV, and comparison of the PYFV polyprotein with those of (**B**) poliovirus 1 and (**C**) foot-and-mouth disease virus. The locations of conserved domains are shown as filled squares (NTP-binding), filled diamonds (protease) or filled circles (polymerase).

where coat proteins have been mapped in the polyprotein. Other cleavage sites are unknown. However, some sequences in the polyprotein have striking similarities to those of domains in other virus proteins that are thought to have NTP-binding, protease or RNA-dependent RNA polymerase functions. The positions are shown on the diagram. Viruses in several families of plant viruses have genomes that are expressed by the cleavage of a large polyprotein. However, the genomes most resembling those of sequiviruses (and of waikaviruses which form the other genus in the family *Sequiviridae*) are those of viruses in the family *Picornaviridae*. Two examples of the polyproteins of such viruses are shown for comparison in **Fig. 1**. In some, the coat proteins are N-terminal; in others there is a protein to the N-terminal side of the coat proteins. The relative positions of the three domains with putative functions in the PYFV polyprotein are the same as in the picornavirus polyproteins (**Fig. 1**).

Sequence Comparisons

Some small regions of the structural and nonstructural proteins of PYFV seem to resemble sequences in picornaviral structural proteins. But there is little overall percent identity much in excess of that between unrelated proteins. This is also true in comparisons between PYFV nonstructural proteins and corresponding proteins of viruses in the genus *Waikavirus*, or of viruses such as comoviruses and nepoviruses (family *Comoviridae*). **Table 1** shows the only values of percent identity between pairs of proteins that were above the background level in comparisons of PYFV proteins and corresponding proteins of other 'plant picornaviruses' (family *Comoviridae*) and viruses in the genera *Enterovirus* and *Hepatovirus* (family *Picornaviridae*).

Evolution

The similarity between the genome expression strategies of sequiviruses and picornaviruses has prompted speculation about common ancestry of these groups. Some picornaviruses have insect hosts, and sequiviruses and waikaviruses are transmitted by insects (see below), albeit in a nonpropagative fashion. This has prompted further speculation about a common origin from an insect virus ancestor. As in most such speculation, there is no evidence to support or to counter these ideas.

Serology

PYFV and DYMV are moderately to highly immunogenic. The virus concentrations in plant extracts are usually too low for reactions to be obtained in gel diffusion tests but satisfactory results can be obtained in other kinds of test, including ELISA and immunosorbent electron microscopy (ISEM). The parsnip and anthriscus serotypes differ by a serological differentiation index of 4–5. Results of ISEM tests suggest the possibility of an extremely distant serological relationship between PYFV and DYMV.

Geographic Distribution

PYFV is reported only from the UK, The Netherlands

Table 1 Comparison of parsnip yellow fleck virus proteins with those of other viruses

	% identity with corresponding polypeptides of PYFV		
Virus	*NTP-binding*	*Polymerase*	*Classification*
Rice tungro spherical	41	49	*Sequiviridae*
Tomato black ring	29	33	*Comoviridae*
Cowpea mosaic	30	40	*Comoviridae*
Poliovirus 1	33	31	*Picornaviridae*
Hepatitis A	30	30	*Picornaviridae*

and Germany. DYMV is reported from these countries and also from Czechoslovakia, Scandinavia and China.

Host Range and Virus Propagation

Both PYFV and DYMV have restricted natural and experimental host ranges. In nature, PYFV is reported only from a few species of Umbelliferae: the parsnip serotype from parsnip (*Pastinaca sativa*), celery (*Apium graveolens*) and hogweed (*Heracleum sphondylium*); the anthriscus serotype from cow parsley (*Anthriscus sylvestris*), carrot (*Daucus carota*) and dill (*Anethum graveolens*). Experimentally, isolates of both PYFV serotypes can infect a few other species of Umbelliferae, notably chervil (*Anthriscus cerefolium*) and coriander (*Coriandrum sativum*), and a few plants in the families Amaranthaceae, Chenopodiaceae, Portulacaceae and Solanaceae. *Chenopodium amaranticolor* and *C. quinoa* are good local lesion assay hosts. *Spinacia oleracea* (spinach) and *Nicotiana clevelandii* (for the parsnip serotype only) are good systemic hosts for maintenance and propagation. *N. benthamiana* is a very sensitive indicator host, developing a lethal systemic wilt and necrosis in 4–7 days.

DYMV has been found naturally only in two species of Compositae (dandelion and lettuce). Experimentally, it has been transmitted to a few species in each of the families Amaranthaceae, Chenopodiaceae, Compositae and Solanaceae. *Chenopodium amaranticolor* and *C. quinoa* are useful both for local lesion assay and as systemic hosts for propagating the virus for purification.

Transmission and Tissue Tropism

Sequiviruses are mechanically transmissible, although mechanical transmission of DYMV to lettuce or dandelion is difficult. The natural vectors of sequiviruses are aphids: *Cavariella aegopodii* for PYFV and *Aulacorthum solani, Myzus ascalonicus, M. ornatus* and *M. persicae* for DYMV. Detailed information on aphid transmission of DYMV is lacking, but PYFV is transmitted only by aphids that also transmit a so-called helper virus (anthriscus yellows virus; AYV). The helper virus must be acquired either before or at the same time as PYFV. The AYV– PYFV complex is transmitted in a semipersistent manner. The minimum acquisition access time (AAT) is about 10–15 min and the minimum inoculation access time (IAT) is 2 min. The transmission efficacy of aphids increased with increasing AAT and IAT of up to 24 h. Adult aphids retain the ability to transmit for up to 4 days after the end of the AAT, but nymphs cease to transmit after molting. Aphids did not transmit virus after injection into the hemocele with purified preparations of PYFV or with extracts of aphids carrying PYFV and AYV. Electron microscopic examination of viruliferous aphids suggested that the virus is carried in the aphid foregut.

Sequiviruses are thought to infect cells in all host tissues. Virus can be acquired by aphids when they feed on mesophyll cells. Sequiviruses are not seed-transmitted.

Pathogenicity

The symptoms in parsnip plants of infection with the parsnip serotype range from yellow flecking to yellow and green mosaic with some stunting. Symptoms can be more severe in infected celery plants. The symptoms of infection of carrot plants with the anthriscus serotype range from mild mottling to severe necrosis, wilting and death.

Available evidence indicates that *Anthriscus sylvestris* is the main wild host of the anthriscus serotype, which infects carrot, and that *Heracleum sphondylium* is the main wild host of the parsnip serotype, which also infects celery. Both these wild hosts are infected symptomlessly. Carrot, celery and parsnip are immune to the helper virus, AYV, so that all the infections observed in these crops are primary and there is no secondary spread within the crop. Nevertheless, PYFV is common in parsnips in the UK. In celery, plants infected with PYFV are usually confined to the edges of fields. In The Netherlands, infection by the anthriscus serotype is uncommon in ware crops of carrot but common in carrot seed crops, in which infection results in 'early-season dieback', which in some years causes severe losses in seed production.

DYMV was so called because it was isolated from dandelion plants showing a bright yellow mosaic with rings and oak leaf patterns. It has also been isolated from lettuce showing a severe necrotic disease: veinal chlorosis and necrosis, thickening and curling of the leaves, severe stunting and prevention of heading. The virus was transmissible from diseased dandelion and lettuce plants to lettuce both by aphids and by manual inoculation. However, evidence that the virus that causes the necrosis in lettuce is the cause of the yellow mosaic in dandelion is either lacking or negative; the appropriateness of the name dandelion yellow mosaic virus for this virus is therefore in some doubt.

Prevention and Control

The diseases caused by PYFV in celery and parsnip, and in ware crops of carrot, do not seem to be

sufficiently serious to warrant specific control measures, although insecticides applied for the control of aphids or carrot fly (*Psila rosae*) may serve to decrease the incidence of PYFV. The disease caused by PYFV in carrot seed crops in The Netherlands is economically important but spraying with systemic biological control insecticide seems of only limited usefulness in preventing infection.

The restricted host range of the AYV helper virus suggests possible control measures by eliminating overwintering weed hosts in the vicinity of susceptible crops.

Little is known about the epidemiology or importance of DYMV. Most outbreaks of necrosis disease in field crops of lettuce occurred in the vicinity of yellow mosaic-diseased dandelion, which suggested that dandelion is an important overwintering host. There is no information about resistance genes.

See also: **Polioviruses (*Picornaviridae*): General features, Molecular biology; Waikaviruses (*Sequiviridae*); Picornaviruses – insect (*Picornaviridae*).**

Further Reading

Elnagar S and Murant AF (1976) The role of the helper virus, anthriscus yellows, in the transmission of parsnip yellow fleck virus by the aphid *Cavariella aegopodii*. *Ann. Appl. Biol.* 84: 169.

Mayo MA, Murant AF, Turnbull-Ross AD *et al* (1995) Family *Sequiviridae*. In: Murphy FA, Fauquet CM, Bishop DHL *et al* (eds) *Virus Taxonomy*. Sixth Report of the International Committee on Taxonomy of Viruses, p. 337. Wien: Springer.

Murant AF (1988) Parsnip yellow fleck virus, type member of a proposed new plant virus group, and a possible second member, dandelion yellow mosaic virus. In: Koenig R (ed.) *The Plant Viruses*. Vol. 3: *Polyhedral Virions with Monopartite RNA Genomes*, p. 273. New York: Plenum Press.

Turnbull-Ross AD, Mayo MA, Reavy B & Murant AF (1993) Sequence analysis of the parsnip yellow fleck virus polyprotein: evidence of affinities with picornaviruses. *J. Gen. Virol.* 74: 555.

Sheep Poxvirus *see* **Poxviruses**

SHRIMP VIRUSES

Philip C Loh, Department of Microbiology, University of Hawaii, Honolulu, Hawaii, USA

Introduction

Since the first shrimp virus *Baculovirus penaei* (BP) was isolated from wild penaeid shrimp (*Penaeus duorarum*) in the early 1970s, a number of penaeid shrimp viruses have assumed great importance because of the severe effect on the growth and sustenance of the penaeid shrimp aquaculture industry. Several of these viruses have been associated with large epizootics and massive mortalities in shrimp farms and hatcheries. The number of penaeid shrimp viruses reported at present belong to six families (**Table 1**). This number, however, is expected to increase and additional studies of viral diseases of penaeid shrimp result in more being isolated and characterized. Many of the penaeid shrimp viruses have only been observed through electron microscopy and have not been extensively characterized in regard to their biological, immunological, biochemical and physical properties.

The Host Animal

Depending on the geographic location, the following penaeid shrimp species have been commercialized on a large scale: *Penaeus monodon* (giant tiger shrimp); *P. vannamei* (whiteleg shrimp); *P. stylirostris* (blue shrimp); *P. japonicus* (kuruma shrimp); *P. chinensis* (orientalis) (fleshy prawn); *P. duorarum* (northern pink shrimp); *P. merguiensis* (banana shrimp); *P. indicus* (Indian white prawn); and *P. setiferus* (northern white shrimp).

The Virus: General Features

Host range

The penaeid shrimp viruses implicated in epizootics and associated with massive mortality in cultured shrimp belong to four groups: *Baculoviridae, Rhabdoviridae, Parvoviridae, Picornaviridae* (**Table 1**).

sufficiently serious to warrant specific control measures, although insecticides applied for the control of aphids or carrot fly (*Psila rosae*) may serve to decrease the incidence of PYFV. The disease caused by PYFV in carrot seed crops in The Netherlands is economically important but spraying with systemic biological control insecticide seems of only limited usefulness in preventing infection.

The restricted host range of the AYV helper virus suggests possible control measures by eliminating overwintering weed hosts in the vicinity of susceptible crops.

Little is known about the epidemiology or importance of DYMV. Most outbreaks of necrosis disease in field crops of lettuce occurred in the vicinity of yellow mosaic-diseased dandelion, which suggested that dandelion is an important overwintering host. There is no information about resistance genes.

See also: **Polioviruses (*Picornaviridae*): General features, Molecular biology; Waikaviruses (*Sequiviridae*); Picornaviruses – insect (*Picornaviridae*).**

Further Reading

Elnagar S and Murant AF (1976) The role of the helper virus, anthriscus yellows, in the transmission of parsnip yellow fleck virus by the aphid *Cavariella aegopodii*. *Ann. Appl. Biol.* 84: 169.

Mayo MA, Murant AF, Turnbull-Ross AD *et al* (1995) Family *Sequiviridae*. In: Murphy FA, Fauquet CM, Bishop DHL *et al* (eds) *Virus Taxonomy*. Sixth Report of the International Committee on Taxonomy of Viruses, p. 337. Wien: Springer.

Murant AF (1988) Parsnip yellow fleck virus, type member of a proposed new plant virus group, and a possible second member, dandelion yellow mosaic virus. In: Koenig R (ed.) *The Plant Viruses*. Vol. 3: *Polyhedral Virions with Monopartite RNA Genomes*, p. 273. New York: Plenum Press.

Turnbull-Ross AD, Mayo MA, Reavy B & Murant AF (1993) Sequence analysis of the parsnip yellow fleck virus polyprotein: evidence of affinities with picornaviruses. *J. Gen. Virol.* 74: 555.

Sheep Poxvirus *see* **Poxviruses**

SHRIMP VIRUSES

Philip C Loh, Department of Microbiology, University of Hawaii, Honolulu, Hawaii, USA

Introduction

Since the first shrimp virus *Baculovirus penaei* (BP) was isolated from wild penaeid shrimp (*Penaeus duorarum*) in the early 1970s, a number of penaeid shrimp viruses have assumed great importance because of the severe effect on the growth and sustenance of the penaeid shrimp aquaculture industry. Several of these viruses have been associated with large epizootics and massive mortalities in shrimp farms and hatcheries. The number of penaeid shrimp viruses reported at present belong to six families (**Table 1**). This number, however, is expected to increase and additional studies of viral diseases of penaeid shrimp result in more being isolated and characterized. Many of the penaeid shrimp viruses have only been observed through electron microscopy and have not been extensively characterized in regard to their biological, immunological, biochemical and physical properties.

The Host Animal

Depending on the geographic location, the following penaeid shrimp species have been commercialized on a large scale: *Penaeus monodon* (giant tiger shrimp); *P. vannamei* (whiteleg shrimp); *P. stylirostris* (blue shrimp); *P. japonicus* (kuruma shrimp); *P. chinensis* (orientalis) (fleshy prawn); *P. duorarum* (northern pink shrimp); *P. merguiensis* (banana shrimp); *P. indicus* (Indian white prawn); and *P. setiferus* (northern white shrimp).

The Virus: General Features

Host range

The penaeid shrimp viruses implicated in epizootics and associated with massive mortality in cultured shrimp belong to four groups: *Baculoviridae, Rhabdoviridae, Parvoviridae, Picornaviridae* (**Table 1**).

Table 1 Penaeid shrimp viruses

Family	*Name/acronym*	*Shape/size (nm)*	*Nucleic acid/sense*	*Envelope*	*Growth in cell culture*
Rhabdoviridae	Rhabdovirus of penaeid shrimp/RPS	Bullet, 70 × 125	ssRNA (−)	+	+
	Yellowhead virus/YHV	Bacilliform, 45 × 160	ssRNA (−)	+	+
Picornaviridae	Taura syndrome virus/TSV	Icosahedron, 31–32	ssRNA (+)	−	−
Reoviridae	Type III reolike virus/REO-III	Icosahedron, 50–70	dsRNA (segmented)	−	−
	Type IV reolike virus/REO-IV				
Togaviridae	Lymphoid organ vacuolization virus/LOVV	?, 55	?	+	−
Parvoviridae	Infectious hypodermal and hematopoietic necrosis virus/IHHNV	Icosahedron, 22–25	ssDNA	−	−
	Hepatopancreatic parvovirus/HPV	Icosahedron, 22–24	ssDNA	−	−
	Lymphoidal parvo-like virus/LPV	Icosahedron, 25–30	?	−	−
Baculoviridae					
Occluded Type A					
	Baculovirus penaei/BP	Rod, 75 × 228	dsDNA	+	−
	Monodon baculovirus/MBV	Rod, 69 × 275	dsDNA	+	+
Nonoccluded Type C					
	Baculoviral midgut necrosis/BMN	Rod, 72 × 310	dsDNA	+	−
	Bacilliform virus/BV	Rod, 83 × 275	dsDNA	+	−
	Chinese baculovirus/CBV	Rod, 120 × 265	dsDNA	+	+
	Systemic ectodermal mesodermal baculovirus/SEMBV	Rod, 121 × 276	dsDNA	+	+
	Whitespot baculovirus/WSBV	Rod, 87 × 330	dsDNA	+	+
	penaeid rod-shaped DNA virus/PRDV	Ovoid, 84 × 226	dsDNA	+	+

The susceptibility of the different species of cultured penaeid shrimp to these viruses varies from infection of only a few to infection of a large number of species. Although the range of these viruses in feral or wild shrimp has not been fully documented, a member of the non-occluded group of baculoviruses, the white spot baculovirus (WSBV), has not only been detected in several species of wild penaeid shrimp but also in other wild decapods. Wild shrimp populations have also been reported to carry the parvovirus, infectious hypodermal and hematopoietic necrosis virus (IHHNV).

Infection and severity

The mortality and morbidity of the penaeid shrimp to several of these pathogenic viruses are affected by age. The older shrimp are generally less susceptible to infection than younger ones. The viruses can infect the penaeid shrimp at various stages of its development, from protozoea to adult, with the highest mortality occurring at the early postlarval stage. Depending on the virus and the species of penaeid shrimp, infected animals exhibit gross physical changes and histopathological aberrations of their organs and tissues. Primary organ and tissue targets may vary with the viral pathogen.

Transmission

There is very little information available on the natural routes of transmission of these viruses. Transmission via the oral route has been demonstrated through feeding of either contaminated foods or infected carcasses and appears to be a dominant route of natural infections. Transmission by way of the gills is another possible route. Although asymptomatic infections have been reported, little is known concerning the mechanism of latent or persistent viral infections in the penaeid host.

Control and prevention

Currently the most effective means of controlling the viral disease problem is to destroy the infected animals, decontaminate the ponds, and start again with virus-free stocks. Although specific-pathogen-free (SPF) shrimp has been developed as a way to control the disease problem, it represents a partial

solution since the animals were tested for only a limited number of viral pathogens. Such SPF animals have been found to be equally susceptible as wild stock to other shrimp viral pathogens.

Growth and assay

Until recently penaeid shrimp viruses were grown only in a sensitive indicator shrimp bioassay system. Primary shrimp cell cultures such as primary lymphoid cell lines are currently used to isolate, grow and assay some of the shrimp viruses. Such primary cell lines have been used in limited studies on the synthesis of viral proteins, viral pathogenesis, of antiviral chemicals, and the development of virus detection/diagnosis protocols. A stable continuous cell line has been prepared by transforming primary shrimp lymphoid cells with the viral oncogene, SV40-large T-antigen. Although the transformed shrimp lymphoid cells exhibited many of the properties of stable, continuous transformed cell lines, they were found to be non-permissive to some of the shrimp viruses. The transformed cells exhibited antiviral activities.

Detection and diagnosis

At present the detection/diagnosis of shrimp viral diseases is still dependent on the clinical history and light and/or electron microscopical examination of affected tissues showing characteristic cytopathology obtained from infected shrimp. Asymptomatic and latent infections can be detected only through the use of either enhancement or bioassay techniques in sensitive indicator shrimp. However, these traditional methodologies have limited sensitivity, require time (days to weeks), specialized equipment, highly trained personnel and high cost. A number of molecular and immunologically based technologies have been recently developed which have facilitated the rapid, specific, sensitive and cost-effective detection/diagnosis of shrimp viral infections. Various methods, such as the nitrocellulose-enzyme immunoassay (NC-EIA), the Western blot and various modifications of the nucleic acid probe (NAP) and polymerase chain reaction (PCR) procedures have been effectively employed.

Basic Properties of Some Penaeid Shrimp Viruses

In this section, the discussion is limited to those shrimp viruses on which there is adequate information concerning their basic properties.

RNA viruses

Rhabdoviridae There are two viruses belonging to this family: the rhabdovirus of penaeid shrimp (RPS) and the yellowhead virus (YHV). The YHV is provisionally grouped in this family.

Rhabdovirus of penaeid shrimp The RPS is the first rhabdovirus to be isolated from penaeid shrimp and also to uniquely infect a continuous fish cell heteroploid line, epithelioma papulosum cyprini (EPS). It was originally isolated from infectious hypodermal and hematopoietic necrosis (IHHN)-diseased and healthy *P. stylirostris* and *P. vannamei* obtained from shrimp farms in Hawaii and Ecuador. However, experimental infections of juvenile penaeids (5–6 g) did not induce histopathological changes characteristically associated with IHHNV infection, nor were clinical or gross manifestations of disease observed. In such animals no mortality occurred and virus replication was demonstrated only in the lymphoid (Oka) organs by plaque assay and immunofluorescence. The affected lymphoid organs, which showed gross cellular changes, were significantly larger in size (6–7 times) than the corresponding organs from uninfected animals and appeared to be the primary target organ of RPS infection. Mortality was observed in younger postlarval (PL) shrimp (0.2 g) experimentally infected by three routes of infection: water-borne (12%), oral feeding (21%) and intramuscular injection (43–50%). The water-borne and oral feeding routes may represent the natural routes of transmission.

Thin-section electron microscopic studies indicated that RPS replicates in the cytoplasm of infected cells and appears to bud from both cytoplasmic vesicles and the plasma membrane. Both thin sections and negative staining studies showed bullet-shaped particles which are enveloped (typical of animal rhabdoviruses). Emanating from the envelope are regularly shaped projections with a knob-like structure at the distal end. Complete virions measured 115–138 × 65–77 nm.

In virus susceptibility studies of several fish cell lines, the heteroploid EPC cell line was determined to be the most susceptible to RPS and had the highest yield of virus. The EPC was found to be especially useful for the primary isolation of RPS. Although several of the other fish cell lines were susceptible, their yields of infectious RPS were much lower (<10%). Single-cycle growth studies of RPS in EPC cells showed an eclipse period of 3 h, followed by a period of exponential growth which was completed by 48 h postinfection (p.i.). Since the virus uniquely replicates in a fish cell line (EPC) causing distinct cytopathic changes, this has enabled the development of a quantitative plaque assay protocol which has greatly facilitated the study of RPS. The efficiency of

plating (EOP) of RPS in EPC was determined to be 30 virus particles per infectious unit. The virus was found to be highly fragile, being sensitive to 20% ethyl ether, low pH, repeated freezing and thawing (3 times), to 37°C (12 h), and storage at −10°C (4 weeks) but was stable at −70°C for several weeks. The buoyant density of RPS in sucrose gradients is 1.19 g cm^{-1}.

The molecular weight of the single RNA species of the RPS genome was determined to be 3.6×10^6 Da (~10.4 kb). The viral RNA has a negative polarity and is sensitive to ribonuclease. Since the replication of RPS in EPC was not inhibited by the DNA antagonist 5-bromo-2′ deoxyuridine (20 μg ml^{-1}), this confirms the RNA genome of the virus.

Analysis by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of RPS proteins revealed the presence of at least four major structural proteins with the following molecular weights (kDa): 165, 65.7, 45.1 and 27.8. The number of structural proteins and the electrophoretic profile of RPS are very similar to those of the prototype rhabdovirus, vesicular stomatitis virus (VSV), and of the fish rhabdovirus carpio (RC) (also named spring viremia of carp virus, SVCV), both of which belong to the genus *Vesiculovirus*. In number of structural proteins and electrophoretic profile, they are different from the lyssatype fish rhabdoviruses, infectious hypodermal necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV). The lyssa variety is easily distinguished from the vesiculo group in that the matrix proteins are composed of two structural polypeptides, M1 and M2, versus a single polypeptide, M, in the vesiculo group. Western blot analysis of the electrophoretically separated RPS structural proteins with anti-RPS polyclonal serum revealed, in addition to the four major proteins, an extra viral protein with a molecular weight of 38 kDa. Based on its molecular weight, this polypeptide was presumed to be nonstructural protein which is present in the vesiculo group of rhabdoviruses. The Western blot technique further revealed that the RPS is partly related to VSV, IHNV and VHSV. The anti-RPS serum identified the G protein of both IHNV and VHSV and the M protein of VSV. Although the anti-RPS serum cross-reacted with the structural proteins of RC, suggesting a close evolutionary relation of these two viruses, the intensity of reaction to RC was much weaker than that observed with RPS. Serologically, RPS is unrelated to IHNV and VHSV and is closely related to but distinguishable from RC when the plaque reduction and neutralization techniques are used.

A solid-phase enzyme-immunoassay protocol, nitrocellulose-enzyme immunoassay/streptavidin–biotin (NC-EIA/SAB), employing a rabbit polyclonal anti-RPS IgG, was developed for the diagnosis and detection of RPS. The NC-EIA/SAB was reported to detect as few as 10 plaque-forming units (PFU) or 300 viral particles (400 pg of viral protein) in experimentally infected shrimp. The protocol has successfully detected natural RPS infections in apparently healthy animals at different stages of development in shrimp farms in Hawaii.

Yellowhead virus The yellowhead virus was named after the disease it caused in the black tiger shrimp, *P. monodon*. Diseased animals showed a characteristic yellow color of the cephalothorax and gills and a pale or bleached appearance. The infection generally resulted in a cumulative mortality of 100% within 3–5 days after the onset of the disease. The etiological agent was initially identified as a baculo-like virus but subsequently was reported to contain RNA and to possess properties not characteristically associated with baculoviruses.

In addition to the original host *P. monodon,* two other penaeid species, *P. stylirostris* and *P. vannamei,* were reported to be highly susceptible to YHV. Intramuscular inoculation of a 10%, w/v, cephalothorax filtrate prepared from naturally infected *P. monodon* Fabricius into subadult (3–10 g) *P. stylirostris* (Stimpson) and *P. vannamei* (Boone) resulted in 100% cumulative mortality within 5–7 days p.i. In such experimentally infected animals, the characteristic light yellowing of the hepatopancreas and gills observed in naturally infected *P. monodon* was not seen. Histopathological examination of naturally and experimentally infected shrimp revealed widespread cellular necrosis in the gills, connective tissues, hemocytes, hematopoietic organs, and lymphoid organ, and strongly indicated a preferential infection of the cells of ectodermal and mesodermal origins.

Primary cultures of shrimp lymphoid cells have been used for the quantitative titration of infectious YHV. This has permitted study of the pathogenesis of YHV in the infected shrimp. The primary targets for infection were the lymphoid organ, gill and head soft tissues, all of which contained 10- to 800-fold higher titers of infectious virus than found in the other tissues and organs tested. Four fish cell lines (fathead minnow or FHM, EPC, chinook salmon embryo or CHSE-214 and brown bullhead or BB) and two insect cell lines (SF9 from *Spodoptera frugiperda* and CRL 1963 from *Drosophila*) were found not susceptible to YHV.

Electron microscopic examination of ultrathin sections of gills and lymphoid tissues from infected animals revealed the presence of numerous enveloped virus particles with bacilliform morphology, measuring 150–200 × 40–50 nm in the cytoplasm of infected

cells. Virions budding out through the cytoplasmic membrane were frequently observed. Cross-sections of the complete virions showed an electron-dense nuclear core which measured 20–30 nm in diameter, surrounded by a trilaminar envelope.

To prepare purified virus the hemolymph was found to yield the cleanest preparation and the largest number of virus particles. Attempts to purify YHV from the gill and head soft tissue met with difficulty because of contaminating vesicles and other membranous cellular materials which banded nonspecifically with the virus in sucrose gradients. The buoyant density of purified YHV ranged from 1.18 to 1.20 g ml^{-1}. This is comparable to the buoyant densities of known rhabdoviruses.

Electron microscopic examination of uranyl acetate-stained, purified YHV revealed peplomer-containing, enveloped, rod-shaped particles measuring 190–200 × 50–60 nm and resembling the plant rhabdoviruses. As with plant rhabdoviruses, the virus particle is fragile and flexible, often assuming pleomorphic forms. The nucleocapsid is arranged in an orderly helical fashion. The helix consists of a coiled tubular structure layered in a regular fashion at right angles to the long axis of the complete virus particle. YHV does not exhibit the bullet-shaped morphology typical of rhabdoviruses infecting vertebrates. However, it closely resembles the bacilliform (with rounded ends) rhabdo-like viruses infecting the blue crab, *Callinectes sapidus*, and the helical, rod-shaped plant rhabdoviruses. In terms of their dimensions and ultrastructure, these rhabdoviruses are very much alike.

Genome analysis of YHV by agarose gel electrophoresis showed a single band of RNA of mol. wt. 8×10^6 Da (~22 kb). The size of the RNA genome is approximately twice that of the rhabdoviruses. The viral RNA is susceptible to ribonuclease (RNase) and is therefore a single-stranded structure. The failure of YHV RNA to be translated in an *in vitro* rabbit reticulocyte translation system strongly indicates that the viral genome possesses a negative polarity.

Structural protein analysis of purified YHV preparations by SDS-PAGE revealed four major bands with the following estimated molecular sizes (kDa): 170, 135, 67 and 22. These bands probably correspond to the large (L), glycoprotein (G), nucleocapsid (N) and matrix (M) proteins of rhabdoviruses. The putative G protein of YHV was determined to be glycosylated.

Western blot analysis of the protein bands using rabbit polyclonal anti-YHV immunoglobulin G (IgG) showed strong reactivity by the antibody with the putative G protein. The proteins of VSV, RPS, and control shrimp tissues did not crossreact with the same antibody preparation. Although the structural protein profile of YHV is similar to that of RPS and other members of the family *Rhabdoviridae*, the molecular sizes of YHV proteins are different. The G protein of YHV is larger (135 kDa versus 90 kDa) and probably accounts for the very prominent peplomers. Furthermore, YHV has a smaller M protein (22 kDa versus 30 kDa), which may account for the high flexibility and fragility of the virus.

In hemagglutination studies, both YHV and VSV were found to agglutinate chicken red blood cells with endpoints of 1:256 and 1:64, respectively. Neither virus was eluted even after 24 h incubation at room temperature, suggesting the formation of a stable complex which lacked receptor-destroying enzymes.

A number of *in vitro* methodologies are available for the detection/diagnosis of YHV infection. An indirect NC-EIA method employing a rabbit polyclonal anti-YHV IgG has been used for the rapid, specific diagnosis and detection of acute and asymptomatic YHV infection of the penaeid shrimp. The solid-phase enzyme immunoassay procedure was capable of detecting as few as 100 $TCID_{50}$ units of virus or an equivalent of 0.4 ng of viral protein. Although gill tissues and lymphoid organs were highly satisfactory sources of YHV antigen for the NC-EIA test, the sampling of the hemolymph which was found to contain a considerable amount of virus, was found to be a more convenient and a less invasive way of monitoring YHV infection in cultured shrimp, particularly invaluable broodstock animals.

Recently a Western blot protocol has been successfully used for the detection of YHV infection in field samples of cultured shrimp from Southeast Asia and the USA. A reverse transcriptase–polymerase chain reaction (RT-PRC) protocol has been used with limited success.

Two of 45 recombinant clones which gave positive hybridization with YHV RNA were negative with the DNA of *P. monodon* and that of the white spot baculovirus. The problem with the probes is that we do not know from which regions of the viral genome they originate. Also, the family of genes making up the YHV genome has not been identified.

Picornaviridae The only member provisionally assigned to this family is the Taura syndrome virus (TSV) which causes a highly infectious disease named Taura syndrome (TS) among the whiteleg shrimp, *P. vannamei*. Epizootics of TS have resulted in extensive economic losses to the shrimp farming industry in the Americas. Several penaeid species have been found to be susceptible to TSV: *P. chinensis, P. aztecus and P. duorarum*. In contrast *P. stylirostris* and *P. setiferus*

were found to be resistant after experimental challenge with TSV.

Taura syndrome generally occurs in juvenile (0.1–5 g) *P. vannamei,* and its clinical effect is mortality (>90%). As a rule, older animals are more resistant.

There is scant information available concerning the presence or distribution of TSV in feral shrimp populations. Several attempts to demonstrate TSV in captured wild broodstock, spawned eggs or naupli have not been successful.

Little is known concerning the natural transmission routes of TSV. Experimental transmission studies have provided strong evidence that the waterborne-oral feeding route may represent the natural route of transmission. Some evidence has been presented to suggest the role of seagulls as the probable transport vector of TSV among shrimp farms.

The buoyant density of TSV in CsCl gradients is 1.338 g ml^{-1}. The virus is a nonenveloped icosahedron with a diameter of 30–32 nm. It has a single-stranded RNA genome which is polyadenylated at the 3′ end. Viral RNA has a positive sense and is approximately 9 kb in length. The viral capsid is made up of three major (55, 40 and 24 kDa) and one minor (58 kDa) structural proteins.

Shrimp with acute natural and experimentally induced TSV infections show a distinct histopathology that consists of multifocal areas of necrosis in the cuticular epithelium and often in the subcuticular connective tissue. Present in these tissues are numerous variably sized eosinophilic to basophilic cytoplasmic inclusion bodies that give TS lesions a 'buckshot' pattern of necrosis which is typical of the disease. These characteristic changes are used as a means of diagnosis of TS. An *in situ* hybridization method using a complementary DNA (cDNA) probe has been developed as a diagnostic tool for the detection of TSV in fixed tissue preparations.

DNA viruses

Occluded baculoviruses The two occluded baculoviruses that have caused serious disease and economic losses in shrimp farms are: Baculovirus penaei (BP) occluded type A also known as *Penaeus vannamei* single nuclear polyhedrosis virus (PVSNPV) and *Penaeus monodon* baculovirus (MBV) occluded type A also known as *Penaeus monodon* single nuclear polyhedrosis virus (PMSNPV).

Baculovirus penaei BP was first described by light and electron microscopy in naturally infected *P. duorarum* (pink shrimp). Since then the virus has been found in several wild and cultured penaeid species: *P. aztecus, P. setiferus, P. vannamei, P. stylirostris, P. penicillatus, P. schmitti, P. paulensis, P. subtilis* and several others. Thus far the virus has been limited in its distribution to the Western hemisphere and Hawaii.

Epizootics of BP can result in high mortality of larval and early postlarval shrimp, particularly in intensive culture systems which facilitate the development and transmission of the disease. However, in larval or late postlarval penaeid shrimp, the effects of BP infection are minimal. Age appears to play a role in susceptibility of the penaeid shrimp to BP.

In the infected animal, BP affects primarily cells of the hepatopancreatic and midgut epithelium. In the hypertrophied nuclei of infected cells in these affected tissues, the newly synthesized progeny may be either free or occluded, with characteristic tetrahedral crystalloid bodies termed occlusion bodies (OBs). The polyhedra are easily seen by light microscopy and may be as large as 17 μm. From one to several polyhedra may occupy a nucleus. The OBs, which are composed primarily of the protein polyhedrin, are used as a diagnostic feature of patent BP infections.

Little is known concerning persistent BP infections in the natural environment. Attempts to demonstrate this phenomenon experimentally in the larvae and postlarvae of *P. vannamei* have not been successful.

A mixed infection involving BP and a reo-like agent has been described. Each shrimp with a reo-like infection also had a BP infection, but the reverse was not always true. Both viruses were observed in the same tissue and occasionally in the same cell.

There is sparse information concerning the biochemical and cellular events involved in the infection cycle of BP. Based on electron microscopical studies the BP virion, presumably after attachment and viropexis or fusion, uncoats or injects its DNA into the host cell nucleus at the nuclear pore. After integration of the virion into the host cell genome, a series of morphologically recognizable sequences of events occur, leading to the eventual production of mature BP and tetrahedral OBs. A distinct intracellular morphological change observed is the appearance of extensive membranous labyrinths (ML) adjacent to the endoplasmic reticulum. The ML appeared to originate from dilated Golgi and endoplasmic reticulum vesicles and from the outer nuclear envelope.

The BP virion is a rod-shaped nucleocapsid surrounded by a trilaminar envelope. Unlike some nuclear polyhedrosis viruses with multiple nucleocapsids per envelope, BP has only one per envelope. The intact, enveloped virions, when banded in CsCl gradient, exhibit a buoyant density of 1.265 g ml^{-1}. On the basis of negative staining, the enveloped virion is 312–320 × 75–87 nm and the nucleocapsid is

approximately 306–312 × 62–68 nm. The complete enveloped virions appear to possess appendage-like structures at both extremities that are assumed to be loose envelope extensions. Similar structures have also been seen among other shrimp baculoviruses, such as the nonoccluded virions. Thin section measurements of the virion reveal a smaller particle of approximately 270–296 × 54–59 nm with nucleocapsid dimensions of 260 × 44.2 nm.

Sucrose-banded OBs, when analyzed by SDS-PAGE using 12% polyacrylamide gels, revealed one major polypeptide with a molecular weight of 52 kDa. Nothing is known about the structural proteins and glycoproteins that compose the complete enveloped virus.

The BP genome is a double-stranded, circular DNA with a molecular weight of 75×10^6 Da. Analysis by electrophoresis in a 1% agarose gel of extracted BP DNA digested with the restriction endonuclease *Bam*HI revealed seven bands with estimated sizes: ≥23, 11.7, 8.2, 4.8, 4.0, 2.9 and 1.1 kb. The largest band probably contained two or more high-molecular-weight bands.

The BP virion is sensitive to a number of physical and chemical conditions. It is completely inactivated under the following conditions: within 30 min at pH 3 but not at pH 11; after 10 min at 60–90°C; after ultraviolet (UV) inactivation for 40 min at a wavelength of 254 nm; and after desiccation for 48 h. On the other hand, it survives 32 parts per thousand sea water at 22°C for 7 days and at 5°C for at least 14 days.

Diagnosis of BP infections has been accomplished in a number of ways: (1) by light microscopic observation of characteristic tetrahedral OBs in wet mount squash preparations of the hepatopancreas, midgut or feces or on histological sections and (2) by the use of the recently developed gene probes that detect BP nucleic acid in infected cells by *in situ* hybridization assay. The latter procedure, which was found to be specific, can also detect BP infections even before the appearance of OBs in wet mount squashes. The probes can detect BP in various species of shrimp from different geographical areas.

More recently, a PCR-based detection procedure was developed for BP. However, this procedure still has, among several caveats, the potential problem of the presence of compounds in shrimp tissues that inhibit the DNA polymerase used in the PCR procedure. It should be added that these recently developed molecular procedures have not been comprehensively evaluated in field studies.

Penaeus monodon baculovirus Since its initial isolation, MBV has been found in a wide variety of both cultured and wild penaeid shrimp species from Asia, Australia, Africa, southern Europe and the Middle East. Reports indicate its presence in shrimp stock in the Americas, but the animals were originally imported from Asia. The virus has been linked to serious diseases and major economic losses in shrimp farms in Southeast Asia and Asia.

A similar agent was found in cultured *P. plebejus* in Australia and was called *Plebejus* baculovirus (PBV). However, on the basis of virus-induced host cell cytopathology and virus morphology, PBV is believed to be a strain of MBV type of viruses rather than a distinct virus type.

MBV is a highly infectious virus that spreads very quickly and causes high larval and juvenile mortality. In adult shrimp the infection is less severe, with the animals showing no significant external signs of disease. In the Indo-Pacific region, MBV has been reported to be a ubiquitous pathogen of cultured *P. monodon*. However, despite its high prevalence and wide distribution, the virion does not appear to be a highly virulent pathogen for *P. monodon*. In disease epizootics, the penaeid shrimp has been frequently found to have mixed infections of MBV and other pathogens. Transmission of MBV is believed to be primarily oral, e.g. from cannibalism. However, other routes of horizontal transmission may occur, such as through contamination of spawned eggs with virus-contaminated feces.

As with all occluded baculoviruses, a principal histopathological and diagnostic feature of MBV infections is the presence of single and multiple, generally spherical OBs, in the hepatopancreas and less often in midgut epithelial cells. The OBs, which have diameters in the range 0.1–20 μm, may be demonstrated in squash preparations of hepatopancreas, midgut, or feces by phase or bright-field microscopy. Different kinds of stains, such as 0.05% aqueous Malachite Green, Acridine Orange, or Phloxine, can be used to enhance visualization of MBV occlusions.

Very little information is available concerning the replication of MBV. A limited ultrastructural study on the morphogenesis of the virions in hepatopancreatic cells revealed certain cytopathic alterations occurring late in the infection, such as nuclear hypertropy, chromatin diminution, loss of nucleolus, formation of virogenic stromata, appearance of many enveloped virions, and appearance and formation of OBs. Another distinct change was the appearance of ML membranes, as was observed in BP infection. Again, the ML was postulated to play two roles in the virion replication cycle: first, as a conduit or transport system for viral structural precursors from the cytoplasm to the nucleoplasm, and second, after this

function is completed, as a mechanism for release of virus and OBs.

Primary shrimp lymphoid cell cultures have been used to support MBV replication. As the result of viral replication, cytopathogenic effects occurred as early as 2–3 days p.i. and became more extensive as the infection progressed. The virus was successfully passaged in primary lymphoid cell cultures at least six times. Unfortunately, no further studies were done until the recent report on the use of primary shrimp lymphoid cells for the growth and assay of YHV and the Chinese baculovirus (or white spot baculovirus).

Electron microscopic examination of uranyl acetate-stained MBV revealed enveloped, rod-shaped particles measuring 265–282 × 68–77 nm and nucleocapsids measuring 250–269 × 62–68 nm. The envelope surface appeared to consist of small, uniformly sized granular structures interspersed with small spikes which were more apparent at the vertices. At the extremities of the envelope were appendage-like structures which were believed to be envelope extensions. Each extremity of the nucleocapsid was enclosed with a double-layered structure, or cap, 16–18 nm thick.

When banded in 30–50% CsCl, complete MBV has a buoyant density of 1.28–1.29 g ml^{-1}, and the OBs have a buoyant density of 1.32–1.33 g ml^{-1}.

The polyhedrin subunits of the spherical MBV polydedron were icosahedral-like structures measuring 22–23 nm in diameter. Analysis of purified MBV OBs by SDS-PAGE and Western blot protocols revealed a single protein band of 62 kDa. The molecular size of the MBV polyhedrin appears to be slightly larger than that of BP (53 kDa), the other occluded baculovirus of penaeid shrimp.

Visualization of MBV DNA by electron microscopy revealed large, supercoiled molecules which were not sufficiently relaxed to allow measurement of the total molecular weight of the genome. However, the viral DNA, after digestion with *Bam*HI endonuclease and electrophoresis in 1% agarose gel, yielded five bands with the following estimated sizes: ≥21, 9, 6.5, 3.5 and 2.8 kb. From these studies, the mol. wt. of the MBV DNA was estimated to be 58–110 × 10^6 kDa (80–160 kb), which falls within the DNA size range of insect baculoviruses. In another study, the molecular size of MBV DNA based on *Eco*RI-cleaved fragments was estimated to be 100–200 kb.

Traditional diagnosis/detection of MBV infection is accomplished by histological examination for the presence of characteristic spherical OBs in hypertrophied nuclei of the hepatopancreas and anterior midgut of the infected animal. Still another source of OBs is shrimp feces. However, these methods do not detect MBV infection at early stages, nor are they adequately sensitive.

Molecular-based methods for the early and specific detection and diagnosis of MBV infections have been developed. The PCR procedure and either *in situ* or dot-blot hybridization techniques employing DNA probes can be used for accurate and early diagnosis or detection of MBV infection.

Non-occluded baculoviruses There are several members belonging to this non-occluded baculovirus group; the classification of all of them has not been officially accepted by the International Committee on Taxonomy of Viruses (ICTV). All of these viruses have caused major epizootics and significant economic losses to the shrimp aquaculture industry.

Baculoviral midgut gland necrosis virus (BMNV) nonoccluded type C also named Penaeus japonicus *nonoccluded baculovirus (PJNOB)* The BMNV is a nonoccluded, gut-infecting virus first isolated in *kuruma* shrimp, *P. japonicus*, larvae. It is highly pathogenic in the early life stages of the shrimp, causing heavy mortality in larval production. Although *P. japonicus* is the natural host for BMNV, other penaeid species, such as *P. monodon*, *P. chinensis*, and *P. semisulcatus*, were found to be experimentally susceptible. Whereas *P. monodon* was found to be highly susceptible, both *P. chinensis* and *P. semisulcatus* showed great resistance to the virus. As with the other shrimp viral pathogens, the waterborne-oral feeding route may represent the natural route of transmission.

Histological examinations of BMNV-infected animals indicate that the midgut and the intestine are the target organs. Infected cells show characteristic nuclear hypertropy and chromatolysis, as well as the absence of OBs which characterize infections by Type A baculoviruses.

Thin-section electron micrographs of the infected nuclei and the midgut lumen reveal numerous rod-shaped, enveloped viral particles, many of which have outer and inner envelopes. The average dimension of the virion was 310 × 72 nm. Purified inner rod-like nucleocapsid structures had capped ends and measured approximately 260 × 50 nm.

No information is available concerning the replication of BMNV at the cellular level.

Viral DNA extracted from purified nucleocapsids was sensitive to digestion with restriction endonucleases *Bam*HI and *Sau*3AI, but not with *Eco*RI, *Pst*I, *Xho*I and *Sal*I. Electrophoretic analysis in agarose gels of the enzyme-digested viral DNA revealed 13 fragments with relative molecular sizes in the range

2.2–27.0 kb. From these results, the mol. wt of viral DNA was estimated to be 85.1×10^6 Da.

Structural protein analysis of nucleocapsid preparation by SDS-PAGE revealed two major proteins with molecular weights of 35 and 14 kDa and three minor bands (mol. wt = 72, 65 and 12 kDa).

Several methods are available for the diagnosis of BMNV infections. Both stained preparations and dark-field microscopic diagnostic methods are used to detect infected, hypertrophied nuclei in squashed preparations of affected tissues such as midgut and intestine. The dark-field microscopic method, because of its simplicity, rapidity, precision and low cost, is the method of choice in shrimp hatcheries in Japan. An immunofluorescent antibody (IFA) procedure has been successfully used to detect BMN-specific virus antigen in smears or sectioned preparations of affected tissues, such as midgut epithelial cells.

White spot baculovirus (WSBV) nonoccluded type C also called systemic ectodermal and mesodermal baculovirus (SEMBV), bacilliform virus (BV), rod-shaped nuclear virus of Penaeus japonicus *(RV-PJ), penaeid rod-shaped DNA virus (PRDV), penaeid hemocytic rod-shaped virus (PHRV) and Chinese baculovirus (CBV)* All these names have been given to the viral isolates obtained from different species of cultured penaeid shrimp from different geographical areas. Until further characterizations of their serological, biochemical and genomic properties are made, the isolates may be considered to be either related strains of the same virus or identical. Although *in situ* hybridization studies have suggested that these nonoccluded baculovirus isolates may be closely related, a recent report has presented evidence to show that they can be distinguished by RFLP (restriction fragment length polymorphism) analyses of the viral genomes. All of these isolates cause epizootics and mass mortality in cultured penaeid shrimp. Diseased shrimp show a characteristically abnormal reddish color together with white spots primarily on the inside surface of the carapace. However, with two experimentally infected penaeid species, *P. stylirostris* and *P. vannamei*, the characteristic white spots were not seen and the reddish color was seen only in the extremities of the appendages. These gross distinctive changes have been used in the diagnosis of WSBV infection. At the cellular level, infected cells showed markedly hypertrophied nuclei. In certain cases, histopathological examination of infected gill tissues may show Cowdry type A nuclear inclusions in hypertrophied nuclei. The natural route of transmission for these virions appears to be the waterborne-oral feeding routes.

Electron microscopical examination of thin sections of affected tissues revealed the presence of large numbers of rod-shaped baculo-like viral particles located primarily in the markedly enlarged nuclei which also showed a loss of integrity of the marginated chromatin material. Viral particles had a multilaminar outer envelope which had a mean size of 265 ± 20 nm in length and 120 ± 10 nm in diameter. The size of the electron-dense nucleocapsid of the virus was 205 ± 12 nm in length and 78 ± 5 nm in diameter. A large number of viruses had double envelopes.

When banded isopycnically in CsCl, the complete virus had a buoyant density of 1.23 g ml^{-1} and the nucleocapsid particle 1.31 g ml^{-1}.

Negatively stained purified CBV particles measured 322–378 nm in length and 130–159 nm in diameter. The inner nucleoprotein core exhibited a unique striated structure and measured 316–350 nm in length and 65–66 nm in diameter. The striations appeared to be the result of the stacking of ring-like structures. These rings consisted of two rows of 12–14 globular units, each measuring 10 nm in diameter.

Genomic analysis of purified CBV revealed a nonsegmented, double-stranded DNA molecule. The reported sizes of the viral DNA genome ranged from 150 kbp to 200 kbp.

Analysis of the structural proteins of purified CBV by SDS-PAGE showed among several, four prominent protein bands with approximate molecular weights of 19, 23.5, 27.5 and 75 kDa. The structural viral proteins were substantiated by Western blot analysis. The 19, 27.5 and 75 kDa structural proteins were found to be nonglycosylated components associated with the viral envelope. The 23.5 kDa protein, also nonglycosylated, was identified with the capsid structure.

For the rapid, sensitive and specific detection/diagnosis of CBV (WSBV) infections both diagnostic probes for *in situ* hybridization and primers for detection by PCR technology have been successfully used. Such studies have also indicated that the gut and the gills of the penaeid shrimp were the primary routes of viral entry and that the lymphoid organ and gills were primary targets for viral replication.

A combined SDS-PAGE/Western blot/EIA protocol has been successfully used for the early detection of CBV in the hemolymph of infected animals. This combination technology has several advantages for the monitoring and surveillance of shrimp populations for virus infections. The sampling of hemolymph is relatively simple and less invasive, particularly for the monitoring of invaluable shrimp broodstocks.

Primary shrimp lymphoid cell cultures have been used in an *in vitro* quantal assay ($TCID_{50}$) for CBV.

Despite limitations associated with primary cell cultures, this assay provides a simple, convenient, reliable and quantitative method for the study of shrimp viruses.

Four fish cell lines (epithelioma papulosum cyprini [EPC]), chinook salmon embryo (CHSE-214), fathead minnow (FHM), and sockeye salmon embryo (SSE-5) and an insect cell line (CRL1963, a *Drosophila* cell line) were resistant to CBV.

A second group of DNA-containing penaeid shrimp viruses causing epizootics are two members of the parvovirus family.

Infectious hypodermal and hematopoietic necrosis virus (IHHNV) The IHHNV is widely distributed, causing severe epizootics and massive mortality in cultured penaeid shrimp, particularly the juveniles of *P. stylirostris*. In other penaeid species, the disease is somewhat less severe. With certain cultured penaeid species, such as *P. vannamei*, a 'runt-deformity syndrome' (RPS) may be the consequence. The affected animals characteristically exhibit greatly reduced growth rates and a variety of cuticular deformities, all of which lessen their market value.

Natural infections by IHHNV have been reported in a number of penaeid species, such as *P. stylirostris*, *P. semisulcatus* and *P. japonicus*. As with the other shrimp viral pathogens, the waterborne-oral feeding route may represent the natural route of transmission. Survivors of IHHN epizootics apparently harbor the virion for life and transmit it to their progeny by vertical and horizontal routes.

Gross clinical symptoms of acute IHHNV infections are not specific. In the infected animal, certain distinguishable histopathological changes occur. Present in affected cells are intranuclear Cowdry type A inclusion bodies (CAI) contained in hypertrophied nuclei in tissues of ectodermal (epidermis, hypodermal epithelium of foregut and hindgut, nerve cord, and nerve ganglia) and mesodermal (hematopoietic organ, antennal gland, gonads, lymphoid organ, connective tissues, and striated muscle) origin.

Purified IHHNV has been prepared from infected penaeid shrimp and banded in CsCl gradients at a buoyant density of $1.40\,g\,ml^{-1}$. Negatively stained, purified virions are nonenveloped icosahedrons with a diameter of 20–22 nm. The virions possess a linear, single-stranded DNA genome of either negative or positive polarity with an estimated size of 4.1 kb. The purified virion is made up of at least four structural polypeptides, VP1 to VP4, with molecular weights of 74, 47, 39 and 37.5 kDa.

To date no cell lines exist which will support the growth of IHHNV. No information is available regarding viral replication at either the cellular or molecular levels.

Several methods are available for the diagnosis of IHHNV infections. The histopathological examination of affected tissues for the presence or absence of intranuclear CAI has provided a fairly reliable diagnosis. However, the formation of CAI may be induced by rather general types of cell injury not involving viruses. Under certain conditions, both DNA- and RNA-containing viruses have been reported to cause CAI. Another method involves enhancement procedures in which the suspected animals are kept under stressful conditions for 2–3 weeks prior to sampling for histologic examination.

For the detection of asymptomatic IHHNV infections, susceptible small juveniles of *P. stylirostris* have been used as indicator shrimp for the presence or absence of the virus.

Gene probes have been developed for the detection/diagnosis of IHHNV infection. The probes have been used in dot-blot and *in situ* hybridization studies and are currently available commercially as a kit.

Hepatopancreatic parvo-like virus (HPV) The HPV is another parvo-like virus that infects a number of cultured and wild *Penaeus* species (*P. chinensis*, *P. merguiensis*, *P. semisulcatus*, *P. monodon*, *P. indicus*, *P. penicillatus*, *P. esculentus*). It has a wide geographic distribution, including the Indo-Pacific area and the Americas. The relationship between all of these reported HPV-type viruses is not known since identification of these virions was based solely on microscopic or histopathological examinations.

Although HPV has been circumstantially implicated in the cause of major disease epizootics, its role as a serious pathogen remains to be clearly defined. This is because of the relative difficulty of diagnosing HPV infections and also because these infections are often accompanied by other viral pathogens which may obscure its importance. Little is known concerning the natural mode of transmission of HPV, although the waterborne-oral route is the most likely. No cell lines are currently available which support the replication of HPV.

In the infected animal, the principal lesion of the disease is characterized by the necrosis and atrophy of the hepatopancreas, which is common to all the penaeid species. Large, prominent, basophilic, Feulgen-positive intranuclear inclusion bodies were often observed in hypertrophied nuclei of hepatopancreatic tubule epithelial cells. These histological changes were used in the diagnosis of HPV infections.

The HPV and the IHHNV are both parvoviruses, but in the permissive host animal they infect different

target tissues: the hepatopancreatic epithelial cells for HPV and all nonenteric tissues for IHHNV.

Electron microscopic analysis of thin sections of HPV-infected cells revealed intranuclear inclusion bodies containing granular virogenic stroma and viral particles 22–24 nm in diameter.

Purified HPV prepared from infected penaeid shrimp and banded in CsCl gradient had a buoyant density of 1.41 g ml^{-1}. Negatively stained, purified virions were nonenveloped, icosahedral particles with a diameter of 22 nm. The virions contained a linear, single-stranded DNA genome of either negative or positive polarity with an estimated molecular size of 5 kb, which, surprisingly, encoded a single protein of 54 kDa.

Gene probes have been used with limited success in *in situ* hybridization assays for the diagnosis of HPV infections. Since the probe did not react positively in the tissues of HPV-infected penaeids from the Indo-Pacific region and the Americas, it is strongly indicated that there are probably several different strains of HPV. The HPV probe did not crossreact with the other shrimp parvovirus, IHHNV. The probe is commercially available as a kit for the diagnosis of HPV.

The following penaeid shrimp viruses have been reported. Most of them remain to be isolated and their relevant properties characterized:

Lymphoidal parvo-like virus (LOV)
Penaeid hemocyte-infecting, nonoccluded baculovirus (PHRV)
Shrimp iridovirus (IRIDO)
Type III reo-like virus (REO-III)
Type-IV reo-like virus (REO-IV)
Lymphoid organ vacuolization virus (LOVV)
Naked star-shaped virus (NSV).

See also: **Baculoviruses (*Baculoviridae*): Granuloviruses, Nucleopolyhedrovirus; Parvoviruses (*Parvoviridae*): Molecular biology, General features; Picornaviruses – insect (*Picornaviridae*); Polioviruses (*Picornaviridae*): General features, Molecular biology.**

Further Reading

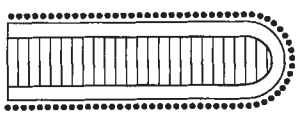
Lightner DV (1993) In McVey JP (ed.) *Handbook of Mariculture*, 2nd edn, vol. 1, p. 393. Boca Raton: CRC Press.

Loh PC (1997) Viral pathogens of the penaeid shrimp. *Adv. Virus Res.* 48: 263.

Shope Fibroma Virus *see* **Poxviruses**

Shope papilloma and bovine viruses *see* **Papillomaviruses – animal**

SIGMA RHABDOVIRUSES (*RHABDOVIRIDAE*)



Danielle Teninges, Centre National de la Recherche Scientifique, Institut de Génétique humaine, Montpellier, France

History

The sigma virus of *Drosophila* is a harmless virus of a harmless insect and probably would never have attracted any attention were it not for the fact that geneticists use carbon dioxide (CO_2) as a mild narcotic for the flies they handle. This gas is generally not noxious and flies recover from narcosis within a few minutes after return to a normal atmosphere. Those infected by the sigma virus, in contrast, remain irreversibly paralyzed and die. CO_2 sensitivity in some *Drosophila* strains was first reported in 1937 by L'Heritier and Tessier as a hereditary trait which was not chromosome-linked. The viral etiology was not suspected at first owing to the complete absence of horizontal transmission of CO_2 sensitivity in natural conditions. Later, L'Heritier observed that inoculation of acellular extracts from CO_2-sensitive flies into resistant flies produced the symptom after an incubation period which increased with the dilution of the inoculum. The size of the agent was deduced from the target size to x-ray inactivation. Studies by electron

target tissues: the hepatopancreatic epithelial cells for HPV and all nonenteric tissues for IHHNV.

Electron microscopic analysis of thin sections of HPV-infected cells revealed intranuclear inclusion bodies containing granular virogenic stroma and viral particles 22–24 nm in diameter.

Purified HPV prepared from infected penaeid shrimp and banded in CsCl gradient had a buoyant density of 1.41 g ml^{-1}. Negatively stained, purified virions were nonenveloped, icosahedral particles with a diameter of 22 nm. The virions contained a linear, single-stranded DNA genome of either negative or positive polarity with an estimated molecular size of 5 kb, which, surprisingly, encoded a single protein of 54 kDa.

Gene probes have been used with limited success in *in situ* hybridization assays for the diagnosis of HPV infections. Since the probe did not react positively in the tissues of HPV-infected penaeids from the Indo-Pacific region and the Americas, it is strongly indicated that there are probably several different strains of HPV. The HPV probe did not crossreact with the other shrimp parvovirus, IHHNV. The probe is commercially available as a kit for the diagnosis of HPV.

The following penaeid shrimp viruses have been reported. Most of them remain to be isolated and their relevant properties characterized:

Lymphoidal parvo-like virus (LOV)
Penaeid hemocyte-infecting, nonoccluded baculovirus (PHRV)
Shrimp iridovirus (IRIDO)
Type III reo-like virus (REO-III)
Type-IV reo-like virus (REO-IV)
Lymphoid organ vacuolization virus (LOVV)
Naked star-shaped virus (NSV).

See also: **Baculoviruses (*Baculoviridae*): Granuloviruses, Nucleopolyhedrovirus; Parvoviruses (*Parvoviridae*): Molecular biology, General features; Picornaviruses – insect (*Picornaviridae*); Polioviruses (*Picornaviridae*): General features, Molecular biology.**

Further Reading

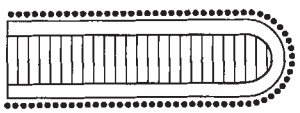
Lightner DV (1993) In McVey JP (ed.) *Handbook of Mariculture*, 2nd edn, vol. 1, p. 393. Boca Raton: CRC Press.

Loh PC (1997) Viral pathogens of the penaeid shrimp. *Adv. Virus Res.* 48: 263.

Shope Fibroma Virus *see* **Poxviruses**

Shope papilloma and bovine viruses *see* **Papillomaviruses – animal**

SIGMA RHABDOVIRUSES (*RHABDOVIRIDAE*)



Danielle Teninges, Centre National de la Recherche Scientifique, Institut de Génétique humaine, Montpellier, France

History

The sigma virus of *Drosophila* is a harmless virus of a harmless insect and probably would never have attracted any attention were it not for the fact that geneticists use carbon dioxide (CO_2) as a mild narcotic for the flies they handle. This gas is generally not noxious and flies recover from narcosis within a few minutes after return to a normal atmosphere. Those infected by the sigma virus, in contrast, remain irreversibly paralyzed and die. CO_2 sensitivity in some *Drosophila* strains was first reported in 1937 by L'Heritier and Tessier as a hereditary trait which was not chromosome-linked. The viral etiology was not suspected at first owing to the complete absence of horizontal transmission of CO_2 sensitivity in natural conditions. Later, L'Heritier observed that inoculation of acellular extracts from CO_2-sensitive flies into resistant flies produced the symptom after an incubation period which increased with the dilution of the inoculum. The size of the agent was deduced from the target size to x-ray inactivation. Studies by electron

microscopy confirmed this information and identified sigma virus as a member of the rhabdovirus family. It has not been assigned to a genus. With its genetic background, *Drosophila melanogaster* was an ideal host to allow genetic analyses of the host factors involved in a virus–insect association and most efforts have been centered on research of this kind.

Pathology

The CO_2 sensitivity symptom is the only sign of disease observed in infected flies. It affects larvae as well as adults and persists throughout their lives. The range of CO_2 concentrations that are lethal to the flies is not frequently met in nature and this cannot account for any counterselection of infected flies. Among inoculated flies the expression of CO_2 sensitivity is correlated to the appearance of infectious material in the central nervous system. Immunocytochemistry reveals the presence of granular inclusions of viral material in the cortex of the cephalic and thoracic ganglia. In hereditarily infected flies, such inclusion bodies are present in all tissues, except muscle. Wild sigma virus-infected flies have been compared to uninfected flies for egg viability, male and female fertility, female longevity and sexual selection. Sigma virus infection only reduced egg viability; other parameters did not show any systematic or significant variation. Survival of adults under winter conditions in France was also studied and showed that the fitness of infected flies to overwintering was slightly lower. Wild sigma virus clones show a very low multiplication rate, which may account for their relative innocuousness. Laboratory strains selected for high multiplication rates generally affect fertility and egg viability in such proportions that when they appear in natural populations they are probably severely counterselected.

In *Drosophila* cells cultivated *in vitro,* sigma virus multiplies without any evidence of a cytopathic effect and a persistent infection is maintained throughout cell transfers.

Since a number of other rhabdoviruses and a bunyavirus (but no flaviviruses) were shown to induce CO_2 sensitivity after inoculation to *Drosophila* flies or to mosquitoes, exposure to CO_2 was proposed as a fast means of screening infected insects in nature.

Virion and Genome Structure

Sigma virus particles exhibit all the structural details of typical rhabdoviruses: a bullet shape, a coiled nucleocapsid, and an envelope derived from the host cell and covered with surface projections. The diameter is 75 nm and the length 200 nm. The presence of shorter particles has also been recorded. The genome structure of a strain selected for high yields in *Drosophila* cell cultures *in vitro* has been studied. It consists of a single segment of approximately 13 000 nucleotides. It encodes six proteins mapped in the following order on the genome strand: 3′-N-P-X-M-G-L-5′ where N is the nucleoprotein and P a phosphoprotein which is the equivalent of the polymerase-associated protein in vesicular stomatitis virus (VSV); gene 3 (X) encodes a protein of unknown function with many potential phosphorylation sites and showing similarities to some of the retroviral reverse transcriptase motifs; M is the matrix protein, G is the glycoprotein and L is the polymerase. The respective lengths predicted for the polypeptides are 450 amino acids for N, 322 for P, 296 for X, 207 for M, and 499 for G. The exact length of L is still unknown but its electrophoretic mobility corresponds to a protein of 250 kDa. The consensus gene-start and gene-end sequences are respectively, CAACANC and $CAUG(A)_7$ in the mRNA sense and thus conform to the consensus AACA and $AUG(A)_7$ observed in all the rhabdoviruses previously analyzed.

Intergenic sequences are thought to intervene in transcriptional control. Some rhabdoviruses like VSV have constant intergenes, whereas others, such as rabies virus, show variation. Sigma virus belongs to the second category and has 36 untranscribed nucleotides which separate N and P, six which separate P and X and four which separate X and M; then G overlaps M, starting 26 nucleotides upstream of the end of the M gene, so the polymerase has to read through the end sequence of the M gene to transcribe the G gene. In spite of this overlap, the transcription results in the regular synthesis of monocistronic M and G mRNAs. The start signal of the L gene is immediately adjacent to the end signal of the G gene. As deduced from UV target size analyses, the transcription of unsegmented negative-strand viruses proceeds sequentially from the 3′ extracistronic region of the genome to the 5′ end. A gene overlap implies that sites of initiation other than the most 3′ proximal one can be used by newly entered polymerases. Gene overlap could be a means of reducing the expression of the two most distal genes from the 3′ end.

Serology and Taxonomy

Fifty animal rhabdoviruses of diverse origins were screened for antigenic crossreactivity. Most of them could be classified in the two large subgroups – the lyssaviruses and the vesiculoviruses. In this study, sigma virus was shown to crossreact with Tupaia, a vesiculovirus isolated from a shrew, and with

Tibrogargan, Humpty Doo and Parry Creek, three lyssaviruses isolated from mosquitoes; it was thus proposed to bridge the gap between the two subgroups. Considering other criteria, such as the scores of similarity between predicted protein sequences, sigma virus appears almost equally distant from the prototypes of both subgroups. The progress of molecular data on other rhabdoviruses may raise the necessity for the definition of new subgroups more closely related to sigma virus.

Virus–Host Interactions

Hereditary transmission is a very efficient means of propagation but its efficiency varies according to the virus genotype and to the sex and genotype of flies. The so called 'stabilized' females transmit the virus to all their descendants with few exceptions. They issue from oocytes that were infected very early, and in which the virus was able to multiply enough to invade all the germline cells from the outset. Their male progeny may also transmit the virus but with a lower efficiency (infection via a spermatozoid is an efficient means of cloning virus particles). The pattern of virus transmission from flies hatched from spermatozoid-infected eggs is different from that of stabilized females: males do not transmit the virus and females may transmit it but only to a small proportion of their descendants. This is an effect of the low concentration of virus genomes in the spermatozoid-infected eggs: cell differentiation and organogenesis probably result in barriers against the penetration of the virus into some tissues. There could be a race during embryogenesis between the virus invasion and the building up of these barriers. Male germ cells which are not infected at the very early stages of embryogenesis (before segmentation) are not invaded at a later stage of development. Female germ cells may be invaded at any stage, including adults, provided that the virus genotype is g^+ (g^- mutants are normally perpetuated in stabilized maternal lines and virions are infectious to somatic cells but they cannot invade the germinal cysts, once isolated by organogenesis). Some stabilized maternal lines (the ultra-rho lines) perpetuate defective viruses which are not infectious and which do not induce CO_2 sensitivity; the only sign of their presence is an immunity to superinfection by homologous virus – immunity which shows exactly the same inheritance pattern as nondefective viruses.

In other stabilized lines, the rho lines, the same phenotype was observed but a still unexplained genetic instability of the viral genomes infecting these lines resulted in the occasional production of fully infectious virions. Some stabilized maternal lines, infected with temperature-sensitive mutants for maturation functions, express exactly the same phenotype as the ultra-rho lines when bred for several generations at nonpermissive temperature, but the CO_2 sensitivity symptom is restored upon return to the permissive temperature. The persistent infection of germ cells through generations by viruses which are defective (or temperature restricted) proves that only the maturation functions are affected in these mutants, not the genome replication. It also proves that viral information may be transmitted by cellular continuity without a necessity for infectious virus production.

Natural populations of *D. melanogaster* are often polymorphic for alleles of genes that confer resistance to sigma virus infection. These restrictive alleles map to six different loci: *ref(1)H*, *ref(2)M*, *ref(2)P*, *ref(3)O*, *ref(3)V* and *ref(3)D* (the number in parentheses represents the *Drosophila* chromosome carrying the gene, the capital letter is the particular name of the gene, and specific alleles of those genes are indicated by an exponent). The refractory loci (*ref*) do not represent a general antiviral system: not only do they not affect other insect viruses but none of them confers resistance to all strains of sigma virus (with a possible exception for *ref(3)V*; see later).

Restrictive effects have been assessed for several parameters of viral infection, such as the mean incubation time required for the expression of CO_2 sensitivity, the probability of initiating infection, the kinetics of virus production (either in flies or in cells cultivated *in vitro*) and the efficiency of virus transmission either by females or by males. A distinction can be made between restriction during either virus maturation or earlier stages of virus production (i.e. genome replication): since hereditary transmission in stabilized maternal lines does not require the production of infective virus, those alleles restricting this transmission necessarily affect the genome replication steps. The four alleles $ref(1)H^h$, $ref(2)M^m$, $ref(2)P^P$ and $ref(3)D^d$ reduce the probability of initiating infection and increase the incubation time for the manifestation of the CO_2 sensitivity symptom. The transmission through the maternal gametes is also reduced very strongly by $ref(1)H^h$ and $ref(2)P^P$, whereas $ref(2)M^m$ and $ref(3)D^d$ exert a weaker action. This implies that the products of these four genes intervene in the replication of the viral genomes. $Ref(3)O^e$ does not affect the hereditary transmission but increases the incubation time of the viral clones sensitive to its action. The allele $ref(1)H^h$ is the only fully dominant allele; flies heterozygous at all other loci express intermediate phenotypes. The $ref(3)V^P$ restrictive allele does not affect the virus multiplication in somatic cells nor in female germ cells and its unique effect is to prevent transmission by

spermatozoa. The specificity of this interaction with sigma virus is still unknown, as to date no viral strains have been found to be resistant. In contrast, the major effects described for the restrictive alleles at other loci apply only to sensitive viral strains.

Flies that are homozygous for either permissive or restrictive alleles are indistinguishable except for the difference in their capacity to permit the multiplication of sigma virus. The most extensively studied refractory gene is *ref(2)P*. Loss of function alleles ($ref(2)P^{null}$) were induced by mutagenesis of a permissive $ref(2)P^{o}$ allele. Homozygous flies for the $ref(2)P^{null}$ alleles are all viable and display no phenotype other than male sterility in particular genetic backgrounds. Some alleles of a gene located on the third chromosome suppress the male sterility of $ref(2)P^{null}$ homozygotes. Such alleles are present in approximately one-third of the flies in natural populations, which means that *ref(2)P* is essential to male fertility in two-thirds of the flies. The mutation exclusively affects the structure of the spermatozoa in which the organization of the mitochondrion and axonema is perturbed. Nevertheless, *ref(2)P* is expressed in a wide variety of tissues: it is expressed in female germ cells as seen from the strong inhibition of the virus transovarian transmission by restrictive alleles; however females are normally fertile, thus the gene is not essential at the cellular level. It is also expressed in somatic cells, as seen, for instance, from the effects on virus yields and CO_2 sensitivity. This expression is autonomous: in organs transplanted into individuals bearing permissive or restrictive alleles, the action of *ref(2)P* on the virus conforms to the organ genotype.

The effect of restrictive alleles is also observed at the single cell level in permanent *in vitro* cell lines. Homozygotes $ref(2)P^{null}$ are permissive to sigma virus infection, thus the *ref(2)P* gene product serves no indispensable function in the virus cycle and hence could be considered as a defense gene. The expression is ubiquitous. All the transcripts found in different tissues result from the same splicing pattern of three exons but different initiation sites are used. As a consequence, the 5′ untranslated region varies in length according to the cell type. The size distribution of the transcripts and their abundance varies according to the tissue, suggesting a tissue-specific regulation of the gene expression at the transcriptional and possibly at the translational level.

The gene encodes a single protein of about 600 amino acids containing a cysteine-rich region resembling a zinc-finger motif. The amino acid sequence is very variable and there is a length polymorphism in the coding region. Restrictive and permissive alleles are codominant, and *ref(2)P* proteins of both types form complexes with the viral P protein. The *ref(2)P* protein crossreacts with antibodies directed against the viral N protein, due to the presence of a common conformation-dependent epitope. The *hap* viral mutants are mutants which escape the restrictive effect of $ref(2)P^{P}$. In the *hap7* mutant, this epitope is not present and the N protein differs from that of the original virus by a single amino acid substitution.

These data could suggest that the *ref(2)P* protein competes with the N protein by means of this epitope to complex the P protein in a form which is unsuitable for its function in the viral transcriptional complex (which requires the N, P and L proteins). However, the restriction mechanism is certainly more complicated, as a transgene containing only the 91 N-terminal amino acids of a restrictive allele, but not the epitope, is sufficient to confer a restrictive phenotype to transformed *Drosophila* lines in the conditions of artificial inoculation, and not in the hereditary transmission process.

Interestingly, a simultaneous adaptation or disadaptation of a number of *hap* mutants to other *ref* genes, such as $ref(1)H^{b}$, $ref(3)O^{e}$ or $ref(2)M^{m}$, was observed. This covariation suggests that the same viral protein interacts with the cellular proteins encoded by these other *ref* genes.

The variability of natural alleles of the *ref(2)P* gene is high and there is a high rate of amino acid replacement to synonymous codon changes. The most recent alleles are the restrictive ones. A possible explanation is that when a restrictive mutant appears and reaches sufficient frequency in a population, the virus evolves rapidly in response to this new allele, which becomes permissive to the adapted virotype; then a new restrictive mutant may arise and be transiently selected.

The time between inoculation and the first detection of virus progeny is about 30 h at 20°C. All the present knowledge about the viral replication process results from the physiological study of temperature-sensitive and host-range mutants. These mutants identify four steps in the growth cycle of the sigma virus. At 20°C, after a rapid phase of adsorption-penetration (1 h), there is a phase lasting about 8 h which corresponds to the temperature-sensitive period of the mutant *hap7*. The next stage, from 4 to 15 h postinfection, is defined by the mutant *ts4*. The mutants *hap7* and *ts4* have been designated early mutants. *Ts4* is defective in hereditary transmission at the restrictive temperature (28°C); *hap7* is not, even at 30°C. The function affected in *hap7* is indispensable before genome replication, and facultative once genome replication has started.

The next stage is defined by mutants such as *ts9*, in which germinal transmission is not temperature sensitive. In such mutants, designated late mutants,

the genome replication functions are not affected and their temperature-sensitive period corresponds to a phase of virus assembly and budding. The functions necessary to initiate infection are not affected in *ts*9 (as deduced from analyses of infectious center decay) but the virions are thermolabile. The *ts* mutations have not yet been assigned to any gene. In analogy to the molecular biology of other rhabdoviruses, some predictions can be made. In VSV only the three proteins of the viral nucleocapsid (N, P and L) are required for the synthesis of monocistronic capped and polyadenylated mRNAs and, in the next step, of full genome length RNAs.

The matrix protein M is required for the transport of newly synthesized nucleocapsids toward the membrane patches in which the glycoprotein G is inserted and for the budding of progeny virions. The sigma virus counterparts of these proteins are likely to play the same roles. If so, the proteins modified in early mutants may be N, P or L, while late mutants may bear temperature-sensitive M or G proteins. We have no clues which could permit the prediction of the phenotypes of mutants affected in the additional protein X. Nevertheless, the absence of cytopathogenicity of the virus, even in the most permissive host genotype, and even in the most sensitive stages of host development, strongly suggests that the sigma virus multiplication rate is self-restricted. It is tempting to speculate that the protein of yet unknown function, or the gene overlap, or both, could account for this feature. According to the first hypothesis, mutants with disfunctioning X protein would produce higher virus yields. Paradoxically, clones with temperature-sensitive mutations of X would be more invasive to the host and thus have enhanced temperature resistance. Viral strains with such a phenotype exist. High yield and high-temperature resistance in sigma virus clones are always correlated with a pathogenic effect on the germline and the loss of hereditary transmission. Such characteristics are shared by the vesiculoviruses (which do not have an X gene) when inoculated into *Drosophila*.

Ecology

The specific CO_2 sensitivity symptom makes the identification of infected flies very easy and has allowed significant exploration of *Drosophila* natural populations. CO_2-sensitive flies were found among several *Drosophila* species throughout the world. The viruses carried by the different fruit fly populations share the major characteristics of sigma virus (hereditary transmission, symptom, etc.) but they may be distinct. The data indicate that such viruses are endemic in all the populations of *Drosophila,* the infected flies being the minority (10–20% in French natural populations). The high proportion of uninfected flies does not correspond to a virus-resistant fraction of the populations, as they and their offspring may become infected experimentally in the laboratory.

The genetic approach to the study of an insect–virus relationship performed with sigma virus underlines the complexity of the interactions and the difficulties involved in the control of all the relevant parameters.

See also: **Rabies virus (*Rhabdoviridae*); Rhabdoviruses (*Rhabdoviridae*): Plant rhabdoviruses, Ungrouped mammalian, bird and fish rhabdoviruses; Vesicular stomatitus viruses (*Rhabdoviridae*).**

Further Reading

Dezélée S, Bras F *et al* (1989) Molecular analysis of ref(2)P, a *Drosophila* gene implicated in sigma rhabdovirus multiplication and necessary for male fertility. *EMBO J.* 8: 3437.

Fleuriet A (1988) Maintenance of a hereditary virus: the sigma virus in populations of its host, *D. melanogaster.* In: Hecht M and Wallace B (eds) *Evolutionary Biology,* p. 2. New York: Plenum Press.

Landes-Devauchelle C, Bras F, Dezélée S *et al* (1995) Gene 2 of the sigma rhabdovirus encodes the P protein and gene 3 encodes a protein related to the reverse transcriptase of retroelements. *Virology* 213: 300.

Wayne ML, Contamine D and Kreitman M (1996) Molecular population genetics of *ref(2)P,* a locus which confers viral resistance in *Drosophila. Mol. Biol. Evol.* 13: 191.

Wyers F, Petitjean AM, Dzu P *et al* (1995) Localization of domains within the *Drosophila* ref(2)p protein involved in the intracellular control of sigma rhabdovirus multiplication. *J. Virol.* 69: 4463.

Simian Hemorhagic Fever Virus *see* **Encephalitis Viruses and Arteriviruses (*Arteriviridae*)**

Simian Herpesvirus *see* **Herpesvirus Saimiri and Ateles**

SIMIAN IMMUNODEFICIENCY VIRUSES (*RETROVIRIDAE*)

Vito G Sasseville and **Ronald C Desrosiers**, Harvard Medical School, New England Regional Primate Research Center, Southborough, Massachusetts, USA

Hillary G Morrison, Marine Biological Laboratory, Woods Hole, Massachusetts, USA

History

Simian immunodeficiency virus (SIV) was first isolated in 1984 from immunodeficient captive rhesus macaques (*Macaca mulatta*) at the New England Regional Primate Research Center (NERPRC). This virus was originally called STLV-III because of its similar morphology, growth characteristics and antigenic properties to the newly described immunosuppressive virus in humans termed HTLV-III, LAV or ARV. When HTLV-III was renamed human immunodeficiency virus (HIV), STLV-III was appropriately changed to SIV. Retrospective studies show that SIV was introduced to the NERPRC via a group of rhesus monkeys with immunosuppressive disease delivered from another primate center 15 years prior to the initial isolation of SIV in 1985. This original cohort of animals was most likely inadvertently infected with SIV from wild-caught sooty mangabey monkeys housed at the same location. Following this initial isolation of SIV from rhesus macaques, SIV was isolated from other captive macaque species (*M. fascicularis, M. nemestrina, M. arctoides*) dying of diseases associated with immunosuppression and from feral asymptomatic African nonhuman primates, including African green monkeys (SIVagm), mandrills (SIVmnd), sooty mangabey monkeys (SIVsmm), red-capped mangabeys (SIVrcm), Sykes' monkeys (SIVsyk) and chimpanzees (SIVcpz).

Taxonomy and Classification

SIVs belong to the *Lentivirus* genus of the *Retroviridae* family. This genus includes the classic ungulate lentiviruses (visna virus of sheep, caprine arthritis encephalitis virus and equine infectious anemia virus) and the immunodeficiency viruses of humans (HIV), monkeys (SIV), cats (FIV) and cattle (BIV). The retroviruses can be subclassified by morphologic and morphogenic criteria. Lentivirus particles are approximately 80–110 nm in size and consist of an RNA genome and viral enzymes enclosed in a core of viral proteins that is encased by a cell-derived membrane spiked with viral envelope glycoproteins. Lentiviruses can be distinguished from other subgroups of retroviruses by the presence of a cylindrical or rod-shaped nucleoid in mature particles. In lymphocytes, lentivirus particles bud from the plasmalemma into the extracellular space, but in macrophages particles often bud from the plasma membrane into cytoplasmic vacuoles where they can accumulate. Lentiviruses also share a similarity in certain biological properties and the organization of their genomes. Unlike many of the other retroviruses, they are not oncogenic. Instead, they produce long-term, persistent infections which eventually lead to chronic debilitating disease. All lentiviruses studied to date replicate and persist in cells of the monocyte/macrophage lineage. In addition to the standard *gag*, *pol* and *env* genes that all retroviruses have, lentiviruses possess a number of additional genes not found in other retroviruses.

The SIVs are named according to the primate species of origin, e.g. SIVmac from macaques or SIVsmm from sooty mangabey monkeys. Based on genetic sequence analysis, five discrete groups of primate lentiviruses have been identified (**Fig. 1**, **Table 1** and see later). These are HIV-1/SIVcpz, HIV-2/SIVsmm/SIVmac, SIVagm, SIVmnd and SIVsyk.

Geographic and Seasonal Distribution

African green monkeys (*Cercopithecus aethiops*), Sykes' monkeys (*Cercopithecus mitis*), sooty mangabey monkeys (*Cercocebus torquatus atys*), red-capped mangabey monkeys (*Cercocebus torquatus torquatus*) and mandrills (*Papio sphinx*) have been shown to be infected with SIV in their natural habitats. Serological evidence suggests that SIV infection of African green monkeys is widely distributed in Africa. Between 20 and 50% of African green monkeys in Kenya, Ethiopia, South Africa and Senegal have antibodies to SIV. However, green monkeys which became established in the Caribbean since the seventeenth century are seronegative. Recent data also indicate that sooty mangabey monkeys in their native habitat in the coastal forests of western Africa are infected with their own highly divergent SIVs. These SIVsmm isolates, which differ markedly from

Table 1 Primate lentivirus nonstructural genes

Gene	*SIVagm*	*SIVsmm/SIVmac/HIV-2*	*SIVmnd*	*SIVsyk*	*HIV-1/SIVcpz*
vpu	−	−	−	−	+
vpx	+	+	−	−	−
vpr	−	+	+	+	+
tat	+	+	+	+	+
rev	+	+	+	+	+
vif	+	+	+	+	+
nef	+	+	+	+	+

one another, are closely related to HIV-2 subtypes D and E from humans in the same areas. These findings suggest that different HIV-2 subtypes may have originated from multiple cross-species transmissions of divergent SIVsmm strains from sooty mangabeys to humans. Macaque monkeys do not harbor SIV in their native Asian habitat, but became infected presumably via contact with SIV-infected sooty mangabeys while in captivity at Regional Primate Research Centers in the United States. Thus, African nonhuman primates and not Asian macaques are the natural hosts of SIV.

Host Range and Virus Propagation

African green and Sykes' monkeys, sooty and red-capped mangabey monkeys and mandrills appear to be natural hosts for their own discrete SIVs. Other species that are susceptible to infection are restricted to primates including humans (SIVhu) on rare occasions. However, immunosuppressive disease appears to be largely restricted to SIV-infected macaque monkeys. The natural routes of transmission for SIV are in general not known, but most evidence suggests horizontal spread via fighting and biting among group-housed macaques. Experimentally, macaques have been infected via intravenous and intramuscular inoculation and by exposure of the genital mucosa. Some SIVs clearly have the capacity for crossing species barriers. For example, SIVsmm is readily able to infect macaque monkeys. In contrast, numerous attempts to infect macaques and other Old World primates with HIV-1 have met with limited success. Baboons (*Papio cynocephalus*), rhesus and cyno-

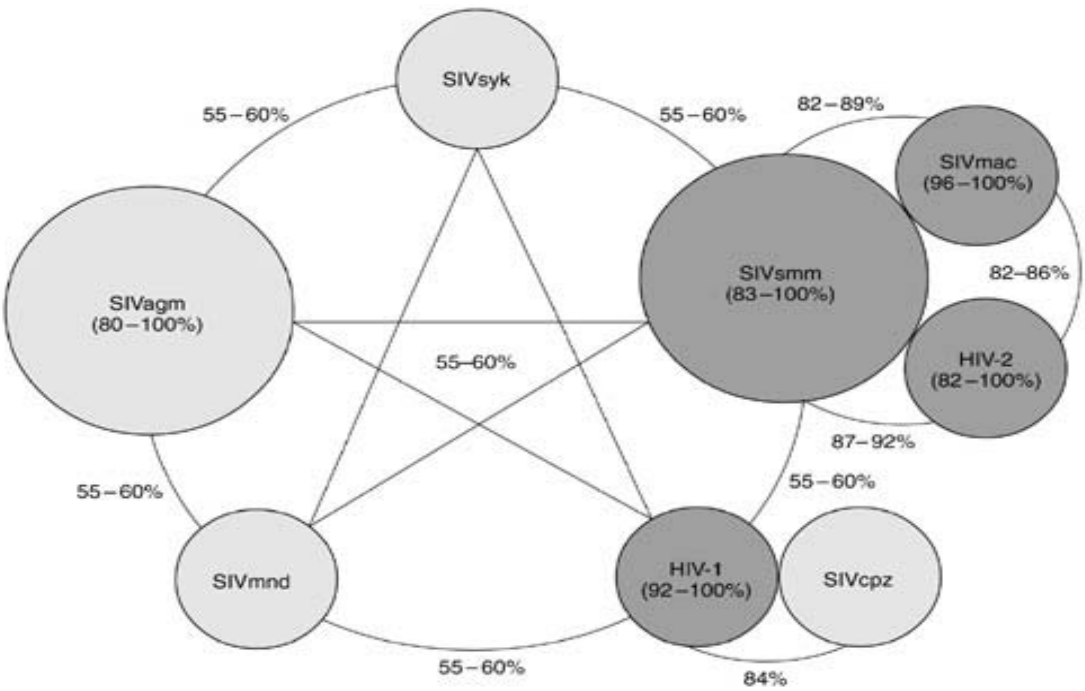


Figure 1 Five groups of primate lentiviruses.

molgus monkeys could not be infected with HIV-1. However, it was discovered that chimpanzees (*Pan troglodytes*) are susceptible to HIV-1 infection, but with the exception of one animal infected for greater than ten years, they have not developed AIDS-like disease. Likewise, pig-tailed macaques (*M. nemestrina*) have been reported to be infected with HIV-1, albeit transiently, but also fail to develop immunosuppression and disease. Although both of these models are important in investigating the interaction of HIV-1 with the host, the lack of clinical disease in most of these animals as well as the expense and endangered species status of chimpanzees limits their usefulness as animal models for AIDS.

SIVs can be propagated in mitogen-stimulated primary peripheral blood mononuclear cells (PBMC), in monocytes/macrophages from primates, and in many cultured cell lines, primarily tumor-derived CD4+ T lymphocytes and monocytes. The acutely pathogenic strains SIVsmmPbj14, SIVmac239YEnef and SIVmac239/R17Y are unusual in their ability to replicate in lymphocytes of resting PBMC cultures without prior lymphocyte activation. In PBMC cultures and many of the cell lines, viral infection results in the fusion of cellular membranes producing large syncytial cells. Syncytium formation, which is mediated by *env*, allows the virus to spread directly from cell to cell. Persistently infected virus-producing cell lines can be established from the cells surviving initial infection. Some isolates also grow well in cultured macrophages derived from lung, blood or bone marrow. As with HIV-1, infection with SIV is predominantly a CD4-mediated event, but CD4 alone is not sufficient to mediate viral entry. Certain chemokine receptors have been shown to be important coreceptors for both SIV and HIV infection. Nonsyncytium-inducing (macrophage tropic) HIV-1 strains predominantly use CCR5, whereas CXCR4 (LESTR/fusin) is utilized primarily by syncytium-inducing (T cell tropic) strains. CCR3, CCR2b, BOB (GPR15) and Bonzo (STRL33) are also utilized by some HIV strains. Similarly, SIV strains have so far been found to utilize CCR5, BOB and Bonzo. As with CD4 interactions with HIV/SIV, coreceptor use is also controlled by *env*. In rare instances, HIV-1 and SIV infection of CD4− cells *in vitro* has been reported. Infection of CD4− cells may be through these or other novel chemokine receptors.

Genetics

Retroviruses contain an RNA genome that replicates via a DNA intermediate. The viral particle contains a diploid genome of single-stranded RNA, linked noncovalently near the 5′ ends of the molecules. The 5′ end of the viral RNA is capped, and the 3′ end is polyadenylated. DNA synthesis by the viral-encoded reverse transcriptase is primed by host tRNA that is base-paired to the viral RNA. The double-stranded (ds) DNA provirus is integrated into the host cell chromosome by a viral-encoded integrase and further events in transcription, translation and assembly are host cell dependent. Particles assemble and bud through the plasma membrane.

All retroviruses possess certain basic features in their genomic organization. Regulatory sequences controlling DNA synthesis, integration, transcription and other functions are located in the long terminal repeat (LTR) at each end of the provirus. LTRs are common to the broader group of eucaryotic transposable elements (retrotransposons) that include the retroviruses. The open reading frames encoding the major structural proteins lie between the LTRs. Genes may be encoded in any of the three possible open reading frames, and overlaps between open reading frames are common. All retroviruses contain genes called *gag* (group-specific antigen) encoding the core proteins, *pol* (polymerase) encoding the viral reverse transcriptase, protease and integrase, and *env* encoding the envelope glycoproteins.

The SIVs similarly contain *gag*, *pol* and *env* genes and use a replication strategy similar to all retroviruses. However, SIVs, HIVs and other lentiviruses contain a number of genes not found in other genera of retroviruses. The SIV genome is approximately 9.6 kb from the 5′ cap to the 3′ polyadenylation site. The sequences of several cloned SIVs have been reported (SIVmac251, SIVmac142, SIVmac239, SIVsmmH4, SIVsmmPBj14, SIVmmGB1, SIVagmTYO1 and SIVcpz). The envelope gene, encoding gp120 and gp41, often contains a premature translation termination signal, resulting in a truncated transmembrane protein. In addition to the major open reading frames *gag*, *pol* and *env*, the human and simian immunodeficiency viruses contain open reading frames for *tat* (transactivator protein), *rev* (regulator of gene expression), *vif* (viral infectivity protein) and *nef* (originally termed negative factor). The *vpu* open reading frame found in HIV-1 and SIVcpz is not present in HIV-2 nor in any of the SIVs. HIV-1/SIVcpz, SIVsmm/HIV-2/SIVmac and SIVmnd contain an open reading frame called *vpr*, which is not found in SIVagm. SIVagm and SIVsmm/HIV-2/SIVmac contain a gene called *vpx* not found in SIVmnd and HIV-1/SIVcpz. Genes *vpx* and *vpr* share sequence similarity and one probably arose from the other via a gene duplication event. The presence of these additional open reading frames in the five discrete groups of primate lentiviruses is summarized in **Table 1**. Several of these additional genes (speci-

fically *vpx*, *vpr* and *nef*) can be deleted without abrogating the ability of the virus to replicate in tissue culture cells, but they certainly must contribute to the virus life cycle *in vivo*. Nef has been found to play an important role in maintaining high virus loads *in vivo* and for disease development. These observations have led to the development of molecular clones of SIV with specific deletions in *vpx*, *vpr* and *nef*, which have been successfully utilized as modified live-virus vaccines in rhesus monkeys.

SIV proteins are translated from a complex population of unspliced, singly spliced and multiply spliced mRNA molecules. The amount of each protein is regulated at least in part by the extent of splicing. The mechanisms that regulate splicing continue to be unraveled, but the interaction of the Rev protein (encoded by fully spliced transcripts) with a region of RNA called the rev-responsive element (RRE) is known to result in the accumulation of full-length transcripts that are translated into the major structural proteins.

SIVs, like other lentiviruses, accumulate genetic changes rapidly *in vivo*, presumably because of errors introduced by the error-prone reverse transcriptase and because of the selective pressures of the host. Lentiviruses may differ from other retroviruses in being able to tolerate greater variation in their envelope glycoproteins and this may contribute to their ability to persist. In one report, the rate of fixation of mutations in the gp120 portion of the envelope gene of SIVmac239 was found to be 8.5×10^{-3} changes per site per year. Mutations in the envelope gene result in antigenic variations that may enable the virus to evade ongoing host immune responses and contribute to its ability to establish persistent infection. Other mutations in the *env* gene appear to affect cell and tissue tropism, for example, by altering the ability of the virus to replicate in macrophages vs lymphocytes. Moreover, recent evidence suggests that env-mediated chemokine receptor usage by HIV changes with time allowing the virus to infect additional populations of leukocytes. Another mechanism that may generate genetic diversity is the production of heterozygous dimer genomes or pseudotype particles in cells that are infected by more than one virus. Endogenous SIV sequences have not been detected in the germ line and gene conversion/recombination events have not been documented.

Evolution

Comparisons of genetic sequences among human and simian immunodeficiency viruses suggest that there are at least five discrete groups of primate lentiviruses in existence (**Figs 1** and **2**): HIV-1 and the closely related SIVcpz; SIVmnd; SIVagm; SIVsmm/SIVmac/HIV-2; and SIVsyk. The evolutionary origin of each of these groups may never be known. It is possible that HIV-1 and HIV-2 evolved from simian viruses and entered the human population by cross-species transmission relatively recently in history. Cross-species transmission between primates in nature or in captivity may have resulted in the generation of new pathogenic variants, with SIVmac infection of macaques possibly being analogous to HIV-2 in humans. However, it is also possible that some of the primate immunodeficiency viruses may have always been present in the corresponding host population but not have been recognized until recently. The SIVs and HIVs are more closely related to one another than to any of the nonprimate lentiviruses. This suggests that the HIVs and SIVs are inherently primate viruses and that they were not derived from rodents, cats, ungulates or other nonprimates via cross-species transmission.

Serologic Relationships and Variability

The *gag* and *pol* genes are the most highly conserved among related primate lentiviruses; sequence comparison of these regions are often used to estimate relatedness rather than serologic tests. Antiserum to the Gag protein is generally crossreactive among different isolates within a group, whereas antiserum to the envelope protein can be used to distinguish between them. Serologic crossreactivity between a member of one group to one in another group is usually weak even to Gag proteins.

Epidemiology

SIV has been found in many species of African nonhuman primates throughout sub-Saharan Africa, but infection does not cause AIDS-like disease. In contrast, SIV infection of Asian macaque monkeys in captivity induces AIDS-like disease similar to that observed in HIV-infected humans. SIV-associated disease has not been seen outside these settings.

Transmission and Tissue Tropism

The routes of transmission of SIV in the wild are not known, but, as with HIV-1, transmission through contact with infected blood seems likely. In nonhuman primates, bite and scratch wounds may be more significant than sexual contact. Macaques can be experimentally infected via intravenous or intramuscular inoculation, or by exposure of the genital mucosa. Like HIV, SIV is tropic for CD4+ cells; both viruses grow preferentially in CD4+ cells, and soluble CD4 or monoclonal antibodies specific to the CD4

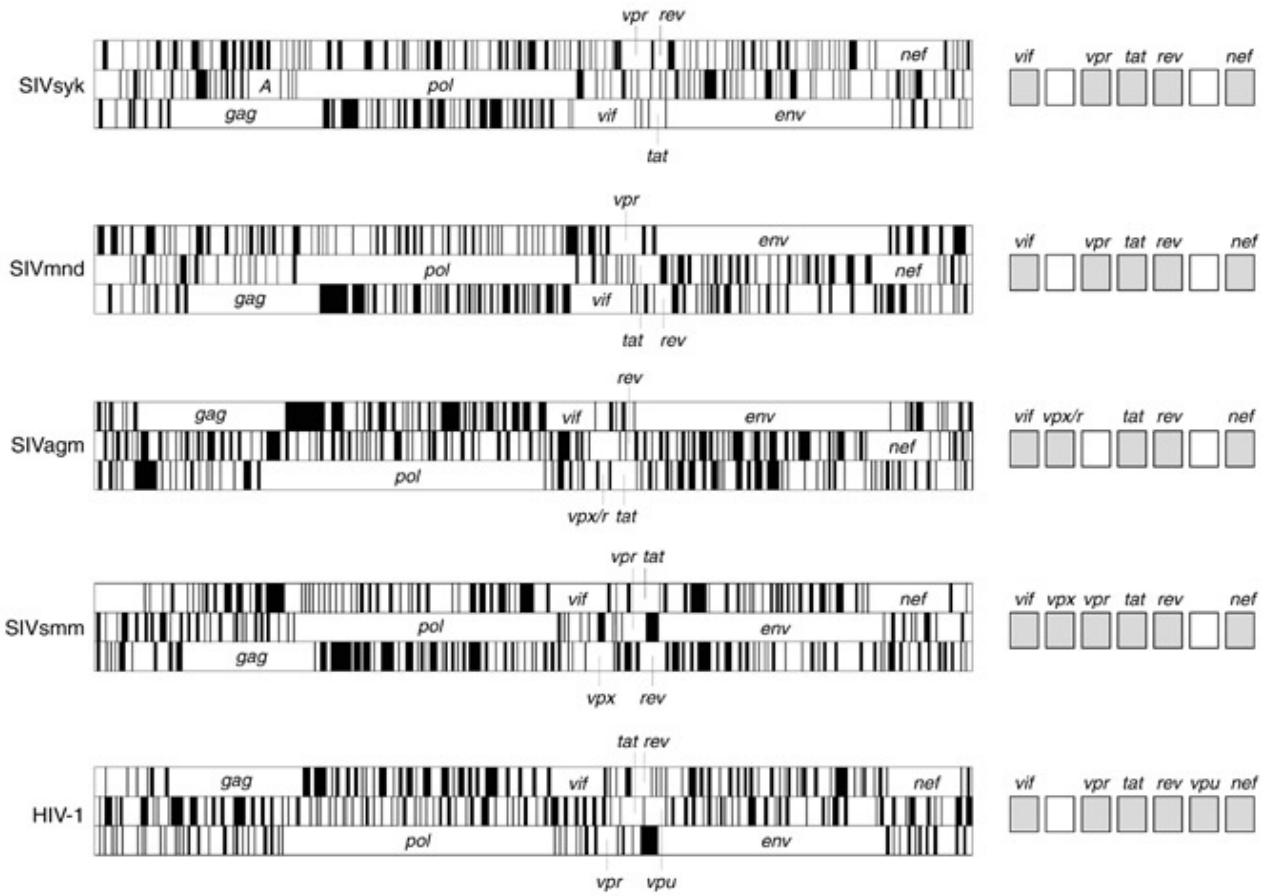


Figure 2 Organization of open reading frames in the five groups of primate lentiviruses. Reproduced from Hirsch VM, Dapolito GA, Goldstein S *et al* (1993) *Journal of Virology*, 67: 1517. Permission granted by American Society for Microbiology.

antigen can block viral infection. Recently, it has been shown that several coreceptors are required for *env*-mediated cell fusion and entry of HIV and SIV into CD4+ cells. These coreceptors are all members of the seven-transmembrane G-protein-coupled receptor family; many serving as chemokine receptors. Of the chemokine receptors used by HIV (CCR5, CCR2b, CCR3, CXCR4, BOB and Bonzo), SIV has so far been found to utilize CCR5, BOB and Bonzo. The primary targets for infection in the macaque are CD4+ helper/inducer T lymphocytes, mononuclear phagocytes, Langerhans cells and dendritic cells of lymphoid organs. Thus, lymphoid tissues (thymus, spleen, lymph nodes, gut-associated lymphoid tissue) are the principal targets of virus infection and replication. Cells of the monocyte/macrophage lineage are also major cell types replicating virus in the infected host. Infection of CD4− cells, such as endothelium, neurons, astrocytes, various epithelial cells and CD34 bone marrow progenitors, has been reported *in vitro*, but infection *in vivo* is controversial.

Pathogenicity

Natural infection of African green and Sykes' monkeys, red-capped and sooty mangabeys and mandrills with their own SIV appears to be non-pathogenic. SIVmac and SIVsmm can be pathogenic in rhesus, cynomolgus, stump-tail (*M. arctoides*) and other macaques. Disease course is dependent on the viral strain used for infection and host immune response to the virus. Acutely pathogenic strains (SIVsmmPBj14, SIVmac239YEnef and SIVmac239/R17Y) can cause death from severe enterocolitis within seven to ten days postinfection. Other strains containing gene deletions (specifically *vpx*, *vpr* and *nef*) are able to replicate *in vitro* and *in vivo*, but are markedly attenuated and rarely cause mortality. However, these clinical outcomes are the exception and most animals infected with a variety of other SIV strains generally succumb within 1–3 years postinfection with immunosuppressive disease. The induction of AIDS-like disease in macaques following infection

with SIVmac is currently used as a model for human AIDS to investigate mechanisms of pathogenesis and to evaluate potential vaccines and therapies.

Clinical Features of Infection

Infection of rhesus macaques with SIV induces both acute and chronic disease syndromes similar to that of HIV-infected humans. Within the first few weeks of infecting macaque monkeys with most strains of SIV, animals develop antigenemia and often have lymphadenopathy of the axillary and inguinal lymph nodes and an erythematous maculopapular rash. A CD8+ T cell lymphocytosis and rise in SIV-specific antibodies correlate with decreased antigenemia. Following the onset of an effective immune response (cell-mediated and humoral), animals enter an asymptomatic period of variable duration. During this time, immune abnormalities include gradual declines in the absolute CD4+ lymphocyte count, CD4/CD8 ratio, and responses to mitogens. Animals can also exhibit chronic diarrhea and wasting, with losses of up to 60% of original body weight. Lymphoid changes are profound and include thymic atrophy and hyperplasia or atrophy of lymphoid tissue (lymph nodes, spleen, gut-associated lymphoid tissue) depending on the stage of the disease. End-stage of SIV infection is characterized by a spectrum of diseases that can be divided into four broad categories: SIV-related inflammatory diseases; opportunistic infections associated with immunosuppression; neoplastic diseases; diseases of unknown pathogenesis. Tropism of SIV strains for monocyte/macrophages correlates with pronounced inflammatory and degenerative changes in the central nervous system (CNS), lung, digestive tract, and other organs independent from pathology associated with opportunistic pathogens. The incidence and characteristics of these lentivirus-induced inflammatory lesions in SIV-infected macaque monkeys closely mimics those observed in HIV-infected patients. CNS involvement (SIV encephalitis) is frequent, with the likelihood of brain lesions appearing to depend on the strain of SIV. At necropsy, 30–50% of SIVmac-infected macaques have a characteristic multinucleate giant cell encephalitis similar to that described for HIV encephalitis. Likewise, the opportunistic pathogens observed in SIV-infected macaques are similar to those observed in HIV-infected patients and include *Mycobacterium avium* complex, cytomegalovirus, rhesus Epstein–Barr virus (rhesus lymphocryptovirus), papovavirus (SV40), adenovirus, *Pneumocystis carinii, Cryptosporidia* sp. and *Toxoplasma gondii*. Neoplastic diseases in SIV-infected macaques are primarily limited to lymphomas, the occurrence of which varies among the different Primate Research Centers. Epizootics of lymphoma in SIV-infected macaques have been associated with co-infection with rhesus Epstein–Barr virus. Diseases of unknown pathogenesis include a nonneoplastic proliferation of lymphoid tissue (lymphoproliferative syndrome), arteritis and arteriopathy.

The course of disease in pigtailed macaques infected with SIVsmmPBj14 virus and rhesus macaques infected with SIVmac239YEnef and SIVmac239/R17Y is extremely rapid in comparison to most other strains of SIV. Macaques infected with these strains usually develop marked lymphoid proliferation, maculopapular rash and profuse, hemorrhagic diarrhea within one week of infection, and can die from severe fluid and electrolyte loss.

Pathology and Histopathology

Pathologic findings in macaques are varied and depend on the clinical course of disease. Within a few weeks of infecting macaque monkeys with most strains of SIV, an erythematous maculopapular eruption can occur, which is histologically identical to that described in primary HIV-1 infection. Vessels of the superficial dermis are congested and contain perivascular infiltrates of lymphocytes and macrophages. SIV nucleic acid and protein have been observed only in biopsies obtained from rhesus macaques infected with the acutely pathogenic strains SIVmac239YEnef and SIVmac239/R17Y, which induce a rash of greater severity and earlier onset than that observed with other strains of SIV.

Lymphoid tissues are the principal targets for SIV and a range of histologic changes are observed including follicular hyperplasia and dysplasia, follicular involution and hyperplasia of the T cell-dependent areas, and lymphoid depletion. These histologic changes represent successive stages in the reaction of lymphoid tissue to persistent SIV infection. In addition, some animals develop a generalized lymphoproliferative syndrome in which lymphoid tissues throughout the body become enlarged due to a nonneoplastic proliferation of lymphocytes. Multiple nodular aggregates of similar cells frequently are found in other organs and tissues of affected animals, including the salivary gland, kidney, lung, liver, bone marrow, skin and thymus.

Approximately 50% of infected animals develop primary lentiviral-induced meningoencephalitis, which is characterized by perivascular infiltrates of macrophages and multinucleated giant cells localized primarily in the white matter. These perivascular macrophages and multinucleated giant cells contain abundant viral nucleic acid and protein and contain

mature lentiviral particles within cytoplasmic vacuoles. These findings are similar to reports on HIV-infected patients with HIV encephalitis. Other SIV-induced lesions in macaques are multinucleate giant cell infiltrates in the lymph nodes, spleen, lung, gastrointestinal tract and other organs.

Other inflammatory lesions, which are histologically distinct from those directly related to SIV, are usually secondary to opportunistic infections. In the CNS, these include SV40-induced progressive multifocal leukoencephalopathy and cytomegalovirus- and *T. gondii*-induced necrosuppurative meningoencephalitis. Rarely, in the absence of a significant inflammatory response to opportunistic infections, the presence of the abnormally large numbers of organisms can cause morbidity and mortality. For instance, *P. carinii* commonly proliferates unabated to the point of filling most alveoli within lung lobes; eventually killing the host.

In SIV-infected macaques, diarrhea can apparently be caused directly by SIV. In these cases, nonspecific enteropathy consisting of blunting of the small intestinal villi, shortening of the crypts of Lieberkuhn, a predominantly mononuclear inflammatory infiltrate within the lamina propria, and attenuation or immaturity of the epithelium is seen. However, diarrhea is most frequently associated with overgrowth of commensal and pathogenic protozoa (i.e. *Cryptosporidia* sp., *Giardia* sp, *Balantidium coli, Trichomonas* sp.) and bacteria (*Mycobacterium avium*). *Cryptosporidia* sp. also infects the tracheal, pancreatic and biliary epithelium. Unexplained arterial lesions have been seen in SIV-infected macaques. These most frequently involve medium- to large-sized pulmonary vessels and include a spectrum of histologic patterns from transmural lymphocytic infiltrates to complete occlusion of the lumen secondary to intimal proliferation and thrombosis. Other organs, such as heart and kidney, may also have SIV-induced abnormalities, but these have not been well characterized.

Immune Response

Depending on viral isolate and host immune response, the disease course in SIV-infected macaque monkeys can be quite variable, as in HIV-infected patients. Experimentally infected animals generally fall into two classes: those that develop a high, persistent humoral and cellular immune response, and those with little or no response. Although the early immune response does limit viral replication, it does not control it. In both HIV-infected people and SIV-infected macaque monkeys, viremia is persistent and sustained by continuous rounds of viral replication throughout the course of infection. Animals in the first group remain persistently infected, develop a protracted disease course similar to AIDS in humans, and generally die one year or longer after infection. Viral load in the blood is variable with higher loads being predictive of rapid disease progression. Approximately 25% of animals do not mount a significant immune response to SIV and viral loads in the blood remain high throughout the course of infection. Generally, these animals die within 3–5 months and are termed rapid progressors. In addition to virus factors, unique host factors may also be important for disease susceptibility and progression.

Prevention and Control

Extensive testing programs have essentially eliminated SIV from captive macaque colonies. However, continued vigilance is needed to maintain breeding colonies free from accidental exposure to the virus. Animals can be conveniently tested serologically for evidence of infection. As rare human cases of SIV infection have been documented, SIV is handled with the same precautions as for work with HIV-1. Disposable surgical gloves and gowns are used, all work with live virus is carried out in a biosafety cabinet, procedures creating aerosols are avoided and use of glass and needles is minimized.

Future Perspectives

The most important role of SIV will be its use in basic research relevant to AIDS. The induction of AIDS in macaques by infectious molecular clones of SIV represents the best existing animal model for AIDS in humans. SIVs are the closest known relatives of the HIVs and the disease induced in macaques is remarkably similar to AIDS in humans. Rhesus and other macaque species are not endangered, can be purchased at a reasonable cost and breed well in captivity. This system can be used to dissect the molecular determinants of AIDS pathogenesis; to define the role of the so-called nonessential genes; to map functional regions of the structural genes such as the envelope; and to evaluate the potential of new treatment and vaccine strategies. Finally, the ongoing investigation of 'new' isolates and their genetic relatedness to existing SIV and HIV isolates may shed light on the origins and evolution of the primate immunodeficiency viruses.

See also: **Autoimmunity; Bovine immunodeficiency virus (*Retroviridae*); Feline immunodeficiency virus (*Retroviridae*); Human immunodeficiency viruses (*Retroviridae*): Anti-retroviral agents,**

General features, Molecular biology; Immune response: Cell mediated immune response, General features; Persistent viral infection; Visna-Maedi viruses (*Retroviridae*).

Further Reading

Desrosiers RC (1990) The simian immunodeficiency viruses. *Annu. Rev. Immunol.* 8: 557.

Desrosiers RC and Ringler DJ (1989) The use of simian immunodeficiency viruses for AIDS research. *Intervirology* 30: 301.

Eichberg W (ed.) (1990) Nonhuman primate models for AIDS II. *J. Med. Primatol.* 19: 161.

Gardner MB (1996) The history of simian AIDS. *J. Med. Primatol.* 25: 148.

King NW (1993) Simian immunodeficiency virus infections. In: Jones TC, Mohr U and Hunt RD (eds) *Nonhuman Primates I*, p. 5. Berlin, Heidelberg: Springer.

SIMIAN VIRUS 40 (*PAPOVAVIRIDAE*)

Janet S Butel, Division of Molecular Virology, Baylor College of Medicine, Houston, USA

History

Simian virus 40 (SV40) was discovered in 1960 as a contaminant of poliovaccines. Hundreds of millions of people worldwide were inadvertently exposed to SV40 in the late 1950s and early 1960s when they were administered contaminated virus vaccines prepared in rhesus macaque kidney cells. Infectious SV40 had unknowingly contaminated batches of both the inactivated and live attenuated forms of the poliovaccine, as well as preparations of some other viral vaccines. Although primary cultures of monkey cells were known to be commonly contaminated with indigenous viruses and safety testing was carried out, SV40 had escaped detection because it failed to induce cytopathic effects in rhesus cells. However, when it was inoculated into African green monkey kidney cells, a prominent cytoplasmic vacuolization developed. Originally christened as 'vacuolating virus', the name was later changed to SV40 to conform with a numerical system of designating simian virus isolates.

Concern about the vaccine contaminations heightened considerably when it was found in 1962 that SV40 was tumorigenic in newborn hamsters and could transform many types of cells in culture. Because of the potential risk to public health posed by the previous distribution of contaminated poliovaccines, SV40 became the focus of intensive investigation. Fortunately, the individuals exposed to SV40-contaminated vaccines appear not to be at higher risk of developing cancer than those who received SV40-free vaccines. However, SV40 DNA is sometimes found in tumors arising in persons too young to have been exposed to the contaminated vaccines. For scientists, SV40 has turned out to be an invaluable tool for dissecting molecular details of eukaryotic cell processes. Numerous techniques now commonly used in molecular biology were pioneered in the SV40 system. It continues to serve as a leading model for basic studies of viral carcinogenesis.

Taxonomy and Classification

SV40 is classified as a member of the *Polyomavirus* genus in the *Papovaviridae* family (**Table 1**). The other well-studied member of the genus is polyoma virus of mice. The group also includes the human polyomaviruses, BKV and JCV, as well as isolates from other species, including hamsters, rabbits, cattle, birds and baboons. The papillomaviruses are classified in the other genus, *Papillomavirus*, in the family. The human and animal polyomaviruses are antigenically distinct, and there is only one recognized serotype for each virus.

The polyomaviruses are small and simple and share certain physical and chemical properties. These include an icosahedral capsid about 45 nm in diameter that contains three viral proteins, the lack of an envelope and a double-stranded (ds) circular covalently closed DNA genome about 5 kbp in size. The outstanding biological characteristics of the polyomaviruses are that they establish persistent infections in natural hosts, stimulate cellular DNA synthesis in infected cells, and are tumorigenic in the appropriate hosts.

Properties of the Virion

SV40 particles are small and spherical, with a diameter of approximately 45 nm. Infectious virions

General features, Molecular biology; Immune response: Cell mediated immune response, General features; Persistent viral infection; Visna-Maedi viruses (*Retroviridae*).

Further Reading

Desrosiers RC (1990) The simian immunodeficiency viruses. *Annu. Rev. Immunol.* 8: 557.

Desrosiers RC and Ringler DJ (1989) The use of simian immunodeficiency viruses for AIDS research. *Intervirology* 30: 301.

Eichberg W (ed.) (1990) Nonhuman primate models for AIDS II. *J. Med. Primatol.* 19: 161.

Gardner MB (1996) The history of simian AIDS. *J. Med. Primatol.* 25: 148.

King NW (1993) Simian immunodeficiency virus infections. In: Jones TC, Mohr U and Hunt RD (eds) *Nonhuman Primates I*, p. 5. Berlin, Heidelberg: Springer.

SIMIAN VIRUS 40 (*PAPOVAVIRIDAE*)

Janet S Butel, Division of Molecular Virology, Baylor College of Medicine, Houston, USA

History

Simian virus 40 (SV40) was discovered in 1960 as a contaminant of poliovaccines. Hundreds of millions of people worldwide were inadvertently exposed to SV40 in the late 1950s and early 1960s when they were administered contaminated virus vaccines prepared in rhesus macaque kidney cells. Infectious SV40 had unknowingly contaminated batches of both the inactivated and live attenuated forms of the poliovaccine, as well as preparations of some other viral vaccines. Although primary cultures of monkey cells were known to be commonly contaminated with indigenous viruses and safety testing was carried out, SV40 had escaped detection because it failed to induce cytopathic effects in rhesus cells. However, when it was inoculated into African green monkey kidney cells, a prominent cytoplasmic vacuolization developed. Originally christened as 'vacuolating virus', the name was later changed to SV40 to conform with a numerical system of designating simian virus isolates.

Concern about the vaccine contaminations heightened considerably when it was found in 1962 that SV40 was tumorigenic in newborn hamsters and could transform many types of cells in culture. Because of the potential risk to public health posed by the previous distribution of contaminated poliovaccines, SV40 became the focus of intensive investigation. Fortunately, the individuals exposed to SV40-contaminated vaccines appear not to be at higher risk of developing cancer than those who received SV40-free vaccines. However, SV40 DNA is sometimes found in tumors arising in persons too young to have been exposed to the contaminated vaccines. For scientists, SV40 has turned out to be an invaluable tool for dissecting molecular details of eukaryotic cell processes. Numerous techniques now commonly used in molecular biology were pioneered in the SV40 system. It continues to serve as a leading model for basic studies of viral carcinogenesis.

Taxonomy and Classification

SV40 is classified as a member of the *Polyomavirus* genus in the *Papovaviridae* family (**Table 1**). The other well-studied member of the genus is polyoma virus of mice. The group also includes the human polyomaviruses, BKV and JCV, as well as isolates from other species, including hamsters, rabbits, cattle, birds and baboons. The papillomaviruses are classified in the other genus, *Papillomavirus*, in the family. The human and animal polyomaviruses are antigenically distinct, and there is only one recognized serotype for each virus.

The polyomaviruses are small and simple and share certain physical and chemical properties. These include an icosahedral capsid about 45 nm in diameter that contains three viral proteins, the lack of an envelope and a double-stranded (ds) circular covalently closed DNA genome about 5 kbp in size. The outstanding biological characteristics of the polyomaviruses are that they establish persistent infections in natural hosts, stimulate cellular DNA synthesis in infected cells, and are tumorigenic in the appropriate hosts.

Properties of the Virion

SV40 particles are small and spherical, with a diameter of approximately 45 nm. Infectious virions

Table 1 Properties of SV40

Classification	Family *Papovaviridae*, genus *Polyomavirus*
Strain variation	Genetically stable; one serotype, multiple strains
Virion	Icosahedral, 45 nm in diameter, no envelope
Genome	Circular covalently closed dsDNA, 5200 bp
Proteins	Three structural proteins, VP1, VP2, VP3; cellular histones condense DNA in virion; nonstructural replication protein, T-antigen, is potent oncoprotein
Replication	In certain primate kidney cells; nucleus; stimulate cell DNA synthesis; long growth cycle
Natural host	Asian macaques, especially the rhesus monkey
Diseases	Asymptomatic persistent infections in natural hosts; neurological disease in immunocompromised hosts; tumors in experimentally infected rodents
Historical note	Contaminant in early poliovaccines administered to millions of people

have a sedimentation coefficient of 240S in sucrose and band at a density of 1.34 g ml^{-1} in CsCl; empty capsids have a density of 1.29 g ml^{-1}. The molecular mass of the SV40 virion has been estimated at 270 kDa. The DNA content is 12.5% (w/w). The major capsid protein (VP1) accounts for 75% of the total virion protein. VP2 and VP3 are minor capsid proteins. Cellular histones (H2A, H2B, H3, H4) are used to condense the viral DNA for packaging and are present in the core of the particle. There is no lipid envelope. SV40 does not agglutinate erythrocytes.

SV40 particles exhibit icosahedral symmetry. The virion is composed of 72 pentameric capsomeres composed of the VP1 protein arranged on a $T = 7d$ icosahedral surface lattice. This puzzling structure (that the hexavalent capsomeres have pentameric substructure) demands nonequivalent contacts between pentamers. This seems to be accomplished by the C-termini of the VP1 polypeptides, which extend as arms from one pentamer and fit into binding sites on adjacent pentamers. The arms can go in different directions, providing the necessary flexibility to build a capsid. The N-terminal arm of VP1 is completely internal in the virus particle. Minor capsid proteins VP2 and VP3 are predominantly internal as well and do not contribute to the basic structure of the virus outer shell.

The virus particles are very resistant to heat inactivation but are relatively labile when heated in the presence of divalent cations. Whereas SV40 is stable at 50°C for hours, incubation in the presence of 1 M $MgCl_2$ at 50°C for 1 h will inactivate the virus. At a higher temperature (60°C), ~99% of infectious virus is inactivated within 30 min in the absence of divalent cations. Purified virions can be disrupted by strong alkaline conditions (pH 10.5), by lower pH (9.2) plus a reducing agent, or by detergent treatment. Intact virus particles are not affected by nucleases, but in the presence of a reducing agent nuclease can enter the virion and cleave the viral DNA. SV40 is efficiently inactivated by UV light irradiation, following single-hit kinetics.

Properties of the Viral Genome

The SV40 genome is a circular covalently closed dsDNA molecule (**Fig. 1**). The native DNA assumes a superhelical configuration (form I) that sediments at 21S in a neutral sucrose gradient. A single-stranded (ss) nick generates relaxed circular dsDNA molecules (form II) that sediment at 16S, whereas a ds break produces linear dsDNA (form III, 14S). Alkaline denaturation of form I DNA produces dense cyclic coils that sediment at 53S. Form II DNA is converted into ss circular (18S) and ss linear (16S) molecules by denaturation. The supercoiled (form I) molecules can be separated from relaxed circular and linear forms by centrifugation of a DNA preparation in CsCl gradients with ethidium bromide. The form I molecules will band in a lower position in the gradient. The DNA forms also separate during electrophoresis in a neutral agarose gel; the supercoiled molecules migrate the fastest, the linear forms move at an intermediate speed, and the relaxed circles migrate the slowest.

The viral DNA both in virions and in infected cells is associated with cellular histones H2A, H2B, H3 and H4. The histones are assembled in 24–26 nucleosomes on the viral DNA. The nucleosome structure and histone composition of the viral minichromosome mimic the chromatin structure of cellular DNA.

SV40 DNA was the first eukaryotic viral genome to be physically mapped by restriction endonuclease analysis (1971) and to be completely sequenced (1978). The DNA of reference strain 776 contains 5243 bp for a calculated molecular weight of 3.5×10^6. The genome is numbered in a clockwise direction from 1 to 5243, the center nucleotide of the unique *BglI* recognition site being assigned as 0/5243. Numbering continues through the late region in the

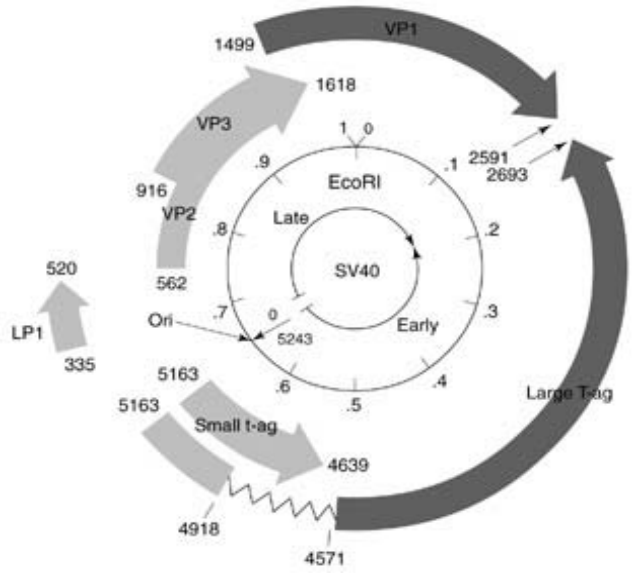


Figure 1 Genetic map of SV40. The thick circle represents the circular SV40 DNA genome. The unique *Eco*RI site is shown at map unit 0/1. Nucleotide numbers begin and end at the origin (*Ori*) of viral DNA replication (0/5243). Boxed arrows indicate the open reading frames that encode the viral proteins. Arrowheads point in the direction of transcription; the beginning and end of each open reading frame is indicated by nucleotide numbers. Various shadings depict different reading frames used for different viral polypeptides. Note that T-ag is coded by two noncontiguous segments on the genome. The genome is divided into 'early' and 'late' regions that are expressed before and after the onset of viral DNA replication, respectively. Only the early region is expressed in transformed cells. (Reproduced with permission from Brooks GF, Butel JS and Ornston LN (1998) *Jawetz, Melnick & Adelberg's Medical Microbiology*, 21st edn. Norwalk, CT: Appleton & Lange.)

sense orientation and the early region in the antisense orientation. The numbering system begins and ends (0/5243) in the middle of the functional origin of DNA replication. The unique *Eco*RI site at nucleotide 1782 was arbitrarily chosen as a point of reference and assigned a value of 0/1.0 on the circular map. Laboratory-adapted strains of SV40 contain a duplication of the 72 bp element in the enhancer region, whereas most natural isolates do not.

SV40 makes maximal use of a limited amount of genetic information, including having compact regulatory sequences and overlapping genes. The genome contains a single origin of replication (core *Ori* = 64 bp in size) embedded within a nontranslated regulatory region. These elements control transcription and replication and span about 400 bp. The SV40 genetic map is divided into two halves, corresponding to regions that are expressed during the early and late stages of infection. These regions represent the 'early' nonstructural genes and the 'late' structural genes, respectively, and are transcribed off opposite strands of the viral DNA.

There is a variable domain at the C terminus of the *T-ag* gene, encompassing about 270 bp. Nucleotide changes within this region can be used to distinguish strains of SV40.

Properties of Viral Proteins

SV40 encodes six gene products: two 'early' nonstructural proteins [large T antigen (T-ag), small t antigen (t-ag)], three 'late' structural proteins (VP1, VP2, VP3) and a maturation protein (LP1 or agnoprotein).

The nonstructural proteins are expressed early in infection, before the onset of viral DNA synthesis. The coding regions of the two T-ags overlap; alternative splicing of viral transcripts determines each protein sequence. Large T-ag of strain 776 (**Table 2**) contains 708 amino acids (~90 kDa), and small t-ag contains 174 residues (~20 kDa). The large and small T-ags share 82 N-terminal amino acids, whereas the remainder of each protein is unique. The T-ag/t-ag common exon contains a 'J domain,' believed to modulate hsc70 activity in the assembly and disassembly of multiprotein complexes.

Large T-ag is an essential replication protein required for initiation of viral DNA synthesis. It stimulates host cells to enter S phase and undergo DNA synthesis and is the SV40 transforming protein. Large T-ag contains a nuclear transport signal (Pro126-Lys-Lys-Lys-Arg-Lys-Val132) that targets the protein into the nucleus. However, about 10% of the T-ag in the cell is found in the cytoplasm and the plasma membrane. The biology of small t-ag is enigmatic. It is a cytoplasmic protein that is not essential for viral replication in cultured cells. It associates with the regulatory and catalytic subunits (36 kDa and 63 kDa) of protein phosphatase 2A and is believed to cause cellular growth stimulation. Perhaps it is required during natural infections by SV40 in host primates.

Large T-ag is a multifunctional protein that is chemically modified in several ways (**Fig. 2**). Its functions in SV40 DNA replication are regulated by phosphorylation. The sites of phosphorylation are clustered near the ends of the molecule, one region lying between residues 106 and 124 and the other between residues 639 and 701. The majority of the phosphorylated residues are serines, although two threonine residues also become phosphorylated. Unlike many oncoproteins, T-ag is not phosphorylated at tyrosine residues.

T-ag is a DNA-binding protein that recognizes multiple copies of the sequence GAGGC in three T-

Table 2 Properties and functions of SV40 T-ag

Structural properties

1. Size:
   - 708 amino acids
   - 82 N-terminal residues shared with t-ag
   - 81 632 Da
   - M_r 90 000–100 000
2. Modifications:
   - Phosphorylation
   - N-terminal acetylation
   - *O*-glycosylation
   - Poly-ADP-ribosylation
   - Palmitylation
   - Adenylation
3. Supramolecular structure
   - Zinc finger
   - Nuclear localization signal
   - J domain
   - Monomers, dimers, higher homooligomers
   - Heterooligomers with transcriptional coactivators (CBP, p300, p400)
   - Heterooligomers with DNA polymerase α; hsc70; cdc-2, cyclin, and tubulin
   - Heterooligomers with tumor suppressor proteins (p53, pRB, p107, p130)

Subcellular distribution

1. Nuclear:
   - Nucleoplasmic
   - Chromatin bound
   - Nuclear matrix associated
2. Plasma membrane:
   - Nonidet P-40 soluble
   - Nonidet P-40 insoluble (plasma membrane lamina)
   - Butanol soluble

Functions

1. Specific DNA binding (viral origin of replication)
2. Initiation of viral DNA replication
3. Autoregulation of viral early transcription
4. Induction of viral late transcription
5. Determination of host range
6. ATPase activity
7. Helicase activity
8. Complex formation with CBP, p300, p400
9. Complex formation with cellular proteins p53, pRB, p107, p130
10. Complex formation with DNA polymerase α
11. Complex formation with heat shock protein hsc70; cdc-2 and cyclin; tubulin
12. Entry of cells into S phase and initiation of cellular DNA replication
13. Induction of cellular gene expression and enzyme synthesis
14. Adenovirus helper function
15. Initiation and maintenance of cellular transformation
16. Induction of immunity to SV40 tumor cells
17. Target for cytotoxic T cells (TSTA)

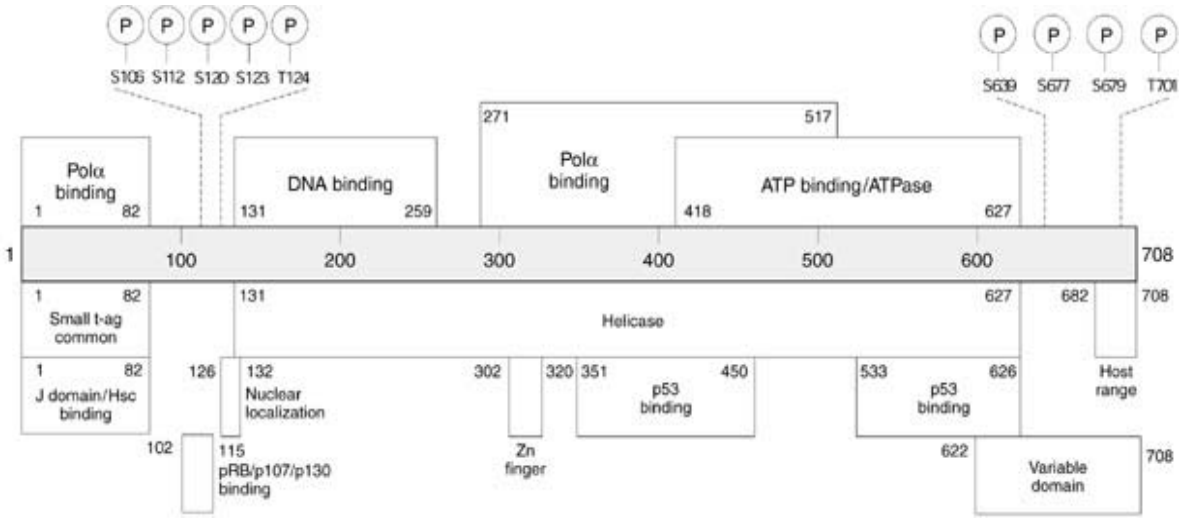


Figure 2 Functional domains of SV40 large T-ag. The numbers given are the amino acid residues using the numbering system for SV40-776. Regions are indicated as follows. Small t-ag common: region of large T-ag encoded in the first exon. The amino acid sequence in this region is common to both large T-ag and small t-ag. Polα binding: regions required for binding to polymerase α-primase. J domain/Hsc binding: region required for binding the heat shock protein hsc70. pRB/p107/p130 binding: region required for binding of the RB tumor suppressor protein, and the RB-related proteins p107 and p130. Nuclear localization: contains the nuclear localization signal. DNA binding: minimal region required for binding to SV40 *Ori* DNA. Helicase: region required for full helicase activity. Zn finger: region which binds zinc ions. p53 binding: regions required for binding the p53 tumor suppressor protein. ATP binding/ATPase: region containing the ATP binding site and ATPase catalytic activity. Host range: region defined as containing the host range and Ad helper functions. Variable domain: region containing amino acid differences among viral strains. The circles containing a P indicate sites of phosphorylation found on large T-ag expressed in mammalian cells. S indicates a serine and T indicates a threonine residue. (Reproduced with permission from Stewart AR, Lednicky JA, Benzick US, Tevethia MJ and Butal JS (1996) Identification of a variable region at the carboxy terminus of SV40 large T-antigen. *Virology* 221: 355.)

ag-binding sites in the viral *Ori*. The phosphorylation of one amino acid, Thr124, is crucial for T-ag to be able to bind to site II and to initiate viral DNA replication. The minimal origin-specific DNA-binding domain of T-ag lies between residues 131 and 259. T-ag is predicted to have a zinc finger motif, typical of DNA-binding proteins, between amino acids 302 and 320. T-ag-specific ATPase and helicase activities are required in addition to DNA-binding activity in order for T-ag to function in initiation of DNA replication. The ATP-binding domain of T-ag is similar in structure to other ATP-binding proteins and is located between residues 418 and 627.

Large T-ag forms complexes with several cellular proteins. Such interactions are involved in T-ag functions in viral DNA replication and induction of cellular DNA synthesis. Target cellular proteins found in heterooligomeric structures with T-ag include transcriptional coactivators (CBP, p300, p400), tumor suppressor proteins (p53, pRB, p107, p130), DNA polymerase α, the molecular chaperone heat-shock protein hsc70, cell-cycle regulatory proteins cdc-2 and cyclin and tubulin. The indicated cellular proteins are not all found in the same T-ag-associated complex; many subpopulations of T-ag exist in a cell.

The variable domain at the C terminus of T-ag encompasses the host range-adenovirus helper function exhibited by SV40 (and mapped to T-ag) in some monkey kidney cell lines. The significance of the variable domain to natural infections by the virus is unknown.

The structural (capsid) proteins are expressed late in infection, after the onset of DNA replication. They are synthesized in much greater abundance than the early proteins. The major capsid protein, VP1, contains 362 amino acids (~45 kDa). The minor structural proteins are VP2 (352 residues, ~38 kDa) and VP3 (234 residues, ~27 kDa). The coding regions for VP2 and VP3 overlap, and they are translated in the same reading frame, so the sequence of VP3 is identical to the C-terminal two-thirds of VP2. VP3 is synthesized by independent initiation of translation via a leaky scanning mechanism. It is not a proteolytic cleavage product of VP2. The N-terminal portion of VP1 is derived from sequences that encode the C-termini of VP2 and VP3. However, VP1 is translated in a different reading frame from a different spliced transcript, so it shares no sequences with VP2 and VP3. VP1 is modified by phosphorylation and acetylation.

The late proteins are required only for the assembly

of progeny virions during lytic infection. They are not involved in the early phases of viral replication. They are synthesized in the cytoplasm and move into the nucleus where particle morphogenesis occurs. The minor capsid proteins contain nuclear transport signals. The VP2/3 signal is Gly-Pro-Asn-Lys-Lys-Lys-Arg-Lys-Leu (VP2, residues 316–324; VP3, residues 198–206). For VP1, two clusters of basic residues within the N-terminal 19 residues are independently important for nuclear targeting. Mutations in VP1 affect capsid assembly and/or virion stability. Mutations in VP2 and VP3 affect the uncoating process, when virions penetrate new host cells.

The agnoprotein LP1 is synthesized late in infection but is not found in virus particles. It is a small (62 residue, ~8 kDa) basic protein involved in particle assembly. It is believed that LP1 interacts with VP1 molecules to inhibit self-polymerization until they interact with viral minichromosomes in the nucleus to form virions.

Replication

Overview of SV40 replication cycle

The SV40 replication cycle is cleanly divided into early and late events, with the onset of viral DNA replication being the dividing landmark. SV40 virions attach to receptors on the cell surface, become internalized, and are transported to the cell nucleus where the viral DNA is uncoated. After uncoating, the half of the genome that contains the early region is transcribed ('early' mRNAs). Viral early proteins (T-ags) are synthesized, cellular genes are expressed, and the cells enter S phase. Viral DNA replication then begins. 'Late' mRNAs are transcribed from the other half of the viral genome (the opposite strand), and viral structural proteins are synthesized. Virus particles are assembled. The majority of progeny virions stay associated with the cell until cell lysis occurs following cell death. The SV40 multiplication cycle is slow, taking 48–72 h. The time course of the virus growth cycle is dependent on the viral multiplicity of infection and the growth state of the host cell at the time of infection.

Strategy of replication of nucleic acid

SV40 DNA is replicated in the cell nucleus as a free unintegrated minichromosome. The only viral components required are the viral origin of replication on the DNA and the T-ag protein; all other factors are provided by the host cell replication machinery. T-ag is required for the initiation of DNA replication. The specific T-ag functions required are its DNA-binding ability and its ATPase/helicase activities. The relative simplicity of the SV40 system has allowed the development of cell-free replication systems and the identification of factors involved in mammalian DNA replication.

T-ag binds to the viral *Ori*, a 64 bp segment that contains binding site II for T-ag. In an ATP-dependent process, T-ag causes localized unwinding of the *Ori* region; cellular single-stranded binding protein is required to stabilize the unwound single strands. The cellular DNA polymerase α–primase complex initiates DNA replication, and replication proceeds bidirectionally, with the two forks advancing at equal rates. The strand growing in the 5′ to 3′ direction is synthesized in a continuous fashion, whereas the strand growing in the 3′ to 5′ direction is synthesized as small pieces (Okazaki fragments). Elongation involves DNA polymerase α, DNA polymerase δ and proliferating cell nuclear antigen. Termination occurs 180° away from the viral *Ori;* topoisomerase II segregates the newly synthesized daughter molecules. Cellular histones are added to the new strands during the process of DNA replication.

Only certain monkey and human cells support SV40 DNA replication. This cell permissiveness seems to depend on the nature of the DNA polymerase α–primase complex.

The onset of viral DNA replication is carefully coordinated with the host cell cycle by the phosphorylation of a specific site on T-ag. The T-ag synthesized soon after viral infection drives the cell to enter S phase. The binding of p53 and pRB cellular proteins is presumably important in this process. A cell cycle-dependent kinase becomes activated and phosphorylates T-ag at Thr124. Only then can T-ag bind to site II in the viral *Ori* and initiate viral DNA replication. This regulation assures that viral DNA does not become unwound for replication until the host cell has entered S phase and the necessary cell replication factors are in place.

Characterization of transcription

Transcription of the viral DNA is carried out by the cellular RNA polymerase II. In the noncoding region of SV40 DNA near the origin of replication are early and late promoter structures and enhancer elements. Early transcription begins at about nucleotide 5237, proceeds in the counterclockwise direction, and ends at the polyadenylation site at nucleotide 2694. The early promoter contains a TATA box about 30 bp upstream of the early RNA initiation site. (This start site is about 70 nucleotides upstream of the initiation codon shared by the early proteins.) There are three G+C-rich regions, the '21 bp repeats,' located 40–103 nucleotides upstream that are binding sites for the Sp1

cellular factor. Even farther upstream are the SV40 enhancer elements, the 72 bp elements, which contain binding sites for other cellular factors that regulate transcription. The primary early transcripts are differentially spliced to generate the mRNAs that code for large T-ag and small t-ag.

There is no requirement for virus-encoded proteins, but early transcription is regulated by T-ag. As the concentration of T-ag increases in the cell, it binds first to site I and then to sites II and III on the viral DNA. Early transcription is repressed when site II is occupied, because the presence of T-ag blocks the binding of RNA polymerase. Therefore, T-ag regulates its own synthesis.

Late transcription begins after viral DNA synthesis is underway. The abundance of late transcripts is much greater than the early transcripts because progeny DNA molecules are utilized as templates. A heterogeneous collection of late mRNAs is made, with late transcription beginning at multiple sites between nucleotides 120 and 482 and proceeding in the clockwise direction, ending at a polyadenylation site at nucleotide 2674. Both the 21 bp repeats and the 72 bp elements have positive effects on late transcription. T-ag *trans*-activates late transcription by an unknown mechanism that does not involve DNA binding. The late transcripts are alternatively spliced into two size classes (19S, 16S). VP1 is synthesized from 16S RNA and both VP2 and VP3 are translated from the 19S species. The agnoprotein is synthesized predominantly from the most abundant species of 16S RNA.

Characterization of translation

Early gene products (T-ag, t-ag) are synthesized from differentially spliced early transcripts. Likewise, the structural proteins (VP1, VP2, VP3) are produced from differentially spliced late transcripts. VP3 is a truncated version of VP2, due to initiation of translation at an internal site on the same species of transcripts. The agnoprotein LP1 is translated from the leader region of late transcripts. The late gene products are produced in much greater abundance than the early proteins, reflecting the relative concentrations of the transcripts.

Post-translational processing

No post-translational cleavages are involved in the production of SV40 proteins. As noted above, T-ag and VP1 are modified in various ways, including phosphorylation.

Uptake and release of virions

SV40 particles attach to receptors on the cell surface. The receptors recognized by virus particles are believed to be the major histocompatibility complex class I molecules.

Attached particles are internalized by endocytosis. Conformational changes are thought to occur that expose the nuclear localization signals on capsid proteins and allow the virions to squeeze through the nuclear pore complex. The capsid disassembles in the nucleus, releasing the viral DNA.

Maturation of progeny virions occurs in the nucleus, where the viral nucleic acid is replicated. Viral proteins are synthesized in the cytoplasm off viral transcripts exported from the nucleus, and the proteins are then transported back into the nucleus. The structural proteins condense around the viral minichromosomes. There is a packaging signal on SV40 DNA that includes the *Ori* and part of the enhancer element. During the maturation process, the agnoprotein is released and is not retained as a component of mature virions. Assembly intermediates that sediment more slowly than extracellular virions have been detected. Certain SV40 T-ag mutants are defective in the assembly of virus particles, but the mechanism is obscure. There are size constraints for packaging DNA; molecules ranging from 3.5 kbp to 5.7 kbp can be encapsidated into SV40 particles.

Some progeny virus is released from the cell by an unknown mechanism, but the majority stays associated with the cell until lysis caused by cell death. Host cells are killed as the result of a variety of effects, including the release of lysosomal enzymes into the cytoplasm and damage to the cell mitochondria. Late in infection, monkey kidney cells develop a characteristic cytopathic effect, cytoplasmic vacuolization. Between 10^4 and 10^5 virus particles are produced by each infected cell.

Geographic and Seasonal Distribution

The geographic distribution of SV40 can only be inferred, as no comprehensive surveys have been conducted. Its distribution in the wild presumably reflects its narrow host range. As far as is known, SV40 is found naturally in wild populations of certain Asian macaque species. Many captive primates can be infected if they have been in contact with an infected macaque. Infections in humans are probably more widespread geographically, as contaminated polio-vaccines were broadly distributed. Nothing is known about seasonal effects on natural infections by SV40.

Host Range and Virus Propagation

Papovaviruses, in general, have a narrow host range,

Table 3 Origin of SV40 strains

Virus strain	*Year isolated*	*Source*
SV40-776	1960	Adenovirus type 1 seed stock prepared in monkey kidney cells
Baylor	1961	Type 2 Sabin poliovaccine prepared in 1956 in monkey kidney cells
VA45-54	1960	Uninoculated green monkey kidney cells
Rh911	ca. 1960	Uninoculated rhesus monkey kidney cells
A2895	ca. 1961	Tumor from hamster injected with rhesus monkey kidney cells
D-128	1962	Uninoculated rhesus monkey kidney cells (Russia)
SVPML-1	1970	Cultured human brain cells from patient with progressive multifocal leukoencephalopathy
SVMEN*	1984	Human meningioma (cloned directly)
SVCPC*	1995	Human choroid plexus carcinoma
SV40-K661	1997	Brain from rhesus monkey coinfected with simian immunodeficiency virus

Data taken from Stewart AR, Lednicky JA and Butel JS (*J. Neurovirol.* 1998; 4: 182) and from Lednicky JA, Arrington AS, Stewart AR *et al* (*J. Virol.* 1998; 72: 3980).
*SVMEN and SVCPC are identical.

with each virus infecting only one or a few closely related species. Based on antibody surveys of wild populations of primates, the natural hosts for SV40 appear to be a few species of Asian macaque monkeys, especially the rhesus (*Macacca mulatta*). In captivity, several related species are easily infected, including the cynomolgus macaque (*M. fascicularis*) and the African green monkey which belongs to the same family as macaques (Cercopithecidae). The virus grows poorly in more distantly related primates. SV40 can infect humans.

SV40 is propagated in tissue culture in established cell lines derived from kidneys of African green monkeys. Characteristic vacuolated cells appear in response to viral replication. The virus grows in rhesus kidney cell lines in which it establishes a persistent infection but produces no cytopathic effects. The SV40 growth cycle is long, compared with those of other virus families.

SV40 does not cause tumors in its natural hosts. To demonstrate its tumorigenic potential, the virus must be inoculated into experimental animals (newborn hamsters are most susceptible). Many types of cells can be transformed in culture, especially those of rodent origin. Primate cells can be transformed, but only if experimental conditions are manipulated to prevent viral replication.

Genetics

SV40 is genetically stable. Sequence variation exists at the C-terminus of the *T-ag* gene among different isolates. Many point mutations, deletions and substitutions have been introduced into the SV40 genome in the course of experimental studies designed to examine mechanisms of gene regulation, viral replication and cell transformation. Adaptation of natural isolates to tissue culture involves the selection of viruses with duplications or rearrangements in the viral regulatory region. The origins of the most well-characterized SV40 strains are listed in **Table 3.** Serial undiluted passage of virus in cultured cells often results in the accumulation of defective-interfering particles containing DNA with extensive deletions and rearrangements. To produce high-titer stocks of virus, serial passage of undiluted preparations should be avoided.

Evolution

Different strains of SV40 can be distinguished on the basis of nucleotide differences in the regulatory region and in the variable domain of the *T-ag* gene. During natural infections, viruses with heterogeneous regulatory regions but a common *T-ag* gene may be generated. During adaptation to tissue culture, a virus with a duplication in the enhancer will usually be selected. The evolutionary origin of SV40 is obscure. Sequence comparisons have revealed short regions of similarity between portions of SV40-encoded gene products and cellular proteins. It may be that the viral proteins are composites of functional domains pirated from cellular progenitors. Because of size constraints imposed on the SV40 genome by capsid architecture, the bulk of the coding sequence for a cellular protein would have to be jettisoned, making identification of origins difficult. Among the polyomaviruses, SV40 is most closely related to BKV by base sequence homology. When all the polyomaviruses are compared, the lowest homologies are found in the noncoding regulatory sequences.

Serologic Relationships and Variability

Only one serotype of SV40 is known. The virus does not undergo noticeable antigenic variation. Perhaps restrictions imposed by the symmetry of the capsid permit only minimal deviation in amino acid sequence of the structural proteins, making most changes lethal for the virus.

There is a genus-specific antigenic determinant on the major capsid protein, VP1, that is shared by all animal and human polyomaviruses. It is internal in the virion, but antibodies are elicited against it by immunization with disrupted capsids or with purified VP1 protein. The determinant is expressed in infected cells. Antibodies against the shared determinant are not neutralizing, as the site is not exposed on the surface of virus particles. Although the structural proteins of SV40 and the two human polyomaviruses are antigenically distinct (with the exception of the genus-specific determinant on VP1), the T-ags of SV40, BKV and JCV show extensive antigenic cross-reactivity.

Epidemiology

Most adults of the Asian macaque species believed to be natural hosts for SV40 have neutralizing antibodies to the virus. Few of the juvenile animals of those species, in the wild, have antibodies. However, in captivity the young animals are readily infected if they have contact with a virus-positive animal.

Serologic surveys have detected SV40-neutralizing antibodies in humans not exposed to contaminated vaccines, with prevalences of 2–10%. Possible cross-reactivity with BKV and JCV does not explain the presence of such antibodies. This suggests that SV40, or an unknown SV40-like agent, is circulating in the human population.

Transmission and Tissue Tropism

SV40 establishes persistent infections in the kidneys of susceptible hosts. The level of virus present may be very low. Modes of transmission are not known, but transmission probably occurs due to virus shed in the urine. Experiments have established that susceptible animals can be infected by the oral, respiratory or subcutaneous routes. Both viremia and viruria occur in infected animals. SV40 may cause neurologic disease in immunocompromised hosts.

The major known source of human exposure to SV40 was via the administration of contaminated viral vaccines before SV40 was recognized. That risk no longer exists. Human exposure could occur by contact with infected monkeys, a situation limited to small numbers of animal handlers. It is presumed that patterns of tissue tropism and transmission similar to those described in monkeys would be observed in humans infected by SV40.

Pathogenicity and Pathology

SV40 infections in normal monkeys appear to be asymptomatic and harmless. However, SV40 has been associated with a fatal case of pulmonary and renal disease, as well as with cases of progressive multifocal leukoencephalopathy, in unhealthy rhesus monkeys. SV40 can cause widespread infections in monkeys suffering from simian AIDS. No tumors have been found in the natural hosts. Transgenic mice carrying wild-type SV40 DNA develop choroid plexus papillomas and die rapidly because of the physiological importance of the tumor site. When foreign tissue-specific regulatory sequences are substituted for the native promoter-enhancer of the virus, SV40 expression can be directed to other tissues in transgenic animals. Tumors usually appear in the targeted tissue and are lethal. In conventional animals, tumors induced by virus injection tend to stay localized and do not invade or metastasize, but rodents bearing such tumors usually succumb due to the tumor load. SV40-induced tumors are usually classified as undifferentiated carcinomas or sarcomas. Intravenous inoculation of SV40 into weanling hamsters induced leukemia, reticulum cell sarcoma and osteogenic sarcoma. SV40 DNA has been detected in several types of human cancers, including brain tumors (especially those from children in the first decade of life), mesotheliomas, osteosarcomas and kidney tumors. The role SV40 may have played in the induction of those tumors is unknown.

Immune Response

SV40, like other members of the *Polyomavirus* genus, induces an asymptomatic, persistent infection in natural hosts. An antibody response to capsid antigen is elicited that can be detected in neutralization assays. It is well documented with the human viruses BKV and JCV that impaired cell-mediated immunity is associated with virus re-activation, showing that viral replication is under the control of the immune system of the host; the same presumably applies to SV40.

Little is known about the immune response of humans to infection by SV40. Small numbers of individuals exposed to contaminated vaccines were analyzed for neutralizing antibody responses to SV40. Humoral responses were variable and dependent on the size of inoculum and route of inoculation. Recent serological surveys have detected SV40 neutralizing

antibody in 2–10% of persons not exposed to SV40-contaminated viral vaccines. Antibodies to SV40 were most often detected in people with some type of immune suppression.

Experimental studies have shown that animals with active infections by SV40 may produce humoral antibodies against the replication oncoprotein, T-ag. The responses were variable and probably reflected the extent of viral replication. It should be noted that a T-ag antibody response could not be used to monitor SV40 infections in humans because of the crossreactivity among the T-ags of SV40 and the human viruses BKV and JCV.

SV40 tumor-bearing animals develop a strong immune response to T-ag. Both humoral and cell-mediated responses occur. No antibodies are produced against capsid antigens, as the structural proteins are not expressed in tumor cells in rodents. Cytotoxic T cells directed against T-ag determinants at the cell membrane help render the animals resistant to the growth of SV40 tumor cells. This system has been an important experimental model for helping to understand the immune response to neoplastic cells in humans.

Interferon is induced only weakly by the polyomaviruses and is not thought to be an important component of the host response to SV40.

Prevention and Control

No control measures are available to prevent SV40 infections.

Future Perspectives

The reports of antibodies to SV40 in humans and the infrequent association of SV40 markers with human tumors suggest that SV40 may be present in the population. If its presence is substantiated, it will be important to determine the natural history of SV40 in humans, including modes of transmission and factors affecting susceptibility to infection. If tumors are produced in humans following exposure to SV40, it will be necessary to develop appropriate control measures to prevent such infections. Because of its small genetic content and dependence on host cell functions, SV40 will continue to be a useful model system for discerning mechanisms of cellular processes, such as mammalian cell DNA replication, cell cycle progression and growth control processes altered in neoplasia.

See also: **Defective interfering viruses; JC and BK viruses (*Papovaviridae*); Persistent viral infection; Polyomaviruses – murine (*Papovaviridae*): General features, Molecular biology; Virus structure: Atomic structure, Principles of virus structure.**

Further Reading

Cole CN (1996) *Polyomavirinae:* the viruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 1997. Philadelphia: Lippincott-Raven.

Liddington RC, Yan Y, Moulai J *et al.* (1991) Structure of simian virus 40 at 3.8-Å resolution. *Nature* 354: 278.

Prives C (1990) The replication functions of SV40 T antigen are regulated by phosphorylation. *Cell* 61: 735.

Shah K and Nathanson N (1976) Human exposure to SV40: review and comment. *Am. J. Epidemiol.* 103: 1.

Stewart AR, Lednicky JA and Butel JS (1998) Sequence analyses of human tumor-associated SV40 DNAs and SV40 viral isolates from monkeys and humans. *J. Neurovirol.* 4: 182.

Tevethia SS (1990) Recognition of simian virus 40 T antigen by cytotoxic T lymphocytes. *Mol. Biol. Med.* 7: 83.

SINDBIS AND SEMLIKI FOREST VIRUSES (*TOGAVIRIDAE*)

Milton J Schlesinger, Department of Molecular Microbiology, Washington University School of Medicine, St Louis, USA

History

Sindbis (SIN) virus was first isolated in 1952 from a group of *Culex univittatus* mosquitoes captured in a light trap in the Sindbis health district 30 km north of Cairo, Egypt. Isolation was by inoculation of triturated mosquitoes into 3-day-old mice. The diameter of the virus was estimated to be 40–48 nm and, based

antibody in 2–10% of persons not exposed to SV40-contaminated viral vaccines. Antibodies to SV40 were most often detected in people with some type of immune suppression.

Experimental studies have shown that animals with active infections by SV40 may produce humoral antibodies against the replication oncoprotein, T-ag. The responses were variable and probably reflected the extent of viral replication. It should be noted that a T-ag antibody response could not be used to monitor SV40 infections in humans because of the crossreactivity among the T-ags of SV40 and the human viruses BKV and JCV.

SV40 tumor-bearing animals develop a strong immune response to T-ag. Both humoral and cell-mediated responses occur. No antibodies are produced against capsid antigens, as the structural proteins are not expressed in tumor cells in rodents. Cytotoxic T cells directed against T-ag determinants at the cell membrane help render the animals resistant to the growth of SV40 tumor cells. This system has been an important experimental model for helping to understand the immune response to neoplastic cells in humans.

Interferon is induced only weakly by the polyomaviruses and is not thought to be an important component of the host response to SV40.

Prevention and Control

No control measures are available to prevent SV40 infections.

Future Perspectives

The reports of antibodies to SV40 in humans and the infrequent association of SV40 markers with human tumors suggest that SV40 may be present in the population. If its presence is substantiated, it will be important to determine the natural history of SV40 in humans, including modes of transmission and factors affecting susceptibility to infection. If tumors are produced in humans following exposure to SV40, it will be necessary to develop appropriate control measures to prevent such infections. Because of its small genetic content and dependence on host cell functions, SV40 will continue to be a useful model system for discerning mechanisms of cellular processes, such as mammalian cell DNA replication, cell cycle progression and growth control processes altered in neoplasia.

See also: **Defective interfering viruses; JC and BK viruses (*Papovaviridae*); Persistent viral infection; Polyomaviruses – murine (*Papovaviridae*): General features, Molecular biology; Virus structure: Atomic structure, Principles of virus structure.**

Further Reading

Cole CN (1996) *Polyomavirinae:* the viruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 1997. Philadelphia: Lippincott-Raven.

Liddington RC, Yan Y, Moulai J *et al.* (1991) Structure of simian virus 40 at 3.8-Å resolution. *Nature* 354: 278.

Prives C (1990) The replication functions of SV40 T antigen are regulated by phosphorylation. *Cell* 61: 735.

Shah K and Nathanson N (1976) Human exposure to SV40: review and comment. *Am. J. Epidemiol.* 103: 1.

Stewart AR, Lednicky JA and Butel JS (1998) Sequence analyses of human tumor-associated SV40 DNAs and SV40 viral isolates from monkeys and humans. *J. Neurovirol.* 4: 182.

Tevethia SS (1990) Recognition of simian virus 40 T antigen by cytotoxic T lymphocytes. *Mol. Biol. Med.* 7: 83.

SINDBIS AND SEMLIKI FOREST VIRUSES (*TOGAVIRIDAE*)

Milton J Schlesinger, Department of Molecular Microbiology, Washington University School of Medicine, St Louis, USA

History

Sindbis (SIN) virus was first isolated in 1952 from a group of *Culex univittatus* mosquitoes captured in a light trap in the Sindbis health district 30 km north of Cairo, Egypt. Isolation was by inoculation of triturated mosquitoes into 3-day-old mice. The diameter of the virus was estimated to be 40–48 nm and, based

on its pathogenesis in neonatal but not adult mice, it was initially classified as a coxsackie-type virus. Additional studies showed it to be distinct from these viruses and it was placed into a separate class.

Semliki Forest virus (SFV) was first isolated in Uganda in 1942 from *Aedes (Aedimorphus) abnormalis*. Its diameter was estimated to be 20–67 nm.

Taxonomy and Classification

SIN and SFV are members of the *Alphavirus* genus of the *Togaviridae* family. Based on sequences of the viral genomes, SIN and SFV are grouped separately and both are distinct from another group that includes Eastern equine and Venezualen equine encephalitis viruses. In the SFV group are ten other alphaviruses; in the SIN group are four other alphaviruses plus Western equine encephalitis virus, which is a genetic recombinant with glycoproteins derived from SIN-like sequences and the balance of the genome from Eastern equine encephalitis virus. SIN and SFV are serologically related to Eastern, Western and Venezualan equine encephalitis viruses. They have also been classified as members of the RNA virus superfamily 1.

Geographic and Seasonal Distribution

SIN strains have been isolated worldwide. Two geographical subgroups exist: European–African and Asian–Australian. Sindbis virus has been isolated in the Near East, Africa, Southeast Asia, India, Borneo, Australia, the former USSR and Czechoslovakia. Viruses closely related to SIN have been isolated in Scandinavia, New Zealand, Brazil and Argentina. SFV and closely related viruses have been isolated from Africa, the former USSR, Australia, Japan, Malaysia and South America. As mosquito-borne viruses, they are more prevalent in tropical regions and their presence is amplified during summer months and during periods of precipitation in tropical areas.

Host Range and Propagation

The natural vectors of SIN include *Culex univittatus, C. antennatus, C. annulirostris, C. pseudovishnue, C. tritaeniorhynchus, C. bitaeniorhynchus* and *Mansonia fuscopennata*. SIN has been isolated also from mites and ticks. Migratory birds are an important host and probably account for the global distribution of these viruses. SIN transmission between birds is from bites of *Ae. albopictus, Ae. aegypti* and *C. pipiens*. SFV is propagated by *Ae. abnormalis* and *Aedes* spp. Transovarial transmission occurs in *Aedes* spp. Both viruses exist in an enzootic cycle involving small wild animals, birds, subhuman primates and mosquitoes. They are arthropod-borne viruses.

Virion Structure

SIN and SFV virions are spherical, 70–80 nm in diameter, with icosahedral symmetry and a $T = 4$ lattice. The internal nucleocapsid or core is spherical, 40 nm in diameter, with icosahedral symmetry and a $T = 4$ lattice. The virus particle (molecular mass of 52 MDa) is composed of 30% lipid, 57% protein, 6% carbohydrate and 7% RNA. The sedimentation coefficient is 280 S and the density is 1.22 g ml^{-1} in sucrose-D_2O. The core's sedimentation coefficient is 140 S. Virions of SFV and SIN are stable to storage at 4°C and to repeated freeze–thawing. Infectious particles rapidly lose activity at 56°C (−1 log/10 min, but varies with a particular strain) and are labile to organic solvents, detergents and thiol reducing agents. The RNA in isolated cores is susceptible to RNase.

The external spikes are arranged as 80 trimers, each consisting of stable heterodimers composed of two glycosylated transmembranal proteins. The spikes are embedded in a lipid bilayer which is derived from the plasma membrane of the infected host cell. Curvature of the membrane is such that the outer area is about 40% greater than the inner leaflet. Cryo-electron micrographs of Sindbis virus are presented in **Fig. 1:** a cross-sectional view has been constructed to show the internal core and the lipid bilayer, which appears nonuniform with an average thickness of 4.8 nm. The spike arrangement indicates a cup-like structure for the trimers which form at their base a protein shell around the lipid bilayer with exposed areas of the latter on the virion surface. An atomic structure of the capsid protein has been determined from x-ray diffraction data to a resolution of 0.3 nm and amino acids from 114 to 264 are folded in a manner closely resembling mammalian serine proteases with the catalytic triad of serine, histidine and aspartate surrounding a hydrophobic pocket occupied by tryptophan.

The genome consists of a nonsegmented, single-stranded RNA with a sedimentation coefficient of 49 S. The RNA is capped at its 5′ end with m^7G and is polyadenylated at its 3′ terminus with average length of 70 A's. Genomic RNA is of the positive orientation and isolated RNA is infectious. The complete sequences of about 12 kb from two strains of SIN and one of the SFV genome have been determined and plasmids containing these sequences in the form of cDNA can be transcribed *in vitro* to yield infectious RNA. The cDNA under control of a eukaryotic promoter is also infectious.

The genome encodes four nonstructural and four

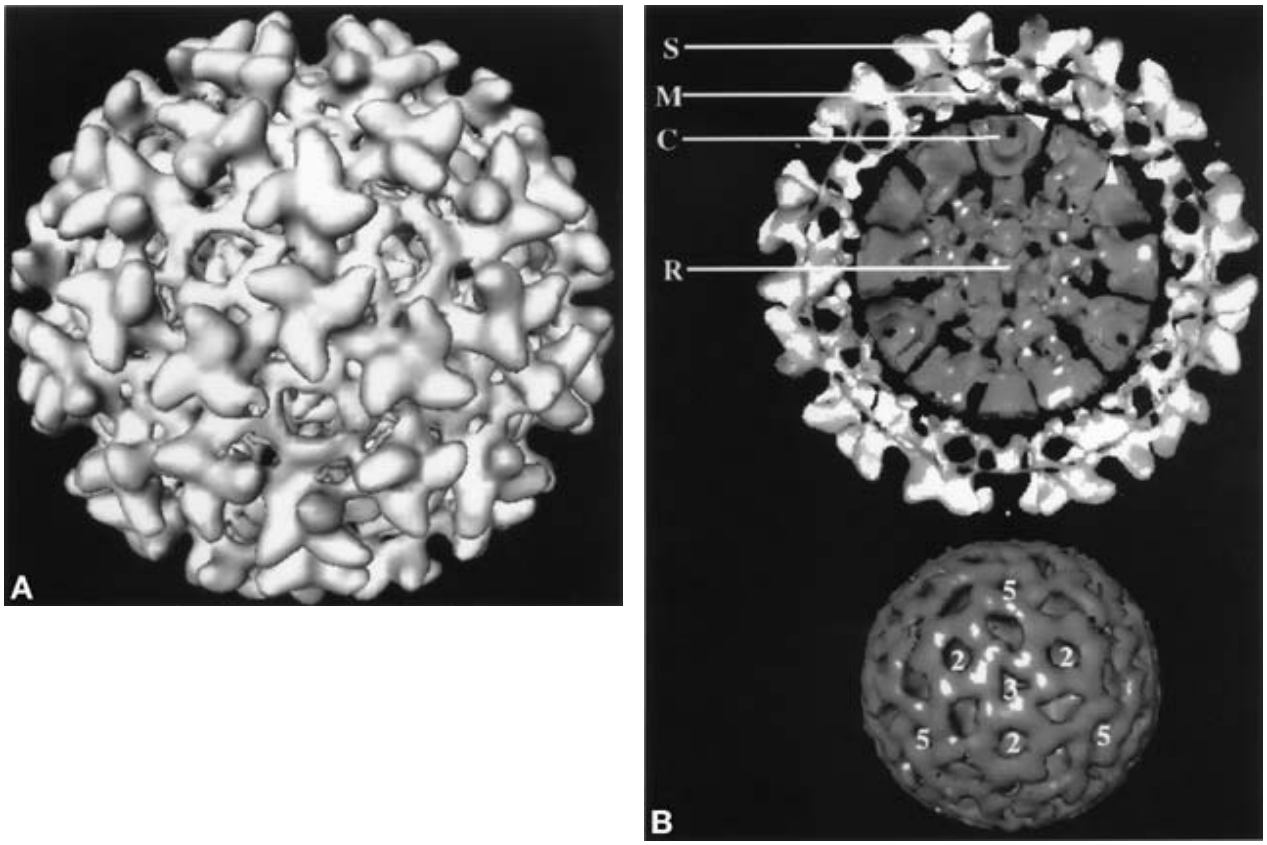


Figure 1 Image reconstruction of Sindbis virus structure based on cryo-electron micrographs. (**A**) Surface view of the virion indicating trimeric spike arrangement. (**B**) Cross-sectional view shows spikes (S), lipid envelope (M), nucleocapsid protein (C) and RNA genome (R). Below, surface of the nucleocapsid with 5, 3 and 2-fold axes of symmetry. (Reproduced with permission from V. Prasad. In: Fields BN *et al* (eds) (1996).) *Fields Virology* 3rd edn, p. 76. Philadelphia: Lippincott-Raven.)

structural proteins. Their sizes and functions are listed in **Table 1.** Four regions in the genome contain *cis*-acting sequences critical to expression and replication of the genome. These include 44 nucleotides at the 5′ terminus, 21 nucleotides at the junction between nonstructural and structural genes, 19 nucleotides at the 3′ terminus and a sequence of 132 nucleotides near the 5′ terminus of the SIN RNA (nt 948) which forms a structure that functions as a packaging signal and selectively binds to the virus capsid. Different sequences in the NSP2 gene appear to serve this function for SFV.

Replication and Molecular Biology

Infection initiates with virus binding to host cell receptors. Attachment is sensitive to ionic conditions and host range mutants can differ in net surface charge of the virion. SIN binds to a highly conserved laminin receptor on mammalian and possibly also on insect cell surfaces. Chicken cells have a high-affinity receptor distinct from laminin and accounting for half of potential receptor sites. Mouse neuronal cells may have two other surface receptors. The virus protein most important for binding to cell receptors is E2, although changes in E1 can affect binding. The E2 sequence between amino acids 170 and 220 is particularly critical. A single amino acid change in SIN at position 55 of E2 from glutamine to histidine confers neurotropism and neurovirulence in rodents. In most cells, the major route for uptake is via clathrin-coated pits and acidified endosomes. Delivery of virus cores into the cell cytoplasm occurs after fusion of the virus membrane with the cell's endosomal membrane, which is mediated by the E1 glycoprotein. At a pH <6, this protein dissociates from the heterodimer to form a homotrimer with a conformation distinct from that on the virion surface. A hydrophobic sequence of amino acids at positions 78–96 has been designated as a fusion domain that is exposed at the lower pH and may initiate formation of a coiled-coiled structure required for fusogenesis. Cholesterol and sphingolipid are essential components in the host cell membrane for virus binding and fusion. Bio-

Table 1 Sizes and functions of SIN and SFV proteins

Protein	*SIN*	*SFV*	*Properties and function(s)*
NSP1	540	537	Methyl and guanyl transferase; 5′ capping activity; specifically required for initiation of (−)RNA transcription; modulation of NSP2 proteinase; membrane associated; palmitylated and mutants lacking fatty acyl groups show different cell membrane distribution
NSP2	807	798	Cysteine protease in the C-terminal half of the protein which functions in *cis* and *trans* cleavages of the NSP polyprotein; nucleoside triphosphatase and RNA helicase motifs in the N-terminal half of the protein; RNA binding activity; specifically required for 26S RNA transcription; accumulates in the nucleolus of infected (or transfected) cells; single site mutations decrease the virus-induced shutoff of host cell protein synthesis
NSP3	556	482	Phosphoprotein (threonine/serine are phosphorylated); variable length in the C-terminus
NSP4	610	614	RNA polymerase; unstable and degraded by the ubiquitin pathway; concentration is tightly regulated
Capsid	264	267	Forms nucleocapsid; folds during synthesis to a serine-like protease that autocatalytically cleaves the capsid from the polyprotein; contains hairpin RNA structure near the N-terminus that enhances translation of virus encoded structural proteins in infected cells; positively-charged N-terminus binds genomic RNA
p62	487	488	Precursor to E2; type 1 transmembrane glycoprotein; palmitylated and glycosylated; cleaved in a *trans*-Golgi vesicle by a furin-type protease; rarely incorporated into virus particles
E3	64	66	N-terminal part of p62 containing the signal sequence; glycosylated; retained on SFV but not on SIN virions
E2	423	422	Component of the heterodimeric virus spike; contains sites for binding to cell receptors and epitopes for neutralizing antibodies; type 1 transmembrane protein with 33 amino acids at its C-terminus in the cytoplasm which forms a motif that binds to the capsid during assembly; glycosylated, palmitylated and, possibly, phosphorylated; amino acid changes in the ecto-domain affect virus assembly, stability, virulence and tropism
6K	55	60	Membrane associated; palmitylated; C-terminus is the signal sequence for E1; small amounts in virions; enhancer for virus assembly and budding
E1	439	438	Component of the heterodimeric virus spike; glycosylated and palmitylated; contains sequences which function in low-pH activated membrane fusion; type 1 transmembrane protein with 2 positively-charged amino acids in the cytoplasm

Values are the number of amino acids in the protein. Note that the p62 protein does not normally appear as a structural protein of the infectious virion.

chemical mechanisms for nucleocapsid uncoating to release RNA are unknown. Within 0.5–1 h postinfection, cells are resistant to superinfection by homologous viruses.

Biosynthetic activities that lead to progeny virions consist of the following steps.

1. Genomic RNA is partially translated to form a polyprotein composed of the four nonstructural proteins, nsP1–4 (**Fig. 2A**). For SIN, most of the polyprotein terminates at the end of the NSP3 gene at an opal stop codon; however, suppression of this codon allows for 20% of the polyprotein to include nsP4. For SFV, the entire nsP1–4 polyprotein is formed. Several stop codons located about two-thirds of the length of the genomic RNA insure that termination of translations stops at the end of the NSP4 gene for both SIN and SFV.
2. A complex consisting of the nsP1–3 polyprotein and nsP4, which has been proteolytically cleaved from nsP1–4 by a *cis*-acting protease encoded in the nsP2 C-terminus, interacts with a 19 nucleotide sequence at the 3′ end of genomic RNA to initiate replication that produces a full length (−)RNA template (**Fig. 2B**). Host cell proteins are also involved in this activity. The replication complex is associated with cellular membranes. Transcription from (−) to (+) RNAs requires modification of the nsP123 complex by the nsP2 protease acting in *trans* to give individual subunits with altered structures (**Fig. 2B**). Proteolytic cleavage of the nsP1–3 complex shuts off (−)RNA synthesis. (−)RNA templates are transcribed into full length (+)RNAs and into a subgenomic species with sequences identical to the 3′ one-third of the virus genomic RNA and a sedimentation coefficient of 26 S (**Fig. 2A**). The minimal promoter for 26S RNA transcription consists of 19 nucleotides upstream and five nucleotides downstream from the transcriptional start site on the (−) RNA. Full-length genome

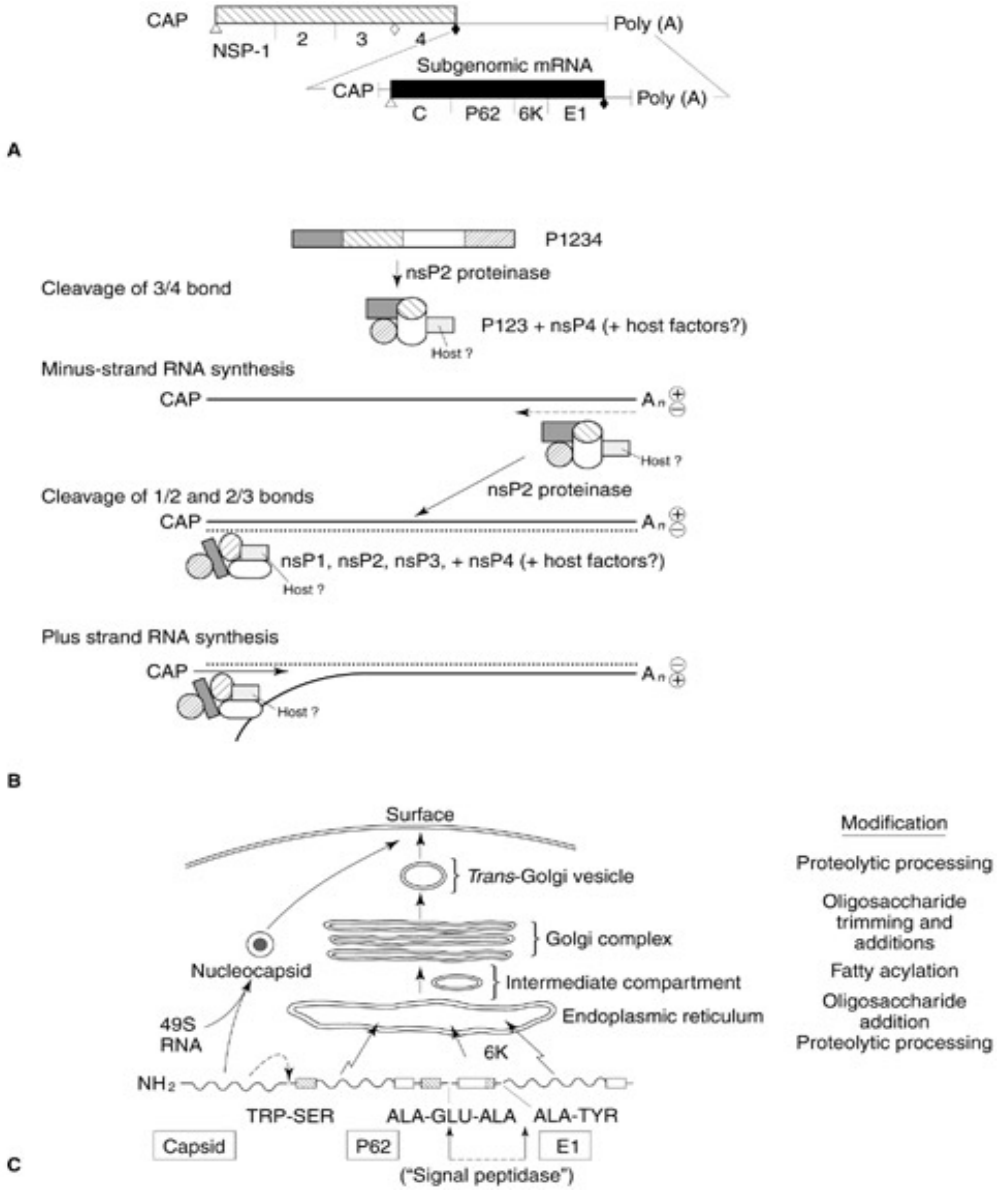


Figure 2 Organization and expression of SIN and SFV genome. (**A**) Order of genes and the sites of initiation (△) and termination (◆) of translation for the genomic and subgenomic mRNAs. In SIN, and opal codon (◇) terminates translation to produce the polyprotein P123. (**B**) Two stages of processing by the nsP2 proteinase of the P1234 polyprotein translated from the genomic mRNA and the function of the different complexes in viral RNA transcription and replication. See text for details. (Reproduced with permission from Strauss and Strauss (1994).) (**C**) Formation, maturation and transport through intracellular membrane vesicles of the viral structural proteins translated from the subgenomic 26S mRNA. Signal sequences for insertion into the membrane are noted by the cross-hatched boxes. (Reproduced with permission from Schlesinger and Schlesinger (1996).)

(+) RNA is packaged with capsid subunits and subgenomic 26S mRNA becomes the predominant RNA translated in the infected cell.

3. 26S RNA encodes the capsid, p62 (E3 + E2), 6K and E1 virus structural proteins and translation initiates at a single site near its 5′ capped end. The first gene translated codes for the capsid protein which folds to form a serine-like protease that autoproteolytically releases capsid protein from the growing nascent polypeptide. A stem–loop structure in the mRNA near the N-terminus of the capsid functions as a translational enhancer in virus-infected cells, yielding very high levels of the virus structural gene products. A transmembranal signal sequence encoded at the N-terminus of the second gene initiates insertion of its protein

product, p62, into the lumen of the endoplasmic reticulum (**Fig. 2C**). Stop-transfer signals followed by additional signal and stop-transfer signals lead to insertion of the C-termini of p62, 6K and E1 and the N-terminus of the 6K into the ER membrane. Host cell signalases cleave at p62–6K and 6K–E1 sites in the ER lumen to give the separate p62, 6K and E1 polypeptides. These three proteins move as a cohort through the intracellular transport vesicles of the host cell where several post-translational modifications occur. The latter include attachment of glycosyl groups to p62 and E1 immediately upon entry into the lumen of the endoplasmic reticulum; a reorientation of the C-terminus of p62 from a transmembranal to a cytoplasmic location, covalent attachment of palmityl groups to cytoplasmically oriented cysteines of p62, 6K and E1 during transport to the Golgi and proteolytic cleavage of the p62 to the E2 glycoprotein in a *trans*-Golgi vesicle by a furin-like protease.

4. Assembly of progeny virus requires two stages: there is the self-association of capsid subunits in the cell cytoplasm to form substructures that bind to specific regions of genomic RNA (nucleotides 945–1076 of SIN and 2737–2993 plus others for SFV) to form nucleocapsids. Capsids can, however, assemble to nucleocapsid-like particles in the absence of RNA. Preformed nucleocapsids bind to arrays of E1–E2 trimers localized to the plasma membrane to initiate the budding process. A motif composed of hydrophobic amino acids in the 33 amino acid cytoplasmic domain of E2 acts as a ligand to bind to a hydrophobic pocket receptor on the surface of capsid subunits. There are 240 such interactions which lead to envelopment of the nucleocapsid by the host cell plasma membrane. Lateral interactions among the glycoproteins are important in the assembly process. The host cell can also influence this assembly; i.e. hyperglycosylated E2 is inhibitory for growth in vertebrate cells but has little effect in assembly in insect cells, and deletion of the 6K protein is relatively nondefective in insect cells but blocks virus assembly in avian cells grown at 40°C. Fusion of the virus lipid bilayer allows for release of progeny virions from the cells. Assembly and budding require cholesterol in the membrane. The process described can occur within 3 h in permissive cells such as chicken embryo fibroblasts and produces thousands of progeny virus which are secreted into the extracellular fluid. Replication is slower in cultured insect cells, with peak production of virus occurring some 15–20 h postinfection.

Pathology and Histopathology

Neither SIN nor SFV are serious pathogens for adult vertebrates unless virus is injected directly into the brain. In rare infections in humans, SIN and SFV produce arthralgia, rash and fever. Human polyarthritis can result from infection by some SIN strains.

SIN is highly pathogenic to embryonated hens' eggs. Within the host organism, the infected cells include neuron and glial cells, striate and smooth muscle cells, cells of lymphoid origin, synovial cells and brown fat cells. Neonatal rodents develop severe neurocytopathology when virus is given intracerebrally or intraperitoneally. In the CNS, focal cystic degeneration, vascular dilatation and neurolysis occur. Strains of SIN that lead to fatal encephalitis after infection of neonatal mice show lesions associated with a severe stress response – including high levels of interferon α/β and toxic cytokines such as tumor necrosis factor α and little evidence of encephalitis. Less virulent strains produce a more classical encephalitis. Adult mice are susceptible to some SIN strains and neuroinvasiveness is attributed to a change in amino acid 55 of the E2 glycoprotein from glutamine to histidine. Other single-site amino acid changes in E2 alter neurovirulence and cytopathology of SIN. Additional changes in the 5′ noncoding region of the genome and at position 190 of the E2 glycoprotein can confer neuroinvasiveness on other isolates. Attenuation of these strains by mutations produces encephalitis but low levels of mortality and low levels of interferon α/β. Comparison of neurological damage in B6 versus SJL mice infected with SFV show more acute encephalomyelitis in the former, which could be correlated with lower levels of several cytokines and higher virus titers early after infection. Cytopathic effects and neurovirulence are dependent also on strains of SFV. Subacute demyelinating disease, chronic benign infection and teratogenesis in pregnant mice have been detected in SFV-infected mice and high mortality for some strains is attributed to replication in the spleen, lymph nodes and liver. Replication in skeletal muscle leads to atrophy, focal necrosis and viremia.

In vertebrate tissue culture, SIN and SFV have a broad host range and are highly cytopathic, inducing apoptosis in many cells. Host cell macromolecular synthesis (protein, DNA and RNA) is shut off early after infection as a result of expression of virus nonstructural genes and high levels of virus RNA. Mutations in the NSP2 gene of SIN can lead to a persistent infection in BHK cells. Blocks in Na^+/K^+-ATPase activity in membranes from SIN-infected cells alters internal ion concentrations and contributes to

cell cytolysis. Virus structural protein synthesis and intracellular vesicle transport are associated with cytopathology.

In the mosquito, replication occurs in epithelial cells of the midgut, in the salivary glands, thoracic muscle and respiratory tissue. Persistent infection occurs in the insect hemolymph, hindgut and tracheole-associated cells, as well as in cultured insect cells. An antiviral hydrophobic peptide is induced by virus infection of cultured insect cells and limits infection but leads to persistent virus replication. Infection of the mosquito also leads to persistent infection.

Genetics

The frequency of mutation is about 10^{-4}–10^{-5}, which is similar to other RNA viruses. Specific nucleotide mutations, however, have been noted in which the frequency is much lower, in the range of 10^{-7}–10^{-8}. Low rates of divergence have been found and attributed to the natural infection and growth of the virus in both insects and vertebrate cells with varying temperatures and host cell metabolic activities. Temperature-sensitive and host-range mutants have been isolated and placed into seven complementation groups. A large number of site-directed and deletion mutations in virtually every gene of these viruses have been prepared and analyzed utilizing the infectious cDNA clones of SIN and SFV. Close to 50% of the amino acids in the structural proteins of SFV are identical to SIN, and 64% are identical between the nonstructural proteins of the two viruses. A chimeric virus with SFV capsid gene and SIN glycoprotein genes is infectious but the reciprocal construct is not.

Defective interfering (DI) particles are generated within six to nine passages in tissue culture cells at high multiplicities of infection. Genomes of DI particles are about one-third the size of wild-type genome and contain scrambled and repeated portions of the genome. Three regions of the wild-type virus genome are conserved in the DI particle: the 5′ domain, a packaging site near the 5′ end of the RNA and 19 bases at the 3′ terminus of the genome RNA.

SIN and SFV undergo recombination; homologous, nonhomologous and aberrant homologous crossovers occur.

SIN and SFV vectors have been developed to express a foreign gene inserted in the subgenomic region and expressed from the 26S mRNA. They contain conserved *cis*-acting sequences and are self-replicating. Helper viruses containing virus structural genes but lacking nonstructural genes and packaging signals are used to package replicon RNA. These virus-like particles can be used to deliver the foreign genes into a susceptible host cell in place of cDNA or RNA.

Evolution

Evolutionary trees have been constructed based on sequences of eight alphaviruses and they show SIN and SFV occupying different subgroupings. SIN-like viruses that were isolated from different geological areas but are related serologically diverge up to 20% in amino acid identity. Western equine encephalitis virus is a recombinant that contains glycoprotein genes derived from a SIN-like virus and the rest of its genome from an Eastern equine encephalitis virus parent. Domains in the SIN nonstructural proteins, nsP1 (methyltransferase), 2 (helicase) and 4 (core RNA polymerase), are homologous to similar domains in tobacco mosaic virus and several other RNA plant viruses in the bromo and tobamo groups. Furthermore, the plant viruses transcribe one or more subgenomic mRNAs, one of which encodes the capsid, in a manner identical to SIN and SFV. There is the suggestion that alphaviruses evolved from recombination with the tobamo group of plant viruses.

Immunological Response

SIN and SFV are highly antigenic and produce high titers of neutralizing antibodies. Humans with antibodies to SIN and SFV have been found worldwide. Cell-mediated immunity and cytokine response also play important roles in clearing virus infections. Benign persistent infections result from infection of *scid* mice, which lack both humoral and cell-mediated immunity.

Two domains of E2 have epitopes to which monoclonal antibodies will bind and block infection (neutralizing). One epitope of E1 generated a monoclonal antibody that is neutralizing and this overlaps with an E2 epitope. Most monoclonal antibodies to E1 are sensitive to the protein's conformation but this is not so with E2, as monoclonal antibodies can react with fragments and denatured protein. Antibodies to some of the latter peptides injected into mice protected them against virus infection.

See also: **Encephalitis viruses (*Flaviviridae*): Encephalitis viruses and related viruses causing hemorrhagic disease; Pathogenesis: Animal viruses; Rubella virus (*Togaviridae*); Viral membranes; Virus structure: Atomic structure, Principles of virus structure.**

Further Reading

Johnston RE and Peters CJ (1995) In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology* 3rd edn, p. 843. Philadelphia: Lippincott-Raven.

Schlesinger S and Schlesinger MJ (1995) In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology* 3rd edn, p. 825. Philadelphia: Lippincott-Raven.

Strauss JH and Strauss EG (1994) The alphaviruses: gene expression, replication and evolution. *Microbiol. Rev.* 58: 491.

SINGLE-STRANDED RNA PHAGES (*LEVIVIRIDAE*)

J van Duin, Gorlaeus Laboratories, Leiden University, Leiden, The Netherlands

Introduction

Since their discovery in 1961 by Loeb and Zinder, the RNA phages have served as a model system to explore a variety of problems in molecular biology. As a source of homogeneous and readily obtainable messenger RNA, they have been particularly helpful in solving questions on regulation of gene expression at the level of translation. The concepts of translational polarity and translational control by repressor proteins resulted from early studies on bacteriophage RNA.

The RNA phages have also provided us with the best defined RNA replication system, which is currently used to study template recognition, *in vitro* RNA recombination and replication.

Available infectious clones have opened the possibility to explore the compromise between the need to fold in a specific way and the need to encode proteins.

Taxonomy and Classification

The RNA coliphages form the family *Leviviridae*. Within this family two genera can be distinguished, *Levivirus* and *Allolevivirus*. The leviviruses, also known as supergroup A, are subdivided into group I and II, whereas the alloleviviruses (supergroup B) consist of the groups III and IV (**Fig. 1**).

The best characterized phage in group I is MS2. Close relatives are R17, f2, M12 and JP501. Somewhat more distant but still in group I is fr, isolated by Hoffmann-Berling from a dung hill. The genomes of MS2, M12, JP501 and fr are completely sequenced and from the others partial sequences are known. In group II, GA and KU1 have been fully sequenced. Other members of this group are JP34, TH1 and BZ13. The prototype of group III is Qβ which, together with M11 and MX1, has been sequenced. Other members include VK and TW18. In group IV SP and NL95 are sequenced representatives. Other strains belonging to group IV are FI, TW19 and TW28. Most of the phages mentioned here are part of the Watanabe Collection at the Keio University, Japan.

Classification into the four groups (**Fig. 1**) was initially based on serological and physicochemical properties, but is presently being replaced by hybridization with group-specific DNA probes. This test recognizes the nucleic acid sequence as the primary criterion for classification. Serotyping is sometimes ambiguous since a few amino acid substitutions in the major coat protein can change the immunological properties dramatically.

Most research has centered around groups I (MS2) and III (Qβ) but it is assumed that features found for group I also hold for group II. Likewise, properties of group III should also exist in group IV.

Virion Structure

In addition to one molecule of positive-strand RNA, each virion contains 180 copies of the coat protein and one copy of the maturation, or A, protein. The group III phages contain in addition about 12 copies of the read-through protein in their capsid. For this reason, the protein shell of the single-stranded (ss) RNA phages is not isomeric like other icosahedral viruses such as poliovirus or satellite tobacco necrosis virus. The diameter of the phage is 26 nm, and the protein shell is about 2 nm wide (**Fig. 2**). The icosahedral shell has a $T = 3$ surface lattice. Crystals of phage MS2, GA, fr and Qβ have been obtained. The coat protein structure of MS2 has been solved to 2.7 Å resolution by x-ray diffraction. Unfortunately, the RNA and the A protein are not seen in the electron

Further Reading

Johnston RE and Peters CJ (1995) In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology* 3rd edn, p. 843. Philadelphia: Lippincott-Raven.

Schlesinger S and Schlesinger MJ (1995) In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology* 3rd edn, p. 825. Philadelphia: Lippincott-Raven.

Strauss JH and Strauss EG (1994) The alphaviruses: gene expression, replication and evolution. *Microbiol. Rev.* 58: 491.

SINGLE-STRANDED RNA PHAGES (*LEVIVIRIDAE*)

J van Duin, Gorlaeus Laboratories, Leiden University, Leiden, The Netherlands

Introduction

Since their discovery in 1961 by Loeb and Zinder, the RNA phages have served as a model system to explore a variety of problems in molecular biology. As a source of homogeneous and readily obtainable messenger RNA, they have been particularly helpful in solving questions on regulation of gene expression at the level of translation. The concepts of translational polarity and translational control by repressor proteins resulted from early studies on bacteriophage RNA.

The RNA phages have also provided us with the best defined RNA replication system, which is currently used to study template recognition, *in vitro* RNA recombination and replication.

Available infectious clones have opened the possibility to explore the compromise between the need to fold in a specific way and the need to encode proteins.

Taxonomy and Classification

The RNA coliphages form the family *Leviviridae*. Within this family two genera can be distinguished, *Levivirus* and *Allolevivirus*. The leviviruses, also known as supergroup A, are subdivided into group I and II, whereas the alloleviviruses (supergroup B) consist of the groups III and IV (**Fig. 1**).

The best characterized phage in group I is MS2. Close relatives are R17, f2, M12 and JP501. Somewhat more distant but still in group I is fr, isolated by Hoffmann-Berling from a dung hill. The genomes of MS2, M12, JP501 and fr are completely sequenced and from the others partial sequences are known. In group II, GA and KU1 have been fully sequenced. Other members of this group are JP34, TH1 and BZ13. The prototype of group III is Qβ which, together with M11 and MX1, has been sequenced. Other members include VK and TW18. In group IV SP and NL95 are sequenced representatives. Other strains belonging to group IV are FI, TW19 and TW28. Most of the phages mentioned here are part of the Watanabe Collection at the Keio University, Japan.

Classification into the four groups (**Fig. 1**) was initially based on serological and physicochemical properties, but is presently being replaced by hybridization with group-specific DNA probes. This test recognizes the nucleic acid sequence as the primary criterion for classification. Serotyping is sometimes ambiguous since a few amino acid substitutions in the major coat protein can change the immunological properties dramatically.

Most research has centered around groups I (MS2) and III (Qβ) but it is assumed that features found for group I also hold for group II. Likewise, properties of group III should also exist in group IV.

Virion Structure

In addition to one molecule of positive-strand RNA, each virion contains 180 copies of the coat protein and one copy of the maturation, or A, protein. The group III phages contain in addition about 12 copies of the read-through protein in their capsid. For this reason, the protein shell of the single-stranded (ss) RNA phages is not isomeric like other icosahedral viruses such as poliovirus or satellite tobacco necrosis virus. The diameter of the phage is 26 nm, and the protein shell is about 2 nm wide (**Fig. 2**). The icosahedral shell has a $T = 3$ surface lattice. Crystals of phage MS2, GA, fr and Qβ have been obtained. The coat protein structure of MS2 has been solved to 2.7 Å resolution by x-ray diffraction. Unfortunately, the RNA and the A protein are not seen in the electron

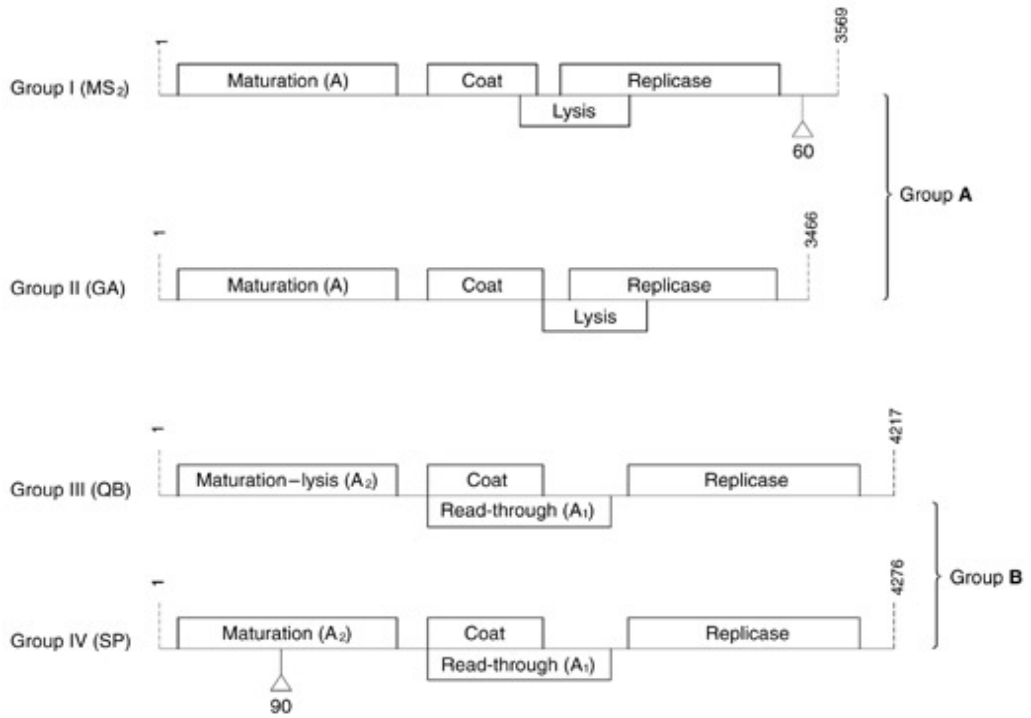


Figure 1 Genetic map of groups I–IV RNA coliphages. Inserts in group I with respect to group II and in group IV with respect to group III are given as triangles.

density map. These molecules are apparently not ordered in the crystal.

The Infection Process

The RNA gains access to the interior of the cell via long, filamentous structures called pili. These can be of various origins and serotypes. In *Escherichia coli*, the sex (or F) pili are employed as vehicle but in *Pseudomonas* and *Caulobacter* polar pili are used for this purpose. Transfer of RNA via pili is not essential for infection, however. Cells that lack pili can be infected if they are converted to spheroplasts.

The attachment of the phage to the sides of the pili proceeds via the maturation (A) protein (**Fig. 3**). The coat protein is dispensable for this process; bacteria can be infected with a binary complex of A protein attached to phage RNA. In group III phages the minimal infection set requires the additional presence of the readthrough (A1) protein.

Contact of the phage with the pilus results in cleavage of the A protein into a 15 kDa and a 24 kDa fragment. This cleavage probably triggers the ordered ejection of the tightly packed RNA from the virus shell. The binding sites of the MS2 A protein on the RNA have been determined at nucleotide regions 400 and 3500, respectively. When cleavage of the A protein occurs between the two RNA binding domains of the protein this would potentially lead to the liberation of the two ends. Conceivably, the 5′ end of the RNA begins to move along the pilus towards the cell. This stage of infection corresponds to the RNase-sensitive step. The two A protein fragments remain associated with the RNA during penetration of the cell envelope. However, it is unlikely that these protein fragments play any further role, since the naked RNA is fully able to generate infectious progeny in spheroplasts. For example, transformation of *E. coli* with phage cDNA containing plasmids leads to productive infection.

Host Range and RNA Phages of Other Genera

RNA phages have also been found in other Gram-negative bacteria. In *Pseudomonas aeruginosa* PP7 and 7S have been characterized and ϕCb5, ϕCb12r, ϕCb8r and ϕCb23r were found in different *Caulobacter* strains. Lately, an *Acinetobacter* RNA phage was identified. As judged by several criteria, these phages must be very similar to the coliphages. They have the same morphology, diameter, and molecular weight range. Sequence comparison shows PP7 to be a member of supergroup A.

Pseudomonas and *Caulobacter* phages enter the cell via polar pili. The dependence of RNA phages on pili

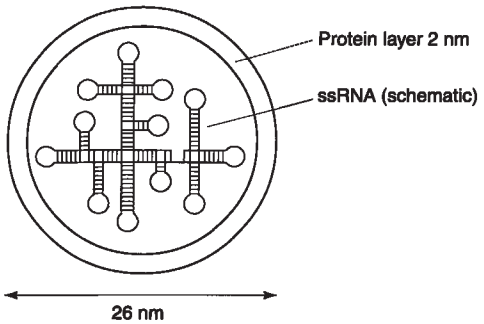


Figure 2 Schematic view of an RNA bacteriophage. The RNA is shown as a highly ordered structure.

as an entry to the bacterial cytoplasm is also reflected in the existence of the ssRNA phage PRR1 that will infect many genera such as *Pseudomonas, E. coli, Salmonella typhimurium,* and *Vibrio cholerae,* provided the host expresses the pili encoded in the drug resistance factor RP (P compatibility group, e.g. RP1, RP4, or R1822). In this connection, it should be mentioned that some members of the *Enterobacteriaceae* have been artificially converted to coliphage sensitivity by introducing the F factor of *E. coli* into *Shigella, Proteus* or *Salmonella.*

Ecology

Furuse has examined and reviewed the geographical distribution of the ssRNA phages as well as their present-day habitat. They are most frequently encountered in sewage and feces of mammals, and their titers in sewage samples may be as high as 10^7 PFU/ml. RNA phages may constitute up to 90% of total coliphages present in these samples, but the number can vary substantially.

The geographical distribution of groups II and III shows a strong bias. In northern Japan, there is a relative abundance of group II over group III (6:1) per sampling site. Moving southward, this ratio drops dramatically until in Southeast Asia group II becomes rare. Furuse has suggested that the north–south gradient is related to differences in climate. Group III (and also groups I and IV) propagates well at 40°C but not at 20°C, whereas for group II the situation is reversed. A problem with this hypothesis is that group II is rare in, for example, The Netherlands where the average temperature is below 20°C.

Although the natural host for ssRNA phages is not known with certainty, it is clear that they survive passing through the gastrointestinal tract of gnotobiotic mice and propagate stably in the intestines if *E. coli* is present as host. Thus *E. coli* can sustain the life cycle of the phage under 'natural' circumstances. In Japan and The Netherlands attempts have been made to determine whether certain phage groups are preferentially associated with certain animal species or with humans. So far, these studies have not been conclusive.

Index Organism

The RNA coliphages and enteroviruses share the same habitat. In addition, because of their structural similarity RNA phages show approximately the same inactivation characteristics as enteroviruses in sewage water treatment processes. For this reason RNA phages can be and are used as index organisms for the possible occurrence of pathogenic enteroviruses.

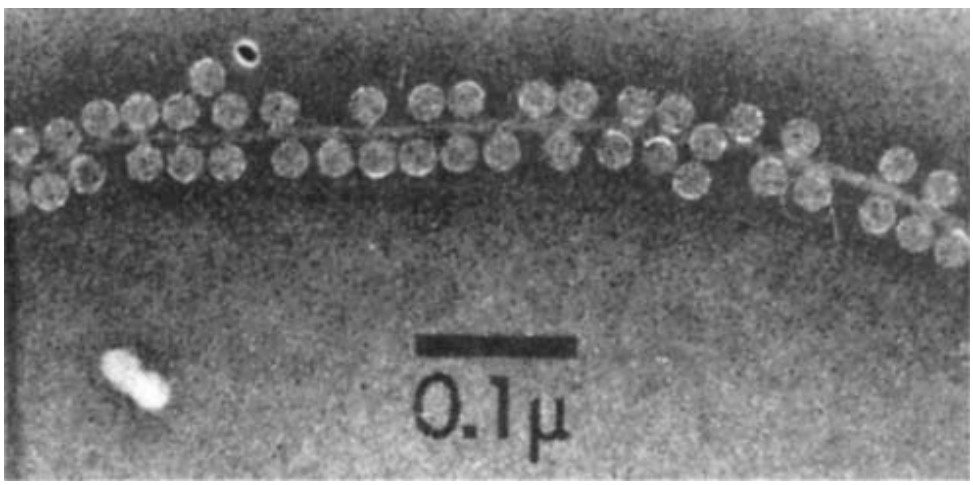


Figure 3 RNA phages attached to the F pilus of *E. coli.* Bar = 0.1 μg. (Reproduced with permission from Zinder ND (1975) Attachment, ejection and penetration stages of the RNA phage infectious process. In: Zinder ND (ed.) *RNA Phages,* p. 89. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.)

RNA phages can be positively identified by specific DNA hybridization probes (see above).

The Genetic Map

A map of the four groups is shown in **Fig. 1**. The conspicuous difference between supergroups A and B is the presence of a readthrough protein in group B. Here, the major coat protein gene ends in a leaky UGA stop codon that is read as tryptophan with a probability of about 5%. This leads to a C-terminally extended coat protein that is incorporated in the capsid and required for infectivity. The other major divergence is the presence of a separate lysis gene in group A. This out-of-frame overlapping gene encodes a hydrophobic peptide about 70 amino acids long, that short-circuits the cytoplasmic membrane of the bacterium. Somehow, loss of membrane potential distorts the balance between components of the bacterial enzyme ensemble that cleaves and extends the peptidoglycan network. This leads to cell lysis.

Genetic differences within the genera are more subtle. In supergroup A the most pronounced one is a 60-nucleotide insertion in the 3′ untranslated leader of group I. A 90-nucleotide insertion in the A2 protein gene of group IV represents the major difference with group III in supergroup B.

The Lysing Protein of Group B Phages

The strong similarity in gene arrangement and control of gene expression between the ssRNA phages is fully lost in the way the phages organize their escape from the wasted host. Bacteria that overproduce the Qβ maturation protein lyse. Qβ mutants carrying an amber mutation in the maturation protein gene do not cause bacteriolysis. Thus it is now thought that the maturation protein of supergroup B has a dual function; it is a constituent of the virion and it triggers cell lysis.

Control of Gene Expression

The appearance of the phage-coded proteins in the infected cell is carefully controlled in timing and amount. For instance, replicase is a minor early product, and the amount of coat protein exceeds by far that of the other proteins. Since no DNA intermediates occur in the life cycle of the phage, control is predominantly exerted at the translational level: it is the RNA secondary structure that regulates access of ribosomes to initiation regions.

The maturation protein is needed at only one copy per virion, and its translation is accordingly kept at a low level. In MS2 the maturation gene can only be translated from a growing strand. Its ribosomal binding site is only accessible during a short period when the growing RNA has not yet reached its equilibrium folding. In the equilibrium structure the RNA is inaccessible. For Qβ the mechanism awaits clarification.

In group A, both replicase (R) and lysis (L) gene are under translational control of the coat gene as witnessed by the observation that early nonsense mutations in the coat gene inhibit expression of the R and L genes. For the replicase the underlying mechanism is base-pairing between the start of the R gene and a coding region of the coat gene. Coat protein-synthesizing ribosomes pass through this region, destroy the pairing and temporarily liberate the R start. Coupling of the lysis gene arises by a local hairpin burying the L start. Exposure is brought about by a ribosome that has arrived at the coat gene terminator codon. After releasing the synthesized coat protein this ribosome can, with a low efficiency, reach the L start site by random movements along the RNA.

The real down regulator of replicase is the coat protein. Once present in sufficient concentration, dimers bind to a hairpin structure that contains the R-start, thereby preventing any ribosome binding. The complex between the hairpin and the coat protein dimer has been modeled by x-ray analysis.

Translational coupling of L and R genes, and the temporary expression of the A gene are not only simple ways to cut down on the product levels, but the designs also serve a more sophisticated purpose. On full-sized RNA the only site independently accessible to ribosomes is the start of the coat gene. As discussed below replicase binding also involves this start site. The resulting competition between ribosome and replicase effectuates that the RNA is either used for replication or for translation.

Apart from the occasional competition by the replicase, the coat protein is not negatively regulated.

Replication

Most of our knowledge on replication has been obtained in the Qβ system, but it is assumed that the principles also apply to the other groups. The replicase holoenzyme contains four different proteins called subunits I to IV, or subunits α, β, γ and δ. Subunit I was identified as ribosomal protein S1, and subunits III and IV are the translation elongation factors EF-Tu and EF-Ts. Thus, three proteins that normally function in the synthesis of proteins are recruited by the phage to assist in RNA synthesis. Subunit II is encoded in the viral genome, and these four subunits all occur in the enzyme complex in not more than one copy. Replication proceeds via the synthesis of a free negative strand. Annealed positive

and negative strands are not a substrate for the replicase enzyme.

The requirements for copying the positive and the negative strand are different. To copy the negative strand, only subunits II, III and IV are required, whereas positive strand replication also needs subunit I. In addition, copying the positive strand is greatly dependent on the product of the bacterial *hfq* gene, called Host Factor. In the uninfected cell one function of this protein seems to be to promote the accessibility of highly structured messengers. In replication its role may be similar, since replication in the absence of Host Factor proceeds only if the base-pairing occluding the 3′ terminus of Qβ RNA is destabilized by substitutions that introduce mismatches. Supergroup A uses a different host factor for copying the positive strand.

Replicase binds to two internal Qβ RNA sequences, called the S and M site. The interaction with the S site provides for the necessary competition with the ribosome, but it is not required for replication itself. Interaction with the M site is essential. It is supposed that in this binary complex the folding of the RNA places the 3′ terminus in the active site of the enzyme. In this concept then, the specificity of the enzyme is derived from RNA structure and not from sequences. From this point of view it is easy to see that Qβ replicase can multiply group IV RNAs as the RNA foldings in groups III and IV are very similar.

Replication of the negative strand depends on structure elements at both the 5′ and 3′ termini and is thus basically different from positive strand copying.

6S RNA and Qβ RNA Variants

Infection of *E. coli* by Qβ leads, apart from phage multiplication, to the accumulation of what has been termed '6S' RNA. This is a nonhomogeneous collection of RNA molecules that vary in size from about 50 to 200 nucleotides and that together with their negative strands serve as templates for Qβ replicase. They do not code for any protein nor do they contribute to the infection process. These molecules arise either by continuous deletion of Qβ RNA sequences or by RNA recombination events using the plethora of RNA fragments present in each cell. Some show homology with Qβ RNA but others do not. All are characterized by a high degree of secondary structure and like all phage RNA have at least three consecutive Cs at their 3′ ends. 6S RNA is also generated on transformation of *E. coli* with the cDNA for Qβ replicase or by the *in vitro* incubation of Qβ replicase with the four nucleotide triphosphates in the absence of added template. The last experiment showed that Qβ replicase is able to perform uninstructed RNA synthesis.

From an evolutionary point of view, it is interesting that these molecules that are not constrained by a coding sequence quickly respond to selective pressure. It is easy to obtain mutants of 6S RNA that are adapted to *in vitro* replication under adverse conditions, such as the presence of ethidium bromide, T1 ribonuclease or limiting amounts of the building blocks. Abbreviated Qβ RNA variants can be prepared *in vitro* by gradually reducing the time allowed for Qβ replication. After some 70 replication rounds the length of Qβ was reduced to about 12%. There seems no basic difference between such truncated Qβ RNA and the RNA present in the defective interfering (DI) virus particles that accompany, for instance, influenza infection. Also here, the abridged molecules once created by replication errors can survive as long as they are templates for the replicase, and their multiplication does not endanger the survival of the virus population as a whole.

The rapid yield to selective pressure by 6S RNA and the Qβ RNA variants reflects the inaccuracy of the Qβ replicase, which has been estimated at between 10^{-3} and 10^{-4} per nucleotide per replication. The presumed absence of a $3' \rightarrow 5'$ exonuclease editing activity in Qβ replicase would be consistent with its relatively low copying fidelity. At the same time the frequency with which deletions occur must also be unusually high.

Rigidity and Plasticity of the Genome Structure

Considering the inaccuracy of phage RNA replication one might expect an endless scale of phage sequences, all fit to survive. This turns out not to be true. The sequences of Qβ and MS2 have, despite many years of laboratory cultivation, not or hardly changed. Similarly it has turned out to be quite difficult to find RNA phages in nature whose sequences diverge substantially from the group prototype. For instance, group I representatives like f2, R17, M12, JP501, all isolated independently in different parts of the world, show more than 90% sequence identity. Thus there seem to be very few solutions that are good enough to coexist. It is assumed that the selection pressure, which discards all potential variants (for instance those having synonymous codons), originates from the contribution of the RNA secondary structure (**Fig. 2**) to phage fitness. Such contributions involve regulatory circuits, RNase resistance, delaying the annealing of positive and negative strands and probably many other parameters.

In spite of this apparent rigidity the genome

structure shows a high degree of flexibility under noncompetitive conditions. Laboratory evolution of phages containing a wanton distortion in RNA structure yields many pseudorevertants presenting us with a large spectrum of structural solutions. These solutions all perform well as long as they do not have to compete with their wild-type counterpart. Then they lose and disappear from the population.

Phylogeny of RNA Phages

An interesting but necessarily most difficult question is that of the origin and kinships of the RNA bacteriophages. It is generally assumed that all of them derive from a common ancester because of the nearly identical genetic organization, the strong resemblance of the replicases, the use of the same host proteins (except Host Factor) as auxiliaries in the copying reaction, and the similarities of several control mechanisms. These properties are more easily explained by divergent than by convergent evolution.

Therapeutic Use of RNA Phages

There is presently renewed interest in DNA phages as combatants of bacterial infections. Early pilot studies showed that *E. coli* adapts to RNA phages by losing its F pili.

Further Reading

Armon, R and Kott Y (1966) Bacteriophages as indicators of pollution. *Crit. Rev. Environ. Sci. Technol.* 26: 299.

Furuse K (1987) Distribution of coliphages in the environment: general considerations. In: Goyal SM (ed.) *Phage Ecology*, p. 87. New York: Wiley.

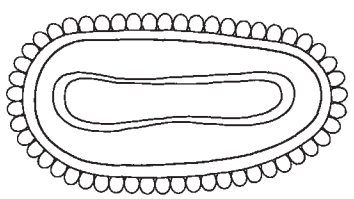
Havelaar HA (1991) Bacteriophages as model viruses in water quality control. IAWPRC Study Group on Health Related Water Microbiology. *Wat. Res.* 25: 529.

Olsthoorn RCL, Licis N and van Duin J (1994) Leeway and constraints in the forced evolution of a regulatory RNA helix. *EMBO J.* 13: 2660.

Valegård K, Murray JB, Stockley PG, Stonehouse NJ and Liljas L (1994) Crystal structure of an RNA bacteriophage coat protein–operator complex. *Nature* 371: 623.

Van Duin J (1988) In: Fraenkel Conrat H and Wagner RR (eds) *The Bacteriophages*. Series *The Viruses*, p. 117. New York: Plenum Press.

SMALLPOX AND MONKEYPOX VIRUSES (*POXVIRIDAE*)



Keith Dumbell, Department of Medical Microbiology, University of Cape Town Medical School, Cape, South Africa

Geoffrey L Smith, Sir William Dunn School of Pathology, University of Oxford, Oxford, UK

Smallpox (Variola) Virus

History

A Chinese account of a disease which was clearly smallpox was written in the 4th century. Over the next two centuries reliable accounts described smallpox in Japan, Korea, India and Egypt. Shortly after this, the Islamic expansion carried smallpox into North Africa and Europe. From the 15th century smallpox was taken to America and southern Africa with the spread of European colonists. According to the Chinese account, smallpox had appeared in China during the first century, but it may have been current in India well before that. A few Egyptian mummies show evidence of a pustular rash which has some resemblance to smallpox, but there are no accounts of such a disease in contemporary Egyptian or Jewish writings.

Taxonomy and classification

Variola (smallpox) viruses belong to the *Orthopoxvirus* genus of the *Chordopoxvirinae* subfamily of DNA viruses in the *Poxviridae* family and have linear, double-stranded DNA genomes of 186 kb. The virions are large (about 250 × 200 nm), brick-shaped particles. Like vaccinia they can be seen as mature, intracellular virions or as enveloped, extracellular virions. When viewed by electron microscopy after dehydration and embedding in Epon, the particles have two lateral bodies and a central core, but the

structure shows a high degree of flexibility under noncompetitive conditions. Laboratory evolution of phages containing a wanton distortion in RNA structure yields many pseudorevertants presenting us with a large spectrum of structural solutions. These solutions all perform well as long as they do not have to compete with their wild-type counterpart. Then they lose and disappear from the population.

Phylogeny of RNA Phages

An interesting but necessarily most difficult question is that of the origin and kinships of the RNA bacteriophages. It is generally assumed that all of them derive from a common ancester because of the nearly identical genetic organization, the strong resemblance of the replicases, the use of the same host proteins (except Host Factor) as auxiliaries in the copying reaction, and the similarities of several control mechanisms. These properties are more easily explained by divergent than by convergent evolution.

Therapeutic Use of RNA Phages

There is presently renewed interest in DNA phages as combatants of bacterial infections. Early pilot studies showed that *E. coli* adapts to RNA phages by losing its F pili.

Further Reading

Armon, R and Kott Y (1966) Bacteriophages as indicators of pollution. *Crit. Rev. Environ. Sci. Technol.* 26: 299.

Furuse K (1987) Distribution of coliphages in the environment: general considerations. In: Goyal SM (ed.) *Phage Ecology*, p. 87. New York: Wiley.

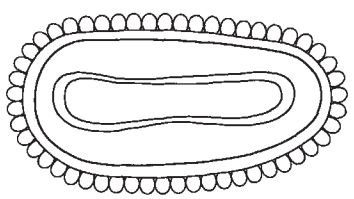
Havelaar HA (1991) Bacteriophages as model viruses in water quality control. IAWPRC Study Group on Health Related Water Microbiology. *Wat. Res.* 25: 529.

Olsthoorn RCL, Licis N and van Duin J (1994) Leeway and constraints in the forced evolution of a regulatory RNA helix. *EMBO J.* 13: 2660.

Valegård K, Murray JB, Stockley PG, Stonehouse NJ and Liljas L (1994) Crystal structure of an RNA bacteriophage coat protein–operator complex. *Nature* 371: 623.

Van Duin J (1988) In: Fraenkel Conrat H and Wagner RR (eds) *The Bacteriophages*. Series *The Viruses*, p. 117. New York: Plenum Press.

SMALLPOX AND MONKEYPOX VIRUSES (*POXVIRIDAE*)



Keith Dumbell, Department of Medical Microbiology, University of Cape Town Medical School, Cape, South Africa

Geoffrey L Smith, Sir William Dunn School of Pathology, University of Oxford, Oxford, UK

Smallpox (Variola) Virus

History

A Chinese account of a disease which was clearly smallpox was written in the 4th century. Over the next two centuries reliable accounts described smallpox in Japan, Korea, India and Egypt. Shortly after this, the Islamic expansion carried smallpox into North Africa and Europe. From the 15th century smallpox was taken to America and southern Africa with the spread of European colonists. According to the Chinese account, smallpox had appeared in China during the first century, but it may have been current in India well before that. A few Egyptian mummies show evidence of a pustular rash which has some resemblance to smallpox, but there are no accounts of such a disease in contemporary Egyptian or Jewish writings.

Taxonomy and classification

Variola (smallpox) viruses belong to the *Orthopoxvirus* genus of the *Chordopoxvirinae* subfamily of DNA viruses in the *Poxviridae* family and have linear, double-stranded DNA genomes of 186 kb. The virions are large (about 250 × 200 nm), brick-shaped particles. Like vaccinia they can be seen as mature, intracellular virions or as enveloped, extracellular virions. When viewed by electron microscopy after dehydration and embedding in Epon, the particles have two lateral bodies and a central core, but the

lateral bodies are not evident when orthopoxviruses are observed by cryoelectron microscopy.

Variola virus has a close antigenic relationship to other orthopoxviruses; it is distinguished as a species by its biological characters and by the characterization of its DNA genome.

Virion structure and properties

What is known about the structure, physical properties and replication of variola virus conforms so closely to the much better studied vaccinia that the reader is referred to that entry. One point of difference is that the variola genome has only a short inverted terminal repeat sequence (ITR) of 725 bp, unlike the 10 kb ITR of vaccinia virus.

Geographic and seasonal distribution

The last natural case of smallpox occurred in 1977; and in 1980 the World Health Organization (WHO) declared that the disease had been eradicated. In its heyday smallpox had a worldwide distribution. The development of effective public health services and the routine practice of vaccination gradually eliminated the disease from North America and most of Europe, but outbreaks, apparently endemic in origin, continued in these parts of the world through the first decades of the 20th century. For a variety of reasons, routine vaccination failed to eradicate smallpox from many tropical and subtropical areas. At the start of the global eradication campaign in 1967 there were still 31 countries in which the disease was endemic.

After smallpox eradication was complete, the number of laboratories maintaining stocks of variola virus was rapidly reduced. At present (1998), variola virus is maintained in just two high-containment laboratories situated in Atlanta, USA and in Novosibirsk, Russia. The WHO is coordinating a program to obtain DNA sequences from representative strains of variola. It is intended that these sequence data shall become the permanent record of this virus and all remaining stocks of the virus shall be destroyed before the end of the 20th century.

Smallpox was maintained entirely by transmission from an active case to another human; latency did not occur. Consequently, active search would reveal cases throughout the year in countries where the disease was endemic. Nevertheless, there were low seasons and high seasons. In tropical areas it seems likely that the seasonal changes in incidence were achieved more through the ease or difficulty of traveling about than from direct effects of climate on survival of virus or susceptibility to it.

Host range and virus propagation

Variola virus had a narrow host range and natural infection was confined to humans. The virus was propagated in laboratories on the chick chorioallantois or in cell cultures, usually of human origin. There was no experimental animal model in which inoculation of variola virus produced clinical disease resembling smallpox and in which the pathology could have been studied. The virus produced a mild disease in some species of monkeys and could be propagated, at least for a time, in baby mice and in rabbits.

Genetics

DNA restriction maps of variola from different geographic areas were remarkably uniform and showed little, if any, variation in viruses from different countries. Consistent differences were found between variola major strains and alastrim strains from South America, but this did not apply to strains of variola minor from Africa. The complete DNA sequence of variola virus strains Bangladesh-1975 and India-1967 and the partial sequences of Harvey-1947, Congo-1970, Somalia-1977 and Garcia-1966 have been determined and are deposited in DNA data banks. There are several instances of some of the individuality of variola virus, but there is at present no evidence that variola encodes any gene which has no counterpart in the genome of another poxvirus.

Comparisons of the entire sequence of a variola virus with that of vaccinia virus has revealed that the central two-thirds of the genomes are highly conserved, with >95% nucleotide identity, but towards the termini the sequences diverge and there are sequences unique to either virus. There are several instances of highly related coding sequences being intact in one virus and truncated or fragmented in the other. Surprisingly, in the majority of cases where coding sequences are disrupted, the sequence is intact in vaccinia and broken in variola. Some open reading frames that at first appeared to be unique to variola have been found to be present in other orthopoxviruses. At present it would appear that the virulence of variola virus for humans probably resided in the combination and variation of multiple genes present in several poxviruses, rather then a 'key' unique virulence gene.

Variola viruses readily recombine with other orthopoxviruses: some of the resulting recombinants were characterized biologically, but work with variola virus ceased before these results were correlated with the exchange of particular segments of the parental genomes.

Evolution

The naturally occurring orthopoxviruses: cowpox, monkeypox and camelpox, currently occupy separate geographic and biologic niches in Europe, Africa and the Middle East. The similarities between the genomes of these three viruses, variola and vaccinia are sufficient to imply a common ancestor. Furthermore, there is evidence that several orthopoxvirus genes, for instance those encoding enzymes for virus transcription and DNA replication, have counterparts in other genera of poxviruses, such as leporipox, capripox, avipox and suipox, molluscipox and yatapox. Two orthopoxviruses that have been identified in America, volepox and raccoonpox, are less closely related to other orthopoxviruses.

Camelpox seems to be most closely related to variola, in biological properties, in some features of the genome, and in its natural infection of only a single species. There is, however, no firm evidence that camelpox virus can infect humans, despite much contact between camels and unvaccinated humans. It is possible that both camelpox and variola viruses having found a single species present in numbers adequate to maintain their survival, have lost the presumed primitive ability of orthopoxviruses to utilize a variety of host species – an ability which has been retained for the survival of cowpox and monkeypox.

Serologic relationships and variability

There was never any evidence for antigenic variability in variola viruses. Indeed it was even difficult to separate variola from other orthopoxviruses by serologic tests. Any strain of vaccinia would protect against variola in any part of the world and in different years. In accord with this, the proteins of the envelope of extracellular virions are highly conserved between vaccinia and variola. Several different antigens of variola virus find their way to the surface of the infected cell before virus release. Because of this and also the length of the incubation period, it is unlikely that minor variation in any particular antigen would confer sufficient advantage to be preferentially selected.

Epidemiology and transmission

The vast majority of smallpox was contracted by direct contact with an infected person. There were rare instances of indirect spread via fomites but animals played no part in sustaining or transmitting the variola virus.

Despite occasional startling incidents, smallpox required close contact for effective transmission and spread mainly within households or to other close contacts.

Those infected with smallpox remained well for the 10–12 days of the incubation period and, during this time, were not infectious to others. In natural smallpox (as opposed to variolation) virus was transmitted by droplet infection. The most usual source was the lesions that occurred in the pharynx or mouth (the enanthem). These ulcerated more quickly than the lesions developing in the skin (the exanthem) and were well situated for distribution to close contacts. Primary lesions were presumed to occur in the respiratory tract but were never seen or demonstrated post mortem. Throat swabs from contacts who subsequently developed smallpox were occasionally found to harbor virus during the incubation period, but there was no evidence that transmission occurred before the end of the incubation period. Nor did those who were subclinically infected pass on the disease.

Cases were potentially infectious until all the lesions had completely healed but virus was not so readily dispersed from dried exudate or scabs. There was no evidence of latency and no spontaneous recurrence. Consequently, once an outbreak had run its course or been controlled in a local community, smallpox would not recur unless reintroduced from outside that community.

Outbreaks varied in severity, but even in a severe outbreak there would be some milder cases. At times whole outbreaks of mild smallpox occurred with little or no fatality. This led to the distinction of two varieties of the disease, variola major and variola minor, and to the presumption that the variola minor outbreaks were caused by a distinct strain of variola virus of lower virulence. In some areas the minor form became established as the endemic strain. The variety of variola minor known as alastrim apparently arose towards the end of the 19th century and produced consistently mild disease. This strain was first described in southeast USA and thence spread through North America, to Europe, to South America and to Australasia. Outbreaks of minor smallpox were still occurring in Europe in the 1950s and in Brazil through the 1960s. Isolates from outbreaks of alastrim in Europe and Brazil shared biologic characters and a DNA restriction pattern which distinguished them from variola major viruses. The position in Africa was less well defined. Outbreaks of major and of minor smallpox continued to occur. Although the endemic variola in some African countries was clinically and epidemiologically variola minor in recent years, virus isolates from these cases could not be distinguished from isolates of variola major virus.

Clinical features

The incubation period of 10–12 days was terminated by the abrupt onset of fever and prostration, often with nausea or vomiting. On the 3rd day of illness a macular rash appeared; this became papular and then vesicular and eventually pustular. The rash tended to be more concentrated on the head and limbs than on the trunk. The lesions were firm and raised but also based deeply in the skin. Scabs formed as the ulcerated pustules dried and gradually separated over 2–3 weeks, leaving depigmented areas. Residual facial scarring, due to destruction of sebaceous glands, was a characteristic feature of the survivors of major smallpox. Blindness was another, not uncommon, sequel. The above description applies to classical cases of major smallpox, in which the fatality rate was 16–30%. Prognosis was worse if the rash was slower in development, with the lesions remaining flat and soft or becoming hemorrhagic. Sometimes the initial prostration was severe, generalized hemorrhagic manifestations appeared on the second or third day and no distinctive rash developed. These patients invariably died, usually between the 5th and 7th day of illness. A more favorable prognosis was signified if the rash evolved more quickly and the lesions were more superficial. This was the usual course of events in people who had been vaccinated, but not recently enough to give complete protection.

When the causative virus was variola minor, prostration was notably less than in patients who had a comparable extent of rash following infection with variola major virus.

Pathology and histopathology

The majority of smallpox lesions were in the skin and mucous membranes of the mouth and oropharynx. The small number of postmortem studies that were done showed few specific lesions in internal organs, except in cases of hemorrhagic smallpox. In the absence of obvious damage in the vital organs it was presumed that death had been due to the profound toxemia. Fuller accounts will be found in the texts cited at the end of this entry.

Immune response

Antibody that neutralized intracellular virus and hemagglutination inhibiting (HI) antibody appeared by about the 6th day of the disease, corresponding to the 18th day after infection. This is slower than the antibody response to primary vaccination and may explain partly why vaccination soon after contact with smallpox often ameliorated the outcome. However, experience with the complications of vaccination showed that a failure to develop cell-mediated immunity was more important than failure to develop antibody. It is presumed that cell-mediated immunity also controlled the development and resolution of smallpox lesions, but this was not adequately investigated.

Recovery left a lasting immunity; although second attacks of smallpox were not unknown. Neutralizing antibody persisted for many years; HI antibody was demonstrable for some months to a few years.

Prevention and control

Successful vaccination gave complete protection against smallpox for at least 3 years and probably 5 or more. Immunity waned after longer intervals, but rarely, if ever, did anyone who had been vaccinated contract fatal smallpox. The International Certificate of vaccination against smallpox was given a validity of 3 years, but those at high risk were normally revaccinated at yearly intervals.

Compulsory routine vaccination should, theoretically, have eradicated smallpox in any area where it was practiced. However, in many countries where the disease was endemic, the fraction of the population that escaped the vaccination program sufficed to maintain a chain of active cases and a reservoir of the virus.

The main factors in containing an outbreak of smallpox were the isolation of cases and the tracing of contacts before the end of their incubation period. These measures were supplemented with vaccination or revaccination in the locality of the outbreak, and were effective in the control and elimination of smallpox introduced into a nonendemic area. The eradication of smallpox from endemic areas was achieved by detecting current outbreaks and containing them. This strategy effectively interrupted the local transmission of the virus and progressively reduced the number of chains of transmission which remained.

Future perspectives

In 1980 the World Health Organization certified officially the eradication of smallpox, and no further cases have come to light in the 18 years that have elapsed since then. This major achievement seems to be firmly established. The only remaining viable variola viruses are the two reference collections of variola strains held in high containment laboratories at Atlanta and Novosibirsk and the possibility that virus might have survived in the bodies of any smallpox victims who were mummified or buried in permafrost conditions. Extrapolation from the known rates of decay of variola virus in smallpox scabs indicates that this possibility is unrealistic.

It has been agreed that the two collections of variola virus strains should be destroyed before the end of the twentieth century. The archival record of variola virus will then be the complete DNA sequence of a small number of representative strains and some fragments of variola DNA which have been cloned into recombinant plasmids. Of course much of the gene pool of variola virus will still remain, in the genomes of other, closely related orthopoxviruses. When the genomes of further orthopoxviruses have been sequenced it will be possible to determine what features of the variola genome were unique to that virus. The section on monkeypox includes a discussion of whether variola virus could emerge again from natural sources.

Monkeypox Virus

History

Monkeypox virus was first described as the cause of an apparently spontaneous outbreak of fever and rash in a colony of cynomolgus monkeys held at the State Serum Institute in Copenhagen in 1958. During the next few years similar outbreaks were reported in monkey colonies in the USA. In 1966 monkeypox infection was introduced into the zoo at Rotterdam. Here the first animals affected were giant anteaters from South America, but the disease spread to various species of apes and monkeys. The viruses isolated from these animals were found to be similar and to represent a species of orthopoxvirus that had not been described before 1958. In two separate incidents in 1970 monkeypox virus was found to have caused a smallpox-like illness in humans in the Democratic Republic of the Congo (formerly Zaire) and in Liberia. Sporadic cases have continued to occur in the African rain forest belt, particularly in the Democratic Republic of the Congo. The virology and epidemiology of monkeypox have been intensively investigated because of the possible threat to the success of smallpox eradication.

Taxonomy and classification

Monkeypox virus is a separate species of the *Orthopoxvirus* genus. Biologically it can be differentiated from other species by the morphology and ceiling temperature of its pocks on the chick chorioallantois and by its pathogenicity to rabbits. Antigenically it crossreacts extensively with other orthopoxviruses but produces a few specific antigens which can be detected with suitably absorbed antisera or monoclonal antibodies. The genome, of approximately 190 kb, has a distinctive restriction map, and DNA sequence data are available for parts of the genome.

Virion properties and replication

There is no reason to believe that replication of monkeypox virus differs significantly from the more extensively studied vaccinia virus.

Geographic and seasonal distribution

The reservoir of monkeypox virus is confined to the tropical rain forest belt of Africa. On current information the reservoir hosts are arboreal rodents, from which sporadic transmissions occur to simians and to humans. Human infections are rare events: most of the reported cases have been in the Democratic Republic of the Congo, which harbors more than half of the present African rain forest. There is no marked seasonal incidence, but some correlation with times of maximum agricultural activity (see Epidemiology).

Partial DNA sequence studies of American and European isolates show identity with isolates from West Africa, thus confirming the presumption that the outbreaks in Europe and America were initiated by subclinical infection in monkeys or other animals exported from West Africa. There have been no outbreaks outside Africa since 1968, following a general tightening of regulations concerning exports of animals. DNA sequence data confirm that the outbreaks in Europe and America were caused by a monkeypox virus originating from West Africa rather than Central Africa.

Host range and virus propagation

Under experimental conditions monkeypox virus is capable of infecting most common laboratory animals and many simian species. The resulting disease is usually mild in African monkeys but Asian and South American monkeys suffer moderate to severe disease. Apes, with the exception of chimpanzees, are seriously affected and the wide range of susceptible exotic animals is illustrated by the two South American giant anteaters which became infected while in transit to the Rotterdam Zoo.

In its natural habitat, monkeypox virus has been recovered from many sporadically infected humans and from one sick squirrel (*Funisciurus anerythrus*) and a chimpanzee. Other information comes from serologic surveys; antibody specific to monkeypox has been detected in a significant proportion of squirrels belonging to the genera *Funisciurus* and *Heliosciurus*. Specific antibody was also detected in seven species of cercopithecus, and also in cercocebus and colobus monkeys. Monkeypox virus was propagated in

laboratories on the chick chorioallantois, where it produced characteristic small pocks with a central hemorrhage. Cell cultures of many different species were susceptible.

Genetics

*Hind*III restriction maps of the monkeypox virus genome confirm the conservation of the central part of the orthopoxvirus genome throughout the genus. Although restriction site maps show more variation in the outer quarters of the genome, DNA fragments from these regions crosshybridize strongly with corresponding fragments of other orthopoxviruses and rule out any long region of sequence that could be unique to monkeypox. There must be short unique stretches to account for the monkeypox-specific antigens that have been demonstrated, but few studies of DNA sequence in monkeypox have yet been reported. There is some variation in restriction site maps of monkeypox isolates from different geographical areas but variants with more dramatic changes in genome structure can readily be isolated in the laboratory. These are the 'white pock' mutants, which also have been described among other orthopoxviruses with a hemorrhagic pock phenotype, such as cowpox.

Evolution

Monkeypox virus, like cowpox virus, appears to be sustained by transmission in small rodents. The present distributions of these viruses do not overlap but the antigenic and genetic similarities among all the orthopoxviruses are such that they must share a common ancestor. Each of these two viruses is known to infect a variety of species under natural conditions, including humans. This could have led to the possibility of further speciation. It has been suggested that monkeypox might be the progenitor of variola. This is ruled out because a gene sequence (ORF) which is present in variola (and vaccinia) has been shown to be degenerate and partially deleted in monkeypox DNA. Also it must be remembered that smallpox appeared in East Asia well before it was current in Africa (see the earlier section on variola virus).

Epidemiology and transmission

In the Democratic Republic of the Congo, sporadic cases of monkeypox infection have continued. Most cases occur in small rural villages in the rain forest belt. The age-specific incidence of primary monkeypox (i.e. excluding spread from person to person) is highest in young children between 1 and 8 years, peaking in the 3–4 year age group. Young children have access to the cleared areas between the village and the forest; in this area also are found squirrels of the genera *Funisciurus* and *Heliosciurus* in which monkeypox antibody is prevalent. The age-specific incidence of secondary cases is more evenly distributed. Most outbreaks are believed to be limited to the primary (and sometimes coprimary) cases. Chains of presumed human-to-human transmission have so far been limited but there is concern that the virus may adapt to become more transmissible in humans. Ongoing surveillance is required. Variation in the reported incidence from area to area and from year to year may well depend on natural fluctuations in the wildlife reservoir but will also be affected by the efficiency of surveillance activities. Transmission from person to person is probably from the enanthem via the respiratory route, but the secondary attack rate among unvaccinated household contacts is much lower than that found in smallpox. Based on experience to date, the basic case reproduction rate does not exceed 1.0 and outbreaks would be self-limiting even in an unvaccinated community.

Clinical features

The incubation period of human monkeypox appears to be about 12 days, the same as for smallpox, but there have been only a few instances where it could be accurately determined. The clinical picture of human monkeypox closely follows that of ordinary smallpox. Most cases in unvaccinated children have been severe, but equivalents of the flat or hemorrhagic types of smallpox have not been encountered. The illness begins with fever, followed in 1–3 days by the rash, and an enanthem is usually present on the oral mucosa. Unlike smallpox, most patients develop a generalized lymphadenopathy or, less often, a regional lymphadenopathy. Mortality in the unvaccinated has been about 11%, though somewhat higher than this in children under 2 years of age. Nearly all deaths have been in children under the age of 9 years.

Pathology and histopathology

This has only been studied in experimental infection of monkeys. Histopathology of the lesions resembled that of smallpox. Following intramuscular inoculation, there was an intense local inflammatory response. Virus spread to the regional lymph nodes and thence to spleen, tonsil and bone marrow. Viremia occurred between the 3rd and 14th days and a generalized rash appeared about the 7th or 8th day.

Immune response

The antibody response to infection with monkeypox virus can conveniently be detected by hemagglutina-

tion inhibition or by ELISA tests. High titers in straight antibody tests suggest a response to monkeypox rather than to vaccination, but a residual titer after absorption with vaccinia virus is required to demonstrate antibody specific to monkeypox. For this reason radioimmunoassay tests have been most useful because of the high titers obtainable from unabsorbed sera by this technique. More specific tests are in course of development.

Prevention and control

Recent vaccination with vaccinia virus effectively protects against monkeypox, and those who had been vaccinated some years previously were susceptible but were less likely to develop severe illness. Widespread routine vaccination would be necessary to protect all who might come into contact with monkeypox; but the incidence of monkeypox is so low that complications arising from the vaccination program might rival the morbidity to be expected from monkeypox itself.

Future perspectives

The main incidence of monkeypox in humans has been in young children. Although this age group is currently unprotected by vaccination, there has not been any evidence of a rising incidence of monkeypox infections. Until recently monkeypox has not presented a public health problem serious enough to require specific action. Significantly more cases of monkeypox have been reported recently, including longer chains of transmission than had previously been recorded. The situation is being closely monitored and recent isolates of the virus will be rigorously compared to see if significant changes have occurred. At this stage it is hard to forecast future developments.

See also: **Cowpox virus (*Poxviridae*); Immune response: Cell mediated immune response, General features; Vaccines and immune response; Vaccinia virus (*Poxviridae*).**

Further Reading

Fenner F, Henderson DA, Arita I, Jezek Z and Ladnyi ID (1988) *Smallpox and its Eradication*. Geneva: World Health Organization.

Fenner F, Wittek R and Dumbell KR (1989) *The Orthopoxviruses*. New York: Academic Press.

Jezek Z and Fenner F (1988) Human monkeypox. *Monogr. Virol.* 17.

SOBEMOVIRUSES

OP Sehgal, University of Missouri, Columbia, Missouri, USA

History

Southern bean mosaic virus (SBMV) is the archetype of the plant virus genus *Sobemovirus* in the family *Tetraviridae*. SBMV was the subject of intensive biological and physicochemical investigations during the 1940s and was the only virus, other than tobacco mosaic and tomato bushy stunt viruses, that had been well characterized at this early period. In the 1970s through the mid 1980s, SBMV served as a prototype on the applicability of small angle x-ray diffraction and neutron scattering in deciphering virion organization. During the same period, studies with SBMV and turnip rosette virus (TRoSV), yielded vital information on the nature of macromolecular forces that govern virion stability.

Taxonomy and Classification

The International Committee on Taxonomy of Viruses recently established the genus *Sobemovirus* in the family *Tetraviridae* (1995). At present, a number of viruses including SBMV and TRoSV are recognized as members of the sobemovirus genus. These are: blueberry shoe string virus (BSSV), cocksfoot mottle virus (CfMV), lucerne transient streak virus (LTSV), rice yellow mottle virus (RYMV), Rottboellia yellow mottle virus (RoYMV), *Solanum nodiflorum* mottle virus (SNMV), sowbane mosaic virus (SoMV), subterranean clover mottle virus (SCMoV) and velvet tobacco mottle virus (VTMoV). Additionally, seven viruses may be considered as probable members pending availability of additional

tion inhibition or by ELISA tests. High titers in straight antibody tests suggest a response to monkeypox rather than to vaccination, but a residual titer after absorption with vaccinia virus is required to demonstrate antibody specific to monkeypox. For this reason radioimmunoassay tests have been most useful because of the high titers obtainable from unabsorbed sera by this technique. More specific tests are in course of development.

Prevention and control

Recent vaccination with vaccinia virus effectively protects against monkeypox, and those who had been vaccinated some years previously were susceptible but were less likely to develop severe illness. Widespread routine vaccination would be necessary to protect all who might come into contact with monkeypox; but the incidence of monkeypox is so low that complications arising from the vaccination program might rival the morbidity to be expected from monkeypox itself.

Future perspectives

The main incidence of monkeypox in humans has been in young children. Although this age group is currently unprotected by vaccination, there has not been any evidence of a rising incidence of monkeypox infections. Until recently monkeypox has not presented a public health problem serious enough to require specific action. Significantly more cases of monkeypox have been reported recently, including longer chains of transmission than had previously been recorded. The situation is being closely monitored and recent isolates of the virus will be rigorously compared to see if significant changes have occurred. At this stage it is hard to forecast future developments.

See also: **Cowpox virus (*Poxviridae*); Immune response: Cell mediated immune response, General features; Vaccines and immune response; Vaccinia virus (*Poxviridae*).**

Further Reading

Fenner F, Henderson DA, Arita I, Jezek Z and Ladnyi ID (1988) *Smallpox and its Eradication*. Geneva: World Health Organization.

Fenner F, Wittek R and Dumbell KR (1989) *The Orthopoxviruses*. New York: Academic Press.

Jezek Z and Fenner F (1988) Human monkeypox. *Monogr. Virol.* 17.

SOBEMOVIRUSES

OP Sehgal, University of Missouri, Columbia, Missouri, USA

History

Southern bean mosaic virus (SBMV) is the archetype of the plant virus genus *Sobemovirus* in the family *Tetraviridae*. SBMV was the subject of intensive biological and physicochemical investigations during the 1940s and was the only virus, other than tobacco mosaic and tomato bushy stunt viruses, that had been well characterized at this early period. In the 1970s through the mid 1980s, SBMV served as a prototype on the applicability of small angle x-ray diffraction and neutron scattering in deciphering virion organization. During the same period, studies with SBMV and turnip rosette virus (TRoSV), yielded vital information on the nature of macromolecular forces that govern virion stability.

Taxonomy and Classification

The International Committee on Taxonomy of Viruses recently established the genus *Sobemovirus* in the family *Tetraviridae* (1995). At present, a number of viruses including SBMV and TRoSV are recognized as members of the sobemovirus genus. These are: blueberry shoe string virus (BSSV), cocksfoot mottle virus (CfMV), lucerne transient streak virus (LTSV), rice yellow mottle virus (RYMV), Rottboellia yellow mottle virus (RoYMV), *Solanum nodiflorum* mottle virus (SNMV), sowbane mosaic virus (SoMV), subterranean clover mottle virus (SCMoV) and velvet tobacco mottle virus (VTMoV). Additionally, seven viruses may be considered as probable members pending availability of additional

Table 1 Geographical distribution, natural hosts and insect vectors of sobemoviruses

Virus	*Geographical distribution*	*Natural hosts*	*Vectors*
SBMV	USA, Central and South America, Africa, Asia	Bean, cowpea	Beetles
BSSV	USA, Canada	*Vaccinium corymbosum*, *V. angustifolium*	Aphids
CfMV	Europe, New Zealand	Cocksfoot, wheat	Beetles
LTSV	Australia, New Zealand, Canada	*Medicago sativa* (lucerne)	Beetles
RYMV	Tropical Africa	*Oryza sativa* (rice)	Beetles
RoYMV	Nigeria	Itchgrass	Unknown
SNMV	Australia	*Solanum nodiflorum*, *S. nigrum*, *S. nitidibaccatum*	Mirid, beetles
SoMV	World-wide	*Chenopodium* spp., apple, grapes, *Atriplex suberecta*	Leafminers, aphids
SCMoV	Australia	Subterranean clover, club clover	Beetles
TRoSV	UK	Turnip, swede	Beetles
VTMoV	Australia	*Nicotiana velutina*	Mirid, beetles
Tentative members			
CYMV	Colombia	*Calopogonium mucunoides* (calopo)	Beetles
CMMV	Europe	Cocksfoot	Aphids
CyMV	Europe, New Zealand	*Cynosurus cristatus*	Aphids
GCFV	Asia, Australia, New Zealand	Ginger	Unknown
PMV	USA, Mexico, Africa	Several grasses	Beetles
RgMV	Japan	*Dactylis glomerata* *Lolium multiflorum*,	Unknown
SsbMV	India	*Sesbania grandiflora*	Unknown

information on their properties. These are: calopo yellow mosaic virus (CYMV), cocksfoot mild mosaic virus (CMMV), cynosurus mottle virus (CyMV), ginger fleck virus (GCFV), panicum mosaic virus (PMV), ryegrass mottle virus (RgMV), and sesbania mosaic virus (SsbMV). Olive latent virus 1, a putative sobemovirus, resembles *Necrovirus* species in its genomic organization and expression and should be included in the genus *Necrovirus*. Other tentative virus species are in the genus.

Geographical Distribution, Natural Hosts and Vectors

Most sobemoviruses have somewhat limited distribution, but as a group, they are found throughout the world (**Table 1**). Individual members have few natural hosts but, as a whole, they infect a wide spectrum of plant species. Eight sobemoviruses affect dicotyledonous species, and an equal number affect monocotyledonous species, but none are transmitted to both. Leaf-eating beetles are their most common vectors.

Properties of the Virion

Sobemovirus virions are isometric with a diameter of *ca.* 25–30 nm (**Fig. 1**). Most virions exclude negative stains from penetrating to the core, a reflection of a highly compact capsid organization. SBMV virions exhibit no structural changes even when inactivated (>99%) by exposure to ultraviolet light, heating, freezing and thawing or nitrous acid. However, EDTA treatment, which removes capsid-associated divalent cations, results in virion 'swelling', and such virions are then rendered permeable to negative stains.

Sobemovirions are nonenveloped, and contain *ca.* 21% RNA and 79% protein. SBMV, LTSV and TRoSV virions contain significant amounts of intimately bound divalent cations (calcium, magnesium). The virions sediment sharply as single components in sucrose gradients, with $s_{20,w}$ values ranging from 109S (PMV) to 120S (BSSV). A characteristic feature of most sobemovirions is that they band homogeneously in cesium chloride (densities ranging from 1.34 to 1.39 g ml^{-1}) but heterogeneously in cesium sulfate gradients. The banding heterogeneity in cesium

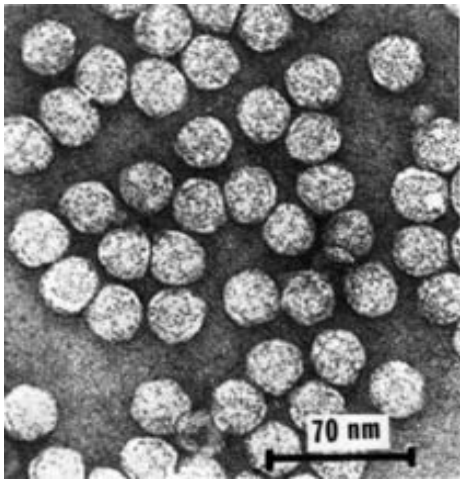


Figure 1 Purified virions of southern bean mosaic virus, bean strain. Uranyl formate was used as the negative stain.

sulfate gradients is a reflection of conformational variations among virions in a population rather than any differences in their chemical composition.

Capsid Organization and Stabilizing Interactions

The sobemovirus capsid is constructed from a protein of about 30 kDa. A proportion of coat protein subunits in SBMV and TRoSV virions exist as stable dimers that are preferentially linked to RNA. The basic SBMV capsid structure is $T = 3$ quasi-symmetry of 180 subunits. High resolution x-ray diffraction has revealed three types of quasi-equivalent subunits, namely A, B and C, which are chemically identical but have different conformations. The A subunits cluster at the fivefold axes, whereas sets of B and C aggregate at the quasi-sixfold vertices. Each subunit consists of a random domain, i.e. the N-terminal 'arm' located towards the virion interior, and the surface domain, which is organized into an eight-stranded antiparallel β barrel and five α helices. Considerable similarity exists between the organization of SBMV capsid with those of tomato bushy stunt, tobacco necrosis, poliovirus type 1 and human rhinoviruses. Sobemovirus virions are extremely stable *in vitro* in their native state. For SBMV and TRoSV, this stability is derived from strong inter-subunit linkages mediated by hydrophobic interactions, divalent cations and pH-dependent bonds. Additionally, protein–RNA linkages contribute to virion stability. Perturbations in the pH-dependent and divalent cation-mediated bonds cause the capsid to relax, rendering it sensitive to proteases, salt or detergents. Only LTSV and CyMV among the sobemoviruses, are sensitive to detergent in their native states.

Properties of the Genome

Sobemovirus genomes are linear, single-stranded RNAs of positive polarities and range in mol. wt from *ca.* 1.3×10^6 to 1.5×10^6. The complete sequences of genomic RNAs of SBMV bean strain (4109 nucleotides (nt)), SBMV cowpea strain (4194 nt), RYMV (4450 nt), CfMV (4038 nt) and LTSV (4275 nt) have been determined. A covalently linked protein (VpG) is present at the 5′ terminus in all of these RNAs; VpG is necessary for the infectivity of SBMV, RYMV and LTSV RNAs. The 5′ end sequence motifs for SBMV and LTSV are ACAAA; for RYMV it is ACAA; whereas for CfMV it is AUAAU. Furthermore, SBMV, LTSV and RYMV genomes contain a polypurine sequence, AG(G)AAA, about 8–10 nt downstream from the ACAA(A) element; for CfMV, the comparable sequence is AGAAAGA. The 3′ ends of sobemoviral genomes lack poly(A) tails and, unlike many other plant viral genomes, are not configured into tRNA like structures.

Encapsidated Subgenomic and Satellite RNAs

Virions of SBMV, GCFV, LTSV, SNMV, SCMoV, TRoSV, VTMoV and CMMV encapsidate minor amounts of heterogeneous subgenomic (sg) or putative sgRNAs. VTMoV virions contain two discrete sgRNAs, RNA-1a (0.63×10^6 Da) and RNA-1b (0.25×10^6 Da), besides the genomic RNA (**Fig. 2**). CfMV virions encapsidate a discrete 0.5×10^6 Da RNA and several intermediate-sized putative sgRNAs. Included among the heterogeneous population of SBMV and TRoSV sgRNAs are the autonomized coat protein cistrons. The sgRNAs of SBMV, RYMV and LTSV possess VpG at their 5′ ends followed by the same sequence motifs as their respective genomic RNAs. LTSV, RYMV, SCMoV, SNMV and VTMoV virions encapsidate discrete, viroid-like satellite (sat) RNAs, in addition to the genomic and subgenomic RNAs. These satRNAs exist in linear and circular forms (**Fig. 2**). Complete sequences are known for satRNAs of LTSV (322 nt), RYMV (220 nt), SCMoV (322 nt and 380 nt), SNMV (377 nt) and VTMoV (366 nt). The satRNAs of SNMV and VTMoV exhibit a high degree of sequence homology and are different from those of SCMoV or LTSV. At 220 nt, RYMV satRNA represents the smallest known, naturally occurring virioid-like RNA. A region comprising 19% of the sequence of RYMV satRNA shows about 93% homology to the

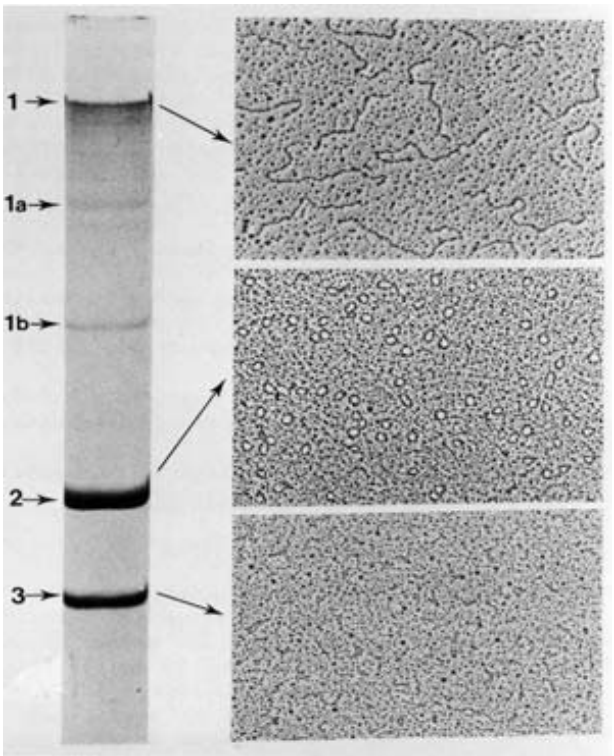


Figure 2 Polyacrylamide gel electrophoresis and electron microscopy of the virion RNAs of velvet tobacco mottle sobemovirus. (Reproduced with permission from Velvet Tobacco Mottle Virus, *AAB Descriptions of Plant Viruses* no. 317, 1986; courtesy of Dr J. W. Randles.)

satRNA of a Canadian LTSV isolate. The sobemoviral satRNAs contain a conserved hammerhead structure which is involved in self-cleavage (ribozyme).

Transmission

Sobemoviruses are transmitted readily with sap inoculation, a reflection of their high endogenous concentration and particle stability. Transmission via contact with leaf abrasion during strong wind is possible, but actual proof is lacking. RYMV exuded with guttation fluid may contaminate irrigation water, which then serves as the inoculum source. Some sobemoviruses, SBMV, SCMoV, SoMV and PMV, are transmitted through the seed.

Insects are the principal vectors of sobemoviruses. SBMV, CfMV, CYMV, PMV, RYMV and TRoSV are transmitted by chrysomelid beetles, whereas SNMV and VToMV are transmitted by coccinellid beetles; SBMV is transmitted also by a coccinellid beetle. The mirid bug, *Cyrtopeltis nicotianae,* is the vector of SNMV and VToMV. Aphids have been implicated in the transmission of BSSV, CMMV and CyMV. SoMV is transmitted by the leafminer fly, *Liriomyza langei;* it is carried mechanically on mouth parts and the ovipositor.

SBMV is acquired by the chrysomelid beetle, *Ceratoma trifurcata,* within a few minutes after feeding and transmitted without a latent period. Virions are present in fairly high concentration in the regurgitant fluid, intestines and hemolymph, but there is no evidence of SBMV multiplication in the vector. The virus persists in beetles for about 5–7 days. It is transmitted through contaminated mouth parts, during regurgitation and with reflexive bleeding. The coccinellid beetle, *Epilachna varivestis,* is an efficient SBMV vector, but the virus is not found in the hemocoel. Obviously, systemic transport within the beetle's body is not a prerequisite for SBMV transmission. CfMV is transmitted by a cereal leaf beetle, *Lema melanopa.* It is excreted in the fecal matter and can cause infection if deposited at freshly damaged feeding sites.

Experimental Host Range and Symptomatology

Most sobemoviruses have restricted host ranges and infect only a few species in one or two plant families. For example, RoYMV infects itchgrass (*Rottboellia cochinchinensis*) and corn (*Zea mays*) in the family Gramineae. The host ranges of SBMV, SCMoV and CYMV are restricted to a few species in the family Leguminosae; CfMV is restricted to the family Gramineae, whereas VToMV is confined to the family Solanaceae. SNMV, LTSV and TRoSV infect a few species in 3–4 different families. Additionally, most sobemoviruses have few hosts in common. For example, of the eight sobemoviruses that affect Gramineae, only PMV and RoYMV are transmitted to corn.

Symptoms induced by sobemoviruses are persistent mosaic, mottle or chlorosis, often accompanied by leaf deformities and stunted plant growth. CYMV produces a striking yellow variegation of leaves. BSSV causes shoestring symptoms in which the leaves are transformed into curled, strap-like structures; additionally, flowers show 'breaking', and immature berries exhibit reddish streaking or vein-banding patterns.

The Infection Process

Sobemoviruses enter through wounds caused by mechanical abrasion or insect feeding and, like other plant viruses, usually establish infection in the directly invaded cells. However, SBMV virions introduced by *E. varivestis* at the feeding site move out rapidly via

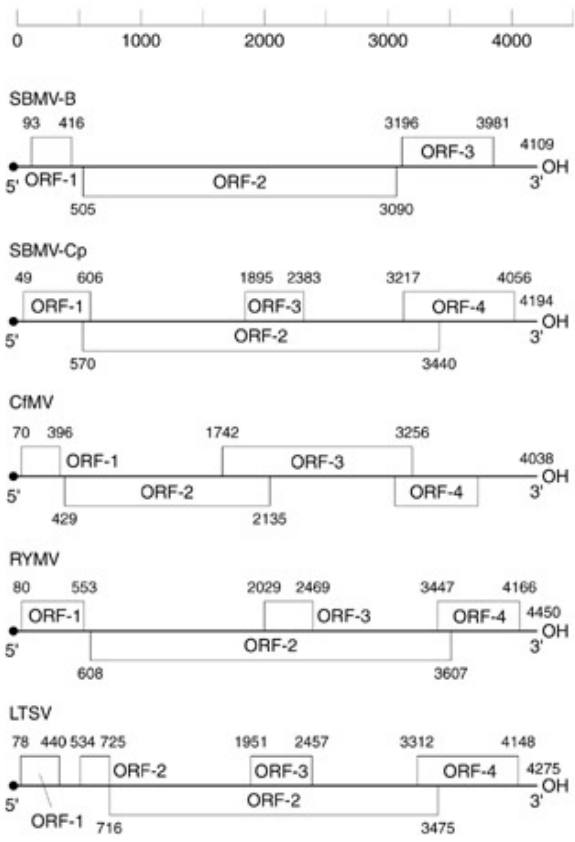


Figure 3 Organization of sobemovirus genomes. The viral RNA is represented by the thick line, and VpG is shown as the solid circle at the 5′ terminus. The open reading frames (ORFs) are shown as boxes.

leaf veins and cause infection in the unwounded cells. Apparently, SBMV virions can enter uninjured cells, although the precise mechanism is unknown. Upon entry into a cell, SBMV virions swell, extruding the 5′ terminus of RNA which is located near the capsid surface. A contact between the exposed RNA region and ribosomes leads to an early translational event, followed by complete capsid disassembly.

Isolated SBMV RNA must exist in a proper configuration to be infective. A marked structural stabilization of SBMV RNA *in situ* occurs when virions are heated, and this altered conformation is retained even when RNA is released from the capsid; this RNA is noninfectious. Upon structural destabilization, however, this RNA regains full infectivity. That SBMV RNA in a conformationally stabilized state is biologically inert, yet is rendered fully competent with denaturation, is a novel and unique phenomenon and underscores the importance of RNA secondary and tertiary structure in the infection process.

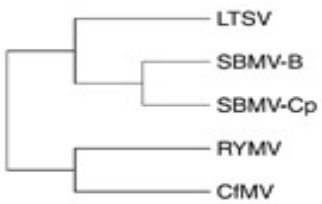


Figure 4 Relationship between coat proteins of sobemoviruses based on alignment by the clustal algorithm of their predicted amino acid sequences.

Replication and Expression of Virion RNAs

Although details are scanty, the replication mode of sobemoviral genomes appears to be typical of viruses that contain ssRNA of positive polarity, i.e. infection by plus strand RNA leads to the production of an intermediate double-stranded RNA complex from which progeny plus-strand RNAs are transcribed. The presence of genomic RNA replicating structures (approx. mol. wt 2.8×10^6) in SBMV and VTMoV infections have been detected in the cytoplasm where most of the virus-specific RNA polymerase activity is located.

Figure 3 is a schematic representation of the expression strategies of sobemoviral genomes. Each genome has three to four open reading frames (ORFs). The size of ORF 1 differs among these viruses and has a potential to encode a 12 kDa to 18 kDa protein; for LTSV ORF 1, two putative coding regions (ORF 1a and ORF 1b) have been identified. The nature or function of protein encoded by ORF 1 is not known, and little sequence similarity exists in this region among the sobemoviruses. ORF 2 encodes a polyprotein containing VpG, serine protease and polymerase domains. The CfMV polyprotein is encoded by two overlapping frames; ORF 2a codes for VpG and serine protease whereas ORF 2b codes for replicase which is expressed via a ribosomal frameshift event. The ORF 3 product has not been identified, and in SBMV-bean strain a comparable ORF is lacking. The viral coat proteins are encoded in ORF 3 of SBMV bean strain and ORF 4 of the other sobemoviruses; however, these coat proteins are expressed from sgRNAs. The transcription initiation sites for some of these sgRNAs have been tentatively identified and are: nt3241 for SBMV cowpea strain; nt3441 for RYMV and nt3285 for LTSV.

The relationship between sobemoviral coat proteins, based on alignment of their predicted amino acid sequences by clustal algorithm, is shown in **Fig. 4**. Among viruses infecting legumes, the SBMV bean and cowpea strains have coat proteins that are more

closely related to each other than to LTSV coat protein. Further, coat proteins of viruses affecting the Gramineae, RYMV and CfMV, appear more closely related between themselves than with the viruses that infect legumes. It will be of interest to ascertain if a similar relationship will hold true when sequence information for coat proteins of other sobemoviruses becomes available.

The replication of sobemoviral satRNAs, which proceeds via the rolling circle model proposed for viroids, is dependent on the presence of a suitable helper sobemovirus. The abilities of sobemoviruses to support satRNA replication, however, differs markedly. LTSV supports replication of satRNAs of SNMV and SCMoV, but SNMV does not support replication of VTMoV satRNA and vice versa. Also, LTSV satRNA replicates in the presence of an appropriate helper sobemovirus in divergent plant species. Thus, it replicates in *Brassica rapa, Raphanus raphanistrum* and *Sinapis arvense* (Brassicaceae) and *Chenopodium amaranticolor* (Chenopodiaceae) in the presence of TRoSV, and in *Triticum aestivum* (wheat) and *Dactylis glomerata* (Gramineae) in the presence of CfMV; all of these plants are nonhosts for LTSV. TRoSV fails to support LTSV satRNA replication in *Thlaspi arvense* or *Nicotiana bigelovii* although these plants are susceptible to TRoSV. Apparently, satRNA replication depends not only on the helper virus polymerase but also on some specific host factor(s).

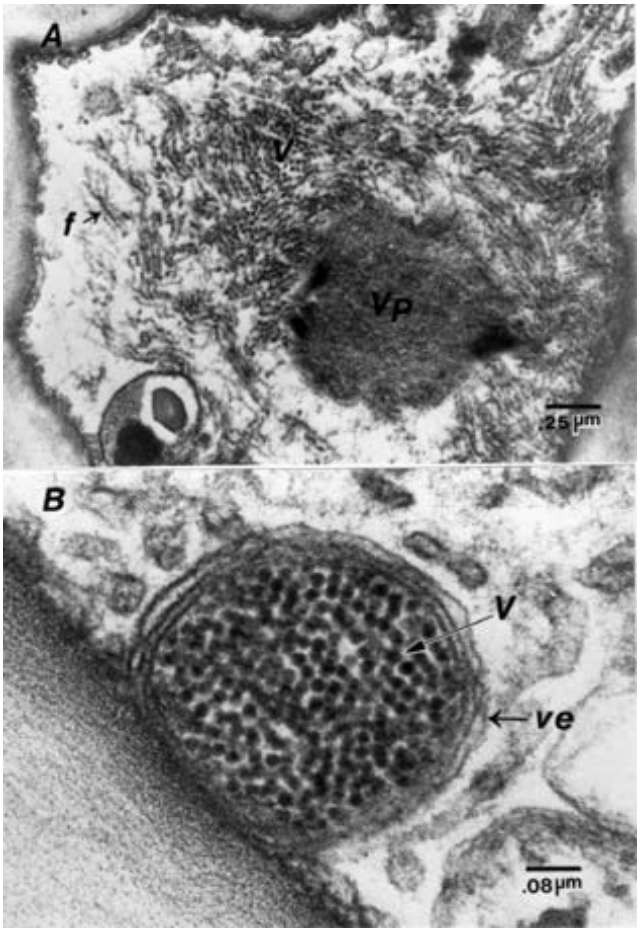


Figure 5 Foliar cells of *Calopogonium muconoides* infected with the calopo sobemovirus. (**A**) Virus-like particles, V, associated with viroplasm, V_P, and fibrils, f. (**B**) Virions, V, enclosed in a vesiculated structure, ve. (Reproduced with permission from Morales FJ (1995) A sobemovirus hindering utilization of *Calopogonium muconoides* as a forage legume in the lowland tropics. *Plant Dis.* 79: 1220.

Virion Assembly

The sequence of events leading to sobemoviral assembly *in vivo* is not understood. Under *in vitro* conditions, however, a few SBMV coat protein subunits (dimers?) bind with RNA, generating a complex which then serves to nucleate the capsid assembly. The coat protein amino-terminal arm appears to be the site with which RNA interacts first to generate this complex. Some evidence suggests that a stretch of 25 nucleotides (nt1410 to nt1436 in ORF 2) on the genomic RNA of SBMV cowpea strain is the coat protein recognition site; this region is highly conserved among sobemoviruses and configures into a stable hairpin structure. Since several subgenomic and satRNAs are encapsidated with great efficiency, these also must possess putative coat protein recognition site(s).

In Vivo Distribution and Cytopathology

Sobemoviruses are present in most plant parts including epidermis, mesophyll, meristem, xylem and phloem. Virions occur in cytoplasm and vacuoles, and except for BSSV, RYMV and CyMV infections, also in the nuclei. They are not found, however, in the mitochondria and chloroplasts. The cytoplasm of infected cells may contain fibrillar material resembling double-stranded replicative form of RNA, which is either enclosed in discrete membranous vesicles or distributed in diffused patches. Sometimes, virions are enclosed in discrete vesiculated structures (**Fig. 5**). In other cases they are arranged in a crystalline array in cytoplasm and vacuole. Bundles of microtubules are seen in cells infected with BSSV, RYMV and SNMV, but their nature is not known.

Serology

Sobemoviruses are strongly immunogenic. In general, they are neither serologically crossreactive nor are they related to viruses belonging to other groups.

However, SNMV and VTMoV are serologically related and so are SBMV, SsbMBV and CYMV; SCMoV and LTSV are distantly related.

SBMV bean and cowpea strains are serologically similar but not identical. Likewise, RYMV isolates from Kenya and the Ivory Coast are dissimilar. Isolates of CfMV and CyMV from New Zealand, but not those from Europe, are serologically related. No serological variations have been observed among the naturally occurring isolates of TRoSV, GCFV, SoMV or LTSV.

Epidemiology and Control

SBMV and RYMV often reach epiphytotic proportions under field conditions. For SBMV, seed-borne inocula and weeds serve as the primary sources of infection. RYMV incidence is considerably higher in areas of continuous rice cultivation than those with interrupted plantings. This suggests that locally present RYMV inocula contribute largely to disease initiation. The beetle vectors move rapidly from plant to plant spreading RYMV. During the off-season, RYMV survives in volunteer and wild rice (*Oryza longistaminata*) plants, regrowths of harvested crops and in ratoons. Some sources of germplasm for resistance towards SBMV and RYMV are available. In view of the worldwide importance of rice and bean/cowpeas in the human diet, the applicability of gene engineering techniques for developing virus-resistant cultivars needs to be exploited.

Conclusions

The sobemoviruses constitute a homogeneous group based on their physicochemical parameters, including stability characteristics, and genomic expression strategies. These viruses possess relatively simple organizations, and their genomes are the smallest among the plant viruses. Furthermore, sobemoviruses represent a class of genetically stable viruses because few naturally occurring variants or strains have been identified. Also, the markedly divergent and non-overlapping host ranges underscore a high level of biological specificity and host plant adaptability of sobemoviruses.

Though the primary structures of the coat proteins of SBMV and tobacco necrosis virus are largely similar, there is only a limited resemblance in their polymerase sequences around the GDD motif. However, considerable similarities exist in the amino acid sequence motifs of the putative polymerase and nucleic acid helicase proteins between sobemoviruses and members of luteovirus subgroup II. Moreover, presence of the ACAAAA element at the 5′ end of genomic and sgRNAs is a property which sobemoviruses share with members of the dianthovirus group and luteovirus subgroup II. LTSV, RYMV, SCMoV, SNMV and VTMoV can be distinguished from other sobemoviruses because they encapsidate satRNAs. That different sobemoviruses can support replication of a given satRNA is a reflection of the nonspecific nature of such an association. In this regard, LTSV satRNA seems most versatile because it interacts with a suitable helper virus in divergent dicotyledonous and monocotyledonous species. Likewise, LTSV, more than any other sobemovirus, is effective in supporting replication and encapsidation of sobemoviral satRNAs. It is rather interesting that satRNA of RYMV, a virus with host range restricted to the monocotyledons, exhibits structural homology with satRNA of LTSV which affects dicotyledonous species. Finally, RYMV satRNA, the smallest viroid-like RNA associated with a plant virus, possesses retroviroid and viroid-like structures making it a probable candidate as an evolutionary bridge between these classes of subviral plant pathogens. Thus, sobemoviral satRNAs offer attractive possibilities for indepth study of molecular interactions between two distinctive biological entities and on the nature and mode of origin of satRNAs associated with plant viruses.

See also: **Dianthoviruses (*Tombusviridae*); Luteovirus; Necroviruses (*Tombusviridae*); Plant virus disease – economic aspects; Vectors: Plant viruses; Virus structure: Principles of virus structure.**

Further Reading

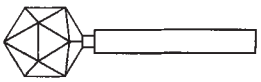
Brunt A, Crabtree A and Gibbs A (1990) *Viruses of Tropical Plants*. Wallingford: CAB International.

Francki RIB, Milne RG and Hatta T (1985) Sobemovirus group. In: *Atlas of Plant Viruses*, vol 1. Boca Raton: CRC Press.

Hull, R (1988) The Sobemovirus group. In: Koenig R (ed.) *The Plant Viruses*, vol. 3, *Polyhedral virions with monopartite RNA genomes*. New York: Plenum.

Sehgal, OP (1995) Sobemoviruses. In: Singh RP, Singh US and Kohmoto K (eds) Pathogenesis and host specificity in plant diseases; histopathological, biochemical, genetic and molecular bases, vol. 3, *Viruses and Viroids*. Oxford: Pergamon.

SPO1 PHAGE (*MYOVIRIDAE*)



Charles R Stewart, Department of Biochemistry & Cell Biology, Rice University, Houston, Texas, USA

History

SPO1 is a large virulent phage of the Gram-positive bacterium *Bacillus subtilis* and is in the genus 'SPO1-like viruses' of the *Myoviridae* family. Shunzo Okubo isolated it from soil in Osaka, Japan, during the early 1960s, and brought it with him to Chicago, where he collaborated with Bernard Strauss and Marvin Stodolsky on the first published studies of SPO1. Peter Geiduschek, Stodolsky's mentor, saw in SPO1 the opportunity to test the generality of the lessons then being learned about sequential gene action in the *Escherichia coli* phage T4, and began a series of experiments which illuminated the regulation of SPO1 gene action and stimulated the interest of many others.

Taxonomy and Evolution

SPO1 is a member of a family of *B. subtilis* phages, whose distinguishing feature is the presence of the unusual base, hydroxymethyluracil, instead of thymine in the DNA. All members of the family, which also includes SP82, Φe, 2C, SP8, H1 and SP5c, appear to be descended from a common ancestor, since they show many striking similarities in structure, restriction maps, genetic maps, gene products and gene regulation. However, they also show many differences in detail, and thus have had the opportunity for significant divergence. Perhaps the most striking similarity is the presence, in SPO1, SP82, 2C, and Φe, of a group I self-splicing intron, discovered by David Shub and his colleagues by testing RNAs from phage-infected cells for their capacity to incorporate labeled GTP. Each of these introns includes an open reading frame that specifies an endonuclease that catalyzes the substitution of its intron for the homologous intron in a related phage genome. The greater efficiency of the SP82 endonuclease, when acting on the SPO1 genome, permits SP82 DNA to exclude closely linked SPO1 alleles from the progeny of a mixed infection. These are among the very few introns known in procaryotes. The presence of introns both here and in T4 has been cited as an argument for the existence of introns in ancient evolutionary times, before the divergence of Gram-positive from Gram-negative bacteria. SPO1 is similar to T4 in many other ways as well, including size, structure, overall organization of the life cycle, and the presence of an unusual pyrimidine.

Virion Structure and Proteins

An SPO1 particle includes a single linear double-stranded DNA molecule and at least 53 different polypeptides, organized into an icosahedral head about 87 nm in diameter, a short neck, and a contractile tail of 19 × 140 nm, ending in a complex base plate 60 nm in diameter.

Genome Structure and Gene Function

The single DNA molecule is 145 kb long, with a 12.4 kb terminal redundancy, and with hydroxymethyluracil (hmUra) completely replacing thymine as the base-pairing partner for adenine. The presence of hmUra gives the DNA a CsCl buoyant density substantially higher than that of other DNAs of similar GC content, which has been useful in making experimental distinctions between phage and host DNAs. The two strands of the phage DNA are physically separable on CsCl, a characteristic that has been used extensively to identify the template strand for specific transcripts. Marmur and Greenspan used that property of SP8 to demonstrate for the first time that specific RNAs are complementary to one strand of the DNA from which they were transcribed.

Figure 1 shows a map of the SPO1 genome. Sixty-three genes have been identified by conditional lethal mutations and/or DNA sequencing, and have been mapped into a single linkage group that spans the entire genome. Conditional lethal mutations have permitted analysis of the functions of 40 of the known genes, showing that genes involved in the same function, such as DNA replication, tail formation, head formation, or virion assembly, tend to be clustered together on the map. The 35 genes that have been sequenced, 24 of which, being in the terminal redundancy, are present in two copies, occupy about 31 kb. The genes specifying the 53 known structural proteins are expected to occupy another 61 kb, on the basis of the molecular weight of the proteins, as estimated by gel electrophoresis. The remaining 53 kb may be estimated to include about 56 genes, assuming similar average size and density, for a total of about 144 genes.

Growth Cycle

Under optimal conditions, SPO1 has an eclipse period of 25–30 min, a latent period of 33–40 min, and a

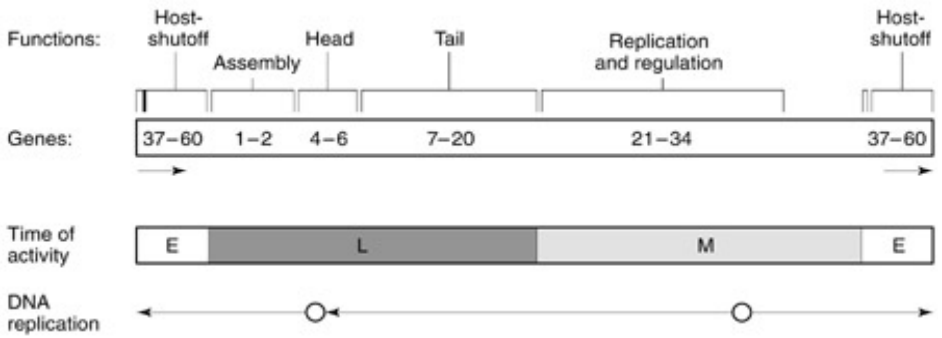


Figure 1 Maps of the SPO1 genome. The 'Genes' box shows the approximate map location of most of the known genes. The position of the terminal redundancy is shown by the arrows under the ends of that box. Genes *37–60*, being located in the terminal redundancy, are indicated twice. Genes *35* and *36*, located in the small portion of the terminal redundancy to the left of gene *37*, are not shown. The clustering of genes according to function is indicated above the 'Genes' box. Most or all of the known genes in each region are believed to specify products involved in the function indicated. Genes for replication and regulation are interspersed among each other, and certain genes are required for both processes. Two genes required for assembly are located in different regions, gene *3* in the head region and gene *35* in the small box to the left of the host-shutoff region. As discussed in the text, the host-shutoff function of genes *37–60* has not been definitively established. Since most of the terminal redundancy has been sequenced, genes *37–60* make up a continuous cluster of known genes. In the rest of the genome, the known genes are interspersed among regions of unknown function, and no genes have been identified between gene *34* and the terminal redundancy. The 'Time of activity' box shows regions predominantly transcribed during early (E), middle (M) or late (L) times, as indicated by the unshaded, lightly shaded and heavily shaded areas, respectively. A minority of each type of transcript is also scattered among the other areas of the genome; for instance, gene *28* is transcribed at early times. The 'DNA replication' line shows the simplest pattern of replication consistent with the data. The two Os represent origins of replication, and the arrows show the directions of replication proceeding from each origin.

burst size of 100–300 phage. Into this brief period is packed a remarkably complex sequence of events. Immediately after infection, some of the SPO1 early genes are turned on, with the others following during the next few minutes. Shortly thereafter occurs the shutoff of host DNA replication and gene expression. By 5 or 6 min after infection, SPO1 middle genes begin turning on and some of the early genes are turned off. The middle genes specify most of the enzymes necessary for SPO1 DNA synthesis, which begins about 10 min after infection. Shortly thereafter, SPO1 late genes begin to function, directing the synthesis of structural proteins, and some of the early and middle genes are turned off. By 25 min after infection, the first infectious phage particles have formed, and lysis begins as early as 33 min, resulting in the destruction of the infected cells. The following sections describe each of these processes in detail.

Regulation of Gene Action

The transitions from early to middle to late gene activity are caused by a cascade of sigma factors. The major host RNA polymerase, with sigma factor A, transcribes from promoters whose −35 and −10 sequences approximate the consensus sequences TTGACA ... TATAAT. The promoters for the SPO1 early genes fit that consensus and thus are transcribed by the host polymerase. One of the early genes, gene *28*, specifies another sigma factor, which substitutes for sigma A on the RNA polymerase, changing the specificity of the polymerase so it then recognizes the promoters of the middle genes, whose consensus sequence is AGGAGA ... TTTNTTT. Two of the middle genes, *33* and *34*, specify proteins which cause the RNA polymerase to recognize the late gene promoters, with consensus sequence CGTTAGA ... GATATT. The sigma factor specified by gene *34* is essential for all late transcription, whereas the accessory protein specified by gene *33* is required for some, but not all, late transcription.

This sequence of events was put together primarily from a series of elegant experiments done by Jan Pero, Peter Geiduschek and their colleagues. RNA pulse-labeled at early, middle or late times showed completely different patterns of hybridization competition and of hybridization to Southern blots of restriction digests of SPO1 DNA. Mutations in gene *28* prevented the transition from the early to the middle patterns, whereas mutations in genes *33* or *34* prevented the middle to late transition. Three different RNA polymerases, A, B and C, were extracted from SPO1-infected cells and used to transcribe SPO1 DNA *in vitro*, producing RNAs which showed the same hybridization patterns as the pulse-labeled early, middle and late RNAs, respectively. Analysis of the polypeptides associated with the three polymerases showed that A contained sigma

A, B contained (instead) the gene *28* product, and C contained (instead) both the gene *33* and gene *34* products.

Thus, the sequential onset of early, middle and late transcription is explained by the sigma cascade. The mechanisms responsible for other regulatory events in the SPO1 life cycle are not yet understood, although, in some cases, gene products that play essential roles have been identified. One of the most interesting is TF1, a type II DNA-binding protein that is synthesized in large quantities during SPO1 infection, and that binds preferentially to specific sites on DNA containing hmUra, causing bending of the DNA. SPO1 mutants deficient in TF1 fail to shut off transcription of certain middle genes and fail to turn on transcription of certain late genes. The bending caused by TF1 may be, in itself, a cause of these regulatory changes, or may make those genes accessible to other regulatory factors.

Certain mutations in two other genes, *22* and *27*, also prevent activity of certain late genes. The effect of the gene *22* mutation may be an indirect result of its prevention of phage DNA synthesis, suggesting that some change in structure, associated with replication, may be necessary for the activation of certain late promoters. The effect of the gene *27* mutation, however, seems to be independent of its effect on replication.

Other regulatory events whose mechanisms are not yet understood include: the delay in the onset of transcription of some early genes relative to others; the turn-on of translation of certain early mRNAs, requiring the activity of one or more SPO1 gene products; the shutoff of two different groups of early genes at two different times; the shutoff of those middle genes for which TF1 is not required; and probably others that are not yet so well defined.

By hybridization of pulse-labeled RNAs to Southern blots of restriction digests of the SPO1 genome, transcription maps have been prepared showing which regions of the genome are transcribed at which times. A summary of the overall trends revealed by such mapping is included in **Fig. 1.** Detailed analysis of individual transcription units, within certain regions of the genome, has been performed by S1 mapping and by *in vitro* transcription, and the results are summarized in the following paragraphs.

Most early transcription takes place in the terminal redundancy, each copy of which contains at least 13 promoters, most of which are very active and some of which are among the strongest promoters known. This high density of active promoters in a duplicated region may be for the purpose of competing effectively with host promoters for RNA polymerase. All 13 promoters direct transcription toward the middle of the redundant region, where two efficient transcription terminators halt transcription from the left and right sides, respectively. Many of the early transcripts of both SPO1 and SP82 undergo processing, by cleavage with RNase III, and six putative sites for RNase III cleavage have been identified within this region of SPO1. This entire region of early transcription has been sequenced, and the roles of its gene products are discussed below.

Most middle transcription occurs in a 60 kb region that occupies most of the right half of the genome. A 28 kb subset of this region includes all five of the regulatory genes mentioned earlier, as well as all the genes known to be required for SPO1 DNA replication. A total of 13 active middle promoters and three relatively weak early promoters have been identified in this region. Each of the early promoters is in a tandem arrangement with a middle promoter, permitting some sequences to be transcribed by both the early-specific and middle-specific polymerases. Eleven of the genes, including all the regulatory genes, have been sequenced.

Most late transcription occurs in the left half of the unique region of the genome. This region includes nearly all the genes known to be directly involved in head or tail morphogenesis, and it is assumed that most late genes specify structural proteins.

DNA Replication

Conditional lethal mutations have identified 10 SPO1 genes as essential for SPO1 DNA replication. Except for gene *28*, most or all of these are middle genes, whose products begin to appear about 7 min after infection. Thus, the time at which replication is initiated, about 10 min after infection, may be determined simply by the time at which all necessary proteins and precursors have accumulated to a sufficient concentration. Genes *23* and *29* are required for the synthesis of hmUra, *32* and *21a* or *b* for initiation of replication, and *22*, *30* and *31* for elongation. Gene *31* specifies the DNA polymerase and is the site of the intron discussed above. Other SPO1 gene products that play a role in replication, but whose genes have not been identified, include DNA gyrase, dCMP deaminase, dTTPase, dTMPase and an inhibitor of thymidylate synthetase.

There are at least two origins of replication, near the opposite ends of the unique region of the SPO1 genome. One growing point proceeds leftward from the right-hand origin, replicating most of the unique region. Another proceeds leftward from the left-hand origin, replicating from there to the end. There must be at least a third growing point to replicate the right end of the genome, but it is not clear whether that

starts above the right-hand origin and replicates rightward or whether there is a third origin, farther to the right, from which replication proceeds bidirectionally. **Figure 1** includes a representation of the simplest interpretation of the data on directions of replication.

As the SPO1 DNA replicates, it forms concatemers of as many as 20 genomes, joined end to end by overlapping terminal redundancies. It has been proposed that concatemer formation is one solution to the inability of DNA polymerase to synthesize the 5′ end of a linear DNA molecule. The two daughters of the replication of a linear molecule would each have protruding 3′ ends which would be complementary to the opposite terminal redundancy. This complementarity would nucleate annealing of the entire terminal redundancies, to form an end-to-end dimer, a process which would be repeated again and again. When the concatemer was broken back down to unit genomes, cleavage would be staggered so as to produce protruding 5′ ends, whose complements could then be synthesized by DNA polymerase. This hypothesis predicts that, after formation of the first dimer, and before the second round of replication has begun, genetic markers in the terminal redundancy should have been replicated only to half the extent of markers in the rest of the genome, a prediction that has been dramatically confirmed, for SPO1, by temperature-shift experiments with temperature-sensitive mutants affected in gene *32*.

Morphogenesis

Little is known about SPO1 morphogenesis. Although many of the known genes are required for head or tail formation or for virus assembly, and 53 proteins have been identified as part of the virus particle, only one gene has been identified with a specific viral protein (gene *6* specifies a particular head protein), and only one morphogenetic process has been studied. The proteolytic processing of a precursor polypeptide to produce the mature form of the major head protein requires the activity of both the gene *5* product and TF1. Nothing is known of the biochemical activity of the gene *5* product, and TF1 may have any of several possible roles. Other type II DNA-binding proteins participate in the wrapping of DNA molecules into chromatin-like structures, suggesting that TF1 might play a similar role in folding SPO1 DNA for packaging into the head, and that processing of the head protein might be an integral part of the packaging process. Alternatively, TF1 might be necessary for expression of gene *5*, or for some other necessary gene.

Effect on the Host Cell

It is to the selective advantage of a virus to shut off the macromolecular syntheses of the host cells, so they will not compete with the comparable viral syntheses for energy, materials and access to biosynthetic machinery. Most synthesis of host DNA, RNA and proteins is shut off within a few minutes after SPO1 infection. The shutoff mechanisms are highly selective, since not only do they have no effect on the synthesis of the comparable phage macromolecules, they also spare certain host syntheses. Host ribosomal RNA continues to be synthesized at nearly normal rates, which seems sensible since the phage has a use for the host ribosomes. The mechanism by which SPO1 distinguishes between host and phage DNAs is not clear. It must be more subtle than the presence or absence of hmUra, since some host genes are unaffected. Unlike T4, which causes the complete degradation of DNA without hydroxymethylcytosine, SPO1 causes no detectable degradation of *B. subtilis* DNA.

Most or all of the 24 early genes in the terminal redundancy are believed to specify components of the host-shutoff machinery, although this belief still awaits definitive confirmation. About one-third of the 24 genes have been tested individually, by expression in uninfected cells, and nearly all are inhibitory to the bacteria, four or five of them to the point of lethality. They affect different host functions, with the two best-characterized acting specifically on RNA polymerase and cell division, respectively. The nucleotide sequence of this 24 gene cluster shows promoters and ribosome-binding sites that are designed for highly efficient expression, and such efficiency has been shown both *in vivo* and *in vitro*. This duplicated cluster of highly expressed genes seems appropriate for specification of the host-shutoff machinery, which must, within a few minutes, cause the cessation of biosyntheses that are occurring at thousands of sites in each infected cell.

Recombination and Mutagenesis

SPO1 has a very active recombination system, producing frequencies of recombination between nearby genetic markers of about 0.001% per base pair. Nothing is known about the mechanisms of recombination or the gene products involved, but the high frequency facilitates several types of experimentation. Cloned SPO1 restriction fragments, as small as 200 bp, undergo significant recombination with the homologous region of the SPO1 genome, resulting in marker rescue of markers as little as 12 bp from the end of the fragment, permitting efficient fine-structure

mapping. Also, new mutations can be constructed *in vitro* and inserted into the SPO1 genome by marker rescue recombination. Mutations of the genes specifying either protein TF1 or E3 were introduced into *B. subtilis* on cloned fragments less than a kilobase in length, and were allowed to recombine with superinfecting wild-type SPO1. By the criterion of plaque-lift hybridization, the frequencies with which the mutations replaced the wild-type alleles ranged from 5×10^{-4} to 4×10^{-3}.

Recombination is also an integral part of the process of transfection by SPO1 DNA (most studies of the phenomenon have been done with the close relative SP82). When a cell is infected with purified DNA, the DNA is damaged by nuclease activity of the host cell. (This activity is inhibited, and therefore causes no problem, during normal infection.) Production of a single intact genome requires recombination between several of the damaged genomes.

Cloning Vehicle

SPO1 can also serve as a cloning vehicle. Entire plasmids, carrying a short region of homology to the SPO1 genome, can be inserted into the SPO1 genome by Campbell-mode integration. At least 5.6 kb of exogenous DNA can be added to the SPO1 genome in this way without apparent effect on the viability of the phage. Although too cumbersome to be used for routine cloning, this procedure offers a way to test the effect on SPO1 infection of adding specific genes to the genome, and to test the effect of the incorporation of hmUra on the functioning of any DNA of interest.

See also: **Bacillus phage ϕ29 (*Podoviridae*); *Bacillus subtilis* phages; History of virology: Bacteriophages; Phage taxonomy and classification; Phages as cloning vehicles; Phages in soil; Recombination of viruses; T4-like phages (*Myoviridae*).**

Further Reading

Geiduschek EP, Schneider GJ and Sayre MH (1990) TF1, a bacteriophage-specific DNA-binding and DNA-bending protein. *J. Struct. Biol.* 104: 84.

Goodrich-Blair H and Shub DA (1996) Beyond homing: competition between intron endonucleases confers a selective advantage on flanking genetic markers. *Cell* 84: 211.

Losick R and Pero J (1981) Cascades of sigma factors. *Cell* 25: 582.

Stewart CR (1993) SPO1 and related bacteriophages. In: Sonenshein SL, Hoch JA and Losick R (eds) *Bacillus subtilis and Other Gram-positive Bacteria*, p. 813. Washington: American Society for Microbiology.

Spongiform Encephalopathies *see* **Prions**

SPUMAVIRUSES (*RETROVIRIDAE*)

Mel Campbell and **Ayalew Mergia**, Department of Medical Pathology, University of California, Davis, California, USA

Philip C Loh, Department of Microbiology, University of Hawaii at Manos, Honolulu, USA

Paul A Luciw, Department of Medical Pathology, University of California, Davis, California, USA

History

Foamy viruses were first recognized in the 1950s as contaminating viral agents which caused cytopathic effects in cultures of rhesus monkey kidney cells. On the basis of morphological, biochemical and genetic properties, foamy viruses are retroviruses; however, recent studies have demonstrated major differences in the replication strategy employed by these viruses and that of conventional retroviruses. Certain aspects of foamy virus replication are similar to hepadnaviruses. Cytopathology in foamy virus-infected monolayer cell cultures is characterized by extensive formation of intracellular vacuoles in multinucleated syncytial cells. By light microscopy, infected cell cultures have a foamy appearance, and hence the Latin term *spuma* for foamy was coined for this group of retroviruses (i.e. spumaviruses). These viruses, also designated syncytium-forming viruses, have been recovered from various mammals, including cats, cows, hamsters, sea

mapping. Also, new mutations can be constructed *in vitro* and inserted into the SPO1 genome by marker rescue recombination. Mutations of the genes specifying either protein TF1 or E3 were introduced into *B. subtilis* on cloned fragments less than a kilobase in length, and were allowed to recombine with superinfecting wild-type SPO1. By the criterion of plaque-lift hybridization, the frequencies with which the mutations replaced the wild-type alleles ranged from 5×10^{-4} to 4×10^{-3}.

Recombination is also an integral part of the process of transfection by SPO1 DNA (most studies of the phenomenon have been done with the close relative SP82). When a cell is infected with purified DNA, the DNA is damaged by nuclease activity of the host cell. (This activity is inhibited, and therefore causes no problem, during normal infection.) Production of a single intact genome requires recombination between several of the damaged genomes.

Cloning Vehicle

SPO1 can also serve as a cloning vehicle. Entire plasmids, carrying a short region of homology to the SPO1 genome, can be inserted into the SPO1 genome by Campbell-mode integration. At least 5.6 kb of exogenous DNA can be added to the SPO1 genome in this way without apparent effect on the viability of the phage. Although too cumbersome to be used for routine cloning, this procedure offers a way to test the effect on SPO1 infection of adding specific genes to the genome, and to test the effect of the incorporation of hmUra on the functioning of any DNA of interest.

See also: **Bacillus phage ϕ29 (*Podoviridae*); *Bacillus subtilis* phages; History of virology: Bacteriophages; Phage taxonomy and classification; Phages as cloning vehicles; Phages in soil; Recombination of viruses; T4-like phages (*Myoviridae*).**

Further Reading

Geiduschek EP, Schneider GJ and Sayre MH (1990) TF1, a bacteriophage-specific DNA-binding and DNA-bending protein. *J. Struct. Biol.* 104: 84.

Goodrich-Blair H and Shub DA (1996) Beyond homing: competition between intron endonucleases confers a selective advantage on flanking genetic markers. *Cell* 84: 211.

Losick R and Pero J (1981) Cascades of sigma factors. *Cell* 25: 582.

Stewart CR (1993) SPO1 and related bacteriophages. In: Sonenshein SL, Hoch JA and Losick R (eds) *Bacillus subtilis and Other Gram-positive Bacteria,* p. 813. Washington: American Society for Microbiology.

Spongiform Encephalopathies *see* **Prions**

SPUMAVIRUSES (*RETROVIRIDAE*)

Mel Campbell and **Ayalew Mergia**, Department of Medical Pathology, University of California, Davis, California, USA

Philip C Loh, Department of Microbiology, University of Hawaii at Manos, Honolulu, USA

Paul A Luciw, Department of Medical Pathology, University of California, Davis, California, USA

History

Foamy viruses were first recognized in the 1950s as contaminating viral agents which caused cytopathic effects in cultures of rhesus monkey kidney cells. On the basis of morphological, biochemical and genetic properties, foamy viruses are retroviruses; however, recent studies have demonstrated major differences in the replication strategy employed by these viruses and that of conventional retroviruses. Certain aspects of foamy virus replication are similar to hepadnaviruses. Cytopathology in foamy virus-infected monolayer cell cultures is characterized by extensive formation of intracellular vacuoles in multinucleated syncytial cells. By light microscopy, infected cell cultures have a foamy appearance, and hence the Latin term *spuma* for foamy was coined for this group of retroviruses (i.e. spumaviruses). These viruses, also designated syncytium-forming viruses, have been recovered from various mammals, including cats, cows, hamsters, sea

Table 1 Foamy virus isolates

Species	*Viral designation*	*No. of serotypes*
Human	Human foamy virus (HFV)	1
Simian/Great Ape	Simian foamy virus (SFV)	11
Feline	Feline foamy virus (FeFV)	2
Bovine	Bovine syncytium-forming virus (BSFV)	1
Murine	Hamster syncytium-forming virus (HaSFV)	1
Otariidine	Sea lion foamy virus	1

Simian foamy virus (SFV) isolates

Species	*Virus serotype*
Prosimian	5
Rhesus macaque	1, 2, 3
African green monkey	1, 2, 3
Baboon	1, 2, 3, 10
New World primates	4, 8, 9
Chimpanzee	6, 7
Gorilla	7
Orangutan	11

Adapted from Mergia A and Luciw PA (1991) Replication and regulation of primate foamy viruses. *Virology* 184: 475.

lions, and from various Asian, African and New World primate species (**Table 1**). In some instances, distinct spumavirus serotypes have been obtained from members of a single species (**Table 1**). In 1971, a spumavirus was isolated from a patient with nasopharyngeal carcinoma, and this virus has been designated human foamy virus (HFV), human spumaretrovirus (HSRV) or human spumavirus (HSpV) (**Fig. 1**).

Taxonomy and Classification

Foamy viruses are classified into the genus *Spumavirus* of the *Retroviridae* family. Foamy virus particles are spherical (100–140 nm diameter) and consist of a ring-shaped nucleoprotein core surrounded by a bilayer membrane envelope. The viral genome is dimeric single-stranded RNA that has positive polarity and encodes three genes for virion polyproteins (i.e. *gag*, *pol* and *env*) and additional open reading frames (ORFs). Extra ORFs, or accessory genes, are also a feature of complex retroviruses such as human immunodeficiency virus (HIV) and human T-lymphotropic virus (HTLV). Foamy viruses replicate via reverse transcription and integrate into host cell DNA; long terminal repeats (LTRs) are located at each end of proviral DNA. Viral-coded reverse transcriptase and integrase are packaged into virions. Reverse transcription is largely completed in the virion prior to entry. Although many features are shared with other retroviruses, comparisons of nucleotide sequences and replication strategy indicate that foamy viruses are a unique and distinct group of retroviruses.

Properties of the Virion

Foamy viruses share several morphological features with retroviruses. Extracellular HFV particles are polymorphic. In addition to naked extracellular preassembled cores, two morphologically different types of enveloped particles are observed. The majority of particles are spherical, with a diameter of 100–120 nm, and contain a 40–55 nm pentagon-shaped electron-lucent nucleoprotein core (i.e. nucleoid) with surface spikes, 10–13 nm in length, on the envelope. A second minor population of particles is larger (120–150 nm diameter), with condensed, amorphic cores. It has been suggested that the former type of particle is an immature form, while the second type represents mature virions. While immature in morphology, these particles are highly infectious. Mature virus particles have a bouyant density of 1.16 gm ml^{-1} in sucrose gradients. Immature viral particles in the cell cytoplasm are ring-shaped, measuring 35–45 nm, and consist of an electron-opaque shell and an inner, less dense center. Prominent envelope glycoprotein spikes up to 15 nm long

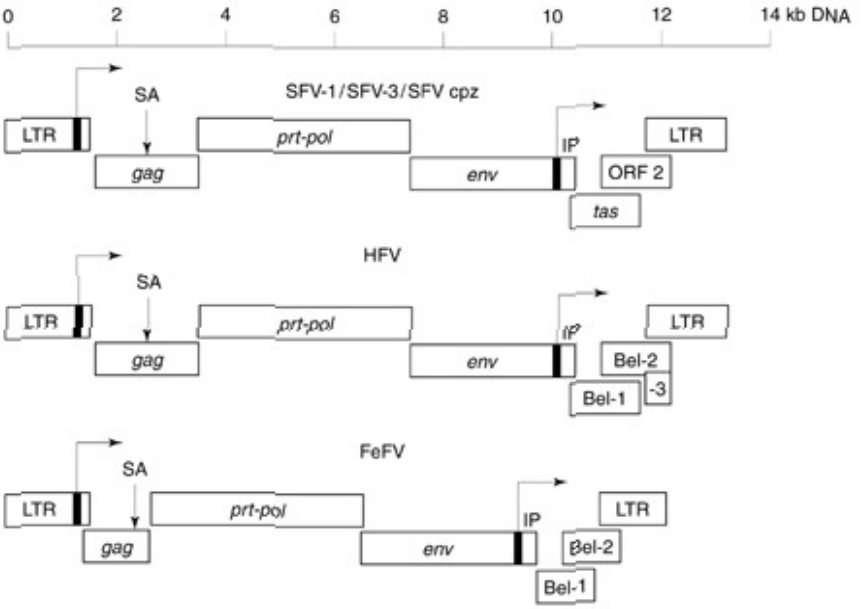


Figure 1 Genetic organization of SFVs, HFV and FeFV. Open reading frames (ORFs) and the long terminal repeats (LTRs) are shown for SFV-1 (rhesus macaque isolate), SFV-3 (African green monkey isolate), SFVcpz (chimpanzee isolate), HFV and FeFV (feline isolate). *tas* and bel-1 are transcriptional transactivators for the respective virus. The genetic organization of SFV-3 and SFVcpz is the same as SFV-1. The horizontal arrows designate viral transcripts that initiate in the 5′ LTR and internal promoter (IP). SA indicates the *prt-pol* splice acceptor.

project out from the virus membrane; the *env* gene encodes the glycoprotein, which has a transmembrane anchor (TM) domain and a surface (SU) domain. The virion core contains two identical copies of single-stranded viral RNA complexed with core proteins derived from the polyprotein precursor encoded by the *gag* gene. Purified extracellular particles contain predominantly unprocessed Gag and Prt-Pol precursor proteins.

Properties of the Genome

Foamy viruses package single-stranded RNA genome in virions. Each virus particle contains two identical copies of genomic RNA; thus, foamy viruses are diploid, as are other retroviruses. Reverse transcription is largely completed in the virion. The retrovirus RNA genome has positive polarity, and the 5′ end contains a cap structure (7-methylguanosine in a 5′–5′ linkage via a triphosphate to a second 2′-*O*-methylated nucleotide). In addition, a poly(A) tail, from about 100 to 200 nucleotides in length, is found at the 3′ end of retroviral RNA genomes. The 5′ cap structure and the 3′ poly(A) tail are features of virion RNA, subgenomic viral transcripts in infected cells, as well as normal cellular mRNA. Virions also contain small RNA molecules derived from the host cell; a cellular transfer RNA species that charges with lysine is bound to a site near the 5′ end of the viral RNA genome and serves as primer for initiating synthesis of plus-strand viral DNA during reverse transcription. In infected cells, the viral genome consists of both unintegrated linear DNA (double-stranded), covalently closed supercoils, and provirus integrated into the host cell genome. LTRs are located at the ends of linear viral DNA molecules; thus, viral genes lie between the LTRs.

Complete nucleotide sequences have been elucidated for SFV-1, SFV-3, SFVcpz, HFV and FeFV; accordingly, the genetic organizations of these foamy viruses are known (**Fig. 1** and **Table 2**). The proviral lengths in base pairs (bp) of SFV-1 (12 952 bp), SFV-3 (13 111 bp), HFV (12 142 bp), SFVcpz (13 246 bp), and FeFV (11 656 bp) are the largest among retroviruses. Sizes for domains in the LTR (i.e. U3, R and U5) and lengths of ORFs are given in **Table 2**. Foamy viruses encode *gag, pol* and *env* genes, each of which specifies a polyprotein precursor for virion proteins. In addition, foamy viruses encode three (SFV-1, SFV-3, FeFV) or two (SFVcpz and HFV) ORFs that extend from the end of the *env* gene into the 3′ LTR (**Fig. 1**). Genomic organizations of SFV-1 and SFV-3 are the same; however, HFV and SFVcpz show an extra ORF (i.e. bel-3; truncated in SFVcpz) (**Fig. 1**). A +1 ribosomal frameshift was presumed to be required for translation through the overlap of the foamy virus *gag* and *prt-pol*

Table 2 Relationships of SFV-1, SFV-3 and HFV[a]

Regions	Length SFV-1/SFV-3/HFV/SFVcpz/FeFV[b]	% amino acid homology[c] SFV-1/HFV	SFV-1/SFV-3	SFV-3/HFV
gag	646/643/648/653/514	48 (66)	67 (78)	46 (65)
prt-pol	1159/1157/1152/1154/1156	76 (85)	84 (91)	73 (83)
env-SU	568/565/567/571/475	64 (80)	66 (80)	62 (78)
env-TM	416/425/415/417/418	73 (88)	84 (92)	73 (85)
bel-1/ORF-1 (tas)	311/301/300/300/209	39 (58)	54 (67)	34 (47)
bel-2/ORF-2	422/395/364/417/357	38 (56)	54 (65)	36 (56)
		% nucleotide homology		
LTR domains	Length SFV-1/SFV-3/HFV/SFVcpz/FeFV[d]	SFV-1/HFV	SFV-1/SFV-3	SFV-3/HFV
U3	1297/1332/777[e]/1423/1070	38	60	34
R	170/217/196/182/134	86	83	81
U5	160/161/150/155/149	84	84	83

[a]Genbank accession numbers for SFV-1 (M33561, M55279, M74039, X58484), SFV-3 (M74895), and HFV (X05591, 05592), SFVcpz (UO4327), FeFV (X98741).

[b]Length in predicted codons.

[c]Comparisons are made on the basis of identical amino acids. The value in parenthesis is the percent homology based on similar amino acids.

[d]Length in nucleotides.

[e]Nonrandom deletions in the HFV LTR U3 region are observed during cell culture; an early passage, nondeleted form of the U3 domain of HFV is the same length as the SFVcpz U3.

translation frames. Translational frameshifting to produce *gag-prt-pol* polyproteins in other retroviruses is by a −1 ribosomal frameshift event. However, recent studies have demonstrated that foamy virus *prt-pol* expression is independent of *gag* synthesis and requires a spliced mRNA which initiates in the 5′ LTR and is subsequently spliced utilizing a splice acceptor in the *gag* gene; *gag* determinants are removed from this *prt-pol* messenger RNA. Predicted protein-coding regions of SFV-1 and SFV-3 show 54–85% homology (**Table 2**). Similar pairwise comparisons of SFV-1 with HFV reveal 38–76% homology, and alignment of SFV-3 and HFV demonstrates 34–78% homology (**Table 2**). In summary, foamy viruses have complex genomes, encoding genes for virion proteins and accessory genes, as do several other retroviruses such as HIV and HTLV. Also, foamy virus *prt-pol* expression is fundamentally different from that of other retroviruses.

Properties of Viral Proteins

Knowledge of foamy virus proteins is based on direct analysis of virions and infected cells and also on comparisons of predicted protein sequences from other retroviruses. Analysis of HFV cores revealed Gag polypeptides with apparent molecular weight of 78 and 74 kDa and a Prt-Pol precursor protein of 135 kDa. Proteolytic processing of foamy virus Gag precursor proteins is inefficient; only a cleavage event near the C-terminus of Gag, which generates p74 from p78, is observed in infected and in purified virions. Prt-Pol cleavage appears to proceed to a greater extent than Gag processing in infected cells with p80 Pol (reverse transcriptase), p40 (integrase) and p15 (nucleocapsid protein) generated from the p135 Prt-Pol precursor. Domains within the *pol* gene for reverse transcriptase, RNAse-H, and integrase are identified by alignment with other retroviruses. The matrix (MA) protein derived from the N-terminus of the *gag* polyprotein precursor has not been identified for foamy viruses. For all other retroviruses, the C-terminus of the *gag* gene specifies a nucleocapsid (NC) protein which has a conserved motif of several cysteine residues; this motif is a metal-binding finger that mediates the interaction of NC protein to the viral RNA genome. Inspection of the predicted sequences of the foamy virus *gag* gene does not reveal a domain with a cysteine motif; instead, sequences near the C-terminus of the *gag* gene encode an NC protein with a high content of glycine and arginine residues (GR boxes). GR boxes are involved in nucleic acid binding and in a transient translocation of the HFV Gag precursor protein to the nucleus of infected

cells. This motif is not conserved in FeFV, although there is some conservation of positively charged residues. FeFV Gag protein is considerably smaller than SFV and HFV Gag.

The SFV-1 *env* glycoprotein is a heterodimer composed of a 70 kDa SU domain and a 30 kDa TM domain. In cells infected with HFV, three viral glycoproteins with molecular weights 170 kDa, 130 kDa and 47 kDa have been identified; respectively, these may represent the *env* precursor, SU and TM domains. Comparison of the predicted amino acid sequences of the *env* genes of SFV-1 and HFV reveals that the *env* proteins are very similar in size as well as in structure; all cysteine residues and the majority of potential glycosylation sites are conserved. The external portion of the TM domain of SFV-1 and HFV is almost twice as large as those in other retrovirus subfamilies. Thus, the TM domain of foamy viruses may be folded into a structure which is fundamentally different from the other retroviruses. An endoplasmic reticulum retrieval signal has been identified in the foamy virus envelope and is conserved among all sequenced foamy viruses; however, its significance remains to be determined.

Physical Properties

Spumaviruses possess lipid bilayer membranes; therefore, stability is similar to that of other viruses with membrane envelopes. Heat and extremes of pH inactivate infectivity, and detergents and organic solvents disrupt virion structure. Ionizing radiation and x-rays inactivate the spumavirus RNA genome. Infectivity is lost after repeated cycles of freezing and thawing and after prolonged storage at 4°C, although virus is relatively stable in the frozen state.

In Vitro Cell Cultures

Single cycle growth studies of foamy viruses in cell culture reveal an eclipse period of 24 h and a maximum yield of virus attained by 72 h postinfection. Approximately 10% of the HFV yield in human diploid cells is released into the medium; the remainder is cell associated. The rate of adsorption of HFV to human embryonic fibroblasts (HEFs) at 37°C is maximal at 3 h postinfection. Nothing is known about cellular receptors in this early stage of infection. After attachment, the viral particle enters into the cell by either viropexis or direct entry. Whether a fusion factor is involved in the penetration process, as seen in other enveloped virus families, remains to be determined.

As with other retroviruses, foamy virus replication is related to host cell division. Similar to oncoviruses, such as murine leukemia virus (MLV), but unlike the lentivirus HIV-1, foamy viruses require host cell proliferation for productive infection. Productive infection is observed only if the target cells pass through mitosis. The semipermissiveness of heteroploid epithelial cell lines to infection with HFV or SFV-3 was determined to be the result of the methylated state of viral DNA. Treatment of such cell lines with the inhibitor of DNA methylation, 5-azacytidine, enhances viral production.

Strategy of Replication of Nucleic Acid

The pattern of foamy virus replication in tissue culture generally conforms to that of other retroviruses; however, differences are noted with respect to viral DNA synthesis. In contrast to conventional retroviruses, a high proportion of infectious HFV particles contain double-stranded DNA similar in size to full-length provirus, suggesting that reverse transcription takes place in the virion, before new rounds of infection. Most retroviruses initiate plus-strand DNA at one site, the 5′ boundary of the 3′ LTR. Foamy viruses utilize this site and may potentially also initiate at an internal site in the *pol* gene; a gap is located in *pol* in the linear duplex viral DNA intermediate. In acutely infected monolayer cells, very large quantities of linear viral DNA molecules are synthesized. These accumulations of viral DNA are presumed to be a consequence of reinfection; permissive cells are presumed to contain numerous cell receptors, and, thus, endogenously synthesized viral *env* glycoprotein may not be present in sufficient amounts to bind to all cell receptors and block superinfection. Several other retroviruses which induce cytopathology in cell culture systems also show large amounts of unintegrated linear viral DNA. Integrated (i.e. proviral) forms of foamy virus DNA have not yet been rigorously identified; however, the fact that these viruses encode an integrase domain in the *pol* gene supports the notion that integration is a critical step in the foamy virus life cycle.

Transcription

The U3 domains of the LTRs of foamy viruses contain *cis*-acting promoter elements (including the TATA box) which are recognized by cellular transcription factors. In addition, a second viral promoter (the internal promoter; IP) is located near the 3′ end of the *env* gene (**Fig. 1**). Both promoters are dependent on the viral transactivator for full activity. Simian foamy viruses have U3 domains that are longer than those of all other retroviruses; thus, the potential is high for many *cis*-acting regulatory

motifs. In the nuclei of infected cells, viral transcription initiates in the 5′ LTR and in the IP, possibly in a temporally regulated manner. The IP appears to be a stronger promoter in infected cells (basal and transactivated levels). Transcripts with the potential to code for the ORF region gene products originate predominantly from the IP. Subgenomic transcripts derived from either the 5′ LTR or the IP are subject to complex splicing. All subgenomic viral transcripts which initiate in the 5′ LTR have a short 5′ leader (51 nucleotides) specified by the R region in the LTR. Both foamy virus promoters demonstrate very low basal levels of activity in transient expression assays, involving transfection of plasmids containing either the LTR or IP, into mammalian tissue culture cells. ORF 1 of SFV-1, SFV-3, SFVcpz and FeFV and bel-1 of HFV are transcriptional transactivator (*tas*) genes that act through *cis*-acting targets in the U3 domain of the respective LTR, or through sequences immediately 5′ to the IP TATA box, to augment levels of viral transcripts. Mutational analysis of cloned viral genomes has demonstrated that the *tas* gene is required for viral replication. The target sequences for *tas* for each virus are upstream from the TATA box. SFV-1 Tas and HFV Bel-1 proteins interact directly with DNA target elements in each promoter. Foamy virus LTRs lack any significant homology with the LTRs of lentiviruses and other retroviruses; in addition, the *tas* genes are unrelated to the regulatory genes of other retroviruses. The Tas-responsive elements in the LTR and IP of each individual virus also show little overall homology other than a conserved TATA box. Thus, the mechanism of transactivation in the foamy virus system is fundamentally different from that for other viruses, and may be different for a given transactivator acting at the 5′ LTR versus the IP. Functions for the potential genes encoded by the remaining ORFs (i.e. ORF 2; Bel-2,-3) are not yet known. Foamy viruses do not appear to contain a Rev/Rex axis as in the lentiviruses or oncoviruses. The additional ORFs are dispensable for replication in tissue culture cells; only the viral transactivator is required.

Translation

A subgenomic mRNA encodes the Prt-Pol precursor which lacks Gag determinants. No Gag-Pol precursor can be detected in foamy virus-infected cells or virions. The Env polyprotein precursor is translated from a subgenomic spliced mRNA. The subgenomic mRNA for Env is presumed to be translated on membrane-bound cytoplasmic polysomes, and other viral mRNA species may be translated on free polysomes in the cytoplasm. Proteins encoded by *bel-2* and *bel-3* have been identified in infected cells. An abundant protein containing N-terminal sequences from the *bel-1* gene and C-terminal sequences from the *bel-2* gene has been detected in infected cells; however, the function of this fusion protein, designated Bet, is not known.

Post-translational Processing

In conventional retroviruses, the viral *gag*, *pol*, and *env* genes encode polyprotein precursors that are processed by specific cleavages. Viral protease produces the matrix (MA), capsid (CA) and nucleocapsid (NC) proteins from the *gag* gene precursor. Little processing of the foamy virus Gag precursor is observed in infected cells or in virions. Only a C-terminal cleavage of p78 Gag to generate p74 Gag is readily observed in foamy virus-infected cells or in virions. The putative MA protein of foamy viruses (and several other retroviruses) has a glycine immediately after the initiator methionine; therefore, MA is predicted to be myristoylated at the N-terminus by a host cell enzyme. Reverse transcriptase and integrase are cleaved from the *pol* gene precursor, presumably by the viral protease. The *env* precursor is glycosylated by cellular enzymes and cleaved into the SU and TM domains; this cleavage site at two basic residues is recognized by a host cell endopeptidase.

Assembly Site, Release, Uptake, Cytopathology

Assembly of nucleoprotein cores appears to occur in the cytoplasm. Intracellular viral particles are ring-shaped, 35–50 nm in diameter, and consist of an electron dense shell and an inner electron-lucent center. Particles mature by budding through the cell plasma membrane.

The receptor(s) for these viruses has not been identified, and the entry mechanism (i.e. direct fusion of viral and cell membranes or receptor-mediated endocytosis) has not been studied.

Cytopathology in monolayer tissue culture cells is characterized by extensive formation of intracellular vacuoles in multinucleated syncytial cells. Certain hematopoietic cell lines can produce high levels of virus without obvious cytopathology for an extended period of culture.

Geographic and Seasonal Distribution

Isolates of FeFV, representing two serotypes, have been identified in domestic cats from three continents (**Table 1**), and an isolate has also been obtained from a European wildcat. FeFV has been identified in

healthy cats as well as in cats with neoplasms or nonneoplastic diseases. BFV infection in cattle populations also appears to be widespread. Four serotypes of SFV have been isolated from Old World monkeys and three additional serotypes are from New World monkeys (**Table 1**). Two unique serotypes of SFV were recovered from chimpanzees and one unique serotype from prosimians (**Table 1**). A recent SFV isolate from orangutan appears to be serologically distinct. In summary, foamy viruses appear to be endemic in some animal species in certain areas. Recent studies have refuted previous seroepidemiological studies which indicated that HFV may infect a small number of individuals in Africa and certain Pacific islands; however, rare zoonotic infection of humans with SFVs has been documented. Nothing is known about the seasonal distribution of foamy viruses in any mammalian species.

Host Range and Virus Propagation

Foamy viruses have a wide host range in cell culture systems. The SFVs replicate and cause cytopathology in epithelial and fibroblastic cells from primates, rodents and chickens. Lymphoid cells can be persistently infected with no visible cytopathic effects on the host cells. Cytopathology in monolayer cells is generally characterized by multinucleated syncytia at early times and extensive vacuolization at later times; inclusion bodies are not observed. HFV replicates in diploid fibroblast-like cell lines but not in heteroploid epithelial-like cell lines. *In vitro,* SFV-1 establishes a carrier state in HEp-2 (human hepatoma) and BHK (baby hamster kidney) cell lines, and a Vero (monkey fibroblast) cell line harboring SFV-3 in a latent state has been described. Manifestation of cytopathology with respect to time after infection *in vitro* depends on the serotype of the virus, titer, passage history and cell type used to propagate virus. Under optimal conditions, SFVs induce cytopathology and reach maximal titers as early as 4–5 days after infection. Virus is spread by the extracellular route and by cell-to-cell transmission.

Genetics

Although the complete sequences of the SFV-1, SFV-3, HFV, SFVcpz and FeFV genomes have been elucidated, the extent of variation within each virus and the potential for recombination between viruses are not known.

Evolution

Foamy viruses are exogenous agents, as uninfected host cells do not contain sequences related to these viruses. Sequence comparisons reveal that SFV-1 (rhesus macaque isolate) and SFV-3 (African green monkey isolate) are more closely related to each other than either is to HFV. HFV and SFVcpz are highly related. A potential progenitor virus(es) for the primate foamy viruses remains to be identified. Evolutionary relationships of the primate foamy viruses with foamy virus isolates from other species are not yet established.

Serologic Relationships and Variability

Serologic relationships have been determined by analyzing properties of virus neutralizing antibodies in sera of naturally infected animals and rabbits immunized with whole virus preparations. Antibodies to foamy virus isolates from primate, feline, bovine or murine (i.e. hamster) species do not crossreact, and these viruses are immunologically unrelated to retroviruses in other genera. At least 11 simian serotypes and two feline serotypes have been identified (**Table 1**). Serologic patterns of the SFVs are complex, in that a single species may have two or more serotypes, and the same serotype has been isolated from different species (**Table 1**). Antibodies to HFV apparently crossreact with several SFVs. The genetic and molecular basis for serological cross-reactivity (and variability) has yet to be determined; these studies will require sequencing of foamy virus genomes coupled with an analysis of immunologic epitopes on viral proteins.

Epidemiology

SFVs are prevalent in captive and wild-caught primates. Surveys of primates reared in captivity suggest that foamy virus prevalence is extremely high, especially in adults, were rates of infection may approach 100%. Previous seroepidemiological studies revealed that HFV may be present in a small number of humans in parts of Africa and the Pacific islands. Recent re-examination of these populations have been unable to confirm these previous findings, which suggested an association of foamy virus with specific human disease, and also failed to find any evidence of naturally occurring human infection with a foamy virus.

Transmission and Tissue Tropism

Mechanisms which account for spread of foamy viruses are not well defined. Vertical transmission has been described in cats, cows and monkeys. FeFV, BFV and SFV have been isolated from throat washings or nasal swabs from the respective hosts. Thus, it is possible for horizontal transmission to

occur by direct contact and, perhaps, by the respiratory route via aerosolization.

SFVs have been isolated from several organs (e.g. brain and lymphoid cells) and fluids of healthy and diseased primates. Analysis of fractionated monkey peripheral blood mononuclear cells indicated foamy virus infection of diverse cell populations, with the highest proviral burden in CD8+ T cells. In their respective hosts, FeFV and BFV are also distributed in many different tissues. A feature of the virus–natural host relationship is that foamy viruses have not been directly observed by electron microscopy or other means in the tissue from which they were isolated. Recovery of virus has always been achieved by culturing cells from the original tissue *in vitro*. The mechanism(s) of virus activation is still unknown.

Pathogenicity

Although several reports provide tentative links between presence of a foamy virus and certain clinical conditions, the vast majority of naturally infected hosts do not display any disease. Epidemiologic studies suggested an association of FeFV with polyarthritis in cats, yet experimentally infected cats remained free of symptoms. HFV has been recovered from several patients with de Quervain subacute thyroiditis, and additional HFVs have been obtained from individuals with other disease conditions; however, none of these viral isolates have been well characterized. The prototype HFV was recovered from an individual in East Africa who had a nasopharyngeal carcinoma. Whether this HFV is a genuine human retrovirus or a simian foamy virus contaminant remains to be resolved by characterization of additional (independent) foamy virus isolates from humans. Sequence analysis of SFVcpz indicated a close relationship between this chimpanzee isolate and HFV, thus HFV may represent a rare zoonotic infection or SFV contaminant. In summary, reports on foamy viruses in humans represent anecdotal observations, and an etiologic role for a putative HFV in human pathology is not yet established.

Seronegative natural hosts experimentally infected with foamy virus develop antibodies to the virus but show no clinical symptoms. Heterologous hosts inoculated with foamy viruses generally do not present with any disease signs. Rabbits experimentally infected with SFV-1 seroconverted and harbored virus in several tissue for 1–3 weeks postinfection. In another study, rabbits infected with SFV-7 by the intravenous route demonstrated transient suppression of cell-mediated immune functions. Progressive encephalopathy and muscular abnormalities were produced in transgenic mice containing a portion of the cloned HFV genome that includes the ORF (or *bel*) region; this region encodes the transcriptional transactivator (*tas*). This observation in the transgenic animal model intimates a pathogenic potential for foamy viruses, perhaps with respect to neurodegenerative diseases. Whether foamy viruses are cofactors for disease for other infectious agents is not known.

Clinical Features of Infection

Naturally and experimentally infected hosts develop antiviral antibodies, and virus is recoverable from a variety of organs by culturing tissue explants *in vitro* to activate viral gene expression. A clear association between foamy avirus infection and disease has not yet been established (see above, Pathogenicity). Extremely rare zoonotic infection of humans, through occupational exposure (animal laboratory workers), has been documented. Infected individuals appear healthy.

Pathology and Histopathology

Currently there is no significant information available on the pathology and histopathology of foamy virus infections in their natural hosts, as clinical disease has not been convincingly associated with these viruses. Histologic analysis of transgenic mice containing portions of the HFV genome reveals patterns of neurological and muscular degeneration (see above, Pathogenicity).

Immune Response

Naturally infected animals have virus-neutralizing antibodies. Accordingly, the presence of these antibodies may account for viral latency or a low-level persistent infection. Other defense mechanisms, such as interferon, may also subdue viral replication in the host. The role of cell-mediated immunity in foamy virus infection remains to be explored.

Prevention and Control of Foamy Viruses

Foamy virus infections of their natural hosts are not known to cause any major clinical disease; consequently, the development of preventive and control measures are not necessary at this time. However, experimental xenotransplants from nonhuman primates to humans raises the possibility of SFV infection in humans.

Future Perspectives

Many fundamental aspects of foamy virus replication

and virus–host interactions remain to be investigated. The emerging picture of foamy virus replication is one with distinct differences from that of conventional retroviruses, with some features reminiscent of hepadnaviruses. The distribution of foamy viruses in a species and the precise modes of transmission are not well established; these studies will be aided by sensitive techniques for detecting viral genomes (e.g. amplification via polymerase chain reaction) and antiviral antibodies (e.g. ELISA systems and immunoblots with genetically engineered viral antigens). The reason(s) for lack of pathology in animals infected with foamy viruses is not known; it is tenable that these viruses may be associated with long-term, chronic degenerative disease(s). In addition, foamy viruses may be cofactors for other infectious agents. In the host, the precise cell types and organs harboring virus and viral genomes are not well defined. Whether infection is 'persistent' or 'latent' remains to be determined. Additional studies are required to elucidate the mechanism of viral transactivation (*via tas/bel*) and to evaluate the effects of cell-activation signals on viral gene expression. The significance of host immune mechanisms for controlling viral replication and pathogenesis is not determined, and a mechanism(s) which accounts for viral cytopathology has not been elucidated. The extent of strain variation, particularly in the *env* genes of primate foamy viruses, is an area for future study. A cellular receptor for attachment of virus particles has not yet been identified. In addition, the broad host range and apparent lack of pathogenesis are factors which may make foamy viruses useful for the development of retroviral vectors for gene transfer. Transduction of a wide variety of vertebrate cells, including primary hematopoietic progenitor cells, by foamy virus vectors has been reported.

See also: **Human immunodeficiency viruses (*Retroviridae*): Molecular biology, Anti-retroviral agents, General features; Human T-cell leukemia viruses (*Retroviridae*): HTLV-1, HTLV-2; Retroviruses – type D (*Retroviridae*).**

Further Reading

Aguzzi A (1993) The foamy virus family: molecular biology, epidemiology, and neuropathology. *Biochim. Biophys. Acta* 1155: 1.

Aguzzi A, Marino S, Tschopp R and Rethwilm A (1996) Regulation of expression and pathogenic potential of human foamy virus in vitro and in transgenic mice. *Curr. Top. Microbiol. Immunol.* 206: 243.

Hooks JJ and Detrick-Hooks B (1981) *Spumavirinae:* foamy virus group infections. In: Kurstak E and Kurstak C (eds) *Comparative Aspects and Diagnosis of Viral Disease,* p. 599. New York: Academic Press.

Rethwilm A (1995) Regulation of foamy virus gene expression. *Curr. Top. Microbiol. Immunol.* 193: 1.

Rethwilm A (1996) Unexpected replication pathways of foamy viruses. *J. Acquir. Immune Defic. Syndr. Hum. Retrovirol.* **13** (suppl. 1): S248.

Yu S, Baldwin DN, Gwynn SR, Yendapalli S and Linial ML (1996) Human foamy virus replication: A pathway distinct from that of retroviruses and hepadnaviruses. *Science* 271: 1579.

Squirrel Fibroma Virus *see* **Poxviruses**

St. Louis Encephalitis Virus *see* **Encephalitis Viruses**

Swine Herpesvirus-1 *see* **Pseudorabies Virus**

Swine Vesicular Exanthema Virus *see* **Caliciviruses**

Swinepox Virus *see* **Poxvirus**

SYNERGISM: PLANT VIRUSES

Vicki Bowman Vance, Department of Biological Sciences, University of South Carolina, Columbia, South Carolina, USA

Mixed Virus Infections are Common in Plants

Mixed virus infections occur in both plant and animal systems, and doubly infected organisms commonly display changes in disease symptoms and in the accumulation of one or both of the co-infecting viruses. However, such infections are relatively uncommon in animal hosts where they are generally associated with depression of the immune response. In contrast, higher plants are frequently infected with multiple viruses. In some cases, the virus interaction is antagonistic and infection by one virus interferes with the subsequent replication or spread of the other virus. This type of interaction is the basis for the phenomenon known as 'cross protection', where a plant is protected from a virulent strain of a virus by prior inoculation with a mild or asymptomatic strain of the same virus. Cross protection is limited to closely related viruses.

In other cases of mixed virus infection in plants, the viral interaction is synergistic and co-infection enhances the replication and/or spread of one or both viruses. The co-infection usually results in a much more severe disease than either virus causes in a single infection, and a number of plant disease syndromes are caused by the interaction of two independent viruses in the same host. In contrast to cross protection, synergistic viral interactions almost always occur between unrelated viruses. Such synergistic interactions offer a unique opportunity to investigate the regulation of viral disease because the accumulation and pathogenicity of one virus are altered when it interacts with a relatively simple genetic element, the genome of the co-infecting virus.

Potyvirus-associated Synergisms

Many synergistic diseases involve a member of the potyvirus group of plant viruses. In these *potyvirus-associated* synergisms, the other virus of the pair may be any of a broad range of unrelated viruses, including pararetroviruses, such as cauliflower mosaic virus, and RNA viruses of both the alphavirus supergroup (for example potato virus X [PVX]) and the picornavirus supergroup (for example cowpea mosaic virus). **Table 1** lists a number of examples of potyvirus-associated synergisms.

Several such potyvirus-associated synergistic diseases have been examined in some detail, and in each, a dramatic increase in host symptoms has been observed in doubly infected plants compared to singly infected plants. The increase in symptoms in doubly infected plants is correlated with an increase in the accumulation of the nonpotyvirus; however, in general, there is no corresponding increase or decrease in the level of the potyvirus.

The PVX/potyvirus Interaction

A model synergism

The interaction between PVX and potato virus Y (PVY, a potyvirus) in tobacco has been well characterized and serves as a model to understand the underlying molecular basis for potyvirus-associated synergistic disease. Plants mechanically inoculated with both viruses develop synergistic disease, which is characterized initially by severe vein clearing and then necrosis of the first systemically infected leaf tissue. The dramatic increase in symptoms in the first systemically infected tissue is correlated with a large (3–10-fold) increase in the level of PVX compared to

Table 1 Potyvirus-associated synergisms

Potyvirus	*Heterologous virus*
Potato virus Y	Potato virus X
Tobacco vein mottling virus	
Tobacco etch virus	
Pepper mottle virus	
Blackeye cowpea mosaic virus	Cucumber mosaic virus
Cowpea aphidborne virus	
Bean yellow mosaic virus	
Zucchini yellow mosaic virus	
Soybean mosaic virus	Bean pod mottle virus Cowpea mosaic virus
Maize dwarf mosaic virus	Maize chlorotic mottle virus
Wheat streak mosaic virus	
Potato virus Y	Tobacco mosaic virus
Turnip mosaic virus	Cauliflower mosaic virus
Maize dwarf mosaic virus	Barley yellow dwarf virus
Tobacco etch virus	Dodder latent mosaic virus

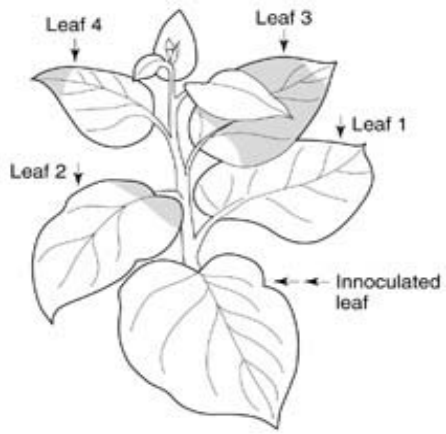


Figure 1 Diagram of a tobacco plant showing the pattern of acute phase synergism symptoms on the bottom of leaf two, all of leaf three and the tip of leaf four above the inoculated leaf. The location of acute phase symptoms is indicated by shading.

the analogous regions of plants singly infected with PVX.

Both the timing of invasion by the two viruses and the developmental state of the host are important for induction of the PVX/PVY synergistic response. PVX must enter the systemically infected cells at a time when PVY is actively replicating and expressing its viral gene products. Outside that window in time, PVX invasion does not lead to the synergistic increase in symptoms or the correlated increase in PVX accumulation. The severe symptoms on first systemically infected leaves are found in a characteristic pattern, confined to the base of the second leaf, over most of the third leaf and at the tip of the fourth leaf above the inoculated leaf (**Fig. 1**). These severely affected tissues are said to be in the acute phase of the synergistic disease. The pattern of acute phase symptoms reflects the developmental state of the plant, since all the affected tissues were at the same level of maturity at the time of viral invasion. The pattern of acute phase disease was one of the first clues that the host may play an important role in the development of synergistic disease. The fact that the timing of PVY invasion was found to be important suggests that an interaction between the potyvirus (or potyvirus gene product) with the host might play a role in mediation of the disease syndrome.

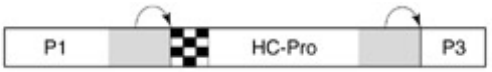
The increase in PVX accumulation in acute phase tissue is due to a change in control of PVX replication. PVX is a (+) strand RNA virus that replicates via production of a complementary RNA, the (−) strand RNA, that is used as a template for production of high levels of the genomic RNA. The genomic RNA encodes five known gene products and serves as mRNA for the 5′-proximal gene product, the 166 kDa putative replicase. The genes that are encoded internally on the genomic RNA are expressed via production of subgenomic mRNAs, each serving as mRNA for the gene encoded at their respective 5′-proximal ends. The virion is rod shaped and consists solely of the genomic RNA encapsidated by the viral coat protein. In the acute phase of PVX/PVY synergism, the levels of infectious PVX particles, PVX genomic RNA, PVX coat protein and PVX coat protein subgenomic mRNA all increase to about the same extent (3–10-fold) over that in the analogous singly infected tissues. In contrast, the level of the PVX complementary RNA, which serves as a template during replication, consistently increases to a level 3-fold above that of the virion and its component parts. Thus, the ratio of (−) strand to (+) strand PVX RNA is increased during synergism and this suggests a change in the regulation of PVX RNA replication. This change in PVX replication does not require replication of the PVY genome, and is mediated by expression of a subset of the PVY genes (see next section).

Although much of the early synergism work centered on the PVX/PVY interaction, a similar synergistic response is induced in co-infections of PVX with several other potyviruses, including the well-studied tobacco vein mottling virus (TVMV) and tobacco etch viruses (TEV). The finding that many different potyviruses could induce the same changes in host response and in PVX replication was important because it was the first hint that the role of the potyvirus in potyvirus-associated synergisms might be the same for all such diseases.

PVX/potyviral synergism is mediated by the potyviral P1/HC-Pro sequence

Two lines of evidence lead to the conclusion that PVX/potyviral synergistic disease does not require replication of the potyviral genomic RNA and that the response is mediated by expression of potyviral sequences in the 5′ proximal one-third of the potyviral genome. The first line of evidence stems from experiments using transgenic plants expressing various subsets of the potyvirus genome. Plants expressing the 5′ proximal region of either the TVMV or the TEV genomic RNA and infected singly with PVX displayed the synergistic increases in symptoms and in PVX RNA replication. These results indicate that the 5′-proximal region of the genomic RNA of either TVMV or TEV is sufficient to mediate synergistic disease.

The second line of evidence stems from experi-



Protein	Domain	Potyvirus function	Synergism △ Replication	Synergism ↑ Symptoms
P1		Replication, polyprotein cleavage	Yes	No
HC-Pro	Zinc finger	Aphid transmission	No	No
	Central	Replication, long distance movement, pathogenicity	Yes	Yes
	Proteinase	Polyprotein cleavage	?	?

Figure 2 Diagram of the potyviral P1/HC-Pro sequence which mediates potyvirus-associated synergisms. Proteinase domains of both P1 and HC-Pro are indicated by shading, whereas the location of the cleavage site for each of these proteinases is indicated by an arrow. The amino terminal zinc finger-like domain of HC-Pro is indicated by a checkerboard pattern. The functions of the P1 and HC-Pro sequences in the potyvirus life cycle, as well as their role in synergism are indicated below the diagram.

ments using PVX as a vector to express this same region of the TEV genome (PVX-5′TEV). An infectious cDNA clone of the PVX genome was engineered to express the TEV sequence under control of a repeated coat protein subgenomic promoter. When inoculated onto tobacco, this engineered PVX induced a characteristic synergistic response with enhancement in both symptoms and in RNA replication. When used to infect tobacco tissue culture cells, expression of the potyviral sequence dramatically prolonged the accumulation of PVX (−) strand RNA over a three-day time-course experiment. This result indicates that the potyviral 5′ proximal sequence induces a synergistic change in PVX RNA replication and confirms that the region is sufficient to mediate synergistic disease.

The region of the potyviral genome that mediates synergism includes the viral genomic 5′ untranslated region (UTR) as well as the coding region for the N-terminal portion of the viral polyprotein, including P1, helper component protease (HC-Pro), and about a quarter of P3 (termed P1/HC-Pro sequence, **Fig. 2**). This region is expressed as polyprotein and subsequently processed by the proteolytic activities of both P1 and HC-Pro.

We can obtain clues for how HC-Pro and P1 might mediate synergism by examining the functions they perform in the TEV infection process (**Fig. 2**). Both HC-Pro and P1 are multifunctional proteins. P1 has proteinase activity that cleaves the potyviral polyprotein, creating the C-terminus of P1 and the N-terminus of HC-Pro. P1 also functions in *trans* as an accessory factor for genome replication and has RNA binding activity. One possibility is that P1 plays a direct role in synergism, perhaps analogous to its role as an accessory factor in TEV replication. Alternatively, it might enhance synergism only indirectly by producing the authentic HC-Pro N-terminus by means of its proteinase activity.

HC-Pro has at least three functional domains: an N-terminal domain required for aphid transmission; a central domain involved in pathogenicity, RNA replication, and leaf-to-leaf movement of the virus through the phloem; and a C-terminal domain required for autoproteolytic processing of the HC-Pro C-terminus. The central domain of HC-Pro is of particular interest because it is involved in the regulation of both pathogenicity and RNA replication of potyviruses, and these are the characteristics that are altered in PVX during synergism.

The potyviral P1/HC-Pro sequence also mediates other potyvirus-associated diseases

The finding that transgenic plants expressing the P1/HC-Pro region of the potyviral genome develop synergistic disease when infected with PVX raised the possibility that many or all potyvirus-associated synergisms might be mediated by this same sequence. To test this hypothesis, two other viruses, tobacco mosaic virus (TMV) and cucumber mosaic virus (CMV), were used to infect transgenic tobacco line U-6B, which expresses the P1/HC-Pro region of the TEV genome. Both viruses can infect tobacco and are known to interact synergistically with a potyvirus. TMV and CMV are both (+) strand RNA viruses in the alphavirus supergroup; however, CMV has a tripartite genome, and many strains also support replication of an associated satellite RNA, whereas the genome of TMV, like that of PVX, is monopartite. The transgenic plants expressing the potyvirus P1/HC-Pro sequence displayed the enhanced symptoms characteristic of synergism when infected singly with either TMV or CMV. **Figure 3** shows enhanced symptoms of PVX, CMV and TMV in the U-6B plants as compared to those in nontransgenic control plants. At the molecular level, infection of the U-6B transgenic resulted in increased accumulation of the genomic RNAs of both of these heterologous viruses. Thus, the response of TMV and CMV to expression of the potyvirus P1/HC-Pro sequence is similar to that previously shown for PVX.

Because both TMV and CMV are capable of

inducing synergistic disease in mixed infections with a member of the potyvirus group, this result supports the hypothesis that all such potyvirus-associated synergistic diseases, which occur in an evolutionarily diverse range of host plants and involve interactions with a large number of viral groups, are mediated by the same potyviral sequence. The fact that expression of this sequence alters disease induction by each of these unrelated heterologous viruses suggests that it affects a step in viral infection that is common to all of these viruses and thus of general significance.

Identification of functional domains within the P1/HC-Pro sequence

The role of P1/HC-Pro in synergism could be mediated by the entire P1/HC-Pro region, either the RNA itself, or the encoded polyprotein. Alternately, the response could be mediated by a part of the RNA sequence or one or a subset of the encoded proteins. Three complementary approaches have been used to delineate the domains of P1/HC-Pro that function in potyviral synergism. The first approach was to use transgenic plants expressing mutant versions of the P1/HC-Pro sequence. Six transgenic lines, three with mutations within the P1 coding region and three with mutations within the HC-Pro coding region, were infected with PVX to assay the ability of the mutant transgene to induce synergism. The three lines with mutations within the P1 coding region all maintained the ability to mediate the synergistic response to PVX. In contrast, two out of three of the mutations within the HC-Pro coding region failed to induce synergistic disease. Both mutations that interfered with the ability to cause synergism were located within the region encoding the central domain of HC-Pro. This result established the importance of the HC-Pro central domain for induction of PVX/potyviral synergistic disease.

A second approach was to use mixed inoculation experiments with a mutant version of TEV. Plants were co-inoculated with PVX and TEV-del-2, a spontaneous deletion mutation of TEV in which the first 65 amino acids of HC-Pro are missing, and assayed for synergistic disease. The mutant TEV interacted with PVX to produce synergistic disease indistinguishable from that produced by mixed infection with wild-type TEV. This result showed that the amino-terminal 65 amino acids of HC-Pro, encoding a zinc-finger-like domain required for aphid transmission of the potyvirus, are dispensable for induction of PVX/TEV synergism.

Although the first two approaches established some sequences required in the induction of synergism, they did not establish the minimal sequence required for the response. The third approach was to use PVX as a vector to express various parts of the P1/HC-Pro sequence and then assay the engineered viruses for the ability to cause disease symptoms and the change in PVX replication characteristic of synergism. This approach revealed that the HC-Pro protein was both necessary and sufficient to cause the synergistic increase in host symptoms. In contrast the synergistic enhancement of PVX replication required both P1 and HC-Pro. This result suggests that the synergistic increase in symptoms and in PVX replication, although correlated in mixed infections, may be mediated via different pathways.

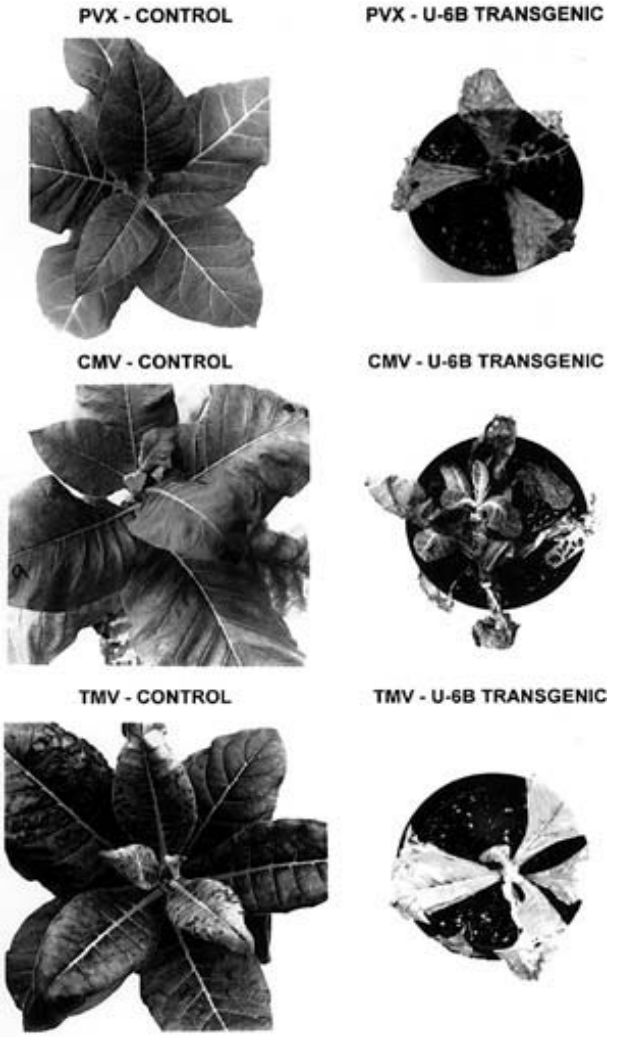


Figure 3 Enhanced pathogenicity of PVX, CMV and TMV in transgenic tobacco expressing high levels of the P1/HC-Pro sequence. The left hand column shows nontransformed tobacco plants infected with PVX (top row), CMV (middle row) or TMV (bottom row). The right hand column shows infection of the P1/HC-Pro expressing transgenic line U-6B infected with the same set of viruses.

Possible mechanisms for potyviral-associated synergism

Potyviral-associated synergisms result in increases in both the accumulation of the nonpotyvirus of the synergistic pair of viruses and in the host symptoms. In the case of PVX/potyviral synergism, these two aspects of the synergistic disease are mediated by different functional domains of the P1/HC-Pro sequence, possibly reflecting action via different pathways. The central domain of HC-Pro is required for both enhanced replication and enhanced symptoms. Since this region of HC-Pro has been shown to prolong replication of the potyvirus in plants and because expression of P1/HC-Pro also prolongs PVX (−) strand RNA accumulation in tobacco culture cells, it is possible that the basis of potyvirus-associated synergism is the ability of HC-Pro to prolong replication not only of potyviruses, but also of a broad range of heterologous viruses.

Although it has been shown that P1/HC-Pro acts to alter replication of PVX, the possibility that the potyviral sequence also facilitates viral movement has not been ruled out. In fact, because the central domain of HC-Pro is required for both movement and replication of the potyvirus and the same domain is also required for the synergistic effect on PVX, a role in movement in addition to the role in replication remains quite possible.

The TEV P1/HC-Pro sequence mediates synergistic disease during infection by viruses from three different groups (PVX, potexvirus; TMV, tobamovirus; CMV, cucumovirus). Therefore, it has been proposed that synergism may occur via an indirect mechanism involving an interaction of the TEV-encoded proteins with one or more host factors common to the different viral infections rather than a direct mechanism involving interactions with different RNAs or proteins from three heterologous viruses.

Two different indirect mechanisms could explain transactivation of viral replication by P1/HC-Pro. The TEV sequence might augment viral replication by enhancing the synthesis, activity or availability of a host factor that affects both TEV and the heterologous viruses in a positive way. It has been shown that host factors may be used as part of the virus replication machinery in plant cells. However, there is no evidence for host factors that act as general positive regulators of virus replication.

Alternatively, the potyviral sequence might interfere with the activity or availability of a negative regulator of viral replication, perhaps part of a host defense system that normally limits the viral infection. Post-transcriptional gene silencing has recently been proposed to act as a general defense system against plant viruses and this system is a candidate for a defense system impacted by P1/HC-Pro. In one post-transcriptional gene silencing model, an RNA targeting system is activated by high level expression of a particular RNA sequence, such as an invading virus. Once activated, the system rapidly destroys the RNA target in a sequence specific manner. The same cellular system is also thought to be involved in post-transcriptional gene silencing of nonviral transgenes in plants. In the case of plant viral synergism, the P1/HC-Pro sequence might interfere with the induction or the action of the post-transcriptional silencing pathway and thus allow a broad range of viruses to overstep the normal host imposed limits of RNA accumulation.

Nonpotyviral Synergisms

A number of plant viral synergisms which do not involve a member of the potyvirus group have been reported (**Table 2**); however, in contrast to the potyvirus-associated synergisms, none of these nonpotyviral synergisms have been well characterized at the molecular level. Probably the best studied of the nonpotyviral synergisms is the interaction of TMV and PVX, which causes the synergistic disease in tomatoes called double streak. This synergistic interaction has been reported to result in an increase in the level of PVX in doubly infected plants. One possibility is that the nonpotyvirus synergisms are mediated by expression of a subset of one viral genome, in a manner similar to that shown for the PVX/potyviral synergism. Interestingly, a high proportion of the nonpotyviral synergisms involve TMV as one member of the pair of interacting viruses, and this raises the possibility that the TMV genome includes a synergism sequence similar in function to the potyviral P1/HC-Pro sequence.

See also: **Vectors: Plant viruses; Potexviruses; Potyviruses (*Potyviridae*).**

Table 2 Nonpotyviral synergisms

Virus pair	
Tobacco mosaic virus	Potato virus X
Tobacco mosaic virus	Cucumber mosaic virus
	Tobacco ringspot virus
Tobacco mosaic virus	Tomato aspermy virus
Cowpea chlorotic mottle virus	Southern bean mosaic virus
Alfalfa mosaic virus	Potato acuba virus

Further Reading

Bennett CW (1953) Interactions between viruses and virus strains. *Adv. Virus Res.* 1: 39.

Dodds JA and Hamilton RI (1976) Structural interactions between viruses as a consequence of mixed infections. *Adv. Virus Res.* 20: 33.

Kassanis B (1963) Interactions of viruses in plants. *Adv. Virus Res.* 10: 219.

Matthews REF (1991) *Plant Virology*, 3rd edn. San Diego, CA: Academic Press.

Rochow WF (1972) The role of mixed infections in the transmission of plant viruses by aphids. *Annu. Rev. Phytopathol.* 10: 101.

Ross AF (1957) Responses of plants to concurrent infection by two or more viruses. *Trans. NY Acad. Sci.* 19: 236.

T

T1-LIKE PHAGES (*SIPHOVIRIDAE*)

JR Christensen, Rochester, New York, USA

Natural History

T1 is one of the seven phages collected by Delbrück and renamed T1 through T7; they all make clear-centered plaques on *Escherichia coli* B. T1 is unrelated to any of the others. Its latent period at 37°C is 13 minutes, and the burst size is about 100. T1 also infects some other laboratory strains of *E. coli* (e.g. K-12 and C) and *Shigella dysenteriae*. Out of 290 clinical isolates of *E. coli*, T1 could replicate in two and kill a third without producing phage.

T1's best-known relative is the *Shigella* phage, D20, with which it readily hybridizes. T1-like phages can be isolated from sewage, but none has received much study. Among laboratory strains of T1, there are a few minor differences, revealed by restriction analysis.

The virion is highly stable in the dry state, so that careless technique may cause T1 (like other phages with this property) to become airborne in the laboratory, and to unexpectedly lyse cultures contaminated accidentally.

The Virion

In the electron microscope, T1 closely resembles lambda, with a polyhedral head about 55–60 nm across, and a long, flexible tail about 7 × 150 nm. Fifteen virion proteins have been recognized. One of these, P7, accounts for about 50% of the total protein, and two additional proteins for another 35%.

The genomic molecule is double-stranded DNA with approximately 48 500 bp. Only the four conventional bases are present. About 0.2% of the cytosine residues and 1.7% of the adenine residues are methylated, at the 5 and 6 positions, respectively. The DNA molecule includes a terminal redundancy of about 2800 ± 530 bp (6%), so that the coding capacity is about 46 000 base pairs. Because of the way the genome is packaged during morphogenesis (see below), there is a limited set of cyclic permutations of the nucleotide sequence within a population of virions.

Early Events in Infection

Adsorption of T1 is a two-step process: an initial, reversible interaction with an outer membrane-spanning protein coded by *fhuA (tonA)*, followed by an irreversible interaction involving the *tonB*-coded protein and energy provided by bacterial metabolism.

The exterior loops of FhuA serve as receptor for a variety of ligands, including several iron compounds, phages T1, T5 and ϕ80, and colicin M. Mutation studies indicate that the various ligands bind to non-identical but partially overlapping portions of FhuA.

It has been proposed that TonB serves to transmit inner-membrane energy to FhuA, altering its conformation, and allowing T1 infection (and the transport of certain other ligands, including ϕ80) to proceed. However, a possibly different role for TonB during T1 infection has been proposed (see below). T5 does not require TonB activity, nor indeed does a host-range mutant of T1, T1*hr*.

During the first 1–2 min after infection, there are marked changes in the membrane properties and in the energy state of the bacterium: there is a large efflux of K^+, the proton motive force (PMF) decreases, but does not vanish, and intracellular ATP levels fall, due to the activity of the proton-translocating ATPase. Those transport systems driven by ATP or by the PMF are inhibited, but the activity of the sugar phosphotransferase systems is stimulated. None of these changes requires phage gene activity.

During this same period of time, entry of phage DNA into the cell and shut-off of host protein synthesis occur. A model that puts these observations

together has been proposed (though there is some dispute about it): preinfection, at least two cation gradients contribute strongly to the energized state of the membrane – K^+ is higher inside the cell, and H^+ is higher outside. The entry of T1 DNA is effected by a proton symport that involves *tonB*. This process tends to deplete the PMF, but partial activity is maintained by H^+ efflux, driven partially by ATP hydrolysis and partially by K^+ symport. The resulting fall in intracellular ATP leads to a fall in GTP. This, and perhaps other changes in the intracellular ionic environment, produces an inhibition of translation of host proteins at the initiation level. (Presumably the translation of phage proteins is resistant to these changes.)

Within a few minutes, a 'resealing' process occurs, although it is not clear that the membrane is restored exactly to its original condition. Resealing and the continued maintenance of the remaining level of the PMF are necessary for the infectious process to continue.

Another early event is the appearance of a phage-coded DNA methyl transferase. Although the specificity of this enzyme is identical to the host's *dam* enzyme, it is quite distinct in other enzymological properties, and T1 DNA shows no hybridization to that of the cloned host gene. The phage-coded enzyme almost totally methylates the adenines occurring within 5′-GATC sequences, even when the phage is grown in *dam* mutants. The biological role of this methylation is unclear.

Transcription

T1 depends on the host RNA polymerase for transcription throughout its cycle. Both early and late, all or nearly all transcripts are read from the same strand of DNA. All major regions of the genome are transcribed early, but there is a relative shift at later times towards the regions coding for virion proteins. The basis for the shift remains unknown. Considerable transcription (but not translation) of host genes continues after infection.

Protein Synthesis

The synthesis of 31 phage-coded proteins has been documented in infected cells. From their combined molecular weights, these account for about 80% of the coding capacity of the genome. Five temporal patterns of synthesis have been noted: proteins synthesized only early; only late; continuously but at a declining rate; continuously and steadily; continuously and at an increasing rate. As with the synthesis of host proteins, much of this regulation is probably at the transitional level.

DNA Synthesis

Among virulent, double-stranded DNA phages, T1 is unusual in that is depends on the *polC*-coded α subunit of Pol III for DNA synthesis. This is a property typical of temperate phages. It also depends on most of the other host-coded proteins involved in the elongation phase of DNA synthesis, except for *dnaB*, but does not require host proteins involved in the initiation of DNA synthesis.

Host DNA synthesis is stopped very early in infection. This inhibition requires protein synthesis, presumably for the expression of a phage gene, but the gene responsible has not been identified.

Two sets of phage genes are required for DNA synthesis. Mutants in genes 1 and 2 are totally defective in DNA synthesis. Presumably, the products of these genes function in initiation of synthesis on T1 templates. The continuing function of both of these genes is required throughout the growth cycle. Lysis of the host is delayed after infection by these mutants, but most phage-coded proteins are produced normally.

Two other genes, 3.5 and 4, encode a recombination function called Grn (for general *r*ecombi*n*ation – pronounced 'green'). This function is obligatory for phage production (see below) but, once expressed, it becomes progressively dispensable as the infection proceeds. Under some conditions, the host's RecE or lambda's Red recombination system can substitute, at least partially, for Grn. Evidence suggests that gene 4 encodes an exonuclease, but no such enzyme has been isolated.

Early in infection, the products of DNA synthesis are linear, monomeric molecules. Later, under the influence of Grn, linear, concatameric molecules are produced; these have a broad distribution of sizes up to about 8- to 10-mers, presumably produced mainly by 'head-to-tail' recombination between homologous redundant ends on two molecules. In the total absence of Grn function, no concatamers are found, and DNA synthesis ceases prematurely, about 6 min into the infection. Why synthesis stops under these conditions is not clear, particularly since, once Grn has been expressed, synthesis of both monomers and concatamers continues even though further Grn activity is blocked. In the total absence of Grn, the cells lyse at the normal time, but no phage are released.

As with other virulent phages, T1 infection leads to degradation of the host's DNA. The liberated material provided about two-thirds of the precursors for the synthesis of T1 DNA. Mutants in gene 2.5 are deficient in host DNA breakdown, but phage DNA synthesis proceeds normally.

However, while phage DNA synthesis can proceed

in the absence of host breakdown, the converse is not true. In T1 there is an unusual functional dependence of the degradation of host DNA upon ongoing synthesis of phage DNA. If phage DNA synthesis is prevented, whether by use of mutants or of naladixic acid, degradation of host DNA does not occur. If degradation has already begun, and a synthesis block is imposed, degradation stops. Thus, no conditions have been found under which free degradation products can be detected. With T4 and other virulent phages that have been studied, if phage DNA synthesis is blocked, host DNA degradation nevertheless occurs, with released materials leaking into the medium.

Morphogenesis

Relatively little is known about the pathways of T1 capsid assembly. Some virion proteins, including P7, are cleaved from larger precursors, as occurs during capsid assembly with several other phages. Mature, DNA-filled heads can join to tails and form infectious particles *in vitro*.

More is known about the manner in which DNA becomes encapsidated. Linear genomic concatamers are the substrate for packaging. The process is initiated at a site called *pac,* located between gene 1 and gene 2 on the map, and processive head-filling proceeds toward gene 1 (leftward, as the map is conventionally represented). As mentioned above, a 'headful' is about 1.06 genomes worth of DNA. Only two or three particles are produced from a single initiation event, so that a limited set of cyclic permutations is produced. Thus, about 18% of the genome will, in some of the particles, be represented twice, once at each end of the packaged DNA molecule.

A mutation, *pip,* located between markers in genes 2.5 and 3 (and which likely represents a separate gene), has a marked influence on DNA packaging. If the host happens to be a lambda lysogen, T1*pip* has an enhanced frequency of initiating packaging 'mistakenly' at a site, *esp*-λ, located on the lambda prophage (see Transduction, below). The *pip* mutant is also deficient in processive packaging, so that almost all of the packaged molecules are initiated at *pac* even though, judging by the small burst size, the efficiency of initiation at *pac* is probably reduced by the mutation.

Mutants in most of the genes involved in head production are grossly defective in processing concatameric DNA to monomeric DNA, suggesting that nearly-complete head structures are required for the maturing of concatamers into monomeric 'headfuls'. However, mutants in gene 12 process concatamers normally, though they do not produce heads; presumably they fill DNA normally into head precursors that are unstable due to the lack of the gene 12 product. Finally, *am*383, the sole mutant in gene 13.3, which maps in the head region and encodes a virion protein, is only partially defective in processing concatamers. It is not clear whether this mutation is phenotypically 'leaky', or whether this observation points to some special role for this gene in DNA packaging.

Extracts, prepared from cells infected with T1 bearing amber mutations in both gene 1 and gene 2, are capable of packaging either homologous or heterologous DNA added *in vitro*. Two packaging pathways have been identified.

If the extract is given homologous DNA extracted from virions of T1 (or T1-like phages), it produces phage. Presumably, the pathway involves two steps: the production of concatamers, via recombination, followed by packaging, initiated at *pac*.

Given heterologous DNA extracted from T3, T7 or lambda *nin,* all of which are about 80% the length of T1 DNA and lack any known *pac*-like sites (*esp*-λ is in the *nin* region), the extract produces the corresponding phage by a *pac*-independent pathway. DNA from wild-type lambda is packaged less efficiently than that from lambda *nin,* suggesting that the second pathway prefers shorter molecules. (The first pathway does not act on wild-type lambda DNA, despite the presence of *esp*-λ, as there are no redundant ends to facilitate concatamer formation.)

Mutants and Maps

The current genetic map (**Fig. 1**) contains 23 essential genes, identified by complementation tests between conditional-lethal mutants, and the nonessential gene 2.5, identified by *tar* mutants (which enhance transduction frequency). These are in numerical order, with fractional gene numbers for those genes identified since the first 18 genes were mapped.

At the left end are the genes discussed above: 1, 2, 2.5, 3.5 and 4, as well as *pac* and *pip,* all of which have roles in DNA metabolism. Curiously, also within this cluster is gene 3, which has no role in DNA metabolism – rather it is essential for tail formation. Perhaps it has a regulatory role, rather than coding for a virion protein.

Next come eight more genes, 5 through 11.5, that are required for the production of phage tails. The *hr* mutation, which allows T1 to infect *tonB* (but not *fhuA*) mutant bacteria, maps just to the left of the available gene 5 markers, and may be within that gene. Finally come ten genes, 12 through 18, required for the production of phage heads. Probably there are

at most a very few undiscovered head or tail genes, judging from the number present in the morphologically similar phage, lambda.

Several tail and head genes, plus the *pac* region, have been cloned in the positive selection vector, pLV59. These clones, which together include about one-third of the total genome, have allowed a comparison of the genetic and physical maps (**Fig. 1**). Genetic markers are relatively far apart at both ends, but especially so at the left end. The occurrence of 'head-to-tail' recombination during concatamer formation would be expected to increase genetic distances between markers at the map ends (this does not mean that the genetic map is 'wrong', merely that it represents a different kind of information). But this effect will not be found to the right of *pac*, and so the wide genetic distances between markers in the region form gene 2 to gene 5 cannot be due to this effect. It is almost certain, however, that more genes remain to be discovered, particularly genes for nonessential functions, as these will not be discovered in collections of conditional lethal mutants. Unless these nonessential genes are scattered among the head and tail genes, which seems highly implausible, their likely location is in the leftward portion of the map.

Restriction and Modification

T1 passes freely among such *E. coli* strains as B, K-12 and C, so it is not subject to either B or K restriction. At one time it was felt that the high level of methylation of T1 DNA might account for this, but as 5′-GATC sequences are the main sequence methylated (at least, the total level of adenine methylation is consistent with the number of such sequences that might be expected on a genome of T1's size), and as this sequence is not part of the recognition sequence for either the B or K restriction enzyme, this seems unlikely.

However, in P1 lysogens, T1 grown in a nonlysogen is subject to restriction and modification; P1-modified T1 plates with full efficiency on both lysogens and nonlysogens. When unmodified T1 infects a lysogen, about 80% of phage DNA is degraded and excreted into the medium within 5 min. After this, degradation stops, and several

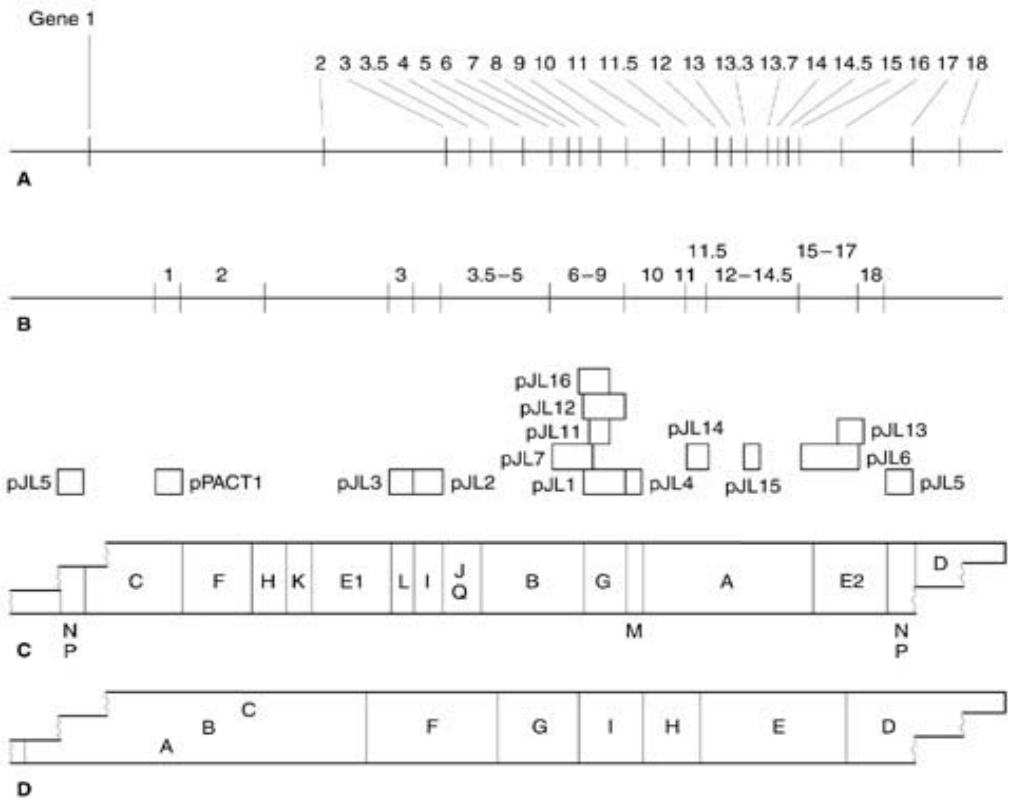


Figure 1 Genetic and physical maps of T1. (**A**) The genetic map, with each gene identified by the *am* or *ts* mutant used to map the gene. (**B**) Positions of the T1 genes on the DNA molecule. Each interval represents the outer limits of the regions occupied by the *am* mutant tested, relative to the physical map below. (**C**) Locations of the T1 cloned fragments relative to the physical map. (**D**) Physical map of T1 DNA with the *Bgl*II (upper) and *Bgl*I (lower) cleavage sites. (Reprinted, with permission, from Liebeschuetz J, Harris RD and Ritchie DA (1987) *J. Gen. Virol.* 68: 2049–2052.)

observations indicate that biologically active fragments of the restricted genome persist in the lysogenic cells for a considerable period of time.

Complementation occurs in 10–25% of the cells when unmodified T1*am* + *hr* and modified T1*amhr* + phage coinfect lysogens. This is true for most of the genes tested but not all. Most of the progeny phage are *amhr* +, so this is not primarily due to marker rescue, though that also occurs (see below). Complementing activity is but little diminished when infection by the modified phage comes 4, or even 9, minutes after infection by the unmodified one.

In co-infection experiments that use plaque-morphology markers to distinguish modified from unmodified phage (no complementation required), recombinational rescue of the alleles of the unmodified parent occurs in a few percent of the cells. Certain markers are rescued more frequently than others are. In three-factor crosses, usually only a single marker is rescued in a given cell. The alleles of the nonmodified phage remain available for rescue for at least 10 min.

In addition, under special conditions, unmodified T1 alone can successfully infect lysogens. The required conditions are high multiplicity of infection (about 10), strong aeration in nutrient medium, and occurrence of protein synthesis during the first few minutes after infection. (These special conditions are not required for the phenomena described above.) Up to 10% of the cells produce phage; this is called cooperative infection. The progeny phage are mostly modified, and they have undergone extensive recombination. Again, dividing the infecting phage into two portions, with up to 6 min intervening, does not interfere with cooperation.

Finally, among the rare (*c.* 1 in 10^4) lysogens that do yield phage after infection by a single, unmodified T1, individual cells lyse and produce their progeny (most of which are unmodified) over a period of 3–5 h.

Transduction

In common with other phage that package DNA by a headful mechanism, T1 is a generalized transducing phage. To demonstrate this, it is necessary to use T1*am* phage (typically a double *am* stock), permissive donors and nonpermissive recipients; otherwise, potential transductants are killed on the assay plate by the large excess of viable, virulent phage in the transducing lysate. The fact that, for a given marker, transduction frequencies are quite reproducible from experiment to experiment makes T1 a good subject for studying various aspects of the transduction process.

Although all markers tested can be transduced, the frequency of transduction varies widely among different markers. It appears likely that packaging of bacterial DNA can be initiated at many sites in the chromosome that mimic, to varying degrees, T1's *pac* site. This idea is strengthened by the discovery of two specific sites that have a special property: markers to one side of the site, but not the other, are transduced at high frequency; this is what would be expected of transductional 'pick-up' initiated at a *pac*-like site. One of these, *esp*, is located between *att*-λ and *gal* on the bacterial chromosome. From this site, *bio* (but not *gal*) markers in nonlysogens, or lambda plaque-forming units (PFUs) in lysogens, are transduced at relatively high frequency. The second site, *esp*-λ, is within lambda, between genes P and Q, and from this site, PFUs are readily transduced from polylysogens. Experiments with tandem heteroimmune dilysogens show that packaging proceeds leftward from this site. While initiation of packaging at *pac*-like sites probably contributes greatly to T1 transduction, *pac*-independent packaging of heterologous DNA can occur *in vitro* (see above), and a similar process may be involved in the transduction of low-frequency markers.

Small plasmids can also be transduced. The frequency is markedly enhanced by cloning *pac* or *esp*-λ into the plasmid. Transducing particles carry head-to-tail multimers of plasmid DNA; perhaps Grn can stimulate circle-into-circle recombination.

The phage-induced degradation of host DNA would be expected to compete with transduction. It does: most of the transducing particles are formed early in the infectious cycle, and *tar* mutations (gene 2.5), which block degradation, enhance the formation of transducing particles.

See also: **Coliphage lambda (*Siphoviridae*).**

Further Reading

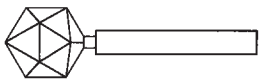
Drexler H (1988) Bacteriophage T1. In: Calendar R (ed.) *The Bacteriophages*, vol. 1, p. 235. New York: Plenum Press.

Figurski DH and Christensen JR (1974) Functional characteristics of the genes of bacteriophage T1. *Virology* 59: 397.

Liebescheutz J, Harris RD and Ritchie DA (1987) Further characterization of phage T1 DNA clones. *J. Gen. Virol.* 68: 2049.

Wagner EF, Auer B and Schweiger M (1983) *Escherichia coli* virus T1: genetic control during viral infection. *Curr. Top. Microbiol. Immunol.* 102: 131.

T4-LIKE PHAGES (*MYOVIRIDAE*)



Gisela Mosig, Department of Molecular Biology, Vanderbilt University, Nashville, Tennessee, USA

History and Overview

Bacteriophages T2, T4 and T6 were among the seven *Escherichia coli* phages ('Snow White and the Seven Dwarfs') selected by Max Delbrück to study fundamentals of viral replication. T2, T4 and T6, which are serologically related are called the 'T-even' phages. The genomes of these phages are contained in large (~170 000 bp) linear, double-stranded (ds) DNA molecules, whose termini contain repetitions of 3–5% of the genome, and are randomly permuted over circular maps (**Fig. 1**). The DNA molecules are packaged in elongated 'heads' of quasi-icosahedral symmetry. The heads are connected to tails whose baseplates and attached tail fibers (**Fig. 2**) are instrumental for recognition, adsorption and injection of the DNA into host bacteria. Differences in the tail fiber regions are important for recognition of different receptors in different host strains, which can be used to distinguish different members of the T-even family.

Since the early days of phage research, many phages from different parts of the world have been classified to belong to this family, based on similar genomic organization, regulatory patterns and sequence similarity of their 'essential' genes and on the presence of hydroxymethylcytosine (HMC) instead of cytosine in their DNA. The HMC residues are glycosylated to different extents in different members of the family. The essential roles of these modifications for the developmental strategy of these phages are discussed below (Restriction-Modification and Exclusion).

T-even phages are some of the most successful molecular parasites. Like all viruses, they depend for their propagation on many vital structures and functions of their hosts, e.g. membranes, energy metabolism, transcriptional and translational machines, and they manage to subvert host functions gradually to their own purposes in an exquisite choreography that allows adaptations to different environmental conditions, including different physiological states of the host. In contrast to many other viruses, they encode their own DNA replication, recombination and repair functions, making them particularly suitable for the study of these fundamental biological processes.

The gradual subversion of host functions to phage propagation is achieved at several interconnected levels:

1. A cascade of phage-induced proteins modifies the host RNA polymerase and its accessory proteins (sigma factors) covalently and noncovalently. These modifications together ultimately turn off all host transcription, and allow timed initiation of transcription from different classes of phage promoters and selective processivity of RNA polymerase on HMC-containing DNA.
2. There are no known T4 transcriptional repressors, but RNA processing by phage and host enzymes, translational repressors and still poorly understood modifications of ribosomes modulate T4 gene expression. Translational modulation is thought to be particularly suitable for physiological adjustments during the rapid development of T-even phages: one growth cycle is finished in less than 30 min at 37°C.
3. The host DNA and host mRNA, present at the time of infection, are rapidly degraded, and the breakdown products are efficiently reused to synthesize phage DNA and RNA.
4. The onset of the first phage DNA replication from specific origins requires host RNA polymerase to generate primers and is thereby connected to physiological regulatory processes of the host. Most subsequent initiations of replication forks depend on DNA primers in intermediates of recombination and on phage-encoded recombination proteins, which are entirely phage controlled.
5. During the later stages of development, DNA packaging proteins compete with replication–recombination proteins for the intracellular phage DNA, thereby coordinating replication and packaging.

These processes are interconnected at several levels. For example, late transcription depends on DNA replication, and in turn influences synthesis of and competitions between recombination, replication and packaging proteins. Together with multiple redundant pathways for these processes, the crossconnections allow a flexible development, which is the recipe for the success of the T-even phages.

Most of the recent work with T-even phages has

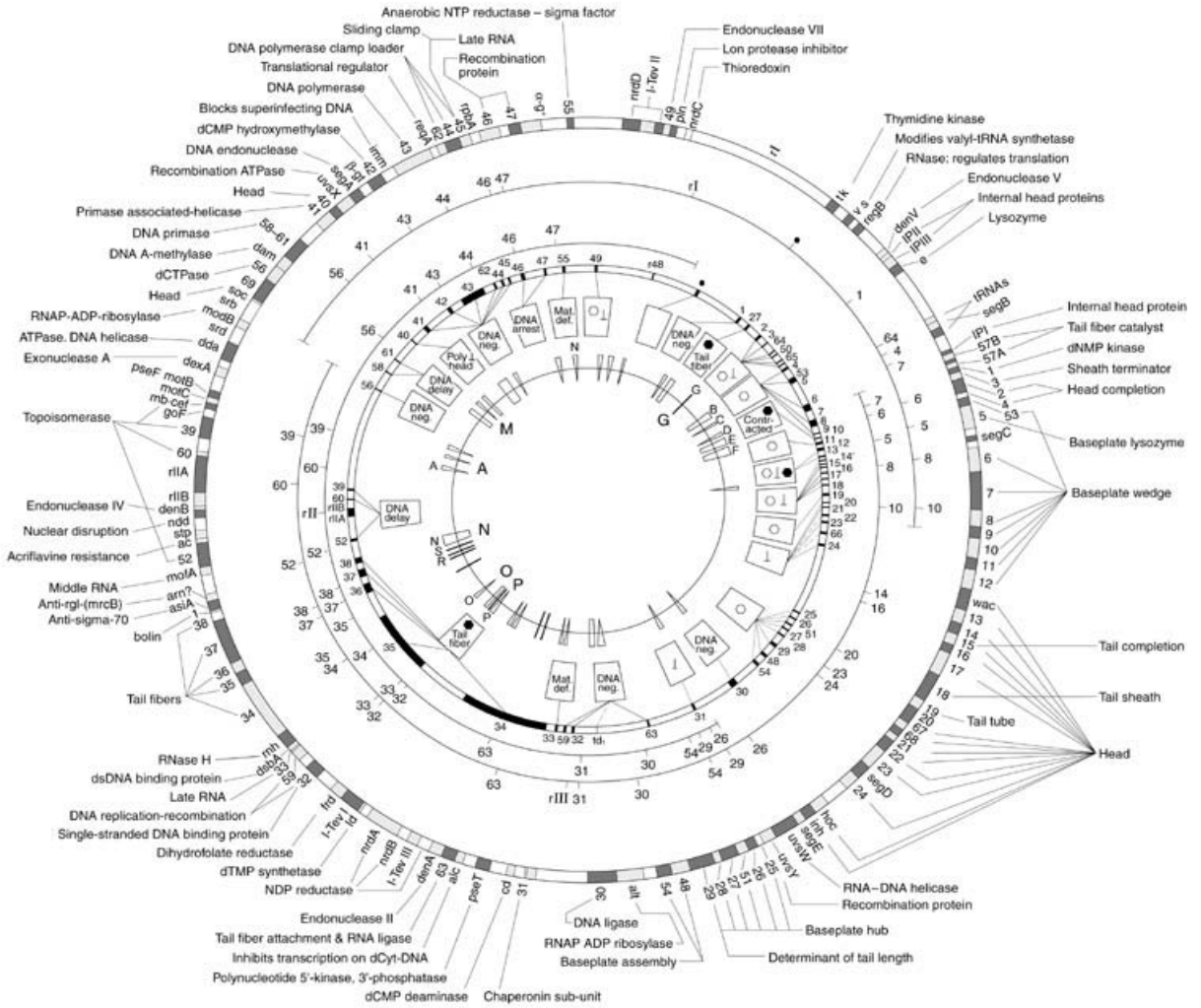


Figure 1 Comparison of several maps of T4 genes. The outermost circle shows the map of known genes and origins of replication, based on the DNA sequence. The next three overlapping circular segments drawn as thin lines show the positions of the indicated genes based on the probability of cutting ends during packaging between these genes and reference markers in *rI*, *rII* and *rIII*. The next circle shows the position of these genes based on recombination frequencies. The rectangles depict mutant phenotypes. The innermost circles represent the heteroduplex loops between T2 and T4 DNA. The substitution loop M is aligned with gene 69 in the outer circle.

been concentrated on T4, mainly because the isolation of a large collection of conditional lethal mutants has provided a powerful impetus for molecular analyses by biochemical and biophysical methods.

Genome Structure and Map

The genome of T4 resides in about 168 000 bp of double-stranded DNA containing glucosylated HMC residues. Using genetic tricks, phage mutants with unmodified cytosine-containing DNA can be isolated. Their DNA has been instrumental in cloning and sequencing the T4 genome.

Mature DNA molecules (chromosomes), packaged into virions, are linear and contain 3–5% of the genome as terminal redundancies at both ends. In contrast, intracellular replicating DNA is highly branched and contains multiple covalently linked (head to tail) copies of the genome. These structures are called 'concatemers'. Mature T4 chromosomes are cut during packaging of intracellular DNA (see below) at nearly random map positions. As a consequence, the ends of different individual chromosomes are almost randomly permuted over the circular genetic map (**Fig. 1**).

Numerous mutations, and their assignments to complementation groups and open reading frames (ORFs) have defined approximately 130 genes with known functions. In contrast to the lambdoid phages, early and late gene clusters and transcription units are

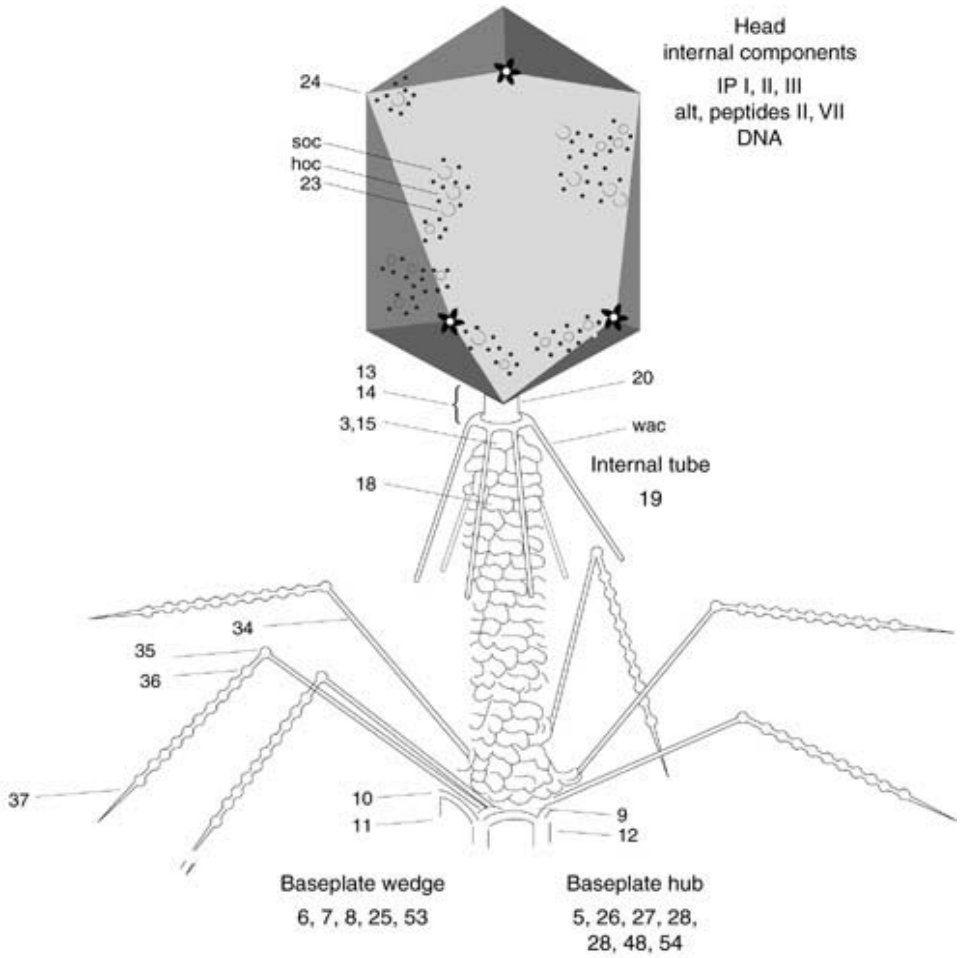


Figure 2 Diagram of the T4 virion, based on electron microscopy at 2–3 nm resolution. Locations of proteins are indicated by the corresponding gene numbers (cf. **Fig. 1**). The portal vertex composed of gp20 is attached to the upper ring of the neck structure inside the head itself. The internal tail tube is inside the sheath and itself contains a structural component in its central channel. The baseplate contains short fibers of gp12; these are shown in stored, folded conformation. (From Karam *et al* (1994) with permission of ASM Press.)

interdigitated, transcribed from multiple promoters, and genes for related or interacting proteins are not necessarily clustered (**Fig. 1**). In this respect, as well as in sequence similarities of certain proteins with the same functions, the T-even phages resemble more the herpesviruses than other phages.

More than 100 additional ORFs were revealed by DNA sequencing; many of them are still in search of functions. There are many overlapping coding regions, many ORFs are very small, and in some regions the two complementary DNA strands code for different proteins. In spite of the large genome size only a few short regions are devoid of coding capacity. These observations suggest that apparently redundant and 'nonessential' genes confer selective advantage, an inference that is supported by other evidence, discussed below.

Particle Structure and its Relationship to Assembly, Infection and Host Range

T-even phages build some of the most complex virus particles, which resemble lunar landing modules (**Fig. 2**). They devote more than 40% of their genetic information to synthesis and assembly of the protein components of these particles. The importance of host functions during assembly became apparent by 'host defective' (*hd*) mutations and compensating phage mutations and led to the discovery of the first chaperonin, now called GroEL.

The complex assembly pathways, revealed by exquisite mutational and biochemical analyses, accommodate efficient packaging of DNA and efficient genome transmission by allowing two-step adsorption to different receptors in the host's cell wall, and

easy release of the DNA during subsequent infection. Complete uptake of phage DNA by a host bacterium is also dependent on membrane potential.

Twenty-four genes are involved in head morphogenesis, more than 25 encode structural components of the tail and tail fibers and five more are needed as morphogenetic catalysts. Heads, tails and tail fibers are assembled in independent pathways and put together after the heads are filled with DNA.

Head shapes and the protein components of heads, tails and baseplates are similar among the T-even phages, except that two nonessential proteins (Hoc and Soc) that decorate and stabilize T4 heads are missing in some members of the family.

Heads

Assembly of heads is complex and has resisted *in vitro* reconstitution. *In vivo* it appears to be initiated on the bacterial membrane. The major head subunits (cleaved gp23) form the bulk of the heads; a related minor cleaved gp24 forms the pentamers at the vertices. Both proteins are converted into an assembly-competent state by a chaperonin composed of a host subunit groEL and a small phage subunit gp31, which takes the place of groES of the bacterial chaperonin. Sequential action of scaffolding proteins and cooperative conformational changes lead to sequential changes in dimensions and shapes of the head precursors while they are being assembled and filled with DNA. Extensive controlled proteolysis by a phage-encoded protease, whose precursor is part of the scaffolding assembly, cleaves head subunits, scaffolding proteins and other packaged proteins, e.g. the ADP-ribosylating Alt protein (see Temporally Controlled Gene Expression During T-even Development, below) and internal proteins bound to the DNA. Together, these processes render head assembly irreversible. Proteolysis *in vivo* is probably triggered by entry of some DNA; *in vitro* it can be achieved by other conditions. In any case, proteolysis is a prelude to rearrangements of the subunits and expansion of the head volume and allows decoration of the heads with Hoc and Soc proteins mentioned above.

Head size is not uniquely determined. The normal T4 head can be described by skewed icosahedral symmetry (T = 13) whose side faces have been elongated (Q = 21). It can accommodate full-length chromosomes with terminal redundancies. Anomalous heads of different sizes and shapes are made in low proportions in wild-type T4 infections, and in higher proportions when infecting phages have mutations in genes for head subunits or scaffolding proteins, or due to incorporation of arginine analogues. Head lengths and shapes are determined at an early assembly stage, prior to formation of the unprocessed proheads.

Small heads ('petites') contain incomplete genomes, which represent nearly random permutations of the genetic map, and recombination between several of them reconstitutes complete genomes with expected frequencies. Most of the larger 'giants' contain oversized chromosomes representing linear concatemers. A small proportion of heads contain more than one DNA molecule; it is not yet known whether these are packaged into the rare anomalous heads containing more than one portal vertex.

The anomalous head sizes and shapes occur because assembly depends on numerous interactions of several proteins, each of which can exist in multiple conformational states and in different concentrations.

Tails

The tails are built like complex cocked mechanical devices with additional catalytic activities. The multifunctional baseplate contains information for building the tail and serves as a perfect valve for DNA entry into bacteria in the next infectious cycle. It consists of a central hub, six outer wedges and six tail spikes, each of these structures being assembled from several different subunits. Baseplate formation combines aspects of catalyzed assembly, self-assembly, concerted conformational changes and proteolysis.

Baseplate components are important for the irreversible second step of phage adsorption. A concerted conformational change of all baseplate components from a hexagon to a star configuration opens a hole in the tail to allow DNA to exit from the particles. It also activates the lysozyme activity of gp5 that actively punctures the host cell wall from the outside.

The tails are of remarkably uniform length, determined by a subunit of the baseplate that acts like a tape measure. Tails have a tubular inner core, through which DNA passes from the head to the baseplate, and an outer sheath that contracts during infection by conformational changes of individual subunits.

Tail fibers

Six bent tail fibers are attached to the tail of each particle. Each fiber consists of two half-fibers whose proteins are joined at an angle. The inner (proximal) half-fibers are attached to the baseplate. The outer (distal) half-fibers contact phage-specific receptors on the surface of the bacterial cell wall during the first, reversible step of adsorption. In many newly formed phage particles this end is transiently attached to the

junction of heads and tails, rendering the fibers less fragile.

Tail fibers of different T-even phages appear superficially similar, but the amino acid sequences and genes are different in the different family members, resulting in recognition of different receptors in the host cell wall. These differences are used to distinguish different T-even phages by their host range. Apparently, illegitimate recombination with other DNA sequences, e.g. those of prophages that reside in the host genome, allow rapid evolution and adaptations to different receptors in different hosts' cell walls under selective pressure.

Temporally Controlled Gene Expression During T-even Development

T-even phages inactivate host translation and transcription in many small steps by multiple redundant mechanisms. The temporal regulation of phage gene expression is likewise exerted at many levels: transcript initiation, elongation and termination; stability, conformation and recognition by ribosomes of the transcripts, and combinations thereof.

Different classes of phage genes are distinguished in terms of timing as early (immediate early, IE), middle (delayed early, DE), or late. Operationally, IE genes are distinguished from the other classes in that they can be transcribed by host RNA polymerase without modification by phage proteins. Expression of all other T-even genes requires phage protein synthesis for several reasons. Successive transcription initiations from early, middle and late promoters are accomplished by a cascade of RNA polymerase modifications: the α subunits are covalently ADP-ribosylated and several accessory proteins bind non-covalently. Moreover, RNA polymerase can be attracted to middle promoters by proteins bound to specific DNA sequences, and it can be activated for initiation at late promoters by another protein bound to DNA, and used in both late transcription and DNA replication, the sliding clamp gp45 (see DNA Replication and Recombination In Vivo, below).

Another classification criterion distinguishes all genes that are expressed prior to the onset of DNA replication as 'prereplicative' or 'early' from 'post-replicative' or 'late' genes whose expression depends on DNA replication.

The distinction between different classes is, however, blurred because most T4 genes are under dual or multiple controls and because of overlapping and interdigitated transcriptional, post-transcriptional and translational control signals. Thus, promoters can be classified as early, middle or late, but genes defy this classification. One example (of ten known T4 regions with overlapping early, middle and late transcripts) is shown in **Fig. 4**. The regulation of T4 gene expression is better described by a web of interacting regulatory networks than by simple progression along a linear timed pathway.

Collectively, prereplicative genes encode: (1) nucleases that degrade the host DNA; (2) enzymes of the deoxyribonucleotide biosynthesis complex; (3) proteins of the replication and recombination machines; (4) proteins that modify the T4 DNA to protect it from degradation by its own nucleases and from other restriction enzymes; (5) several tRNAs; (6) proteins that modify structure and function of the host RNA polymerase; (7) at least one RNase (RegB protein) that selectively destroys certain early transcripts; (8) proteins that repress translation (e.g. RegA protein). In addition some prereplicative transcripts serve as primers for leading strand DNA synthesis in origins of replication (see below).

A T4-encoded sigma factor, gp55, associated with host core RNA polymerase, directs transcription from late promoters. Late transcription requires several additional proteins and concomitant DNA replication, further discussed below.

The late genes code for virion components, for some DNA repair and recombination proteins, and proteins that cut and package the complex vegetative DNA into preformed heads. A soluble lysozyme, different from but evolutionarily related to the baseplate lysozyme, lyses the host bacteria to release the progeny phage particles. Late genes that are under multiple controls (e.g. **Fig. 4**) can be expressed early, particularly when the RNA is broken or when infections occur at high temperatures, i.e. conditions that allow access of ribosomes to the translation initiation regions of late genes in the early RNA.

Transcription

The first set of promoters, early promoters, resembles the consensus sequence of *E. coli* promoters with additional information content (**Fig. 3a**). They are recognized by the *E. coli* RNA polymerase containing the major sigma factor σ^{70}, at a time when the host DNA is still largely intact. T4's early promoters are preferred to *E. coli* promoters, apparently not due to gene dosage effects. Several factors are thought to contribute to such preferential transcription of T4 versus *E. coli* genes:

1. Many early T4 promoters contain upstream polyA tracts, functioning as bendable sequences or as 'promoter UP elements'.
2. Arg265 of one α subunit of the host's RNA polymerase is ADP-ribosylated immediately after infection by the T4 Alt protein that is packaged

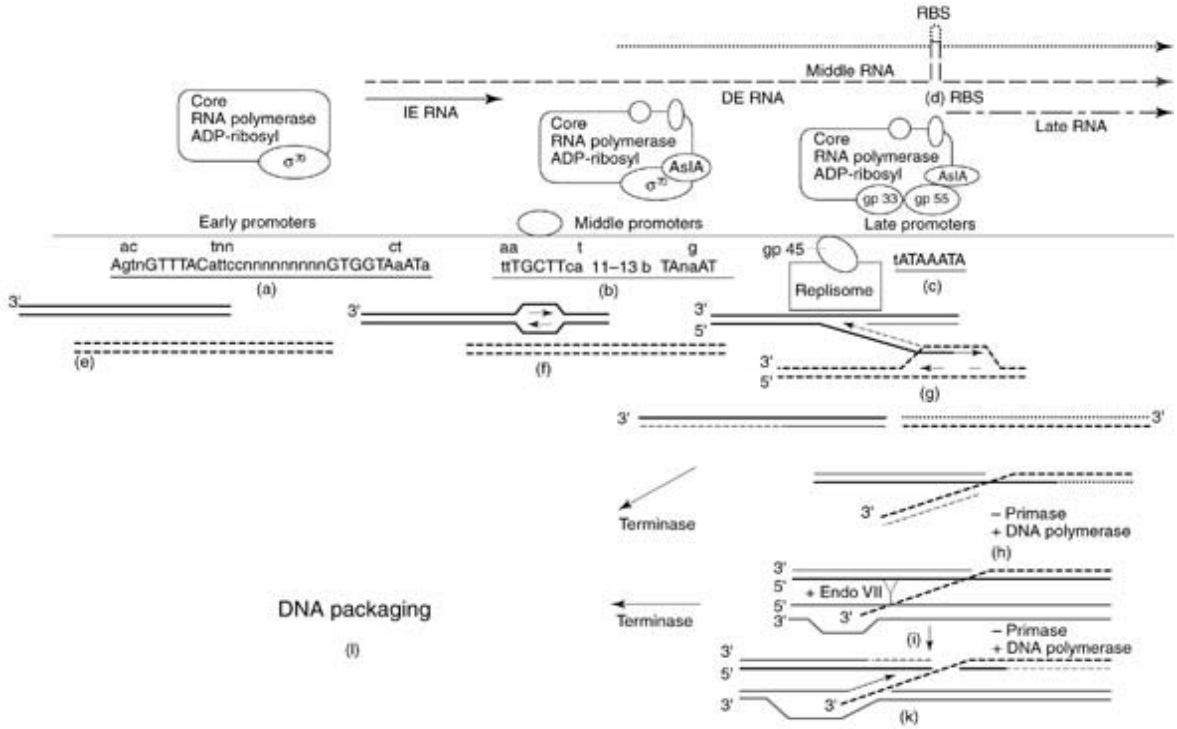


Figure 3 A diagram of transcription, DNA replication, recombination and packaging during T4 development (progressing in time after infection from left to right). The upper panel (a)–(d) shows overlapping early, middle and late transcripts in a 'generic' region, the modifications of host RNA polymerase after infection and the consensus sequences of early, middle and late T4 promoters that are recognized by different forms of the RNA polymerase. Ribosome-binding sites for late proteins in prereplicative transcripts are sequestered in hairpins (d); see also **Fig. 4**. The lower panel shows different stages of DNA replication and recombination. (e) Two infecting permuted T4 chromosomes. (f) Bidirectional origin initiation in one of them. Leading strand synthesis is primed by RNA polymerase-generated transcripts; lagging strand synthesis is primed by short RNAs synthesized by primase. As soon as the first growing point reaches an end (only one is shown), the partially single-stranded 3′ terminus invades the homologous region of another chromosome (g) or the terminal redundancy of the same chromosome (not shown). Join–copy replication is initiated from the invading 3′ DNA end. When an endonuclease cuts at the recombinational junction, join–cut–copy recombination can be initiated from the 3′ ends to allow copying of single-stranded segments of an invading DNA. Together both processes generate branched concatemers, which become increasingly more complex by reiterations. This DNA is processed to mature, unbranched chromosomes during packaging. Parental molecules are drawn as bold lines, filled with different patterns. Newly synthesized DNA is drawn as thin lines. Discontinuous synthesis of Okazaki pieces is indicated by dashed lines, continuous synthesis by solid lines. Arrowheads indicate the directions of RNA or DNA synthesis. (Modified from Mosig *et al* (1995).)

and injected with the phage DNA. This Arg265 is located at the dimer interface of the two α-subunits and it is important for activation of many strong *E. coli* promoters including those for ribosomal RNA. Its modification affects transcription in several ways.

3. At the time of infection, the host DNA is associated with nonspecific (e.g. HU, NS) or semi-specific (e.g. IHF, FIS) DNA binding proteins. In contrast, the infecting phage DNA is at first largely free and may be much more readily accessible to the host's RNA polymerase.

Host transcription is further reduced and the transition from host to phage transcription is accelerated by products of several early phage genes. Some of them disrupt the host nucleoid. Alc protein selectively inhibits transcript elongation on the host DNA but allows elongation on phage DNA, whose cytosine residues are hydroxymethylated and glycosylated (see below). The *asiA* product binds to the C-terminal segment of σ^{70}, interfering with transcription from all host promoters with standard -35 regions, as well as from T4 early promoters. However, phage infection can proceed because most prereplicative genes can also be transcribed from middle promoters (**Fig. 3**).

MotA protein bound to mot-boxes in middle promoters (**Fig. 3b**) recognizes Ast-A-modified RNA polymerase and allows initiation from middle promoters and expression of most prereplicative genes after the AsiA protein has inhibited initiation

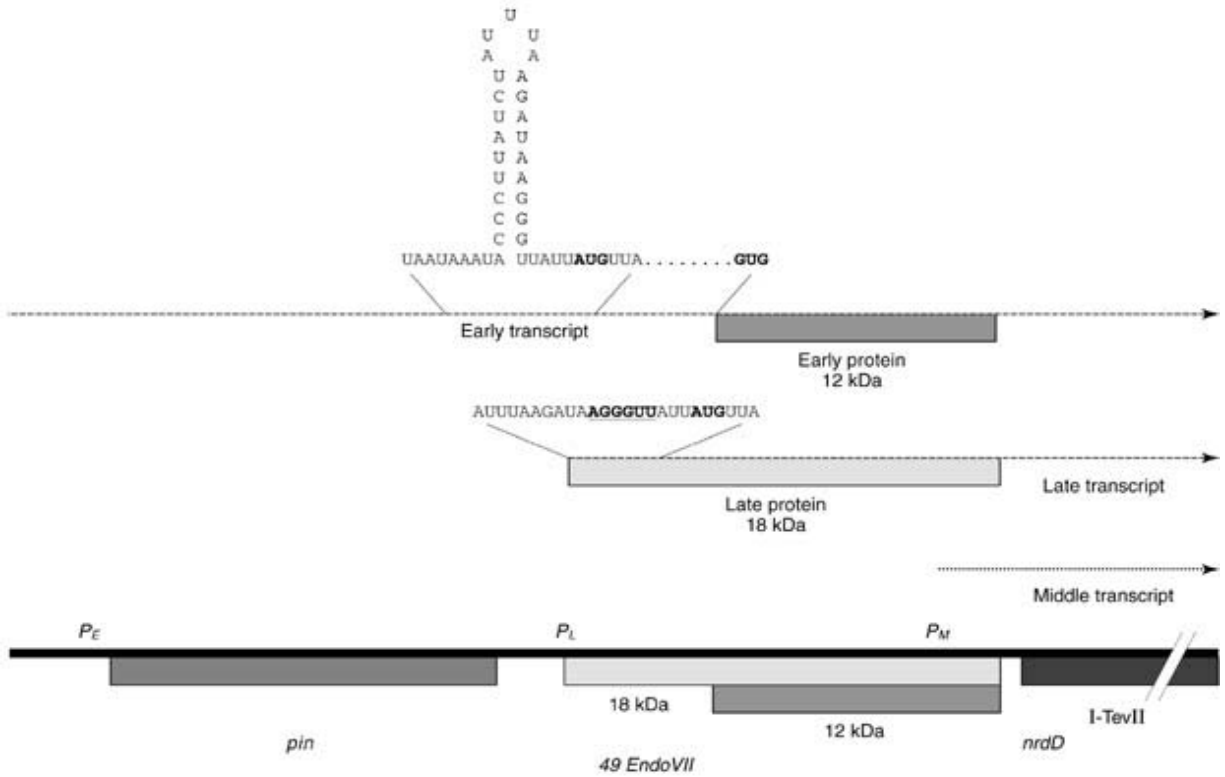


Figure 4 An example of interwoven transcriptional and post-transcriptional controls in the T4 gene *49* region (cf. **Fig. 1**). The locations of early (P_E), middle (P_M), and late (P_L) promoters and the protein-encoding segments of genes *pin*, *49* and *nrdD* are marked. Overlapping transcripts are distinguished by differentially patterned horizontal lined with arrows. The *nrdD* transcripts encode two proteins, one from spliced and the other (a truncated peptide) from unspliced RNA. I-TevII is a self-splicing intron. The late gene *49* transcript is predominantly translated into the 18 kDa EndoVII. In the larger, early transcript the Shine–Dalgarno sequence is sequestered in a hairpin. A shorter 12 kDa protein can be initiated in frame from an internal GUG. (Modified from Karam *et al* (1994).)

from early promoters. Initiation from early and middle promoters can overlap in time, depending on the proportions of RNA polymerase molecules that have been modified. Initiation from late promoters (**Fig. 3c**) requires a phage-encoded sigma factor (gp55), activation by gp45, the sliding clamp of the T4 replisome, and an adapter protein (gp33). In addition it requires concomitant DNA replication, or 'uncoupling' from DNA replication by combinations of certain recombination- and ligase-defective mutations. The critical common aspect of the latter conditions is that single-strand interruptions in the DNA provide entry sites for the sliding clamp, gp45, that is loaded on to the DNA by a complex of gp44 and gp62. The sliding clamp has to track along DNA to activate RNA polymerase bound at late promoters by T4 σ^{gp55}, to form open complexes and initiate transcription.

As indicated in **Figs 3** and **4**, most T4 genes can be transcribed from several promoters belonging to different classes. Genes downstream of any promoter may be poorly expressed because of premature *rho*-dependent transcription termination or because of RNA processing or degradation, or because the ribosome binding sites are sequestered by secondary structures.

Certain *rho* (transcription termination factor) mutations (also called host defective, *hdF* or *nusD*) of *E. coli* prevent growth of wild-type T4 by causing premature termination of many T4 transcripts. Mutations that allow growth in these specific *rho* mutants have been found in three nonessential T4 genes. It was initially thought that the corresponding T4 genes encode transcriptional antiterminators, but current evidence suggests that at least one of them allows T4 growth in the (*nusD*) *rho* mutants, because it stabilizes the few functional transcripts that have not been prematurely terminated.

Several additional phage proteins with unknown functions associate with host RNA polymerase. They are probably important in facilitating transcription initiation and elongation after phage infection under certain stress conditions.

The timing of differential gene expression during

T4 development is modulated by specific nucleases; for example, a T4 *regB*-encoded ribonuclease cleaves early transcripts rather selectively in the ribosome-binding sites of certain early T4 genes, thereby reducing expression of certain early T4 genes, and presumably of some host genes, at the post-transcriptional level. Moreover, several host nucleases and autocatalytic cleavages are important in processing the precursor RNAs for eight T4-encoded tRNAs and two tRNA-like structures of unknown function. The T4 tRNAs supplement host tRNAs during translation and are important for codons that are rare in *E. coli*, but frequent in T4; thereby they help maintain the different codon usages and AT contents of phage and host.

The introns in at least three T4 mRNAs (for thymidilate synthetase, *td*, and aerobic and anaerobic nucleotide reductase genes *nrdB* and *nrdD* respectively, have to be excised by self-splicing. Numerous mutations define the active centers of these ribozymes in exquisite detail.

Translational controls

Due to the interspersion of early and late genes on the T4 map (**Fig. 1**) and the 'sloppiness' of the interdigitated T4 transcription patterns shown in **Figs 3** and **4**, many early and middle transcripts are extended into late genes. Nevertheless, few or no corresponding late proteins are synthesized early at normal growth temperatures of 37°C or below. At higher temperatures there is some early expression of these late genes *in vivo* and early RNA sheared *in vitro* can direct synthesis of these late proteins. It is remarkable that expression of all of ten such late genes investigated so far follows similar patterns: in the long early transcripts a hairpin sequesters the translation initiation region, either the Shine–Dalgarno sequence or the initiation codon, or both. An additional late promoter immediately upstream of the late gene directs synthesis of transcripts that cannot form the hairpin, and these transcripts are efficiently translated (**Figs 3d** and **4**). Remarkably, phage evolution has conserved the early transcription of these late genes, while incorporating other means of preventing their translation. Pausing of RNA polymerase at such hairpins might facilitate access of the T4 σ^{gp55} required for initiation from the late promoters.

Three additional regulatory systems of T4 use translational controls. Expressions of gene *32* for the major single-stranded DNA binding protein (SSB) involved in DNA replication, recombination and repair, and of gene *43* for DNA polymerase, are autogenously regulated by translational repression. A more general, nonessential translational repressor (RegA protein), which binds to several specific transcripts, can reduce translation of several T4 replication proteins and of some host proteins. This repression is most apparent under experimental conditions that prolong early and delay late transcription.

DNA Replication and Recombination *In Vivo*

DNA replication and recombination are tightly interwoven, following several different redundant pathways. The T-even phages provided the first and most compelling evidence for recombination-dependent DNA replication. Redundant alternative modes of replication and recombination ensure that both processes work under many different conditions and during different stages of development. Known interrelationships are shown diagrammatically in **Fig. 3.**

The first round(s) of DNA replication are initiated from one of several potential origins. Because of the circular permutation of chromosomal ends (**Fig. 3e**), in each individual chromosome any origin is located at different distances from the ends. In most chromosomes only one origin is used, perhaps due to limited abundance of replisome components. Different origins share the requirement for transcription from early or middle promoters, generating large transcripts that can serve as primers for leading strand DNA synthesis at one of several sites approximately 1 kb downstream of the promoter. However, each of the four origins that have been closely investigated has a different sequence and overall structure, presumably related to preferred usage under different conditions. Three origins (*A*, *F* and *G*) require transcription from middle promoters, *oriE* depends on an early promoter. **Fig. 3f** depicts initiation from an arbitrary generic origin.

The transition from σ^{70}-dependent prereplicative transcription to T4 σ^{gp55}-dependent late transcription inhibits initiation from these origins, either by default (because the RNA polymerase no longer recognizes origin promoters), or because late protein(s), e.g. UvsW protein, actively prevent transcripts from serving as primers for DNA synthesis, or for both reasons.

Subsequent DNA replication is initiated from intermediates of homologous recombination (**Fig. 3g, i**). The recombinational intermediates are mainly formed by invasion of a single-stranded terminus into the homologous region of another molecule (or at the other end of the same molecule). When T4 chromosomes are broken by radiation damage or by certain

nucleases, single-stranded termini of internal breaks can initiate recombination similarly as natural ends of infecting chromosomes. This is a major reason for the recombinogenic effects of radiation damage.

The recombination intermediates can initiate replication from the 3′ ends of the invading single strand (join–copy recombination, **Fig. 3g**), or, after an endonucleolytic cut at the junction from a 3′ end in the invaded strand (join–cut–copy recombination, **Fig. 3i**). The join–copy mode can start as soon as a growing point has reached an end. The join–cut–copy mode requires an additional endonucleolytic cut, which probably depends on one or more late proteins, and therefore occurs later. This mode can bypass the requirement for primase or topoisomerase in T4 DNA replication and can be used when these enzymes are limiting, e.g. late in infection. Ultimately, reiterations of these processes generate a highly branched concatemeric network in which no individual chromosomes can be distinguished.

Of course, not all recombination junctions need to be converted to replication forks. Some T4 recombination can occur, albeit with delay, when DNA replication is inhibited. Electron micrographs of such T4 DNA intermediates provided the first compelling evidence for the importance of branch migration in homologous recombination. Under these conditions, no viable progeny is produced, because no packagable concatemers are formed; there is little, if any, late transcription, and there are no heads to be filled.

DNA Replication *In Vitro*

Virtuoso biochemical and biophysical characterization of replication proteins, in combination with genetic experimentation, has led to an understanding of functions and interactions of the basic replication proteins in the replisome, a biological machine that moves the replication fork, or through which the replicating DNA is passaged. Seven proteins, corresponding to genes *43* (DNA polymerase), *44* and *62* (sliding clamp loader), *45* (sliding clamp), *41* (DNA helicase), *61* (primase to synthesize primers for lagging strand synthesis) and *32* (single-stranded DNA-binding protein), form an active complex that replicates model templates with *in vivo*-like speed. Leading and lagging strand synthesis are coupled by interactions of primase-helicase with DNA polymerase. These basic reactions and protein functions are similar in all procaryotic and eucaryotic systems; in fact, some of the T4 proteins can partially substitute in eucaryotic *in vitro* systems.

Recombination-dependent initiation by a join–copy mechanism has also been achieved. Consistent with genetic analyses, these *in vitro* reactions require several recombination proteins in addition to the seven basic replication proteins just mentioned: the T4 RecA analogue gpUvsX, the single-stranded DNA binding proteins gpUvsY and gp*59*. The latter protein loads the gene *41* helicase, an enzyme that is important for branch migration in addition to its unwinding function at the replication fork. Initiation by the join–cut–copy mechanism and origin initiation have not yet been achieved *in vitro*.

Many of the T4-encoded DNA enzymes, most importantly DNA ligase, kinase and polymerase, are now standard components of cloning procedures and kits.

DNA Packaging

The ends of mature T4 chromosomes are nearly randomly permuted over the map, and 3–5% of the genome is repeated at both ends as so-called terminal redundancies. Mature chromosomes are generated from the branched concatemeric vegetative DNA during packaging by a terminase, a heteromeric protein encoded by genes *16* and *17* that associates with DNA and with gp20 at the portal vertex of the head (**Fig. 1**) and uses ATPase activities for the head-filling process. Gene *17* produces several proteins of different sizes by initiation from in-frame internal initiation codons. Several of these proteins have nuclease activity; the largest one also binds non-specifically to single-stranded DNA segments. There is controversial evidence as to whether T4 has sequence specific *pac* sites or whether it initiates packaging at such random single-stranded DNA segments. Perhaps both processes can initiate packaging. If the first initiation of packaging is a relatively rare event, processive packaging of 103–105% genome lengths to fill the preformed heads can account for the random circular permutation of the ends in mature virion DNA by either mechanism. Endonuclease VII which cuts Holliday and Y junctions and mismatched base pairs *in vitro* is required *in vivo* to trim the branches of vegetative DNA. It can associate with gp20 and retain nuclease DNA ligase, endonuclease V and topoisomerase are also required, presumably to provide uninterrupted double-stranded DNA as packaged chromosomes.

An *in vitro* packaging system has been developed to package large pieces of foreign DNA.

Restriction-Modification and Exclusion

In the following discussion the term 'restriction' is used in its broadest meaning, not limited to type II restriction enzymes. The complex modification and restriction of T4 DNA and of other DNA by T4 can

best be rationalized as the result of an ongoing evolutionary process that includes exchanges between the phage, its host and prophages resident in the host.

T-even phages destroy dCTP, synthesize dHMCTP and use the latter for DNA synthesis. This modification protects the T4 DNA against T4-encoded restriction endonucleases II and IV that degrade the host DNA as part of the parasitic strategy to usurp the host. However, HMC residues render DNA susceptible to the Mcr restriction systems of the host. These host functions were the first restriction systems (then called Rgl) discovered. They are now called McrA and McrB, because they restrict DNA containing methylcytosine or hydroxymethylcytosine. These restriction functions are overcome when the HMC residues are glycosylated. In T4 DNA, all HMC residues are modified; 70% with α- and 30% with β-glycosyl linkages. In T2 and T6 DNA, there are no α-glycosyltransferases, and 25% of the HMC residues remain unglycosylated. T6 contains many diglycosylated residues. In addition, a T4-encoded early anti-restriction protein (Arn) protects nonglycosylated T4 DNA against one but not all of these host restriction enzymes.

T2 and T4, but not T6, encode a Dam methylase that methylates 0.5–1.5% of the adenine residues at the N^6 positions, mostly but not exclusively at GATC sequences. These enzymes exhibit patches of similarity at the protein level with the *E. coli* Dam methylase and the *Dpn*II methylase of *Diplococcus pneumoniae*. The only proven physiological role of adenine methylation is protection against the phage P1 restriction system, when there is no HMC glycosylation.

Intriguingly, several other 'host' genes that exclude T4 by various strategies are located in resident prophages or their defective derivatives.

The Mcr A system of K12 mentioned above resides in a cryptic prophage-like element, *e14* that is not present in all *E. coli* strains.

Another protein of *e14*, Lit, in combination with a short internal peptide (*gol*) of the major T4 head protein, gp23, cleaves the host's elongation factor EF Tu, thereby inhibiting translation of all late T4 proteins.

The classical example of phage exclusions by genes of resident prophages is that of T4 *rII* mutants in lambda lysogens by lambda's *rexA* and *rexB* genes. This exclusion was elegantly used in Benzer's classical analyses of structure and function of a gene. It occurs at the time of transition from join–copy to join–cut–copy recombination mentioned in the section on DNA replication and recombination, and it involves several enzymes important in the latter mechanism, as well as a putative ion channel produced by lambda's Rex proteins. Probably the otherwise nonessential RII proteins counteract this restriction, but the molecular mechanism is still unknown.

Another cryptic DNA element of certain *E. coli* strains, *prr*, encodes a PrrC protein that excludes T4 RNA ligase/polynucleotide kinase-deficient mutants. PrrC protein is a cryptic RNA endonuclease that is activated by the small (26 residues) T4 Stp protein to cleave the anticodon loop of an essential host $tRNA^{Lys.}$ T4 RNA ligase/polynucleotide kinase can repair this damage, but in the absence of RNA ligase the cleavage of this tRNA is lethal to T4 protein synthesis. Intriguingly, the *prrC* gene is located between three genes of a type IC restriction cassette. The corresponding proteins are thought to inhibit PrrC RNase activity in uninfected cells.

Phage P2 lysogens exclude T4 by two mechanisms: the Tin protein poisons the single-stranded DNA binding protein gp32 that is essential for all T4 DNA replication and recombination, and the P2 Old protein can degrade DNA from ends, nicks and gaps (although the ends of the infecting T4 chromosomes are probably protected by bound T4 gp2).

Evolution

Sequences and map positions of the 'essential' genes whose products have the same functions of most T-even phages are similar. In contrast, genomes of different members of the family have different 'nonessential' genes interspersed between these essential genes (**Fig. 1**). The heterologies contribute to apparent exclusions of alleles of one phage by another, and to the species barriers between different members of the family. They first became evident as insertion or substitution loops in electron micrographs of heteroduplex DNA prepared *in vitro* by annealing single strands of T2, T4 and T6 DNA, and have been confirmed by sequence comparisons in many cases.

In some cases the sequence divergence reflects gene amplifications and permutations of duplicated sequences. The tail fiber genes of different T-even phages appear to have diverged by illegitimate recombinations with genes of other phages, including prophages residing in the host genome. Substitutions of sequence blocks of individual genes by foreign sequences can account for the variability between different members of the family. In turn, these substitutions allow adsorption to different hosts with different receptors, accounting for the remarkable coevolution of viral and host genomes.

Such illegitimate recombinations are not limited to genes for recognition proteins. Illegitimate pairing of partially homologous sequences and join–copy and

join–cut–copy recombination (discussed above) were apparently involved in horizontal gene transfer of nonessential genes adjacent to the essential dCTPase gene, and probably other genes as well. Although inactivating their functions has little or no consequences for phage development in the laboratory, we surmise that these genes are important for viral growth and survival under different physiological conditions, in different hosts with different receptors or containing different prophages, and in the face of various restriction systems imposed by different hosts.

Future Perspectives

The T-even phages have been instrumental in first formulations of several fundamental biological concepts: (1) the unambiguous recognition of nucleic acids as genetic material; (2) the operational distinctions in defining the gene by mutational, recombinational or functional analyses (the concepts of muton, recon and cistron); (3) the demonstration of mRNA; (4) the nature of the triplet code, and the importance of initiation and nonsense codons; (5) homologous recombination as exchange between DNA molecules, and the importance of heterozygotes in this process; (6) the role of homologous recombination in initiating DNA replication; (7) restriction and modification of DNA as important aspects of host–virus interactions; (8) light-dependent and light-independent DNA repair mechanisms; (9) the importance of pathways of macromolecular assemblies (protein machines) in morphogenesis and DNA metabolism; (10) the presence of self-splicing introns and mobile endonucleases in prokaryotes; and (11) the facility of ribosomes to skip unspliced introns in mRNA during translation.

Recent progress towards understanding the importance of redundant pathways and proteins for fundamental processes is bound to lead to better appreciation of the functional significance of web-like interconnections between different processes for development, for virus–virus and virus–host interactions, and for evolution.

See also: **SPO1 phage (*Myoviridae*); History of virology: Bacteriophages; T1-like phages (*Siphoviridae*); Salmonella phage P22 (*Podoviridae*).**

Further Reading

Brody EN, Kassavetis GA, Ouhammouch M *et al* (1995) Old phage, new insights: two recently recognized mechanisms of transcriptional regulation in bacteriophage T4 development. *FEMS Microbiol. Lett.* 128: 1.

Geiduschek, EP (1997) Paths to activation of transcription. *Science* 275: 1614–1616.

Karam JD, Drake JW, Kreuzer KN, *et al* (eds) (1994) *Molecular Biology of Bacteriophage T4*. American Society of Microbiology: ASM Press: Washington, DC.

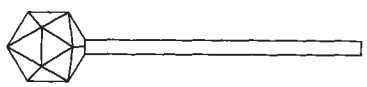
Kutter E, Gachechiladze K, Poglazov A *et al* (1996) Evolution of T4-related phages. *Virus Genes* 11: 213.

Mosig G (1998) Recombination and recombination-dependent DNA replication in bacteriophage T4. *Annu. Rev. Genet.* 32: 379–413.

Mosig G and Eiserling F (1988) Phage T4: structure and metabolism. In: Calendar R (ed.) *The Bacteriophages*, vol. 2, p. 521. New York: Plenum Press.

Mosig G, Colowick N, Gruidl ME, Chang A and Harvey AJ (1995) Multiple initiation mechanisms adapt phage T4 DNA replication to physiological changes during T4's development. *FEMS Microbiol. Rev.* 17: 83.

T5-LIKE PHAGES (*SIPHOVIRIDAE*)



D. James McCorquodale, Midwestern University, Downers Grove, Illinois, USA

Classification and Morphology

Bacteriophage T5 and its relatives BF23, PB, BG3 and 29-alpha are in the T5-like viruses genus of the *Siphoviridae* family. They have a general morphology that consists of an icosahedral head and a long noncontractile flexible tail. The head of T5 has an average diameter of 90 nm. The tail is attached to one of the head apices via a head–tail linker protein and has three L-shaped tail fibers attached at a site near its distal end. A ring-like structure is formed at this site as a result of the attachment of these tail fibers. The tubular tail undergoes a transition at the tail fiber attachment site to a conical form, which tapers into a single straight tail fiber. The tail has a diameter of 12 nm with a length of 190 nm. The cone (12 nm) plus

join–cut–copy recombination (discussed above) were apparently involved in horizontal gene transfer of nonessential genes adjacent to the essential dCTPase gene, and probably other genes as well. Although inactivating their functions has little or no consequences for phage development in the laboratory, we surmise that these genes are important for viral growth and survival under different physiological conditions, in different hosts with different receptors or containing different prophages, and in the face of various restriction systems imposed by different hosts.

Future Perspectives

The T-even phages have been instrumental in first formulations of several fundamental biological concepts: (1) the unambiguous recognition of nucleic acids as genetic material; (2) the operational distinctions in defining the gene by mutational, recombinational or functional analyses (the concepts of muton, recon and cistron); (3) the demonstration of mRNA; (4) the nature of the triplet code, and the importance of initiation and nonsense codons; (5) homologous recombination as exchange between DNA molecules, and the importance of heterozygotes in this process; (6) the role of homologous recombination in initiating DNA replication; (7) restriction and modification of DNA as important aspects of host–virus interactions; (8) light-dependent and light-independent DNA repair mechanisms; (9) the importance of pathways of macromolecular assemblies (protein machines) in morphogenesis and DNA metabolism; (10) the presence of self-splicing introns and mobile endonucleases in prokaryotes; and (11) the facility of ribosomes to skip unspliced introns in mRNA during translation.

Recent progress towards understanding the importance of redundant pathways and proteins for fundamental processes is bound to lead to better appreciation of the functional significance of web-like interconnections between different processes for development, for virus–virus and virus–host interactions, and for evolution.

See also: **SPO1 phage (*Myoviridae*); History of virology: Bacteriophages; T1-like phages (*Siphoviridae*); Salmonella phage P22 (*Podoviridae*).**

Further Reading

Brody EN, Kassavetis GA, Ouhammouch M *et al* (1995) Old phage, new insights: two recently recognized mechanisms of transcriptional regulation in bacteriophage T4 development. *FEMS Microbiol. Lett.* 128: 1.

Geiduschek, EP (1997) Paths to activation of transcription. *Science* 275: 1614–1616.

Karam JD, Drake JW, Kreuzer KN, *et al* (eds) (1994) *Molecular Biology of Bacteriophage T4*. American Society of Microbiology: ASM Press: Washington, DC.

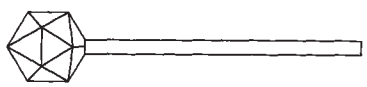
Kutter E, Gachechiladze K, Poglazov A *et al* (1996) Evolution of T4-related phages. *Virus Genes* 11: 213.

Mosig G (1998) Recombination and recombination-dependent DNA replication in bacteriophage T4. *Annu. Rev. Genet.* 32: 379–413.

Mosig G and Eiserling F (1988) Phage T4: structure and metabolism. In: Calendar R (ed.) *The Bacteriophages*, vol. 2, p. 521. New York: Plenum Press.

Mosig G, Colowick N, Gruidl ME, Chang A and Harvey AJ (1995) Multiple initiation mechanisms adapt phage T4 DNA replication to physiological changes during T4's development. *FEMS Microbiol. Rev.* 17: 83.

T5-LIKE PHAGES (*SIPHOVIRIDAE*)



D. James McCorquodale, Midwestern University, Downers Grove, Illinois, USA

Classification and Morphology

Bacteriophage T5 and its relatives BF23, PB, BG3 and 29-alpha are in the T5-like viruses genus of the *Siphoviridae* family. They have a general morphology that consists of an icosahedral head and a long noncontractile flexible tail. The head of T5 has an average diameter of 90 nm. The tail is attached to one of the head apices via a head–tail linker protein and has three L-shaped tail fibers attached at a site near its distal end. A ring-like structure is formed at this site as a result of the attachment of these tail fibers. The tubular tail undergoes a transition at the tail fiber attachment site to a conical form, which tapers into a single straight tail fiber. The tail has a diameter of 12 nm with a length of 190 nm. The cone (12 nm) plus

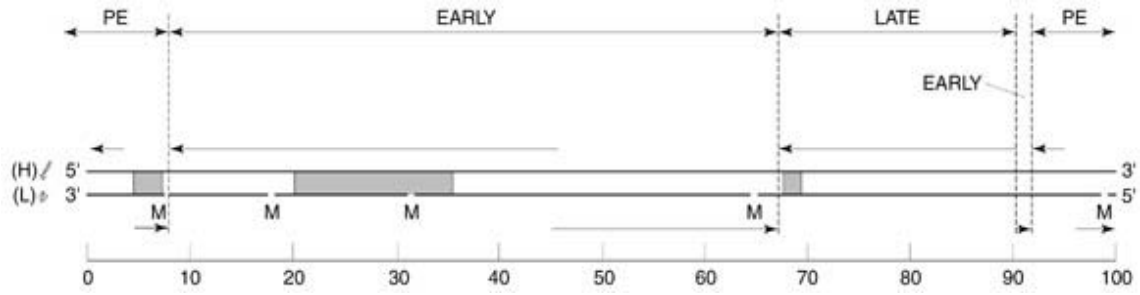


Figure 1 Structure and organization of the genome of T5 and BF23. The heavy double lines represent the two DNA strands of the double-stranded genome. H and L indicate the relative bouyant density of the upper un-nicked strand to that of the lower nicked strand. The script '1' and 'r' indicate leftward transcription from the upper strand and rightward transcription from the lower strand, in agreement with the 3′-5′ polarities of the two strands. Major nicks in the lower strand are indicated by gaps in the heavy line and the letter 'M'. Deletable regions are indicated by shaded regions between the two heavy lines. The distribution of pre-early, early and late genes along the genome is indicated by name and by lines with arrowheads at both ends. The direction of transcription for each class of genes is indicated by directional arrows above and below the two heavy lines. The 0–100 scale at the bottom divides the genome into percentages from left to right.

the single straight tail fiber (50 nm) bring the total length of the tail to about 250 nm. The phage protein (Oad) that binds irreversibly to the host receptor (FhuA) is located in the conical region. The tail is hollow from the head to the cone-shaped structure and provides part of the route for transfer of phage DNA from the phage head to the host cell. A total of 15 different polypeptides have been detected in mature T5 phage particles and the number of copies of each polypeptide per particle depends on the part of the phage structure that it forms. For example, the major head polypeptide is present at 730 copies per particle whereas the straight tail fiber polypeptide is present at only five copies per particle.

Structure of the Genome

The DNA within mature T5 or BF23 particles is linear, double-stranded, and about 121 300 bp long (**Fig. 1**). It contains only the four common bases, adenine, guanine, cytosine and thymine, none of which are methylated. Its AT content is 61%. Two features of the DNA stand out. First, it has unusually long direct terminal repetitions of about 10 100 bp and second, it is nicked at precise sites, and all nicks are in one strand only. The nicks consist of a missing phosphoester bond between the 5′-phosphate group of one nucleotide residue and the 3′-OH group of its adjacent nucleotide residue. Thus, these nicks can be ligated with DNA ligase. The nicks are introduced by a site-specific nicking enzyme coded by genes *sciA* and *sciB*, which map at the right end of the 'late' region of the genetic map (see **Fig. 1**). Nicks are classified as 'major' or 'minor', with major nicks occurring in virtually all phage DNA molecules and minor nicks occurring in only a small fraction of these molecules.

The frequency of the major nicks (four per 111 200 bp) predicts a recognition sequence of seven or eight nucleotides. A prominent sequence on the 5′ side of nicks in T5 DNA is 5′-GCGCGGTG-3′, and the sequence in the unnicked strand around the major nick at 64.8% of the length of DNA from the left end of both T5 and BF23 DNA is 5′-CCCGCGCCC-3′. The left end is defined as the end that always enters the host cell first during normal infections (**Fig. 1**). Thus the sequence most efficiently recognized by the nicking enzyme appears to be

```
intact strand: 5′  (C) | CCGCGCC | C 3′
nicked strand: 3′ G(T) | GGCGCGG | G 5′
                       ↑
                      nick
```

The boxed in sequence would generate major nicks whereas minor variations in this sequence would presumably generate minor nicks.

The DNA can be deleted in three regions without affecting viability (**Fig. 1**). The major deletable region is between positions 20.0 and 35.7%. Although this region spans about 19 kb, only about 13.3 kb can be deleted from the DNA and still be packaged. Larger deletions yield DNA too small to be packaged. This deletable region contains genes that code for at least one tRNA for each of the 20 amino acids found in proteins. Another deletable region is between positions 4.1 and 7.1% (about 3.6 kb) and is therefore in the terminal repetition. It follows that most genes in the right half of the terminal repetition are unessential. The third deletable region is between positions 67.8 and 69.5% and is within a nonessential gene that codes for the L-shaped tail fibers of the

mature phage. Because regions of the T5 and BF23 genomes can be deleted, these phages could be used as cloning vehicles for inserts up to about 19 kb. However, such use has not been developed. Nevertheless, some T5 promoters, because of their strength (see below), are currently being used in expression vectors.

The nucleotide sequences that have been determined in T5 DNA plus those determined in BF23 DNA (not counting overlapping sequences) come to about 30% of their genomes, or about 36 400 nucleotides. Most of this sequencing has been done in the region from 58.3 to 92.2%, which includes some early but mostly late genes, and includes T5 genes *D7-8-9* (the phage DNA polymerase), *D10* (a putative helicase), *D11, D12* and *D13* (nucleoside triphosphate-binding proteins, probably involved with DNA replication, recombination and/or repair), *D14, D15* (a 5′-exonuclease), the *dut* gene (dUTPase), the *1tf* gene (L-shaped tail fibers), the *oad* gene, the *11p* gene (lipoprotein), and BF23 genes 25 (equivalent to T5 gene N4) and 24 (tail proteins). Four other interesting regions of T5 or BF23 have been sequenced. About 1000 bp have been sequenced in the region where the first step of DNA transfer stops (see below), which is very close to the right end of the left terminal repetition (positions 7.4–8.3%). Present in this sequence are several direct repeats and palindromes, DnaA-binding sites and simple repeats with a periodicity suggestive of DNA bending. Another sequence of about 1100 bp (positions 2.4–3.3%) defines gene *A2–A3*, an unidentified open reading frame (ORF) on its right, and the beginning of gene *A1* on its left. The third region (positions 22.4–27.8%) that has been sequenced codes for tRNAs, but contains short ORFs interspersed between the tRNA genes. Lastly, a sequence of about 180 bp defining the left end of BF23 DNA (90% homologous to that of T5 DNA) encodes a strong transciptional stop signal just after the termination codon for the *dmp* gene (5′-deoxyribonucleoside-5′-monophosphatase), which is the leftmost gene in the terminal repetitions.

The genetic maps for both T5 and BF23 have been correlated with their physical genomes such that pre-early genes are located from 0 to 8.3%, and are repeated between 91.7 and 100%, i.e. pre-early genes are in the terminal repetitions. Early genes are located between positions 8.3 and 67.2% as well as in a very short region between 90.5 and 91.5%. Late genes are located between 67.2 and 90.5%.

Restriction maps for T5 DNA have been developed for the restriction endonucleases *Bal*I, *Bam*HI, *Bgl*I, *Bst*EII, *Eco*RI, *Hin*dIII, *Hpa*I, *Kpn*I, *Pst*I, *Sac*I, *Sal*I, *Sma*I, *Sst*I and *Xho*I, and for BF23 DNA for *Bal*I, *Bam*HI, *Eco*RI, *Hpa*I and *Sal*I.

The Infection Process

Attachment of T5 to host cells is facilitated by the L-shaped tail fibers which bind rapidly and reversibly to polymannose O antigens on the outer surface of the outer membrane of the host cell, and move the phage across the outer surface until a receptor is located. The host cell receptor for T5 is *fhuA*, the receptor for ferrichrome, and for BF23 is *btuB*, the receptor for vitamin B_{12}. Irreversible binding occurs between the phage Oad protein and these host receptors, and is accompanied by a covalent cross-linking of three copies of a minor tail protein, pb4. The straight tail fiber, pb2, rearranges to form a channel as does the host cell receptor, thereby providing the route of entry of phage DNA into the host cell. The straight tail fiber is long enough to span the outer membrane, the periplasm, and the inner membrane, but it may also draw the outer membrane to the inner membrane to create contact points where the phage DNA actually enters the host cell through the newly formed channel. Transfer of phage DNA by both T5 and BF23 is unique in that they transfer their DNA in two steps. The left terminal repetition is always transferred first after which DNA transfer stops. DNA transfer resumes only after the pre-early genes in the left terminal repetition have been expressed. The remaining 92% of the phage DNA is then quickly transferred to the host cell such that late genes follow the entry of early genes by only a few seconds.

During transfer of the left terminal repetition (the first step of DNA transfer), the entry channel for DNA is open and low-molecular-weight substances such as K^+ and phosphate leak out of the host cell so that partial depolarization of the periplasmic membrane ensues. After transfer of the left terminal repetition, the channel is closed by Ca^{2+} and the periplasmic membrane repolarizes. When the channel is reopened for transfer of the remaining 92% of the DNA, low molecular weight components of the host cell again leak out and another partial depolarization of the periplasmic membrane occurs. Ca^{2+} again closes the channel, the periplasmic membrane repolarizes, and a successful infection is established. The T5 and BF23 systems therefore both have a Ca^{2+}-requirement, and cannot progress when the concentration of Ca^{2+} is below 0.1 mM.

Pre-early Genes

Since genes in the terminal repetition (**Fig. 1**) are the first phage genes to be expressed in the infected host cell, they have been termed 'pre-early'. Pre-early genes that have been identified include *dmp* (coding for a deoxyribonucleoside-5′-monophosphatase), *A1* (cod-

ing for a protein required for completion of DNA transfer, for shutdown of expression of pre-early genes, and for degradation of host DNA), and *A2-A3* (coding for a protein that is also required for completion of DNA transfer and that binds to DNA, lipopolysaccharide, and host RNA polymerase). Other functions induced by genes in the terminal repetition include the inactivation of host restriction endonucleases, of the host cell reactivation system, of host DNA methylases as well as the total inhibition of host DNA, RNA and protein synthesis. The product of gene *A2-A3* (gp*A2-A3*) is also crucially involved in the abortive response that ensues when either T5 or BF23 infects host cells that harbor a ColIb plasmid or, in some cases, a ColIa plasmid.

Effect on Host Cell Metabolism

Infection by T5 or BF23 results in a rapid and complete degradation of host DNA to individual deoxyribonucleotides, and therefore the synthesis of host DNA, RNA and protein ceases soon after infection. Mutations in gene *A1* prevent this degradation and cells infected with *A1* mutants continue synthesis of host proteins for at least 60 min. The deoxyribonucleotides derived from host DNA are partially degraded to ribonucleosides, free bases and deoxyribose-1-phosphate. The free bases and deoxyribonucleosides are secreted by the infected cell so that all deoxyribonucleoside triphosphates used in the synthesis of phage DNA are synthesized via *de novo* pathways of nucleotide anabolism. A possible reason for the clearance of all nucleotides derived from host DNA is that the phage-induced nuclease that degrades host DNA may only attack methylated DNA. Phage DNA is not methylated and so would be protected from attack, but if any methylated bases derived from host DNA were incorporated into phage DNA, it would be attacked. Thus, the elimination of host-derived bases would be a requirement for a successful infection. This suggestion would also require that a phage function inactivate host DNA methylases, which, as stated above, is known to occur after infection by T5.

Another requirement for a successful infection is the inactivation of host cell restriction endonucleases. Neither T5 nor BF23 DNA contain *Eco*R1 or other common restriction sites in their terminal repetitions but do have them in the central nonredundant portion of their genomes. Thus, inactivation of host restriction endonucleases by the product of one or more pre-early genes, and therefore prior to entry of the portion of the genome containing susceptible restriction sites, allows the susceptible portion of the phage genome to escape the action of host restriction endonucleases. If, on the other hand, the terminal repetition contains even a single restriction site that is cleaved by a host restriction endonuclease, the infection is unsuccessful.

Early Genes

After pre-early genes are expressed, phage DNA transfer resumes and early but not late genes begin their expression as soon as the rest of the phage DNA enters the host cell. Early genes code mostly for enzymes and proteins required for biosynthesis of deoxyribonucleotides, replication of phage DNA and regulation of transcription. Early gene expression begins about 5 min after infection at 37°C, and continues in the case of some early genes until about 20 min after infection, but in the case of other early genes until lysis. Thus, early genes can be divided into two subclasses on the basis of their period of expression.

Products of early T5 genes that have been identified include DNA polymerase (*D9*), deoxynucleoside monophosphokinase (*dnk*), dihydrofolate reductase (*B3*), 5′-exonuclease (*D15*), ribonucleotide reductase (possibly *B1* or *B2*), thioredoxin, thymidylate synthase (*thy*), dUTPase (*dut*), tRNAs (genes within the major deletable region), lipoprotein (*11p*), and RNA polymerase modifying proteins (*C2*, *D5* and *14* and *10* in the case of BF23). This array of early gene products enables the infected cell to initiate phage DNA replication, which begins about 8–9 min after infection and continues until lysis.

Late Genes

Expression of late genes begins at 10–12 min after infection and continues until lysis. Most late genes code for structural proteins of the mature phage particle, and when they begin to accumulate, phage morphogenesis begins using the phage DNA that had begun replication earlier. The eclipse period for T5 and BF23 is about 20 min. Thus, there is a well-regulated temporal sequence for the synthesis of phage DNA and of pre-early, early and late proteins which corresponds to the same temporal sequence for the synthesis of mRNAs. Although most late genes code for structural proteins of the mature phage particle, two late genes (*sci*A and *sci*B) code for the protein that introduces nicks into the phage DNA. Lysis of the infected cell presumably depends on a late gene which codes for a lysis protein, but this has yet to be demonstrated for the T5 system. Lysis exposes the progeny phage to phage receptors liberated from the lysed host cells. To counteract the inactivation of progeny phage by these receptors, the product of early

gene *11p* (a lipoprotein) combines with liberated host receptors to inactivate them.

Regulation of Transcription

The temporal appearance of phage-specified proteins in T5- or BF23-infected cells is regulated at the level of transcription (**Fig. 1**). However, since all classes of T5 or BF23 genes (pre-early, early and late) are efficiently transcribed *in vitro* by unmodified host RNA polymerase (with sigma-70), and this capacity for transcription is the same whether nicked or ligated phage DNA is used as a template, the temporal expression of phage genes *in vivo* must be regulated by mechanisms that prevent the simultaneous expression of all classes of genes if the phage DNA entered the cell in one step. This regulation appears to be accomplished in part by sequential modifications of the host RNA polymerase. On the other hand, expression of pre-early genes is temporally separated from early and late gene expression because of the two-step mechanism of phage DNA transfer, whereby pre-early genes enter the host cell first and must be expressed before early and late genes are able to enter the cell. Pre-early genes are therefore transcribed *in vivo* by the pre-existing unmodified host RNA polymerase. The first modification to host RNA polymerase is the binding of the pre-early proteins coded by gene *A2-A3* (gpA2-A3) and gene *A1* (gpA1). The modification by gpA1 causes shutdown of pre-early gene transcription, whereas the modification by gpA2-A3 prevents the premature transcription of late genes when the phage DNA carrying early and late genes enters the host cell after pre-early genes are expressed. It follows that early genes, but not late genes, can be transcribed by host RNA polymerase modified by gpA2-A3 and gpA1. Transcription of late genes would then require a further modification of RNA polymerase by early gene products, and for T5 they are gpC2 and a 15 kDa protein, whereas for BF23 they are gp10 and gp14. GpA2-A3 is then displaced from the RNA polymerase and late transcription proceeds with the resulting gpC2-15 kDa (for T5) or gp10-gp14 (for BF23) modified RNA polymerase.

DNA Replication

T5 DNA contains multiple origins of replication, which suggests that its DNA is replicated linearly. However, T5 DNA is found in a circular form in infected cells, and the length of such circles is equal to a genome length minus one terminal repetition. Formation of these circles could therefore arise from a recombinational event between the terminal repetitions of a single incoming parental DNA. The occurrence of circles suggests a rolling circle model of DNA replication. Sedimentation studies of replicating T5 DNA from infected cells shows a fast-sedimenting fraction, which could be linear concatemers or the rolling circle intermediate, and a slow-sedimenting fraction that corresponds to genome-length T5 DNA. These findings suggest that excision of genome-length DNA from larger precursors and packaging of phage DNA into immature heads proceed independently of one another.

Morphogenesis

Morphogenesis follows two separate pathways, head formation and tail formation. The immature head is filled with a genome length of phage DNA that is cut from a linear concatemer consisting of phage genomes minus the length of one terminal repetition. The most likely mechanism for excising exactly one genome from such a linear concatemer is by means of two staggered single-strand cuts at each internal terminal repetition in order to generate genome-length DNA with each terminal repetition having a 3′-recess. Filling of each 3′-recess would then generate full, completely double-stranded phage DNA.

Both head and tail morphogenesis involve cleavage of polypeptides that contribute to the formation of these structures. Tails can be connected to heads *in vitro*, but packaging of T5 or BF23 DNA *in vitro* has not yet been accomplished.

Abortive infection in ColIb Hosts

If T5 or BF23 infects a host cell harboring the colicinogenic plasmid, ColIb (or some ColIa plasmids), the infection is abortive. In such infections, the phage adsorbs to the host cell normally, and the phage DNA is transferred into the host cell in the usual two-step manner without being degraded. Pre-early genes are expressed and shutdown normally, but early genes barely begin expression before the host cell prematurely lyses, resulting in death of both the host cell and the infecting phage. Gene products from the phage, the host cell and the plasmid are necessary for this abortive response. The phage gene is pre-early gene *A2-A3*, which binds to both DNA and host RNA polymerase. The host cell genes involved are *cmrA* and *cmrB*, which map suspiciously close to *trkA* and *trkB*, respectively, which code for potassium transport proteins located in the cell membrane. The plasmid gene is *abi* (*ab*ortive *i*nfection), which could code for a polypeptide of 114 amino acids that is strongly hydrophobic and may therefore interact with cell membranes. The putative Abi protein, however, has not yet been detected and is presumably synthe-

sized in very small amounts. How the gene products from three sources interact to cause the abortive response has yet to be elucidated.

Transfection

Transfection of spheroplasts by naked T5 or BF23 DNA provides a means to assess the functional importance, other than that of phage DNA transfer, of the two pre-early proteins, gpA1 and gpA2-A3, which are normally required for transfer of phage DNA into the host cell. Transfection bypasses the normal mechanism, and by so doing, phage DNA with an amber mutation in either gene *A1* or *A2-A3* can produce intact phage (still with the original amber mutation) when transfected into su^- spheroplasts. The efficiency of such phage production is considerably lower than that produced from wild-type phage DNA, but still much higher than that produced from phage DNA with an amber mutation in an essential gene such as T5 DNA polymerase. For a DNA with an amber mutation in gene *A2-A3*, the efficiency was 16%, for one in gene *A1* it was 1.4%, and for one in the essential gene *D9* it was 0%. It thus appears that the role of gpA2-A3 in preventing premature transcription of late genes is important, but not as important as the role of gpA1 in shutting down host macromolecular synthesis by degrading host DNA plus shutting off pre-early transcription.

Interestingly, transfection by T5 or BF23 DNA is a two-hit process in wild-type spheroplasts. Since in transfection, the entire DNA molecule enters the spheroplast in one step, the first DNA molecule to enter inactivates hostile host functions but its non-redundant region is largely degraded. However, such a spheroplast should now be able to accept a second DNA molecule without degradation and a successful infection would follow. One hostile host function appears to be the RecB nuclease since transfection is a one-hit process in $recB^-$ hosts.

Cloning Genes from T5 or BF23

Many restriction fragments from T5 or BF23 DNA are not directly clonable because they either code for lethal products or contain such strong promoters that the cells harboring them cannot survive. The strength of some T5 promoters has prompted their use in expression vectors, some of which are commercially available. Fragments that have been cloned are largely from the region from 58 to 92%, which includes mostly late structural genes with some early genes, and from 21 to 36%, which includes all the tRNA genes that are expressed during the early period. A small fragment from 2.1 to 3.4% in the pre-early region has also been cloned.

T5 genes that have been overproduced from an expression vector include gene *D7-8-9* (coding for T5 DNA polymerase), gene *D15* (coding for T5 5′-exonuclease), and *11p* (coding for a lipoprotein that inactivates host cell receptors). BF23 genes *24* and *25* (coding for a minor and major tail protein, respectively) have also been cloned, sequenced, and expressed.

Future Perspectives

Contributions from the T5 and BF23 systems include the identification and use of some of their gene products. Pre-early gene products that inactivate specific host functions, including restriction endonucleases and DNA methylating enzymes, should show interesting mechanisms of action and prove experimentally useful. Similarly, the availability of the 'nicking' enzyme would add to our battery of enzymes for manipulation of DNA. The nucleotide sequence of all T5 and BF23 promoters and their strength of binding to unmodified and modified forms of host RNA polymerase should sharpen our understanding of promoter function. The elucidation of the mechanims by which the two-step transfer of phage DNA to host cells and the ColIb-directed abortive response is accomplished will probably reveal some unique cellular interactions. Finally, the complete nucleotide sequence of T5 and BF23 genomes would greatly help our understanding of this system.

See also: **Host-controlled modification and restriction; Phage taxonomy and classification; Phages as cloning vehicles; Replication of viruses.**

Further Reading

Bonhivers M and Letellier L (1995) Calcium controls phage T5 infection at the level of the *Escherichia coli* cytoplasmic membrane. *FEBS Lett.* 374: 169.

Bujard H, Niemann A, Brevnig K *et al* (1982) The interaction of *E. coli* RNA polymerase with promoters of high signal strength. In: Rodriguez RL and Chamberlin MJ (eds) *Promoters Structure and Function*, p. 121. New York: Praeger.

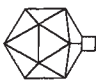
Decker K, Krauel V, Meesmann A and Heller KJ (1994) Lytic conversion of *Escherichia coli* by bacteriophage T5: blocking of the FhuA receptor protein by a lipoprotein expressed early during infection. *Mol. Microbiol.* 12: 321.

Duckworth DH, Glenn J and McCorquodale DJ (1981) Inhibition of bacteriophage replication by extrachromosomal genetic elements. *Microbiol. Rev.* 45: 52.

McCorquodale DJ and Warner HR (1988) Bacteriophage

T5 and related phages. In: Calendar R (ed.) *The Bacteriophages*, p. 439. New York: Plenum.
Nakayama S-I, Kaneko T, Ishimaru H, Moriwaki H and Mizobuchi K (1994) Cloning, sequencing, and expression of bacteriophage BF23 late genes 24 and 25 encoding tail proteins. *J. Bacteriol.* 176: 7280.

T7-LIKE PHAGES (*PODOVIRIDAE*)



Ian J Molineux, Department of Microbiology, University of Texas, Austin, Texas, USA

General Properties, Ecology and Evolution

Bacteriophage T7 is the prototype of a group of virulent phages having a $T = 7$ icosahedral head approximately 60 nm in diameter, a stubby, noncontractile tail (about 20 nm in length and 10 nm wide) plus six thin tail fibers. It is the type species of the genus 'T7-like phages' in the *Podoviridae* family. The distinguishing characteristic of the group is the synthesis of an RNA polymerase that is both resistant to the antibiotic rifampin and highly specific for phage promoters. T7-like phages that infect one of a variety of Gram-negative bacteria have been described, but none are yet known that infect Gram-positive hosts. Most studies have been performed on the coliphage T7 and properties of other phages are usually described relative to those of T7.

T7 does not form plaques on most *Escherichia coli* strains newly isolated from nature because it does not adsorb to smooth or capsulated bacteria. In *E. coli* B the primary receptor for T7 is the R-core portion of the lipopolysaccharide (LPS) of the outer membrane; in smooth strains the R-core is inaccessible to the phage gp17 tail fibers, which specify the adsorption host-range. The precise adsorption component may be different on *E. coli* K-12 or C strains, on which T7 grows equally well. T7-like phages are known that grow on smooth bacteria; virion-associated hydrolases may degrade cell surface polysaccharides to allow a gp17 homologue to access the primary receptor.

T7 and T3 were isolated in 1945 as phages that grew on *E. coli* B. Similar coliphages have been isolated from different parts of the world and about 60 representatives are known that can be subdivided by the promoter specificity of the phage-coded RNA polymerase. Recombination between phages in a subdivision is very efficient, recombination between those in different subdivisions is extremely rare but undoubtedly highly significant in the evolution of specific phages. Electron microscopic analyses of heteroduplexed DNA of T7-like phages showed varying degrees of homology, some regions exhibiting >90% sequence identity and others with no apparent similarity. These observations suggest that a given phage is the result of multiple recombination events between many T7-like phages.

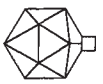
Genetic Structure

The genetic map of T7 is based on the nucleotide sequence of 39 937 bp. Numbers define genes, ordered sequentially from the genetic left end of the DNA (**Fig. 1**). Three classes of genes have been identified: class I, or early, genes are expressed until about 8 min after infection at 30°C; class II genes are expressed from about 6 to 15 min after infection; and class III genes are expressed from about 8 min until lysis (about 25 min at 30°C). Fifty-six known or potential T7 genes have been described – less than half are essential for phage growth on usual laboratory strains but mutant hosts have allowed the functions of several other genes to be elucidated. There are indications that a few genes may be remnants of homing endonucleases or other mobile elements. More than 90% of the genome is coding and most of the remainder contains recognizable genetic signals. Little overlapping of genes occurs; by means of an internal in-frame initiation gene *4* specifies two distinct polypeptides and programmed ribosomal frameshifting yields two products from gene *0.6*, gene *5.5* (yielding a *5.5–5.7* fusion), and gene *10*. Only the gene *10* frameshift has been well characterized; both gp10A and the longer, −1 frameshifted, gp10B are assembled into wild-type particles but either alone suffices for viability. The frameshifted protein may be biologically significant since a comparable T3 gp10B exists even though the frameshifting sequences have diverged from T7. Other T7-like phages are also thought to contain two forms of their major capsid protein.

Genetic signals include promoters and terminators

T5 and related phages. In: Calendar R (ed.) *The Bacteriophages*, p. 439. New York: Plenum.
Nakayama S-I, Kaneko T, Ishimaru H, Moriwaki H and Mizobuchi K (1994) Cloning, sequencing, and expression of bacteriophage BF23 late genes 24 and 25 encoding tail proteins. *J. Bacteriol.* 176: 7280.

T7-LIKE PHAGES (*PODOVIRIDAE*)



Ian J Molineux, Department of Microbiology, University of Texas, Austin, Texas, USA

General Properties, Ecology and Evolution

Bacteriophage T7 is the prototype of a group of virulent phages having a $T = 7$ icosahedral head approximately 60 nm in diameter, a stubby, noncontractile tail (about 20 nm in length and 10 nm wide) plus six thin tail fibers. It is the type species of the genus 'T7-like phages' in the *Podoviridae* family. The distinguishing characteristic of the group is the synthesis of an RNA polymerase that is both resistant to the antibiotic rifampin and highly specific for phage promoters. T7-like phages that infect one of a variety of Gram-negative bacteria have been described, but none are yet known that infect Gram-positive hosts. Most studies have been performed on the coliphage T7 and properties of other phages are usually described relative to those of T7.

T7 does not form plaques on most *Escherichia coli* strains newly isolated from nature because it does not adsorb to smooth or capsulated bacteria. In *E. coli* B the primary receptor for T7 is the R-core portion of the lipopolysaccharide (LPS) of the outer membrane; in smooth strains the R-core is inaccessible to the phage gp17 tail fibers, which specify the adsorption host-range. The precise adsorption component may be different on *E. coli* K-12 or C strains, on which T7 grows equally well. T7-like phages are known that grow on smooth bacteria; virion-associated hydrolases may degrade cell surface polysaccharides to allow a gp17 homologue to access the primary receptor.

T7 and T3 were isolated in 1945 as phages that grew on *E. coli* B. Similar coliphages have been isolated from different parts of the world and about 60 representatives are known that can be subdivided by the promoter specificity of the phage-coded RNA polymerase. Recombination between phages in a subdivision is very efficient, recombination between those in different subdivisions is extremely rare but undoubtedly highly significant in the evolution of specific phages. Electron microscopic analyses of heteroduplexed DNA of T7-like phages showed varying degrees of homology, some regions exhibiting >90% sequence identity and others with no apparent similarity. These observations suggest that a given phage is the result of multiple recombination events between many T7-like phages.

Genetic Structure

The genetic map of T7 is based on the nucleotide sequence of 39 937 bp. Numbers define genes, ordered sequentially from the genetic left end of the DNA (**Fig. 1**). Three classes of genes have been identified: class I, or early, genes are expressed until about 8 min after infection at 30°C; class II genes are expressed from about 6 to 15 min after infection; and class III genes are expressed from about 8 min until lysis (about 25 min at 30°C). Fifty-six known or potential T7 genes have been described – less than half are essential for phage growth on usual laboratory strains but mutant hosts have allowed the functions of several other genes to be elucidated. There are indications that a few genes may be remnants of homing endonucleases or other mobile elements. More than 90% of the genome is coding and most of the remainder contains recognizable genetic signals. Little overlapping of genes occurs; by means of an internal in-frame initiation gene *4* specifies two distinct polypeptides and programmed ribosomal frameshifting yields two products from gene *0.6*, gene *5.5* (yielding a *5.5–5.7* fusion), and gene *10*. Only the gene *10* frameshift has been well characterized; both gp10A and the longer, −1 frameshifted, gp10B are assembled into wild-type particles but either alone suffices for viability. The frameshifted protein may be biologically significant since a comparable T3 gp10B exists even though the frameshifting sequences have diverged from T7. Other T7-like phages are also thought to contain two forms of their major capsid protein.

Genetic signals include promoters and terminators

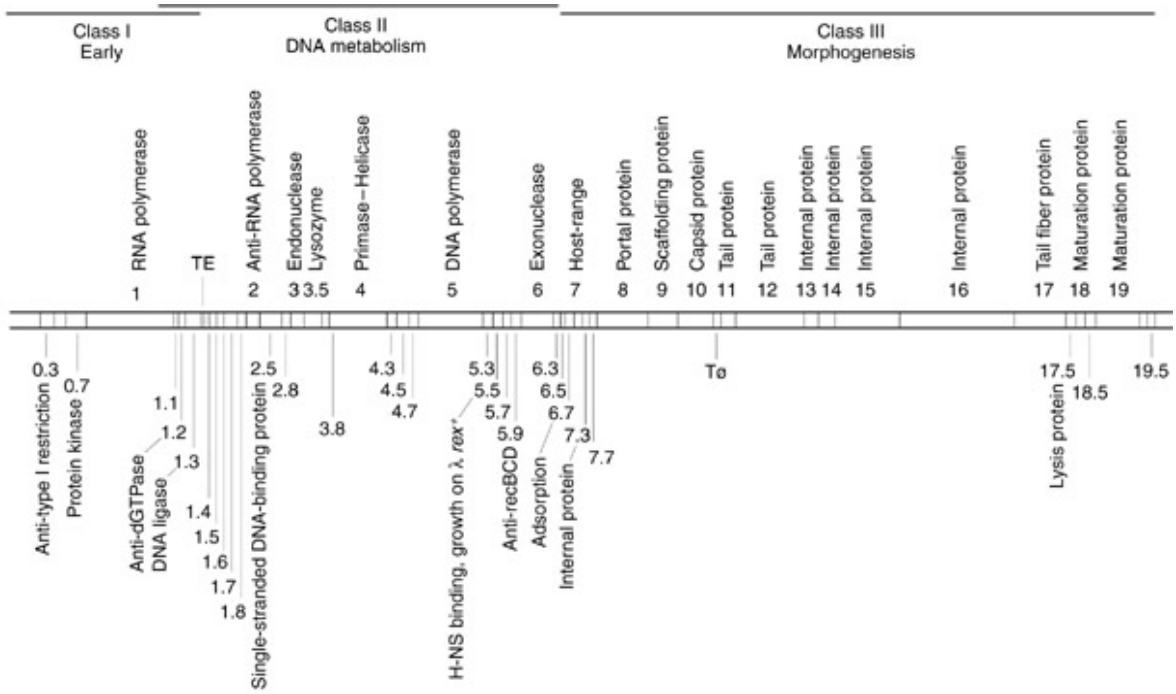


Figure 1 Genetic map of bacteriophage T7.

for the host and phage RNA polymerases, RNase III recognition sequences, the primary origin of DNA replication, and a terminal repetition of 160 bp used in forming concatemers during replication. The terminal repeat of different T7-like genomes is different in both length and sequence. The sequence GATC (a major regulatory sequence in *E. coli*) is distinctly underrepresented; this sequence, statistically expected 156 times in a 40 kb genome, occurs only six times in T7, 10 times in T3, and not at all in BA14. T7 and T3 inactivate type I restriction enzymes but remain susceptible to cells harboring type II and III enzymes. However, susceptibility to the latter enzymes may not be universal among the T7 group: some members contain restriction sites in their genomes but grow despite the presence of the cognate enzymes. The phage gene(s) responsible for type II and III enzyme inactivation are unknown.

Class I genes

Class I genes are those transcribed by *E. coli* RNA polymerase immediately after infection; the biochemical functions of five gene products are known. Type I restriction enzymes are inactivated by gp0.3 (which, for some T7-like phages, also harbors *S*-adenosyl methionine hydrolase activity). gp0.7 is a serine–threonine protein kinase that phosphorylates many host proteins, including RNA polymerase, RNase III, some ribosomal proteins and several translation factors. gp0.7 is also responsible for shut-off of host-catalyzed transcription, although this function is separable from kinase activity. Since host-catalyzed transcription is inactivated and the upstream T7 promoter ϕOL is poorly utilized during infection, early phage genes are also shutoff. Although class I mRNAs are stable, their translation (and those of residual host mRNAs) abruptly ceases about 8 min after infection; how inhibition is achieved is unknown.

gp1 is the T7 RNA polymerase, gp1.2 inhibits *E. coli* dGTPase, and gp1.3 is DNA ligase. Of class I genes, only gene *1* is essential, the remainder are required only in certain hosts or under adverse conditions. In the first few minutes of infection, T7 gene products thus inactivate some potentially deleterious host enzymes and, via inhibition of *E. coli*, and synthesis of T7, RNA polymerase, subsume all intracellular nucleotides and the translation machinery for the exclusive benefit of phage development.

Class II and class III genes

Class II and III genes are transcribed by T7 RNA polymerase; they are distinguished by a selective shut-off of class II transcription and translation about 15 min after infection. Class II genes function primarily in T7 DNA metabolism; they comprise the last three class I genes, *1.1–1.3*, plus genes *1.4–6.3*. In addition to their role in concatemer formation and

resolution, the gp3 endonuclease and the gp6 exonuclease also degrade host DNA to mononucleotides; these are subsequently utilized for T7 DNA synthesis. More than 80% of the nucleotides in progeny phage originated from the host chromosome. *In vitro*, gp3 and gp6 degrade T7 DNA and their activities must be regulated *in vivo;* regulation may involve gp2 but the mechanism is not well understood. The gene 3.5 lysozyme is also multifunctional: its location among the class II genes reflects its role in replication and transcriptional regulation rather than in cell lysis.

Expression of the late class III genes is independent of DNA replication; times of appearance, rates of synthesis and final accumulation of class III proteins are unaffected by preventing phage replication. Essentially all class III genes are involved in phage morphogenesis and maturation or in cell lysis.

Phage Particle

The phage capsid is composed of 415 copies of gp10A or gp10B. At one vertex of the icosahedron, 12 molecules of gp8 form a portal through which the phage genome can pass. The portal also serves as the connector for the phage tail, consisting of six copies of gp12 and 12 or 18 copies of gp11. Six tail fibers, each comprised of gp17 trimers, are attached to the tail just below its junction with the capsid. The particle also contains several internal proteins – three forming a hollow cylindrical core structure, coaxial with the tail, that is attached to the inner surface of the capsid and the portal. This internal core is important during both DNA packaging and ejection. Functions of other particle proteins are incompletely understood, although gp6.7 is essential for adsorption and gp13 for an early stage of infection. Cryoelectron microscopy shows the 40 kb T7 genome spooled around the tail-specified axis in a close-packed, quasi-crystalline array.

T7 Infection Cycle

Aside from lysis, the T7 infection cycle has been modeled as a linked set of differential equations, directly derived from experimental data. The computer simulation accurately displays genome translocation into the cell, gene expression and its regulation, DNA replication and morphogenesis.

DNA Ejection

Following adsorption to *E. coli* K-12 the infection is initiated by degradation of the internal head proteins gp7.3 and gp13. The stubby T7 tail cannot directly penetrate the cell cytoplasm and a channel across the outer envelope must form to allow DNA translocation. The internal core of the head dissociates and its constituent proteins enter the cell (**Fig. 2**). gp14 localizes to the outer membrane; the N-terminal region of gp16 shares homology with a bacterial lytic transglycosylase and may enlarge a hole through the peptidoglycan. This presumed activity of gp16 is important only when cells are at high density or low temperature, conditions likely favoring increased crosslinking of the peptidoglycan. Much of the 143 kDa gp16 presumably spans the periplasm but the protein also penetrates the cytoplasmic membrane to complete the DNA translocation channel. gp15 appears to be cytoplasmic at this stage of infection but its function is unknown.

Only when channel assembly is complete does the phage genome exit the capsid. gp16 appears to be one component of an enzymatic motor, fueled by the proton motive force, that ratchets T7 DNA at a constant rate from the capsid into the cell. About 850 bp enters the cytoplasm by this mechanism; in the absence of transcription DNA translocation normally aborts at this stage. Therefore, neither the classic syringe model for phage DNA ejection nor the concept of packaged DNA resembling a compressed coiled spring apply to T7. It should be noted that these two widely held ideas have no direct experimental support in any phage–host system.

Within the 850 bp that enter the cell lie three *E. coli* RNA polymerase promoters. Transcription from these promoters causes 19% of the genome to be pulled into the cell, simultaneously causing expression of the nine early genes. One of these codes for T7 RNA polymerase, responsible not only for translocating the remaining 81% of the genome but also for all class II and class III gene expression. Complete internalization of the T7 genome occupies one-third of the latent period; the slow transcriptional mode of entry both ensures gp0.3 synthesis with consequent inactivation of type I restriction enzymes before cognate sites enter the cell, and may also help regulate temporal gene expression.

Transcription

All transcription of T7 DNA *in vivo* goes from left to right on the genetic map (**Fig. 3**). The three *E. coli* RNA polymerase promoters are all active, the A1 promoter being among the strongest known. Additional minor promoters identified by *in vitro* transcription studies or predicted from the nucleotide sequence are not known to be biologically significant except for mutant phages. Transcription from the major promoters usually terminates at the early terminator TE to produce three RNAs differing at

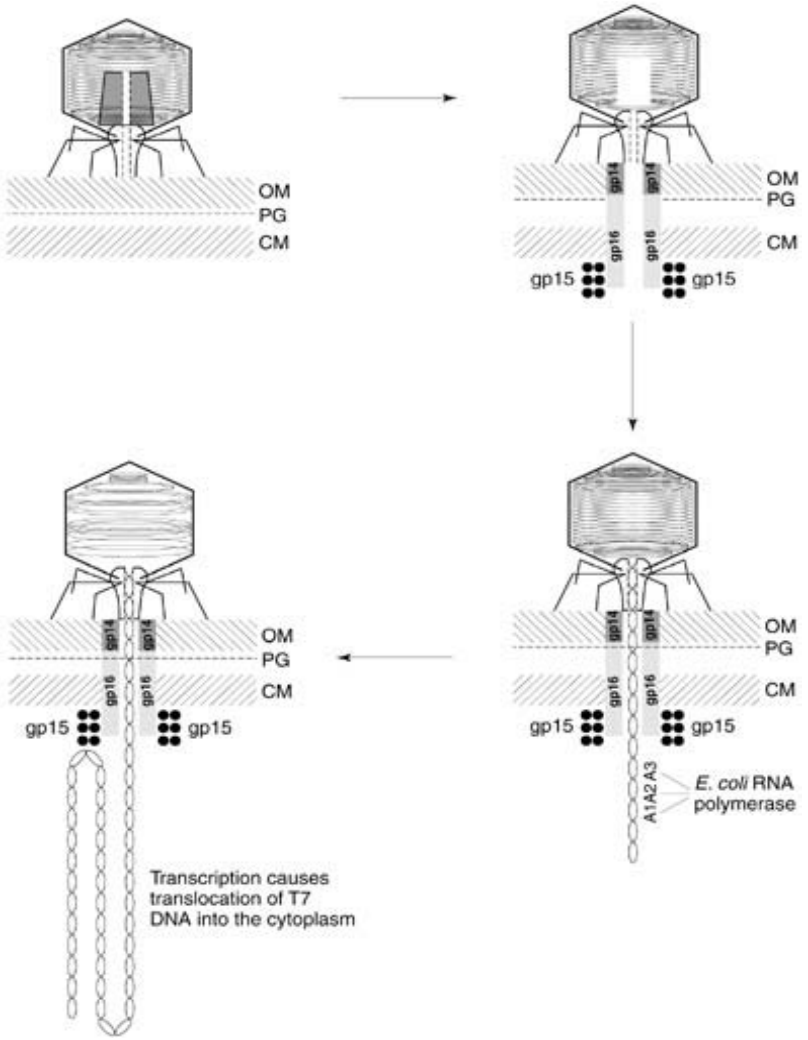


Figure 2 T7 DNA translocation into the bacterial cytoplasm. OM, outer membrane; PG, peptidoglycan; CM, cytoplasmic membrane.

their 5′ ends but containing identical coding information. Transcription that reads through TE terminates inefficiently near the 3′ end of gene *3.5*, distal to gene *10* at the T7 RNA polymerase terminator, or at the genome end.

Processing of primary transcripts by the host enzyme RNase III is a major feature of T7-infected cells, although nonessential for phage development. Early RNAs are processed into a noncoding initiator RNA and five mRNAs, only one of which is monocistronic. Processing of late transcripts also occurs but all RNase III-generated RNAs remain polycistronic. The stability of T7 RNAs during infection is likely due, in part, to the formation of base-paired structures at their 3′ ends by RNase III processing.

T7 RNA polymerase promoters consist of a highly conserved 23 bp segment that runs from −17 to +6, relative to the transcription start. Seventeen promoters exist in the T7 genome, ten expressing class II and class III genes and five expressing only class III genes. The *φOL* and *φOR* promoters may function more in replication or maturation than in mRNA synthesis but neither are essential for phage viability. T3 contains a similar, though not identical, set of promoters.

Transcription from class II promoters and the first three class III promoters terminates distal to gene *10* at T*φ* and results in a nested set of polycistronic RNAs that differ at their 5′ ends. Termination at T*φ* is about 80% efficient – in T7 the essential genes *11* and *12* are expressed only from readthrough RNAs; these, together with transcripts from *φ13* and *φ17*, terminate near the genome end.

The significance of synthesizing such a complex array of transcripts, in particular over the class II

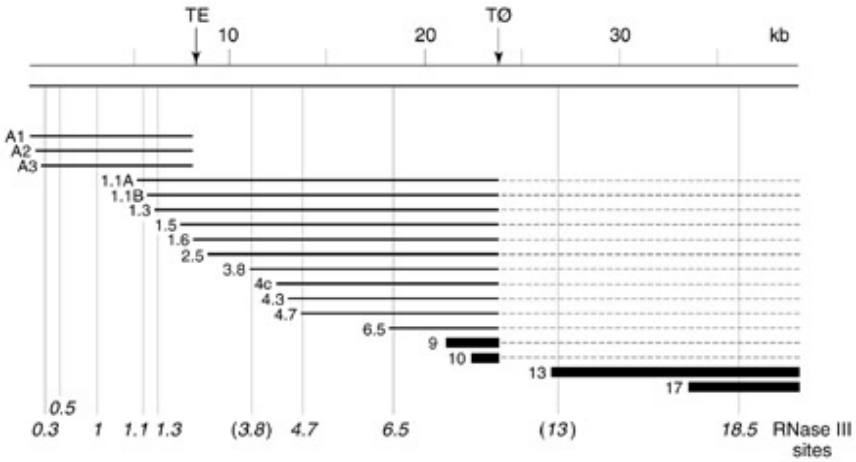


Figure 3 T7 RNAs. Promoters are indicated at the 5′ ends of transcripts. Horizontal dashed lines represent the major readthrough RNAs. Vertical lines indicate the positions of cleavage by RNase III; sites are named by the gene following the site. Sites where cleavage is inefficient are in parentheses.

region, is unclear; however, it does provide a means of selectively producing large quantities of gene *9* and especially gene *10* RNAs. These are partly responsible for the high levels of gp9 and especially gp10 in the infected cell. A leader sequence on gene *10* mRNA and the initial gene *10* codons both also appear designed to maximize gp10 synthesis: during infection perhaps 100 000 gp10 molecules can be made from a single T7 genome in about 20 min.

The sequences of the five class III promoters are identical; these promoters are stronger than class II promoters, whose sequences differ from consensus at 2–7 positions. Promoter strength differences are manifest by the frequency of abortive initiation, which is higher at class II than at class III promoters. gp3.5 lysozyme also complexes with T7 RNA polymerase to inhibit the conformational change necessary for polymerase to switch from the initiation to the processive elongation mode of synthesis. One consequence of gp3.5–gp1 complex formation is then to effect the selective shut-off of class II transcription. Since gp3.5 is itself a class II product, its synthesis is autoregulating and high-level expression of gene *3.5* inhibits phage growth. gp3.5–gp1 complex formation also stimulates DNA replication, perhaps by providing primers via abortive transcription initiation over an origin sequence or by facilitating replication complex assembly on the separated strands of the transcription bubble. Transcripts originating from *φOR* encounter a strong transcriptional pause site that is prolonged by gp3.5 on concatemeric DNA immediately 3′ of the terminal repeat. Enhanced pausing at this site may aid assembly of the machinery for DNA packaging. Gene *3.5* is thus central to several aspects of T7 development.

Promoter specificities

The RNA polymerases coded by other T7-like phages are similar to that of T7. A single amino acid change allows T7 RNA polymerase to specifically recognize T3 promoters, and *vice versa*. RNA polymerases coded by *Salmonella* phage SP6 and *Klebsiella* phage K11 have diverged more extensively from the T7 enzyme, although their respective promoters retain substantial homology to T7. However, there is little recognition of these enzymes for noncognate promoters. Of likely evolutionary significance is the homology between these phage-coded enzymes and the *Saccharomyces cerevisiae* mitochondrial RNA polymerase.

DNA Replication

Replication *in vivo* requires T7 RNA polymerase, DNA polymerase, primase/helicase, single-stranded DNA binding protein (gp2.5), endonuclease and exonuclease. There is no requirement for topoisomerase activity as no closed circular species of the T7 genome form. Even though *E. coli* RNA polymerase is transcriptionally inactive when replication begins, it can interfere with DNA maturation and packaging and gp2 is required to alleviate inhibition. Other than thioredoxin, which forms a 1:1 complex with gp5 in T7 DNA polymerase, no host proteins are known to be required for phage replication. A crystallographic structure of T7 DNA polymerase, bound to a primer-template and nucleoside triphosphate, has been reported.

Replication *in vivo* is normally initiated at an A+T-rich region located 15% from the left end,

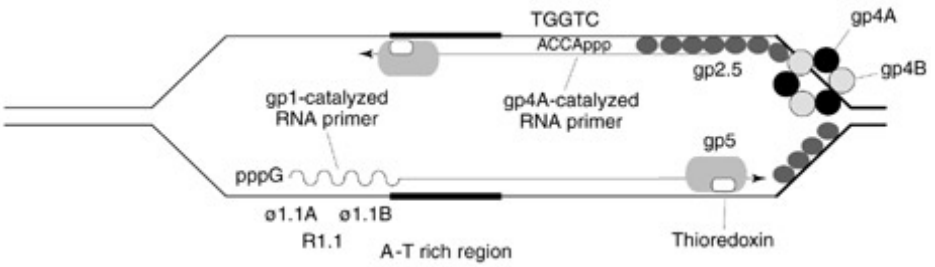


Figure 4 Initial events at the primary origin of DNA replication. Not all protein:proteins are indicated.

although deletion mutants lacking this origin grow well in most laboratory hosts. Replication is bidirectional on the linear genome, the replication 'bubble' enlarges, providing a Y-shaped molecule, before further elongation yields two linear molecules (**Fig. 4**). Replicated linear molecules necessarily contain unreplicated 3′ ends; T7 circumvents replicated genome erosion by forming linear concatemers via its terminal 160 bp repeats. As replication proceeds, fast-sedimenting DNA molecules appear that consist mainly of linear concatemers and branched, recombining molecules. The fast-sedimenting DNA complex, containing >100 genome equivalents, is ultimately converted by the gp3 resolvase into concatemeric molecules that are substrates for the packaging machinery. Although incorporation of thymidine into T7 DNA can be detected for about 20 min, the >200-fold increase in T7 DNA mass resulting from replication is achieved much more rapidly, fluctuations in nucleotide pool sizes as bacterial DNA is degraded and reutilized confound analyses of precursor incorporation data. The rate of T7 DNA mass increase is suggestive of numerous replication forks, consistent with the idea that recombination intermediates may be converted into replication forks, as is the case with T4. Recombination rates in T7-infected cells are very high; wild-type recombinants have been detected between mutants altered in adjacent nucleotides. Recombination is independent of the host RecA but requires most T7 replication proteins. Single strands may be formed by gp6 exonuclease or during replication; strand exchange is catalyzed by gp2.5 and the gp4 helicase, and gp3 resolves Holliday junctions.

Plasmid-based assays *in vivo* have revealed secondary replication origins that may function in later stages of phage replication. Plasmids containing the promoters *ϕ6.5* and *ϕ13* are replicated following T7 infection as efficiently as plasmids containing *ϕ1.1A* or *ϕ1.1B* and the primary origin; those containing *ϕOR* are replicated even more. Furthermore, plasmids containing both *ϕOR* and a packaging signal are packaged after replication, yielding particles that transduce the plasmid DNA. Plasmids containing other promoters are, at best, poorly replicated after infection, but what makes a promoter functional in DNA replication is not understood.

In vitro, bidirectional replication from the primary origin has been achieved using purified T7 RNA and DNA polymerases, gp2.5, and the heterohexameric primase–helicase complex of gp4A and gp4B. RNA polymerase synthesizes primers of 10–60 nucleotides from both promoters immediately upstream of the origin. The gp4 hexamer forms a sliding clamp that translocates 5′ to 3′ to unwind unreplicated DNA using ribo- or deoxy-NTP (preferring dTTP *in vitro*) hydrolysis for energy. The clamp also interacts with both gp2.5 and DNA polymerase, making DNA synthesis more processive. Whereas gp4B has only helicase activity, gp4A also serves as a primase; the N-terminal 63 residues not present in gp4B contain a zinc-finger motif that interacts with the sequences 3′-CTGG(G/T)-5′ or 3′-CTGTG-5′ in single-stranded DNA. Primers, 5′-ACC(A/C) or 5′-ACAC are synthesized that initiate lagging strand DNA synthesis. gp2.5 interacts with both DNA polymerase and primase–helicase to stimulate lagging strand synthesis and to promote bidirectional replication from the primary origin. Although *E. coli* SSB stimulates T7 DNA polymerase activity *in vitro*, it cannot substitute for gp2.5 for bidirectional replication from the primary origin and T7 SSB is an essential protein.

Capsid Assembly and DNA Packaging

Detailed information on morphogenesis has been obtained with both T7 and T3. It is assumed that the mechanism of particle assembly is common to both phages.

Packaging of T7 DNA starts with the assembly of a DNA-free procapsid. The major capsid protein gp10 assembles around a gp9 scaffold in a reaction that has been accomplished *in vitro* using purified proteins. One vertex of the icosahedral procapsid is modified by the addition of gp8 to form the portal. In addition, the procapsid contains the internal protein core, and also the maturation protein gp19. The latter is likely outside the procapsid, as it recognizes the gp8 portal

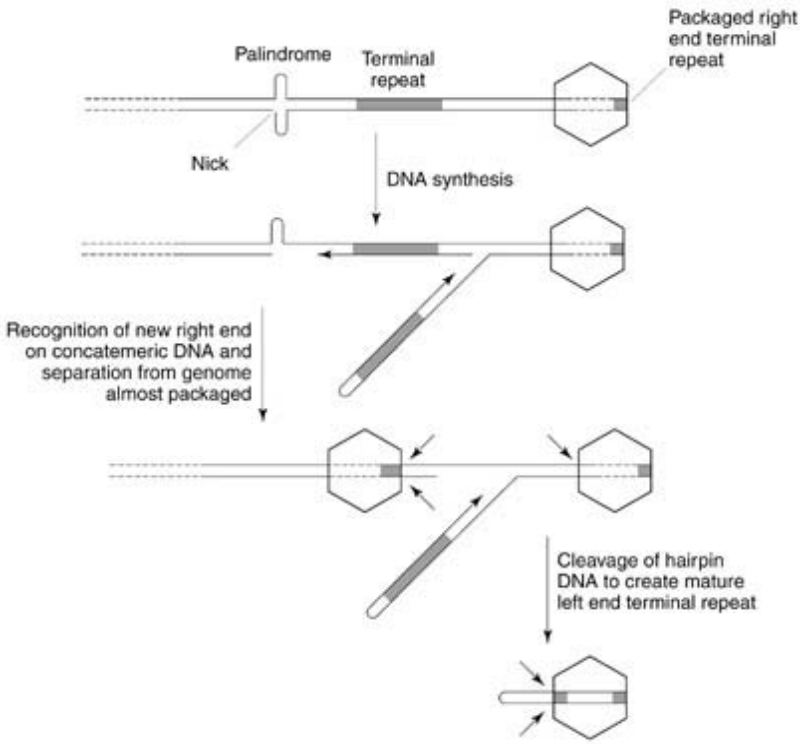


Figure 5 Pathway for duplication of the terminal repeat during packaging from concatemeric DNA.

protein and, perhaps in conjunction with gp18, interacts with concatemeric DNA before packaging. Both gp18 and gp19 are required for packaging, though neither are found in the mature phage particle. Packaging of a single genome from concatemeric DNA is estimated to take about 90 s, a rate that has been achieved *in vitro*.

During packaging of DNA, the procapsid undergoes a conformational change characterized by an increase in size and a conversion from rounded to icosahedral morphology. The gp9 scaffolding protein likely exits the procapsid as these changes occur. After association of the procapsid with concatemeric DNA, packaging proceeds from a genomic right end leftwards in an incompletely understood process. A series of reactions is necessary for duplication of the 160 bp terminal repeat that in concatemers is present in only one copy between genomes. The DNA is nicked by an unknown nuclease at a palindromic sequence located to the left of the right terminal repeat, creating a hairpin primer for DNA polymerase (**Fig. 5**). Extending the hairpin through the terminal repeat and into the genome being packaged provides duplex DNA that could be converted into a mature left end. On the remaining concatemeric DNA, primase-initiated synthesis on the displaced strand proceeds through the terminal repeat, providing sequences that could be converted into the mature right end of the next genome to be packaged. However, the nicking site and palindromic sequences are nonessential, although the obligatory alterations in the packaging mechanism are unknown.

The nuclease recognizing the termini of mature T7 DNA is unknown, although gp19 possesses a nonspecific endonucleolytic activity that is suppressed by gp18, and both proteins are required to create the mature termini. T7 RNA polymerase is also required for DNA packaging; it seems likely that gp3.5-enhanced pausing of transcription near the concatemer junction may recruit gp19 for initiation of packaging.

The final stage of phage development is lysis of the host cell, a process poorly understood. Genes *3.5* and *17.5* are required, perhaps together with another component (gp18.5 and/or nucleic acid?). gp17.5 may be a holin that disrupts the cell membrane, allowing gp3.5 lysozyme to lyse the cell or to release phage from cell debris. The potential role of DNA in lysis is unclear, but replication-defective mutants are as lysis defective as gene *3.5* amber mutants, even though replication does not affect gene expression.

Host Functions in T7 Development

A number of natural *E. coli* or other enterobacterial hosts are nonpermissive for many members of the T7

group of phages. In most cases the host genes involved are unknown. Several prophages or resident plasmids are known to have the potential to inhibit growth of T7 by a process(es) distinct from adsorption or DNA restriction; however, the incoming phage often contains a gene that prevents inhibition. The λ *rex* genes exclude certain missense mutants of T7, perhaps by a comparable (but unknown) mechanism to that of exclusion of T4 *r* II mutants. The Col Ib plasmid inhibits growth of *0.7* mutants; the basis of exclusion is not understood but may involve the failure to inactivate *E. coli* RNA polymerase. Most T7-like phages are also excluded from growth in F plasmid-containing cells, although T3 is an exception. The *pifA* gene of F interferes with the normal functions of T7 genes *1.2* and *10*; interaction of either gene with *pifA* causes inhibition of all macromolecular synthesis and membrane functions. The rapid loss of metabolic potential of the abortively infected, F-containing *E. coli* suggests that some key cellular component(s) is inactivated by the interaction of T7 and *pif* genes. This component is unidentified but can be protected from PifA and gp10 (or gp1.2) by increased synthesis of the *E. coli* membrane protein FxsA, a protein with no known independent function.

Expression Systems Based on T7 RNA Polymerase

The specificity of T7 RNA polymerase for its promoter has allowed the development of high-level, regulated expression systems for cloned DNA in both procaryotes and eucaryotes. Typically, the gene *10* promoter is employed, with or without gene *10* translational start sequences; T7 RNA polymerase is supplied from the cloned gene or by phage infection. Epitope-tagged expression vectors to aid in product purification are available. A phage display system based on the capsid protein accommodating both high and low levels of display has also been developed.

Future Perspectives

The utility of the expression and display systems alone will ensure continued research on bacteriophage T7. Our understanding of, in particular, mechanisms of DNA replication and transcription will continue to be furthered using T7 as a model. The mechanisms of DNA translocation across membranes and the morphogenesis and structure of complex nucleoprotein assemblages are two additional fields of research where the T7 model can be expected to make significant contributions.

Further Reading

Cerritelli ME, Cheng B, Rosenberg AH *et al* (1997) Encapsidated conformation of bacteriophage T7 DNA. *Cell* 91: 271.

Chung Y-B, Nardone C and Hinkle DC (1990) Bacteriophage T7 DNA packaging. *J. Mol. Biol.* 216: 939.

Dunn JJ and Studier FW (1983) Complete nucleotide sequence of bacteriophage T7 DNA and the locations of T7 genetic elements. *J. Mol. Biol.* 166: 477.

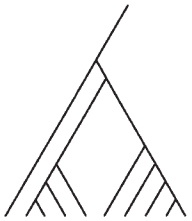
García LR and Molineux IJ (1996) Transcription-independent DNA translocation of bacteriophage T7 DNA into *Escherichia coli*. *J. Bacteriol.* 178: 6921.

Kong D and Richardson CC (1996) Single-stranded DNA binding protein and DNA helicase of bacteriophage T7 mediate homologous DNA strand exchange. *EMBO J.* 15: 2010.

Molineux IJ (1991) Host–interactions: recent developments in the genetics of abortive phage infections. *New Biol.* 1991 3: 230.

Tanapox Virus *see* **Yabapox Viruses**

TAXONOMY, CLASSIFICATION AND NOMENCLATURE OF VIRUSES



Claude M. Fauquet, The Scripps Research Institute, Division of Plant Biology, La Jolla, California, USA

History of Virus Classification and Virus Nomenclature

Humans feel the need to classify natural entities and the viruses are no exception. As in other biological systems, virus classification is an approximate and imperfect exercise. Like any other type of classification, it is a totally artificial and human-driven activity without any natural base. However, science requires workable descriptions of living systems and their constituent parts, and, when achieved properly, classifications are extremely useful for showing similar characteristics and properties across populations. Unfortunately for virus taxonomy no fossil record exists and so evolutionary relationships are very speculative, meaning that only a logical and precise virus classification can provide indications of the evolution of viruses. Appropriately chosen classification criteria are also informative in the case of newly discovered viruses. In theory, nomenclature and classification are totally independent, but for viruses both issues are often considered at the same time. As a result, taxonomic names for the viruses have always been the subject of passionate discussions and the taxonomic status of viruses is a sensitive and critical issue.

Virus classification is a relatively new exercise, as the first evidence for existence of a virus was only presented at the end of the nineteenth century by Beijerinck in 1898. It was not until 1927 that Johnson, a plant virologist, drew attention to the need for a system of virus nomenclature and classification. First efforts to classify viruses utilized a range of ecological and biological criteria, including pathogenic properties in the case of human and animal viruses, and symptoms for plant viruses. For example, viruses sharing the pathogenic properties causing hepatitis (e.g. hepatitis A virus, hepatitis B virus, yellow fever virus, and Rift Valley fever virus) were grouped together as 'the hepatitis viruses'. Virology developed substantially in the 1930s and early classifications for the viruses reflected these advances. In 1939, Holmes published a classification of plant viruses dependent on host reactions and differential host species, using a binomial-trinomial nomenclature based on the name of the infected plant; however, only 89 viruses were described and classified in this way. With the development of electron microscopy and biochemical studies in the 1950s, the first virus groupings based on common virion properties emerged: like the Herpesvirus group described by Andrewes in 1954, the Myxovirus group by Andrewes *et al*, in 1955, and the Poxvirus group by Fenner and Burnet in 1957. During this period there was also an explosion of newly discovered viruses; in response, several individuals and committees independently proposed virus classification systems but none was widely adopted by the scientific community. It became obvious that only an international association of virologists could propose a comprehensive and universally acceptable system of virus classification.

At the 1966 International Congress for Microbiology held in Moscow, the International Committee on Nomenclature of Viruses (ICNV) was established by an international group of 43 virologists. An international organization was set up with the aim of developing a taxonomy and nomenclature system for all viruses that would be recognized worldwide. The name of the ICNV was changed in 1974 to the more appropriate International Committee on Taxonomy of Viruses (ICTV), which remains active today. ICTV, the unique official committee of the Virology Division, is now considered the international official body for all matters related to taxonomy and nomenclature of viruses.

Since the founding of the ICTV, all virologists have agreed that the hundreds of viruses isolated from different organisms should be classified together in a unique system, but separate from other microorganisms such as fungi, bacteria and mycoplasma. However, there was much controversy on the way to do it. Lwoff, Horne and Tournier argued for the adoption of a system classifying viruses into subphyla, classes, orders, suborders and families. Descending hierarchical divisions would have been based on nucleic acid type (DNA or RNA), strandedness (single or double), presence or absence of an envelope, capsid symmetry, and so on. The hierarchy of this system has never been recognized by the ICTV; nevertheless, the types of criteria used became the basis of the universal taxonomy system now in place, and all ICTV reports have used this scheme. Until 1990, no hierarchical

classification level higher than the family was used, however, the system has recently begun to move in this direction. A first order, *Mononegavirales*, was accepted in 1990, and another two, *Caudovirales* and *Nidovirales*, were adopted in 1996. In its nonlinnean structure, the scheme is quite different from that used in the taxonomy of bacteria and other organisms. Nevertheless, the usefulness of the scheme is being demonstrated by its wide application. It has replaced all competing classification schemes for all viruses and no one would now dispute with the ICTV the international mandate to name and classify viruses.

Since its establishment, a total of seven virus taxonomic reports (also known by the names of the ICTV Presidents acting as Editors in Chief of the reports) have been published by the ICTV: Wildy in 1971; Fenner in 1976; Matthews in 1979; Matthews in 1982; Francki *et al* in 1991; Murphy *et al* in 1995; and van Regenmortel *et al* in 1999. At the first meeting in Mexico City in 1970, two families with a corresponding two genera and 24 floating genera were adopted to begin the grouping of the vertebrate, invertebrate and bacterial viruses. In addition, 16 plant virus groups were designated, as reported by Matthews in 1983. The fifth ICTV report, edited by Francki *et al* in 1991, described one order, 40 families, nine subfamilies, 102 genera, two floating genera and two subgenera for vertebrate, invertebrate, bacterial and fungal viruses, and 32 groups and seven subgroups for plant viruses. While most virologists shifted to placing viruses in families and genera, plant virologists retained the term 'groups' until 1993. It was only in 1995, as described in the sixth ICTV report, that the ICTV proposed a uniform system for all viruses, with two orders, 50 families, nine subfamilies, 126 genera, 23 floating genera and four subgenera encompassing 2644 assigned viruses. Most recently, at the 28th meeting of the ICTV in March 1998 in San Diego, California, the Universal Virus Classification was adopted; this comprises three orders, 56 families, nine subfamilies, 203 genera, 30 floating genera and a total of 3954 species, strains and/or serotypes of species and tentative species. It is a general trend that the number of described taxa and the number of species of viruses is increasing steadily, easily explained by the increasing complexity of the virus classification and by the amount of data available to demarcate viruses.

With precise and complete descriptions available for a large number of virus families, this classification now constitutes a valuable source of information for new 'unknown' viruses. Therefore, the ICTV classification is not only a taxonomic exercise for virus evolutionists but also a valuable diagnostic tool and educational system for virologists, teachers, medical doctors and epidemiologists.

How does the ICTV operate?

The ICTV is a committee of the Virology Division, which is in turn part of the International Union of Microbiological Societies. The ICTV is a nonprofit-making organization composed of prominent virologists representing countries from throughout the world who work to designate virus names and taxa through a democratic process. The ICTV operates through a number of committees, subcommittees and study groups consisting of more than 492 eminent virologists with expertise in viruses infecting humans, animals, insects, protozoa, archaea, bacteria, mycoplasma, fungi, algae, yeasts and plants. Taxonomic proposals are initiated and formulated by individuals or by the study groups. These proposals are revised and accepted by the corresponding subcommittees and presented for executive committee approval. All decisions are then ratified at a plenary session (or also now by postal vote) held at each Virology Congress where all members of ICTV and more than 50 representatives of national microbiological societies are represented. At present, there are 47 study groups working in concert with six subcommittees – namely, the vertebrate, invertebrate, plant, bacteria, fungus and virus data subcommittees. The ICTV does not impose any taxonomic terms or taxa but ensures that all propositions are compatible with ICTV rules for homogeneity and consistency. The ICTV regularly publishes reports describing all existing virus taxa with a list of classified viruses as well as descriptions of virus families and genera. An Internet web site, where the most important information relative to virus taxonomy is made available, is updated regularly. The sixth report was published by Murphy *et al* (1995) and the seventh by van Regenmortel *et al* (1999).

The increasing number of virus species and virus strains being identified, together with the explosion of data on many descriptive aspects of viruses and viral diseases, and particularly sequence data, has led the ICTV to launch an international virus database project. This project, termed ICTVdB, is scheduled to be fully operational and accessible to the scientific community around the year 2000. The ICTVdB, in addition to the taxonomic descriptions of all the taxa, will comprise all the information available about each virus species, and later each virus strain, for all the descriptors necessary to identify and recognize all viruses.

A Universal System for Virus Classification

There are currently two systems in use for classifying organisms: the linnean and the adansonian systems.

The former is the monothetic hierarchical classification applied by Linnaeus to plants and animals, while the adansonian is a polythetic hierarchical system initially proposed by Adanson in 1763. In 1984 Maurin and collaborators suggested applying the linnean classification system to the viruses. Although convenient to use, this system has shortcomings when applied to the classification of viruses. Firstly, it is difficult to appreciate the validity of a particular criteria. For example, it may not be appropriate to use the number of genomic components as a hierarchical criteria. Secondly, there are no obvious reasons for prioritizing criteria, and in consequence it is difficult to rank all the available criteria. For instance, is the nature of the genome (DNA/RNA) more important than the sense of the coding sequence of the genome or the shape of the virus particles?

The adansonian system considers all available criteria at once and makes several classifications, taking the criteria into consideration successively. The criteria leading to the same classifications are considered as correlated and are therefore not discriminatory. Subsequently, a subset of criteria are considered, and the process is repeated until all criteria can be ranked to provide the best discrimination of the species. This system has not been used frequently in the past owing to its labor-intensive nature, but this situation has changed as a result of the power and availability of today's computer technology. Furthermore, qualitative and quantitative data can be simultaneously considered when generating such a classification. In the case of viruses, it was determined by Harrison and collaborators in 1971 that at least 60 characters could be used for a complete virus description (**Table 1**). Thus, the limiting factor for applying the adansonian system is now not its labor-intensive nature but the lack of data for many of the viruses.

In addition, the increasing number of viral nucleic acid sequences being reported, in combination with the appropriate computer software, allows the comparison of viruses to generate different phylogenetic trees, according to the gene or set of genes used, as for example proposed by Koonin in 1991, Dolja and Koonin in 1991 and Dolja *et al* in 1991. However, to date, none of them has satisfactorily provided a clear classification of all viruses. A multidimensional classification, taking into account all the criteria necessary to describe viruses, would probably be the most appropriate way of representing a virus classification, but again the shortcomings of data for some viruses would prevent the use of this system in the foreseeable future.

For almost 25 years, the ICTV has been classifying viruses essentially at the family and genus levels using a nonsystematic polythetic approach. Viruses were clustered first in genera and then in families. A subset of characters, including physicochemical, structural, genomic and biological criteria, is then used to compare and group viruses. This subset of characters may change from one family to another, according to the availability of the data and the importance of a particular character for a particular family. It is obvious that there is no homogeneity in this respect throughout the virus classification and that virologists weigh the criteria differently in this subjective process, leading to the generation of a nonhomogeneous classification. Nevertheless, over time we can see stability of the current ICTV classification at the genus and family level. When sequence, genomic organization and replicative cycle data are subsequently used for taxonomic purposes, they usually confirm the actual classification. It is also obvious that hierarchical classifications above the family level will encounter conflicts between phenotypic and genotypic criteria and that virologists will have to consider the entire classification process in order to progress in this direction.

Currently, and for practical reasons only, virus classification is structured according to the presentation indicated in **Tables 2** and **3**. This 'Order of Presentation of the Viruses' does not reflect any hierarchical or phylogenetic classification but only a convenient order of presentation of the virus taxa. Since a taxonomic structure above the level of family (with the exception of the orders *Mononegavirales*, *Caudovirales* and *Nidovirales*) has not been developed extensively, any listing must be arbitrary. The order of presentation of virus families and genera follows four criteria: (1) the nature of the viral nucleic acid; (2) the strandedness of the nucleic acid; (3) the use of a reverse transcription process (DNA or RNA); and (4) the positive or negative sense of gene coding on the encapsidated genome. These four criteria give rise to six clusters comprising the 86 families and floating genera of viruses. In the past, two other criteria were also taken in account: the presence or absence of a lipid envelope and the segmentation of the genome as mono-, bi-, tri-, tetra- or multipartite. However, it has become clear that the presence of an envelope was entirely related to the nature of the host and that families could comprise genera having viruses with segmented or nonsegmented genomes, but sharing all other properties, including genome organization and sequence homology. These criteria have been therefore abandoned.

The Virus Species Concept and its Application

In 1991 the ICTV accepted the concept that viruses

Table 1 Virus family descriptors used in virus taxonomy

I Virion properties

A Morphology properties of virions

1 Size
2 Shape
3 Presence or absence of an envelope and peplomers
4 Capsomeric symmetry and structure

B Physical properties of virions

1 Molecular mass
2 Buoyant density
3 Sedimentation coefficient
4 pH stability
5 Thermal stability
6 Cation (Mg^{2+}, Mn^{2+}) stability
7 Solvent stability
8 Detergent stability
9 Radiation stability

C Properties of genome

1 Type of nucleic acid DNA or RNA
2 Strandedness: single-stranded or double-stranded
3 Linear or circular
4 Sense: positive, negative or ambisense
5 Number of segments
6 Size of genome or genome segments
7 Presence or absence and type of 5′ terminal cap
8 Presence or absence of 5′ terminal covalently linked polypeptide
9 Presence or absence of 3′ terminal poly(A) tract (or other specific tract)
10 Nucleotide sequence comparisons

D Properties of proteins

1 Number
2 Size
3 Functional activities (especially virion transcriptase, virion reverse transcriptase, virion hemagglutinin, virion neuraminidase, virion fusion protein)
4 Amino acid sequence comparisons

E Lipids

1 Presence or absence
2 Nature

F Carbohydrates

1 Presence or absence
2 Nature

II Genome organization and replication

1 Genome organization
2 Strategy of replication of nucleic acid
3 Characteristics of transcription
4 Characteristics of translation and post-translational processing
5 Site of accumulation of virion proteins, site of assembly, site of maturation and release
6 Cytopathology, inclusion body formation

III Antigenic properties

1 Serological relationships
2 Mapping epitopes

IV Biological properties

1 Host range, natural and experimental
2 Pathogenicity, association with disease
3 Tissue tropisms, pathology, histopathology

Table 1 Continued

4 Mode of transmission in nature
5 Vector relationships
6 Geographic distribution

Adapted from ICTV guidelines for family descriptions.

exist as species, in a similar manner to other organisms, and adopted a definition for a virus species proposed by van Regenmortel in 1990: 'A virus species is a polythetic class of viruses that constitutes a replicating lineage and occupies a particular ecological niche.' This simple definition and the position taken by the ICTV has already had, and will continue to have, a profound effect on virus classification. Effectively, in the sixth ICTV report virus names were indicated in 'List of species' but they were in fact a 'List of virus names' with undefined taxonomic status. In the seventh ICTV report, according to the polythetic nature of the species definition, a 'List of species-demarcating criteria' is provided for each genus, indicating how virus species can be identified in this particular genus. Viruses are then differentiated in species and tentative species according to this list of criteria and the availability of information to demarcate the species.

First, it is intended to define for each genus the criteria demarcating a virus species, and, second, to compare these criteria from one genus to the next, searching for homogeneity throughout the virus classification. Naturally this list of criteria should follow the polythetic nature of the species definition and more than one criteria should be used to determine a new species. It is obvious that most of the criteria in the list of demarcating criteria are shared amongst the different genera, within and across families; namely, host range, serological relationships, vector transmission type, tissue tropism, genome rearrangement and sequence homology (**Table 4**). However, if the types of criteria are similar, the levels of demarcation clearly differ from one family to another. This may reflect differences in appreciation from one family to another but also the differential ranking of a particular criterion in different families. The huge differences (up to 30%) in sequences among nucleoproteins of species of lentiviruses does not have the same biological significance as small differences in capsid protein sequences (1–10%) of species of potyviruses, and therefore universal levels of sequence identity for similar genes may not exist for viruses! The levels of demarcation may even change from one gene to another within the same family. Homogenization of the application of the species definition concept throughout the virus definition will be the next challenge of ICTV for the eighth report to be published by 2002. This, in turn, will contribute to homogeneity of the genus and family demarcation criteria (**Table 4**) and will permit creation of new families or merging of existing families. However, it is important to note that the nature of the demarcating criteria at the genus level will probably not change as these have passed the test of time. Despite the fact that they were mostly established using biochemical and structural criteria, they remained valid when correlated with genome organization and sequence data.

The Universal Virus Classification

The present universal system of virus taxonomy is set arbitrarily at hierarchical levels of order, family (in some cases subfamily), genus and species. Lower hierarchical levels, such as subspecies, strain, serotype, variant, pathotype and isolate are established by international specialty groups and/or by culture collections, but not by the ICTV. However some of them may be indicated in the ICTV report for information or because in the past these names were listed as 'viruses' in previous reports.

Virus species

The species taxon is always regarded as the most important taxonomic level in classification but it has proved to be the most difficult to apply to the viruses. ICTV definition of a virus species was long considered to be 'a concept that will normally be represented by a cluster of strains from a variety of sources, or a population of strains from a particular source, which have in common a set or pattern of correlating stable properties that separates the cluster from other clusters of strains' as stated by Matthews in 1982 and by Francki *et al* in 1991. This was a general definition, which was in fact not very useful for practically delineating species in a particular family. Furthermore, this definition directly addressed the definition of a virus strain, which had never been attempted in the history of virus taxonomy. In 1991, the ICTV Executive Committee accepted a definition proposed by van Regenmortel in 1990 (see above). This definition states: 'A virus species is a polythetic class of viruses that constitutes a replicating lineage

Table 2 Order of presentation of the viruses

Order	*Family*	*Subfamily*	*Genus*	*Type species*	*Host*
The DNA viruses					
The dsDNA viruses					
Caudovirales	*Myoviridae*				
			"T4-like viruses"[a]	*Enterobacteria phage T4*	Bacteria
			"P1-like viruses"	*Enterobacteria phage P1*	Bacteria
			"P2-like viruses"	*Enterobacteria phage P2*	Bacteria
			"Mu-like viruses"	*Enterobacteria phage Mu*	Bacteria
			"SP01-like viruses"	*Bacillus phage SP01*	Bacteria
			"ϕH-like viruses"	*Halobacterium virus ϕH*	Archaea
	Siphoviridae				
			"λ-like viruses"	*Enterobacteria phage λ*	Bacteria
			"T1-like viruses"	*Enterobacteria phage T1*	Bacteria
			"T5-like viruses"	*Enterobacteria phage T5*	Bacteria
			"L5-like viruses"	*Mycobacterium phage L5*	Bacteria
			"c2-like viruses"	*Lactococcus phage c2*	Bacteria
			"ψM1-like viruses"	*Methanobacterium virus ψM1*	Archaea
	Podoviridae				
			"T7-like viruses"	*Enterobacteria phage T7*	Bacteria
			"P22-like viruses"	*Enterobacteria phage P22*	Bacteria
			"ϕ29-like viruses"	*Bacillus phage ϕ29*	Bacteria
	Tectiviridae		*Tectivirus*	*Enterobacteria phage PRD1*	Bacteria
	Corticoviridae		*Corticovirus*	*Alteromonas phage PM2*	Bacteria
	Plasmaviridae		*Plasmavirus*	*Acholeplasma phage L2*	Mycoplasma
	Lipothrixviridae		*Lipothrixvirus*	*Thermoproteus virus 1*	Archaea
	Rudiviridae		*Rudivirus*	*Sulfolobus virus SIRV1*	Archaea
	Fuselloviridae		*Fusellovirus*	*Sulfolobus virus SSV1*	Archaea
			"SNDV-like viruses"	*Sulfolobus virus SNDV*	Archaea
	Poxviridae				
		Chordopoxvirinae			
			Orthopoxvirus	*Vaccinia virus*	Vertebrates
			Parapoxvirus	*Orf virus*	Vertebrates
			Avipoxvirus	*Fowlpox virus*	Vertebrates
			Capripoxvirus	*Sheeppox virus*	Vertebrates

Table 2 Continued

Order	Family	Subfamily	Genus	Type species	Host
			Leporipoxvirus	*Myxoma virus*	Vertebrates
			Suipoxvirus	*Swinepox virus*	Vertebrates
			Molluscipoxvirus	*Molluscum contagiosum virus*	Vertebrates
			Yatapoxvirus	*Yaba monkey tumor virus*	Vertebrates
		Entomopoxvirinae			
			Entomopoxvirus A	*Melolontha melolontha entomopoxvirus*	Invertebrates
			Entomopoxvirus B	*Amsacta moorei entomopoxvirus*	Invertebrates
			Entomopoxvirus C	*Chironomus luridus entomopoxvirus*	Invertebrates
	Asfarviridae		*Asfivirus*	*African swine fever virus*	Vertebrates[b]
	Iridoviridae				
			Iridovirus	*Chilo iridescent virus*	Invertebrates
			Chloriridovirus	*Mosquito iridescent virus*	Invertebrates
			Ranavirus	*Frog virus 3*	Vertebrates
			Lymphocystivirus	*Flounder virus*	Vertebrates
	Phycodnaviridae				
			Chlorovirus	*Paramecium bursaria Chlorella virus 1*	Algae
			Prasinovirus	*Micromonas pusilla virus SP1*	Algae
			Prymnesiovirus	*Chrysochromulina brevifilum virus*	Algae
			Phaeovirus	*Ectocarpus siliculosus virus 1*	Algae
	Baculoviridae				
			Nucleopolyhedrovirus	*Autographa californica nucleopolyhedrovirus*	Invertebrates
			Granulovirus	*Cydia pomonella granulovirus*	Invertebrates
	Herpesviridae				
		Alphaherpesvirinae			
			Simplexvirus	*Human herpesvirus 1*	Vertebrates
			Varicellovirus	*Human herpesvirus 3*	Vertebrates
			"Marek's disease-like viruses"	*Marek's disease virus*	Vertebrates
			"ILTV-like viruses"	*Infectious laryngotracheitis virus*	Vertebrates
		Betaherpesvirinae			
			Cytomegalovirus	*Human herpesvirus 5*	Vertebrates
			Muromegalovirus	*Mouse cytomegalovirus 1*	Vertebrates
			Roseolovirus	*Human herpesvirus 6*	Vertebrates

Table 2 Continued

Order	Family	Subfamily	Genus	Type species	Host
		Gammaherpesvirinae			
			Lymphocryptovirus	*Human herpesvirus 4*	Vertebrates
			Rhadinovirus	*Ateline herpesvirus 2*	Vertebrates
			"Ictalurid herpes-like viruses"	*Ictalurid herpesvirus 1*	Vertebrates
	Adenoviridae				
			Mastadenovirus	*Human adenovirus 2*	Vertebrates
			Aviadenovirus	*Fowl adenovirus 1*	Vertebrates
			Rhizidiovirus	*Rhizidiomyces virus*	Fungi
	Polyomaviridae				
			Polyomavirus	*Murine polyomavirus*	Vertebrates
	Papillomaviridae				
			Papillomavirus	*Cottontail rabbit papillomavirus*	Vertebrates
	Polydnaviridae				
			Ichnovirus	*Campoletis sonorensis virus*	Invertebrates
			Bracovirus	*Cotesia melanoscela virus*	Invertebrates
	Ascoviridae		*Ascovirus*	*Spodoptera frugiperda ascovirus*	Invertebrates
The ssDNA viruses					
	Inoviridae				
			Inovirus	*Coliphage fd*	Bacteria
			Plectrovirus	*Acholeplasma phage L51*	Mycoplasma
	Microviridae				
			Microvirus	*Coliphage ϕX174*	Bacteria
			Spiromicrovirus	*Spiroplasma phage 4*	Spiroplasma
			Bdellomicrovirus	*Bdellovibrio phage MAC1*	Bacteria
			Chlamydiamicrovirus	*Chlamydia phage 1*	Bacteria
	Geminiviridae				
			Mastrevirus	*Maize streak virus*	Plants
			Curtovirus	*Beet curly top virus*	Plants
			Begomovirus	*Bean golden mosaic virus*	Plants
	Circoviridae		*Circovirus*	*Chicken anemia virus*	Vertebrates
			Nanovirus	*Subterranean clover stunt virus*	Plants

Table 2 Continued

Order	Family	Subfamily	Genus	Type species	Host
	Parvoviridae				
		Parvovirinae			
			Parvovirus	*Mice minute virus*	Vertebrates
			Erythrovirus	*B19 virus*	Vertebrates
			Dependovirus	*Adeno-associated virus 2*	Vertebrates
		Densovirinae			
			Densovirus	*Junonia coenia densovirus*	Invertebrates
			Iteravirus	*Bombyx mori densovirus*	Invertebrates
			Brevidensovirus	*Aedes aegypti densovirus*	Invertebrates
The DNA and RNA reverse transcribing viruses					
	Hepadnaviridae				
			Orthohepadnavirus	*Hepatitis B virus*	Vertebrates
			Avihepadnavirus	*Duck hepatitis B virus*	Vertebrates
	Caulimoviridae				
			Caulimovirus	*Cauliflower mosaic virus*	Plants
			"PVCV-like viruses"	*Petunia vein-clearing virus*	Plants
			"SbCMV-like viruses"	*Soybean chlorotic mottle virus*	Plants
			"CsVMV-like viruses"	*Cassava vein mosaic virus*	Plants
			Badnavirus	*Commelina yellow mottle virus*	Plants
			"RTBV-like viruses"	*Rice tungro bacilliform virus*	Plants
	Pseudoviridae				
			Pseudovirus	*Saccharomyces cerevisiae Ty1 virus*	Yeast, Plants
			Hemivirus	*Drosophila melanogaster copia virus*	Yeast, Invertebrates
	Metaviridae				
			Metavirus	*Saccharomyces cerevisiae Ty3 virus*	Yeast, Plants, Invertebrates
			Errantivirus	*Drosophila melanogaster gypsy virus*	Invertebrates
	Retroviridae				
			Alpharetrovirus	*Avian leukosis virus*	Vertebrates
			Betaretrovirus	*Mason-Pfizer monkey virus*	Vertebrates
			Gammaretrovirus	*Mouse mammary tumor virus*	Vertebrates
			Deltaretrovirus	*Bovine leukemia virus*	Vertebrates
			Epsilonretrovirus	*Walleye dermal sarcoma virus*	Vertebrates
			Lentivirus	*Human immunodeficiency virus 1*	Vertebrates
			Spumavirus	*Human spumavirus*	Vertebrates

Table 2 Continued

Order	*Family*	*Subfamily*	*Genus*	*Type species*	*Host*
The RNA viruses					
The dsRNA viruses					
	Cystoviridae		*Cystovirus*	*Pseudomonas phage ϕ6*	Bacteria
	Reoviridae				
			Orthoreovirus	*Reovirus 3*	Vertebrates
			Orbivirus	*Bluetongue virus 1*	Vertebrates
			Rotavirus	*Simian rotavirus SA11*	Vertebrates
			Coltivirus	*Colorado tick fever virus*	Vertebrates
			Aquareovirus	*Golden shiner virus*	Vertebrates
			Cypovirus	*Bombyx mori cypovirus 1*	Invertebrates
			Fijivirus	*Fiji disease virus*	Plants
			Phytoreovirus	*Wound tumor virus*	Plants
			Oryzavirus	*Rice ragged stunt virus*	Plants
	Birnaviridae				
			Aquabirnavirus	*Infectious pancreatic necrosis virus*	Vertebrates
			Avibirnavirus	*Infectious bursal disease virus*	Vertebrates
			Entomobirnavirus	*Drosophila X virus*	Invertebrates
	Totiviridae				
			Totivirus	*Saccharomyces cerevisiae virus L-A*	Fungi
			Giardiavirus	*Giardia lamblia virus*	Protozoa
			Leishmaniavirus	*Leishmania RNA virus 1-1*	Protozoa
	Partitiviridae				
			Partitivirus	*Gaeumannomyces graminis virus 019/6-A*	Fungi
			Chrysovirus	*Penicillium chrysogenum virus*	Fungi
			Alphacryptovirus	*White clover cryptic virus 1*	Plants
			Betacryptovirus	*White clover cryptic virus 2*	Plants
	Hypoviridae		*Hypovirus*	*Cryphonectria hypovirus 1-EP713*	Fungi
			Varicosavirus	*Lettuce big-vein virus*	Plants
The negative-stranded ssRNA viruses					
Mononegavirales					
	Bornaviridae				
			Bornavirus	*Borna disease virus*	Vertebrates

Table 2 Continued

Order	Family	Subfamily	Genus	Type species	Host
	Filoviridae				
			"Ebola-like viruses" Zaïre	*Ebola virus*	Vertebrates
			"Marburg-like viruses"	*Marburg virus*	Vertebrates
	Paramyxoviridae				
		Paramyxovirinae			
			Respirovirus	*Human parainfluenza virus 1*	Vertebrates
			Morbillivirus	*Measles virus*	Vertebrates
			Rubulavirus	*Mumps virus*	Vertebrates
		Pneumovirinae			
			Pneumovirus	*Human respiratory syncytial virus*	Vertebrates
			Metapneumovirus	*Turkey rhinotracheitis virus*	Vertebrates
	Rhabdoviridae				
			Vesiculovirus	*Vesicular stomatitis Indiana virus*	Vertebrates
			Lyssavirus	*Rabies virus*	Vertebrates
			Ephemerovirus	*Bovine ephemeral fever virus*	Vertebrates
			Novirhabdovirus	*Infectious hematopoietic necrosis virus*	Vertebrates
			Cytorhabdovirus	*Lettuce necrotic yellows virus*	Plants
			Nucleorhabdovirus	*Potato yellow dwarf virus*	Plants
	Orthomyxoviridae				
			Influenzavirus A	*Influenza A virus*	Vertebrates
			Influenzavirus B	*Influenza B virus*	Vertebrates
			Influenzavirus C	*Influenza C virus*	Vertebrates
			Thogotovirus	*Thogoto virus*	Vertebrates
	Bunyaviridae				
			Bunyavirus	*Bunyamwera virus*	Vertebrates
			Hantavirus	*Hantaan virus*	Vertebrates
			Nairovirus	*Nairobi sheep disease virus*	Vertebrates
			Phlebovirus	*Sandfly fever Sicilian virus*	Vertebrates
			Tospovirus	*Tomato spotted wilt virus*	Plants
			Tenuivirus	*Rice stripe virus*	Plants
			Ophiovirus	*Citrus psorosis virus*	Plants
	Arenaviridae		*Arenavirus*	*Lymphocytic choriomeningitis virus*	Vertebrates
			Deltavirus	*Hepatitis delta virus*	Vertebrates

Table 2 Continued

Order	Family	Subfamily	Genus	Type species	Host
The positive-stranded ssRNA viruses					
	Leviviridae				
			Levivirus	*Enterobacteria phage MS2*	Bacteria
			Allolevivirus	*Enterobacteria phage Qβ*	Bacteria
	Narnaviridae				
			Narnavirus	*Saccharomyces cerevisiae 20S narnavirus*	Yeast
			Mitovirus	*Cryphonectria parasitica NB631 virus*	Yeast
	Picornaviridae				
			Enterovirus	*Poliovirus 1*	Vertebrates
			Rhinovirus	*Human rhinovirus 1A*	Vertebrates
			Hepatovirus	*Hepatitis A virus*	Vertebrates
			Cardiovirus	*Encephalomyocarditis virus*	Vertebrates
			Aphthovirus	*Foot-and-mouth disease virus O*	Vertebrates
			Parechovirus	*Human echovirus 22*	Vertebrates
			"Cricket paralysis-like viruses"	*Cricket paralysis virus*	Invertebrates
	Sequiviridae				
			Sequivirus	*Parsnip yellow fleck virus*	Plants
			Waikavirus	*Rice tungro spherical virus*	Plants
	Comoviridae				
			Comovirus	*Cowpea mosaic virus*	Plants
			Fabavirus	*Broad bean wilt virus 1*	Plants
			Nepovirus	*Tobacco ringspot virus*	Plants
	Potyviridae				
			Potyvirus	*Potato virus Y*	Plants
			Rymovirus	*Ryegrass mosaic virus*	Plants
			Macluravirus	*Maclura mosaic virus*	Plants
			Ipomovirus	*Sweet potato mild mottle virus*	Plants
			Bymovirus	*Barley yellow mosaic virus*	Plants
			Tritimovirus	*Wheat streak mosaic virus*	Plants
	Caliciviridae				
			Vesivirus	*Swine vesicular exanthema virus*	Vertebrates
			Lagovirus	*Rabbit hemorrhagic disease virus*	Vertebrates

Table 2 Continued

Order	Family	Subfamily	Genus	Type species	Host
			"Norwalk-like viruses"	*Norwalk virus*	Vertebrates
			"Sapporo-like viruses"	*Sapporo virus*	Vertebrates
			"Hepatitis E-like viruses"	*Hepatitis E virus*	Vertebrates
	Astroviridae		*Astrovirus*	*Human astrovirus 1*	Vertebrates
	Nodaviridae				
			Alphanodavirus	*Nodamura virus*	Invertebrates
			Betanodavirus	*Striped jack nervous necrosis virus*	Vertebrates
	Tetraviridae				
			Betatetravirus	*Nudaurelia capensis β virus*	Invertebrates
			Omegatetravirus	*Nudaurelia capensis ω virus*	Invertebrates
			Sobemovirus	*Southern bean mosaic virus*	Plants
			Marafivirus	*Maize rayado fino virus*	Plants
	Luteoviridae				
			Luteovirus	*Barley yellow dwarf virus – MAV*	Plants
			Polerovirus	*Potato leafroll virus*	Plants
			Enamovirus	*Pea enation mosaic virus 1*	Plants
			Umbravirus	*Carrot mottle virus*	Plants
	Tombusviridae				
			Avenavirus	*Oat chlorotic stunt virus*	Plants
			Aureusvirus	*Pothos latent virus*	Plants
			Carmovirus	*Carnation mottle virus*	Plants
			Dianthovirus	*Carnation ringspot virus*	Plants
			Machlomovirus	*Maize chlorotic mottle virus*	Plants
			Necrovirus	*Tobacco necrosis virus*	Plants
			Panicovirus	*Panicum mosaic virus*	Plants
			Tombusvirus	*Tomato bushy stunt virus*	Plants
Nidovirales					
	Coronaviridae				
			Coronavirus	*Avian infectious bronchitis virus*	Vertebrates
			Torovirus	*Berne virus*	Vertebrates
	Arteriviridae				
			Arterivirus	*Equine arteritis virus*	Vertebrates

Table 2 Continued

Order	Family	Subfamily	Genus	Type species	Host
	Flaviviridae				
			Flavivirus	*Yellow fever virus*	Vertebrates
			Pestivirus	*Bovine diarrhea virus*	Vertebrates
			Hepacivirus	*Hepatitis C virus*	Vertebrates
	Togaviridae				
			Alphavirus	*Sindbis virus*	Vertebrates
			Rubivirus	*Rubella virus*	Vertebrates
			Tobamovirus	*Tobacco mosaic virus*	Plants
			Tobravirus	*Tobacco rattle virus*	Plants
			Hordeivirus	*Barley stripe mosaic virus*	Plants
			Furovirus	*Soil-borne wheat mosaic virus*	Plants
			Pomovirus	*Potato mop-top virus*	Plants
			Pecluvirus	*Peanut clump virus*	Plants
			Benyvirus	*Beet necrotic yellow vein virus*	Plants
	Bromoviridae				
			Alfamovirus	*Alfalfa mosaic virus*	Plants
			Bromovirus	*Brome mosaic virus*	Plants
			Cucumovirus	*Cucumber mosaic virus*	Plants
			Ilarvirus	*Tobacco streak virus*	Plants
			Oleavirus	*Olive latent virus 2*	Plants
			Ourmiavirus	*Ourmia melon virus*	Plants
			Idaeovirus	*Rasberry bushy dwarf virus*	Plants
	Closteroviridae				
			Closterovirus	*Beet yellows virus*	Plants
			Crinivirus	*Lettuce infectious yellows virus*	Plants
			Capillovirus	*Apple stem grooving virus*	Plants
			Trichovirus	*Apple chlorotic leaf spot virus*	Plants
			Vitivirus	*Grapevine virus A*	Plants
			Tymovirus	*Turnip yellow mosaic virus*	Plants
			Carlavirus	*Carnation latent virus*	Plants

Table 2 Continued

Order	*Family*	*Subfamily*	*Genus*	*Type species*	*Host*
			Potexvirus	*Potato virus X*	Plants
			Allexivirus	*Shallot virus X*	Plants
			Foveavirus	*Apple stem pitting virus*	Plants
	Barnaviridae		*Barnavirus*	*Mushroom bacilliform virus*	Fungi
Unassigned viruses					

The subviral agents: viroids, satellites and agents of spongiform encephalopathies (prions)

Subviral agent	*Family*	*Genus*	*Type species*	*Host*
Viroids				
	Pospiviroidae			
		Pospiviroid	*Potato spindle tuber viroid*	Plants
		Hostuviroid	*Hop stunt viroid*	Plants
		Cocadviroid	*Coconut cadang-cadang viroid*	Plants
		Apscaviroid	*Apple scar skin viroid*	Plants
		Coleviroid	*Coleus blumei viroid 1*	Plants
	Avsunviroidae			
		Avsunviroid	*Avocado sunblotch viroid*	Plants
		Pelamoviroid	*Peach latent mosaic virus*	Plants
Satellites				Plants Invertebrates Fungi
Prions				Vertebrates Fungi

[a] Quotes are used to denote taxon names that are not approved ICTV international names, and are thus temporary until formal names are approved.
[b] Vertebrate arthropod-borne viruses are listed according to their vertebrate hosts.

Table 3 Orders, families and floating genera of viruses according to the seventh ICTV report (1999)

								Number of species		
Criteria	*Order*	*Family*	*Floating genus*	*Morphology*	*Genome configuration*	*Genome size (kb)*	*Virus host*	*Species*	*Strains/ serotypes*	*Tentative*	*Total*
dsDNA	*Caudovirales*	*Myoviridae*		Tailed phage	1 linear	336	Bacteria, archaea	15	23	117	155
		Siphoviridae		Tailed phage	1 linear	53	Bacteria, archaea	7	0	137	144
		Podoviridae		Tailed phage	1 linear	40	Bacteria, archaea	8	12	66	86
		Tectiviridae		Isometric	1 linear	16	Bacteria	4	0	38	42
		Corticoviridae		Isometric	1 circular supercoiled	10	Bacteria	1	0	2	3
		Plasmaviridae		Pleomorphic	1 circular	12	Mycoplasma	1	0	7	8
		Lipothrixviridae		Rod	1 linear	16	Archaea	2	0	0	2
		Rudiviridae		Rod	1 linear	33–36	Archaea	2	0	1	3
		Fuselloviridae		Lemon-shape	1 circular supercoiled	15	Archaea	1	0	0	1
			"SNDV-like viruses"	Droplet-shape	1 circular	20	Archaea	1	0	0	1
		Poxviridae		Ovoid	1 linear	130–375	Vertebrate, invertebrate	62	8	23	93
		Asfarviridae		Isometric	1 circular	170–190	Vertebrate	1	0	0	1
		Iridoviridae		Isometric	1 linear	160–400	Vertebrate, invertebrate	17	4	3	24
		Phycodnaviridae		Isometric	1 linear	250–350	Algae	27	0	38	65
		Baculoviridae		Bacilliform	1 circular supercoiled	90–230	Invertebrate	17	6	7	30
		Herpesviridae		Isometric	1 linear	120–220	Vertebrate	56	0	65	121
		Adenoviridae		Isometric	1 linear	32–48	Vertebrate	26	102	35	163
			Rhizidiovirus	Isometric	1 linear	27	Fungus	1	0	0	1
		Polyomaviridae		Isometric	1 circular	5	Vertebrate	12	4	0	16
		Papillomaviridae		Isometric	1 circular	6.8–8.4	Vertebrate	7	0	88	95
		Polydnaviridae		Rod, fusiform	1 circular supercoiled	2–28	Invertebrate	59	0	0	59
		Ascoviridae		Ovoid and bacilliform	1 circular	100–180	Invertebrate	3	0	1	4
								330	**159**	**628**	**117**

Table 3 Continued

Criteria	Order	Family	Floating genus	Morphology	Genome configuration	Genome size (kb)	Virus host	Number of species			
								Species	Strains/ serotypes	Tentative	Total
ssDNA		*Inoviridae*		Rod	1 circular	7–20	Bacteria, mycoplasma	36	7	5	48
		Microviridae		Isometric	1 circular	6	Bacteria, spiroplasma	7	0	33	40
		Geminivirus		Isometric	1 or 2 circular	3–6	Plant	94	2	10	106
		Circoviridae		Isometric	1 circular	1.7–2.3	Vertebrate	3	0	1	4
			Nanovirus	Isometric	6–9 circular	6–9	Plant	4	0	1	5
		Parvoviridae		Isometric	1 – strand	6–8	Vertebrate, invertebrate	38	0	16	54
								182	**9**	**66**	**257**
ssDNA RT		*Hepadnaviridae*		Isometric	1 circular – strand	3	Vertebrate	5	0	2	7
dsDNA RT		*Caulimoviridae*		Isometric, bacilliform	1 circular	8	Plant	26	0	8	34
ssRNA RT		*Pseudoviridae*		Ovoid	1 linear	5–8	Yeast, plant	15	0	0	15
ssRNA RT		*Metaviridae*		Isometric	1 linear	4–10	Yeast, fungus, Invertebrate	18	0	1	19
ssRNA RT		*Retroviridae*		Spherical	dimer 1 + segment	7–10	Vertebrate	59	44	2	105
								123	**44**	**13**	**180**
dsRNA		*Cystoviridae*		Isometric	3 segments	17	Bacteria	1	0	0	1
		Reoviridae		Isometric	10–12 segments	19–62	Vertebrate, invertebrate, plant	62	256	39	357
		Birnaviridae		Isometric	2 segments	6	Vertebrate, invertebrate	4	21	1	26
		Totiviridae		Isometric	1 segment	5–7	Fungus, protozoa	18	0	5	23
		Partitiviridae		Isometric	2 segments	3–10	Fungus, plant	30	0	15	45
		Hypoviridae		Pleomorphic	1 segment	9–13	Fungus	3	0	2	5
			Varicosavirus	Rod	2 segments	14	Plant	1	0	3	4
								119	**277**	**65**	**461**
Negative ssRNA	*Mononegavirales*	*Bornaviridae*		Spherical	1 – segment	9	Vertebrate	1	0	1	2
		Filoviridae		Bacilliform	1 – segment	13	Vertebrate	5	19	0	24

Table 3 Continued

Criteria	Order	Family	Floating genus	Morphology	Genome configuration	Genome size (kb)	Virus host	Number of species			
								Species	Strains/ serotypes	Tentative	Total
		Paramyxoviridae		Helical	1 – segment	15–16	Vertebrate	31	5	2	38
		Rhabdoviridae		Bacilliform	1 – segment	10–13	Vertebrate, plant	37	0	142	179
		Orthomyxoviridae		Helical	8 – segments	13–14	Vertebrate	5	1	0	6
		Bunyaviridae		Spherical	3 – segments	12–23	Vertebrate, plant	93	236	66	395
			Tenuivirus	Filaments	4 –?segments	15–19	Plant	6	0	5	11
			Ophiovirus	Filaments	3 – segments	12	Plant	3	0	0	3
		Arenaviridae		Spherical	2 – segments	11	Vertebrate	19	27	2	48
			Deltavirus	Spherical	1 circular – strand	1.7	Vertebrate	1	0	0	1
								201	**288**	**218**	**707**
Positive ssRNA		*Leviviridae*		Isometric	1 + segment	3–4	Bacteria	4	18	35	57
		Narnaviridae		Ribonucleic complex	1 + segment	2.5	Yeast	3	0	0	3
		Picornaviridae		Isometric	1 + segment	7–8.5	Vertebrate	16	105	137	258
			"CrPV-like viruses"	Isometric	1 + segment	9–10	Invertebrate	5	0	0	5
		Sequiviridae		Isometric	1 + segment	9–12	Plant	5	0	0	5
		Comoviridae		Isometric	2 + segments	9–16	Plant	50	0	9	59
		Potyviridae		Rod	1 or 2 + segments	8–12	Plant	106	0	92	198
		Caliciviridae		Isometric	1 + segment	8	Vertebrate	6	40	8	54
			"HEV-like viruses"	Isometric	1 + segment	7	Vertebrate	1	0	0	1
		Astroviridae		Isometric	1 + segment	7–8	Vertebrate	6	13	0	19
		Nodaviridae		Isometric	2 + segments	5	Vertebrate, invertebrate	14	0	0	14
		Tetraviridae		Isometric	1 + segment	5	Invertebrate	9	0	0	9
			Sobemovirus	Isometric	1 + segment	4	Plant	11	0	3	14
			Marafivirus	Isometric	1 + segment	6–7	Plant	3	0	0	3
		Luteoviridae		Isometric	1 or 2 + segment	6–9	Plant	8	0	11	19
			Umbravirus	No particles	1 + segment	4	Plant	7	0	15	22
		Tombusviridae		Isometric	1 or 2 + segment	4–5.5	Plant	38	0	11	49

Table 3 Continued

Criteria	Order	Family	Floating genus	Morphology	Genome configuration	Genome size (kb)	Virus host	Number of species			
								Species	Strains/ serotypes	Tentative	Total
	Nidovirales	*Coronaviridae*		Pleomorphic	1 + segment	28–33	Vertebrate	16	5	1	22
		Arteriviridae		Spherical	1 + segment	13–16	Vertebrate	4	0	0	4
		Flaviviridae		Isometric	1 + segment	10–12	Vertebrate	57	47	6	110
		Togaviridae		Isometric	1 + segment	10–13	Vertebrate	23	6	0	29
ssRNA Positive sense			*Tobamovirus*	Rod	1 + segment	6	Plant	16	0	3	19
			Tobravirus	Rod	2 + segments	9–11	Plant	3	0	0	3
			Hordeivirus	Rod	3 + segments	10	Plant	4	0	0	4
			Furovirus	Rod	2 + segments	9–11	Plant	1	0	4	5
			Pomovirus	Rod	3 + segments	12	Plant	4	0	0	4
			Pecluvirus	Rod	2 + segments	10	Plant	2	0	0	2
			Benyvirus	Rod	4 (or 5) + segments	14–16	Plant	2	0	0	2
		Bromoviridae		Isometric, bacilliform	3 + segments	8–9	Plant	28	0	0	28
			Ourmiavirus	Bacilliform	3 + segments	4–5	Plant	3	0	0	3
			Idaeovirus		3 + segments	8	Plant	1		0	1
		Closteroviridae		Rod	1 or 2 + segments	15–19	Plant	18	0	16	34
			Capillovirus	Rod	1 + segment	7	Plant	3	0	1	4
			Trichovirus	Rod	1 + segment	7.5	Plant	3	0	1	4
			Vitivirus	Rod	1 + segment	7.5	Plant	4	0	1	5
			Tymovirus	Isometric	1 + segment	6	Plant	20	0	1	21
			Carlavirus	Rod	1 + segment	7–8	Plant	31	0	29	60
			Potexvirus	Rod	1 + segment	6	Plant	26	0	18	44
			Allexivirus	Rod	1 + segment	9	Plant	6	0	3	9
			Foveavirus	Rod	1 + segment	8–9	Plant	2	0	1	3
		Barnaviridae		Bacilliform	1 + segment	4	Fungus	1	0	1	2
								565	**234**	**403**	**1202**
		Unassigned viruses		—	—	—	All	30	0	0	30
								1550	**1011**	**1393**	**3954**
		Viroids		—	—	—	Plant	27	0	8	35
		Satellites		—	—	—	Plant	33	0	6	39

Table 4 List of criteria demarcating different virus taxa

I Order
Common properties between several families including:
Biochemical composition
Virus replication strategy
Particle structure (to some extent)
General genome organization
II Family
Common properties between several genera including:
Biochemical composition
Virus replication strategy
Nature of the particle structure
Genome organization
III Genus
Common properties within a genus including:
Virus replication strategy
Genome size, organization and/or number of segments
Sequence homologies (hybridization properties)
Vector transmission
IV Species
Common properties within a species including:
Genome rearrangement
Sequence homologies (hybridization properties)
Serological relationships
Vector transmission
Host range
Pathogenicity
Tissue tropism
Geographical distribution

and occupies a particular ecological niche.' The major advantage in this definition is that it can accommodate the inherent variability of viruses and is not dependent on the existence of a unique characteristic. Members of a polythetic class are defined by more than one property and no single property is absolutely essential and necessary. Thus in each family it might be possible to determine the set of properties of the class 'species' and to check if the family members are species of this family or if they belong to a lower taxonomic level. The ICTV is currently conducting this exercise throughout all virus families. This exercise should ultimately result in an excellent evaluation of a precise definition of each virus species in the entire classification.

Several practical matters are related to the definition of a virus species with the goal of improving the usefulness of virus classification. These include: (1) homogeneity of the different taxa; (2) diagnosis-related matters; (3) virus collections; (4) evolution studies; (5) biotechnology; (6) sequence database projects; (7) virus database projects; and now (8) intellectual property rights.

Virus families and genera

There is no formal definition for a genus, but it is commonly considered as: 'a population of virus species that share common characteristics and are different from other populations of species'. Although this definition is somewhat elusive, this level of classification seems enduring and useful; some genera have been moved from one family to another over the years, but the composition and description of the genera has remained stable. The characters defining a genus differ from one family to another and there is a tendency to create genera with fewer differences between them. Upon examination, there is more and more evidence that the members of a genus have a common evolutionary origin. The use of subgenera has been abandoned in current virus classification.

Notwithstanding the creation of the ICTV, plant

virologists continued to classify plant viruses in 'groups', refusing to place them in genera and families. However, owing to obvious similarities, plant reoviruses and rhabdoviruses had been integrated into the families *Reoviridae* and *Rhabdoviridae* (**Table 2**). This position was mostly due to plant virologists' refusal to accept binomial nomenclature. Since this form of nomenclature was withdrawn from the ICTV classification rules in 1995, they subsequently accepted the placing of plant viruses into species, genera and families as shown in the sixth ICTV report. However, there are still 30 of so-called 'floating genera' that do not pertain to any family. This is mostly due to the fact that plant virologists prefer to accumulate data on virus species and genera before clustering appropriate genera in families. It is remarkable that this attitude has also been adopted by other virologists as a convenient way of classifying viruses, without having to move genera out of families when it becomes apparent that they are part of a distinct family. For example, the members of the floating genus 'cricket paralysis-like viruses' share enough properties with picornaviruses to be included in the family *Picornaviridae;* however, they also possess properties that would justify their classification in a separate family. Only new data or new viruses will permit a definitive position, therefore for the time being it remains a floating genus. Similarly the same strategy is used to create a floating genus 'ictalurid herpes-like viruses', within the family *Herpesviridae*, although in this case it is a floating genus within the family because of uncertainty as to whether the members of this genus should be classified in one of the existing subfamilies or to a new subfamily.

Virus orders

As mentioned above, the higher hierarchy levels for virus classification are extremely difficult to establish. Despite several propositions in the past, only three have been accepted: *Caudovirales, Mononegavirales* and *Nidovirales*. The first virus order, *Mononegavirales*, was established in 1990 and comprises the nonsegmented single-stranded RNA negative-sense viruses, namely the families *Bornaviridae, Filoviridae, Paramyxoviridae* and *Rhabdoviridae*. This decision was taken because of the great similarity of many criteria between these families, including their replication strategy. A second order, *Caudovirales*, contains all the families of double-stranded DNA phages possessing a tail, including the families *Myoviridae, Podoviridae* and *Siphoviridae*. A third order, *Nidovirales*, comprising the families *Coronaviridae* and *Arteriviridae*, was accepted in 1996 because of the impossibility of grouping together these two taxonomic entities, which share many properties and yet are so different, as a single family. Many members of the ICTV advocate the creation of many more orders, but it has been decided to proceed cautiously to avoid creation of short-lived orders. The creation of formal taxa higher than the orders, for example, kingdoms, classes and subclasses, has not been considered by the ICTV.

Virus Taxa Descriptions

Virus classification continues to evolve with the technologies available for describing viruses. The first wave of descriptions, those before 1940, mostly took into account the visual symptoms of the diseases caused by viruses, along with their modes of transmission. A second wave, between 1940 and 1970, brought together an enormous amount of information from studies of virion morphology (electron microscopy, structural data), biology (serology and virus properties) and physicochemical properties of viruses (nature and size of genome, number and size of viral proteins). Since 1970, the third wave of virus descriptions has included genome and replicative information as well as molecular relationships with virus hosts. There is a correlative modification of the list of virus descriptors and **Table 1** lists the family and genera descriptors which are used in the current ICTV report. **Figures 1–5** are diagrammatic representations of families and genera of viruses infecting vertebrates, invertebrates, plants, fungi, yeasts, protozoa and bacteria. The most recent wave of information used to classify viruses is naturally nucleotide and amino acid sequences. It is becoming more and more prevalent in virus taxonomy, as exemplified by the presence of a significant number of 'phylogenetic trees' in the seventh ICTV report, and by the huge number of scientific publications on this topic. Some scientists promote the concept of 'quantitative taxonomy' aimed at demonstrating that virus sequences contain all the coding information required for all the biological properties of the viruses. This is in complete agreement with the polythetic concept of the virus species definition, as demonstrated for example by Padidam *et al* in 1995, van Regenmortel *et al* in 1997, Hyppia *et al* in 1998, and Aleman *et al* in 1999.

The impact of descriptions on virus classification has been particularly influenced by electron microscopy and of the negative staining technique for virions. This technique had an immediate influence on diagnostics and classification of viruses. With negative staining, viruses could be identified from poorly purified preparations of all tissue types, and informa-

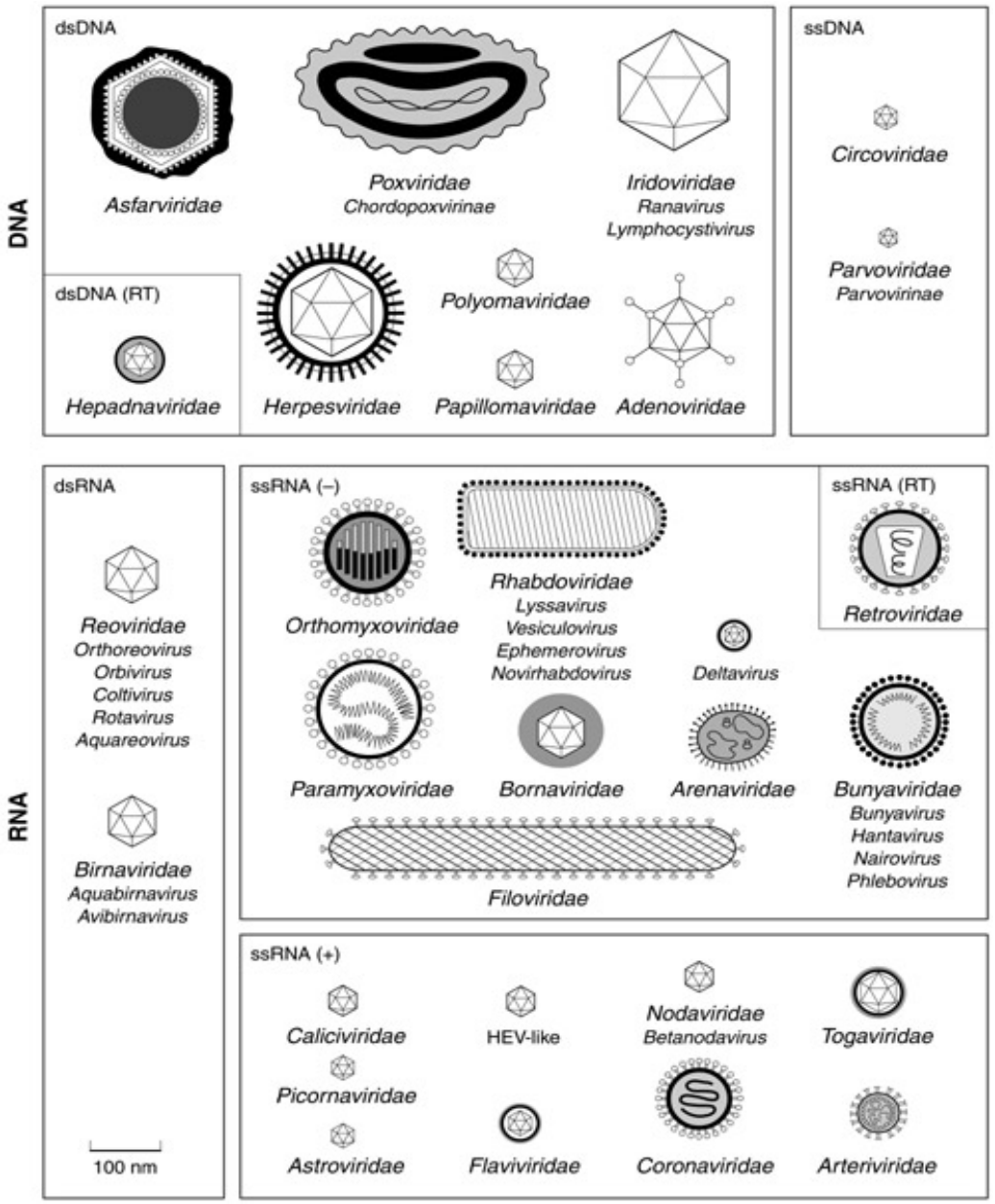


Figure 1 Families and genera of viruses infecting vertebrates.

tion about size, shape, structure and symmetry could be quickly provided. As a result, virology progressed simultaneously for all viruses infecting animals, insects, plants and bacteria. Thin sections of infected tissues brought a new dimension to virus classification by providing information about virion morphogenesis and cytopathogenic effects. These techniques, in conjunction with the determination of the nature of the genome, provided a major source of information for the system of virus classification established in the 1980s, as shown by the large number of viruses listed in the fifth ICTV report in 1989.

In many instances the properties of viruses belonging to the same genus are correlated. Thus, the classification of a few of them will likely be sufficient to allow the classification of a new virus into an established genus. For example, a plant virus with filamentous particles of 700–850 nm and transmitted by aphids is likely to be a member of the genus *Potyvirus*. Establishment of new genera in the future will require more information. Most of the properties listed in **Table 3** will have to be precisely analyzed to warrant the formation of a new genus.

Table 3 lists 45 different categories of properties but each category includes many items. Lists of virus descriptors usually comprise 1000–2000 descriptors.

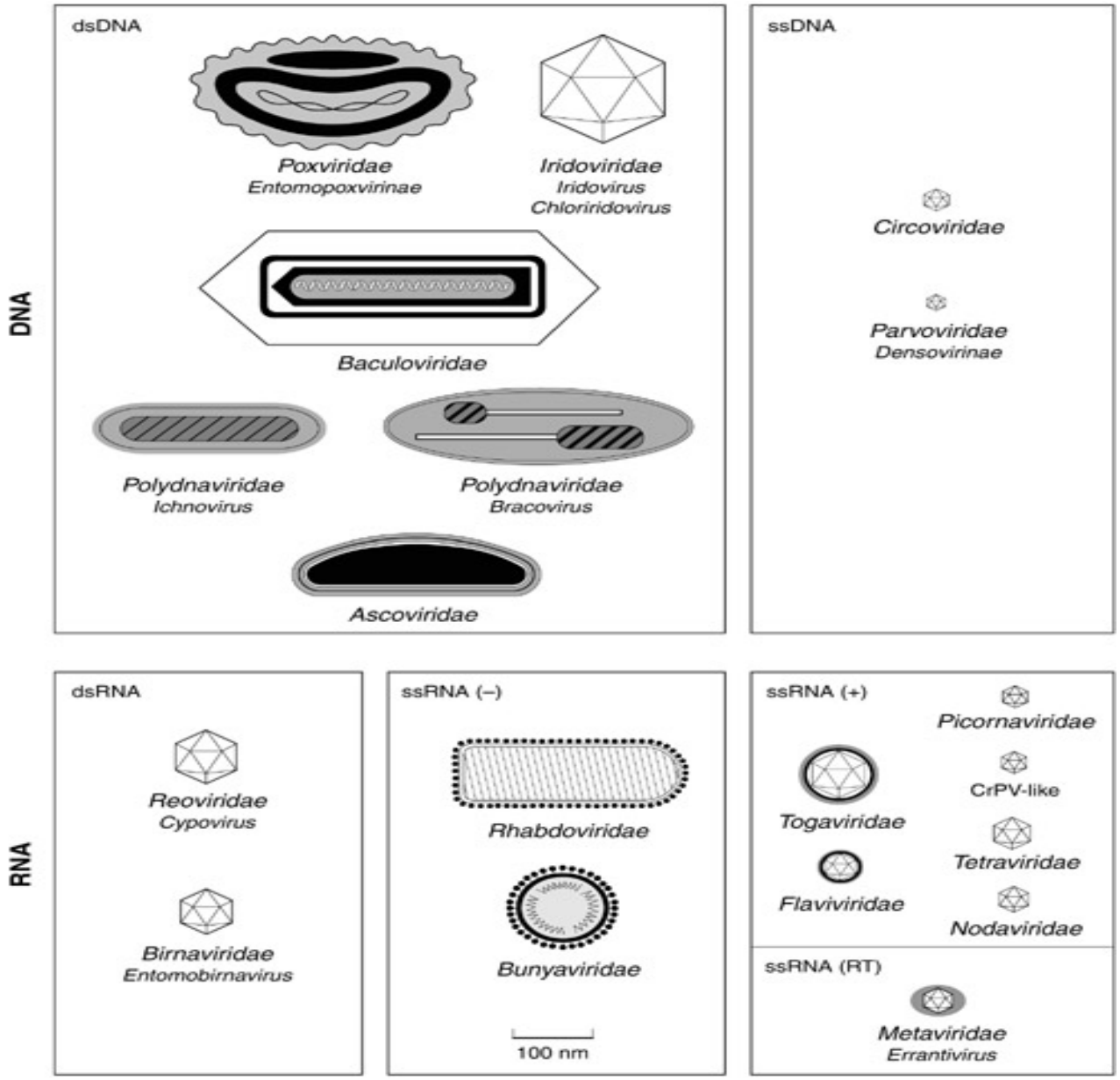


Figure 2 Families and genera of viruses infecting invertebrates.

The establishment of a universal list of virus descriptors is under way and should be adopted by ICTV around 2000 with the establishment of the ICTVdB. It will contain a common set of descriptors for *all* viruses and subsets for specific viruses in relation to their specific hosts (human, animal, insect, plant and bacterial).

A Uniform Nomenclature of Viral Taxa

When a genus is approved by ICTV, a type species is designated. However, none of these type species have received a new international name and only English names are used. Latinized binomial names for virus names have been supported by animal and human virologists of ICTV for many years, but have never been implemented. This suggestion was in fact withdrawn from ICTV nomenclature rules in 1990 and consequently such names as *Herpesvirus varicella* or *Polyomavirus hominis* should not be used. For several years, plant virologists have adopted a different nomenclature, using the vernacular name of a virus but replacing the word 'virus' by the genus name; for example, *Cucumber mosaic cucumovirus* and *Tobacco mosaic tobamovirus*. Though this usage is favored by many scientists, and examples of such practice can

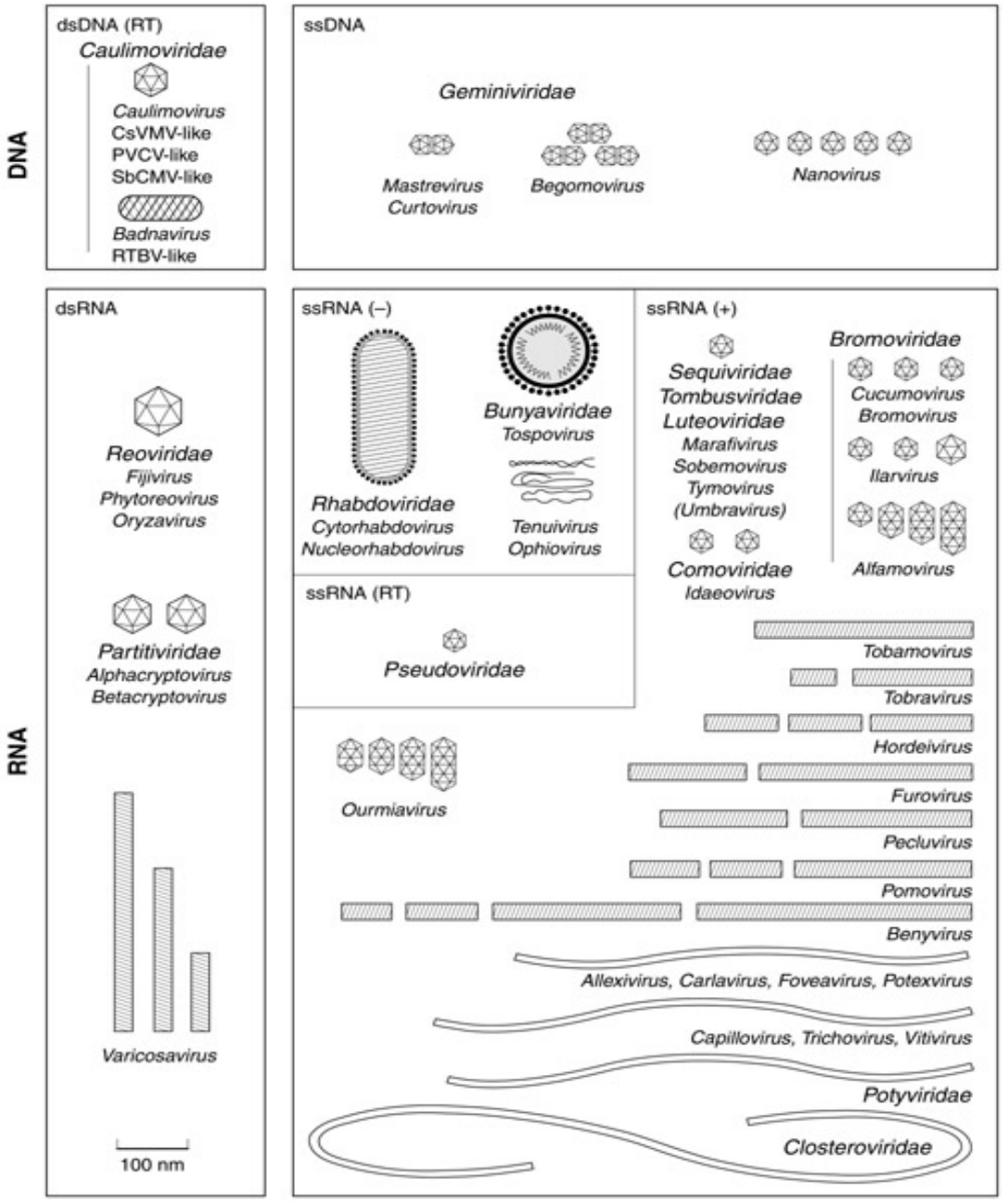


Figure 3 Families and genera of viruses infecting plants.

be found for human, animal and insect viruses (e.g. *Human rhinovirus, Canine calicivirus, Acheta densovirus*...), it has not been universally adopted by the ICTV.

The ICTV has set rules for virus nomenclature and orthography of taxonomic names that are regularly revisited and improved. The last word of international virus species names is 'virus', the international genus names universally end in '...virus', the international subfamily names end in '...virinae', the international family names end with '...viridae', and the international order names are ending in '...virales'. In formal taxonomic usage, the virus order, family, subfamily, genus and species names are all printed in italics (or underlined) and the first letter is capitalized. For all taxa except the species names, new names are created *de novo* following ICTV guidelines, but in the case of virus names English vernacular form is used. In formal usage, the name of the taxon precedes the name of the taxonomic unit; for example, 'the family *Picornaviridae*' or 'the genus *Rhinovirus*'. In informal vernacular usage, order,

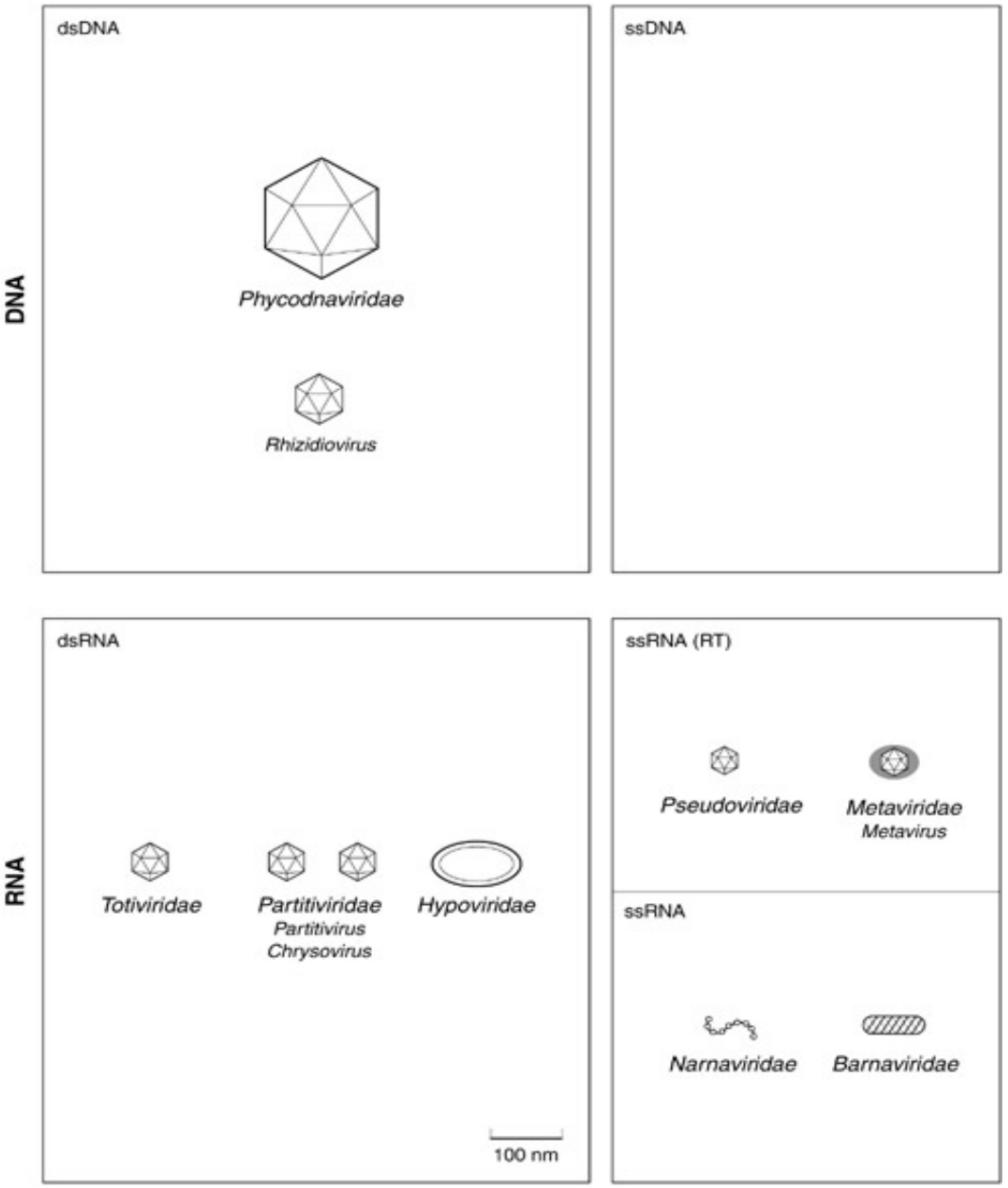


Figure 4 Families of viruses infecting algae, fungi, yeasts and protozoa.

family, subfamily, genus and species names are written in lower case Roman script; they are not capitalized nor italicized (or underlined). Additionally, in informal usage, the name of the taxon should not include the formal suffix, and it should follow the term for the taxonomic unit; for example, 'the mononegavirales order', 'the adenovirus family', 'the avihepadnavirus genus' or 'the tobacco mosaic virus' species. Virus names are often abbreviated for convenient reasons, but ICTV has not set up guidelines to generate such abbreviations. The ICTV reports list abbreviations most commonly used by specialists and the ICTV reports help virologists to identify duplicates of abbreviations in order to decrease the number of such duplicates. In 1988 plant virologists initiated the publication of such lists and have indicated guidelines for the creation of new virus names and new abbreviations. These guidelines were last published in 1991 by Fauquet and Martelli and will be updated again in 1999.

To avoid ambiguous virus identifications, it has been recommended to journal editors that published papers follow ICTV guidelines for proper virus identification and nomenclature, and that viruses should be cited

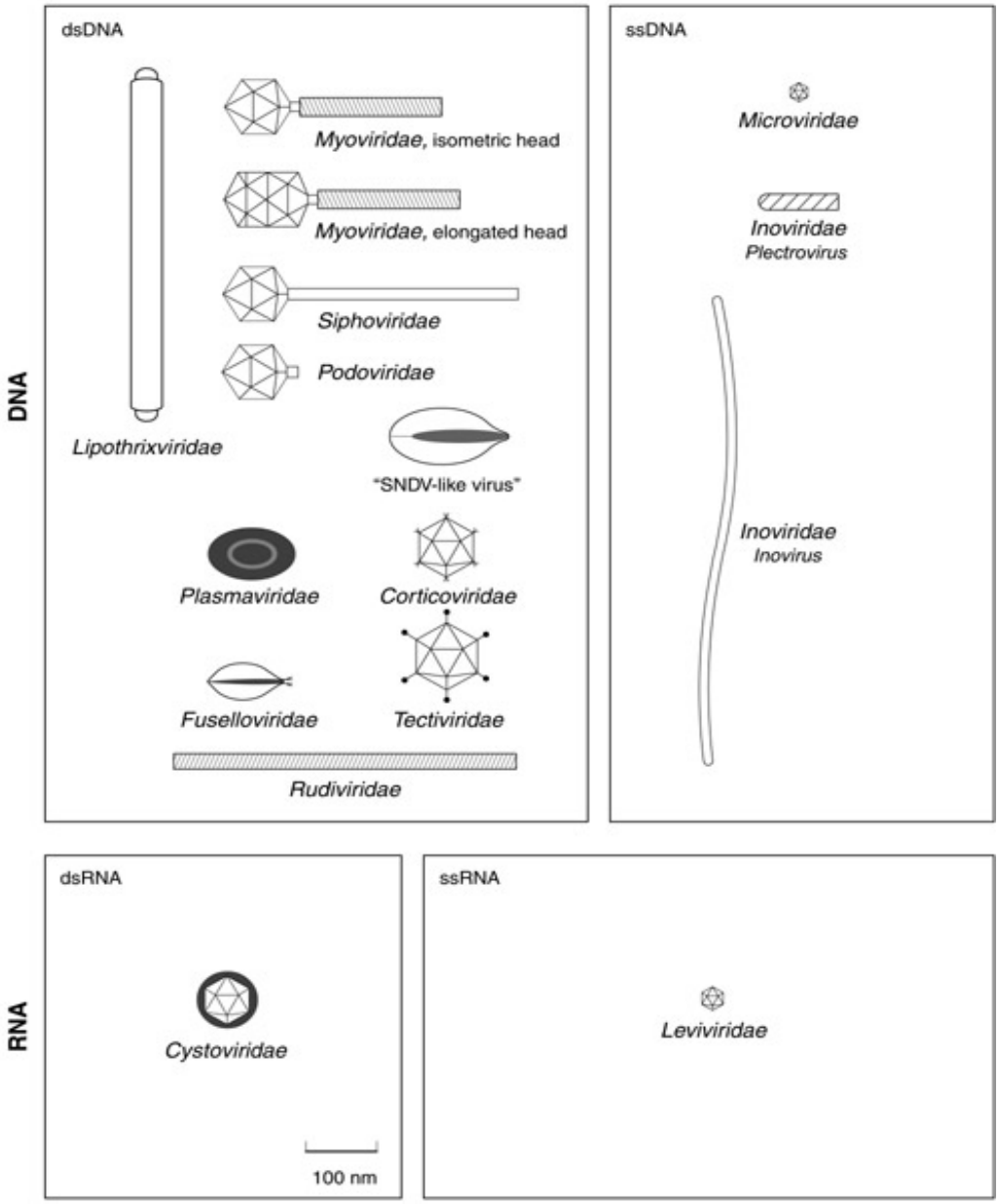


Figure 5 Families and genera of viruses infecting bacteria.

with their full taxonomic terminology when they are first mentioned in an article. For example:

- Order *Caudovirales*, family *Podoviridae*, genus 'T7-like viruses', species *Enterobacteria phage T7*.
- Order *Mononegavirales*, family *Paramyxoviridae*, subfamily *Paramyxovirinae*, genus *Rubulavirus*, species *Mumps virus*.
- Order *Nidovirales*, family *Coronaviridae*, genus *Coronavirus*, species *Avian infectious bronchitis virus*.
- Family *Iridoviridae*, genus *Iridovirus*, species *Chilo iridescent virus*.
- Family *Picornaviridae*, genus *Enterovirus*, species *Poliovirus*, serotype Human poliovirus 1.
- Genus *Tobamovirus*, species *Tobacco mosaic virus*.

See also: **Phage taxonomy and classification; Virus structure: Atomic structure, Principles of virus structure.**

Further Reading

Fauquet CM and Martelli GP (1995) Up-dated ICTV list of names and abbreviations of viruses, viroids and satellites infecting plants. *Arch. Virol.* 140: 393.

Francki RIB, Milne RG and Hatta T (1985) *Atlas of Plant Viruses*. Boca Raton, CRC Press.

Francki RIB, Fauquet CM, Knudson DL and Brown F (1991) *Classification and Nomenclature of Viruses*. Fifth Report of the International Committee on Taxonomy of Viruses. Vienna: Springer.

Lwoff A, Horne R and Tournier P (1962) *A system of viruses*. Cold Spring Harb. Symp. Quant. Biol.

Matthews REF (1983) *The History of Viral Taxonomy. A Critical Appraisal of Viral Taxonomy*, pp 1–35. Boca Raton: CRC Press.

Murphy FA, Fauquet CM, Bishop DHL *et al* (eds) (1995) *Virus Taxonomy*. Sixth Report of the International Committee on Taxonomy of Viruses. Vienna: Springer.

van Regenmortel MHV, Bishop DHL, Fauquet CM *et al* (1997) Guidelines to the demarcation of virus species. *Arch. Virol.* 142: 1505.

van Regenmortel MHV, Fauquet CM, Bishop DHL *et al* (eds) (1999) *Virus Taxonomy*. Seventh Report of the International Committee on Taxonomy of Viruses. New York: Academic Press.

TENUIVIRUSES

Bryce W. Falk, Department of Plant Pathology, University of California, Davis, California, USA

History

The viruses in the genus *Tenuivirus* (tenuiviruses) have been recognized as important plant pathogens since the early 1900s. However, in 1977 it was first recognized that rice stripe virus (RSV)-infected rice (*Oryza sativa* L.) plants contained unusual fine-stranded particles. These appeared as circular or branched filaments and were distinctly different from particles associated with other plant viruses. Subsequent work showed that plants infected by several other viruses contained similar particles. This and the somewhat unique biological properties shared by these viruses led to the early grouping together of RSV and other plant viruses, including maize stripe virus (MSpV) and rice hoja blanca virus (RHBV). Since 1995, RSV and related viruses have been grouped together by the International Committee on Taxonomy of Viruses (ICTV) as members of the genus *Tenuivirus* (tenuiviruses).

Taxonomy and Classification

There are currently six species within the genus *Tenuivirus*, and at least five tentative species (**Table 1**). The definitive tenuiviruses are: rice stripe virus (RSV; the type species of the genus); MSpV; RHBV; rice grassy stunt virus (RGSV); *Echinochloa* hoja blanca virus (EHBV) and *Urochloa* hoja blanca virus (UHBV). All tenuiviruses exhibit similar properties, and in recent years molecular biological analyses have shown that they are quite distinct from other currently recognized plant viruses. The genus *Tenuivirus* is not placed within a formally recognized virus family; however, tenuiviruses appear in some ways to be more closely related to viruses in the genus *Phlebovirus* of the family *Bunyaviridae*, than they are to other plant viruses.

Biology, Host Range and Vector Transmission

The plant host ranges of all tenuiviruses are limited to monocotyledenous plant species within the family Poaceae, including many plants which are important food crops (i.e. rice (*Oryza sativa* L.) and maize (*Zea mays* L.)). The symptoms induced in infected plants are generally similar for the different tenuiviruses and includes general leaf striping, a distinct white coloring of the leaf stripes and stunting (**Fig. 1**).

Tenuiviruses generally are not mechanically transmissible to their plant hosts; however, mechanical transmission has been reported in limited instances but only under specific conditions. All tenuiviruses are transmitted to plants by specific planthoppers (Homoptera: Delphacidae, see **Table 1** and **Fig. 2**). Transmission of a given tenuivirus is specific and may be limited to planthoppers of a single species. Compared to other vectors of plant viruses (i.e. aphids or whiteflies), delphacid planthoppers are not generally thought of as common vectors; however, they are perfectly adapted to be vectors of tenuiviruses. Delphacid planthoppers which vector tenuiviruses

Further Reading

Fauquet CM and Martelli GP (1995) Up-dated ICTV list of names and abbreviations of viruses, viroids and satellites infecting plants. *Arch. Virol.* 140: 393.

Francki RIB, Milne RG and Hatta T (1985) *Atlas of Plant Viruses*. Boca Raton, CRC Press.

Francki RIB, Fauquet CM, Knudson DL and Brown F (1991) *Classification and Nomenclature of Viruses*. Fifth Report of the International Committee on Taxonomy of Viruses. Vienna: Springer.

Lwoff A, Horne R and Tournier P (1962) *A system of viruses*. Cold Spring Harb. Symp. Quant. Biol.

Matthews REF (1983) *The History of Viral Taxonomy. A Critical Appraisal of Viral Taxonomy*, pp 1–35. Boca Raton: CRC Press.

Murphy FA, Fauquet CM, Bishop DHL *et al* (eds) (1995) *Virus Taxonomy*. Sixth Report of the International Committee on Taxonomy of Viruses. Vienna: Springer.

van Regenmortel MHV, Bishop DHL, Fauquet CM *et al* (1997) Guidelines to the demarcation of virus species. *Arch. Virol.* 142: 1505.

van Regenmortel MHV, Fauquet CM, Bishop DHL *et al* (eds) (1999) *Virus Taxonomy*. Seventh Report of the International Committee on Taxonomy of Viruses. New York: Academic Press.

TENUIVIRUSES

Bryce W. Falk, Department of Plant Pathology, University of California, Davis, California, USA

History

The viruses in the genus *Tenuivirus* (tenuiviruses) have been recognized as important plant pathogens since the early 1900s. However, in 1977 it was first recognized that rice stripe virus (RSV)-infected rice (*Oryza sativa* L.) plants contained unusual fine-stranded particles. These appeared as circular or branched filaments and were distinctly different from particles associated with other plant viruses. Subsequent work showed that plants infected by several other viruses contained similar particles. This and the somewhat unique biological properties shared by these viruses led to the early grouping together of RSV and other plant viruses, including maize stripe virus (MSpV) and rice hoja blanca virus (RHBV). Since 1995, RSV and related viruses have been grouped together by the International Committee on Taxonomy of Viruses (ICTV) as members of the genus *Tenuivirus* (tenuiviruses).

Taxonomy and Classification

There are currently six species within the genus *Tenuivirus*, and at least five tentative species (**Table 1**). The definitive tenuiviruses are: rice stripe virus (RSV; the type species of the genus); MSpV; RHBV; rice grassy stunt virus (RGSV); *Echinochloa* hoja blanca virus (EHBV) and *Urochloa* hoja blanca virus (UHBV). All tenuiviruses exhibit similar properties, and in recent years molecular biological analyses have shown that they are quite distinct from other currently recognized plant viruses. The genus *Tenuivirus* is not placed within a formally recognized virus family; however, tenuiviruses appear in some ways to be more closely related to viruses in the genus *Phlebovirus* of the family *Bunyaviridae*, than they are to other plant viruses.

Biology, Host Range and Vector Transmission

The plant host ranges of all tenuiviruses are limited to monocotyledenous plant species within the family Poaceae, including many plants which are important food crops (i.e. rice (*Oryza sativa* L.) and maize (*Zea mays* L.)). The symptoms induced in infected plants are generally similar for the different tenuiviruses and includes general leaf striping, a distinct white coloring of the leaf stripes and stunting (**Fig. 1**).

Tenuiviruses generally are not mechanically transmissible to their plant hosts; however, mechanical transmission has been reported in limited instances but only under specific conditions. All tenuiviruses are transmitted to plants by specific planthoppers (Homoptera: Delphacidae, see **Table 1** and **Fig. 2**). Transmission of a given tenuivirus is specific and may be limited to planthoppers of a single species. Compared to other vectors of plant viruses (i.e. aphids or whiteflies), delphacid planthoppers are not generally thought of as common vectors; however, they are perfectly adapted to be vectors of tenuiviruses. Delphacid planthoppers which vector tenuiviruses

Table 1 Tenuiviruses, their planthopper vectors and plant hosts, and geographic range

Virus	*Vector*[a]	*Plant hosts*[b]	*Range*[c]
Rice stripe virus (RSV)	*Laodelphax striatellus*	Rice (*Oryza sativa*)	Asia, Far East
Maize stripe virus (MSpV)	*Peregrinus maidis*	Maize (*Zea mays*)	Worldwide tropics and subtropics
Rice grassy stunt virus (RGSV)	*Nilaparvata lugens*	Rice	Asia, Far East
Rice hoja blanca virus (RHBV)	*Tagosodes oryzicola*	Rice	American tropics and subtropics
Echinochloa hoja blanca virus (EHBV)	*Tagosodes cubanus*	Rice, *Echinochloa colona*	American tropics and subtropics
Urochloa hoja blanca virus (UHBV)	*Caenodelphax teapae*	*Urochloa* spp.	

[a] All Tenuivirus insect vectors are planthoppers in the Delphacidae. The name indicates the main vector.
[b] Indicates the primary economically important host. All tenuiviruses have more extensive experimental host ranges (Falk and Tsai, 1998).
[c] Indicates the main natural geographic distribution.

are phloem feeders, and colonize plants within the family Poaceae. The planthopper vector can acquire the corresponding tenuivirus by feeding from a virus-infected plant and, once acquired, the planthopper generally retains the ability to transmit the corresponding tenuivirus for the remainder of its life. Immediately after acquisition there is a defined incubation period before subsequent transmission to new host plants is possible. This incubation period is fairly long, measured in days, and is suggestive that viral multiplication in the planthopper is a prerequisite before the tenuivirus can subsequently be transmitted to new plant hosts. Further evidence supporting the replication of tenuiviruses in their planthopper vectors is that RHBV, RSV and MSpV can be transovarially transmitted from viruliferous female planthoppers to their progeny, and RHBV can be paternally transmitted to offspring.

Proof for multiplication of tenuiviruses in their planthopper vectors has been demonstrated by using different approaches. For example, RHBV has been maintained in *Tagosodes orizicola* planthoppers for up to ten generations, and RSV has been maintained in *Laodelphax striatellus* planthoppers for 40 generations. Serological analyses have been used to show definitively that MSpV antigens increase over time in *Peregrinus maidis* planthoppers after MSpV acquisition (either by plant feeding or intrathoracic injec-

Figure 1 Two *Zea mays* plants showing late and early symptoms, respectively, of infection by the tenuivirus maize stripe virus (MSpV). Leaves in late infections can turn completely white, while in early infections white stripes begin to appear along veins. (With permission, from the *Annual Review of Phytopathology*, Volume 36, copyright 1998, by Annual Reviews.)

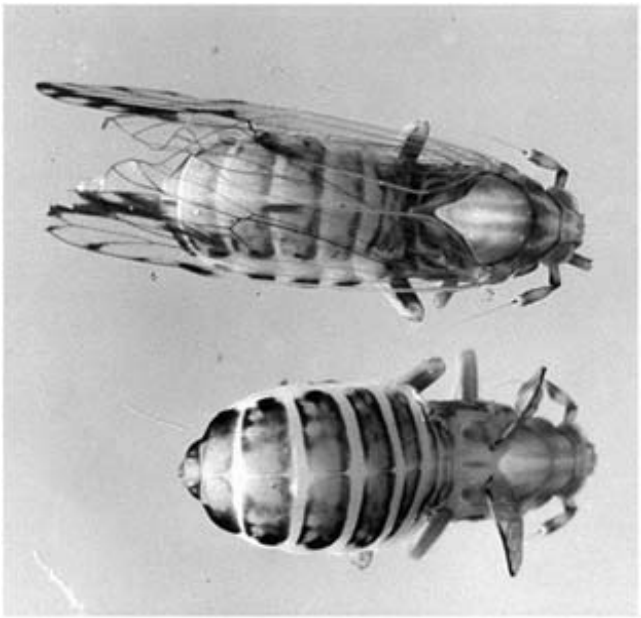


Figure 2 *Peregrinus maidis*, the delphacid planthopper vector of MSpV. The upper planthopper is the long-wing adult form; the lower insect is the short-wing adult. (With permission, from the *Annual Review of Phytopathology*, Volume 36, copyright 1998, by Annual Reviews.)

tion), providing conclusive evidence that MSpV replicates in *P. maidis*.

Even though transmission of a given tenuivirus is specific, it appears that vector specificity may be even further affected by the genetic composition within the vector species population. There are 'transmitters' and 'nontransmitters' for RHBV within *T. oryzicola* populations. The transmitters are competent RHBV vectors, while the nontransmitters are not, and transmitters are capable of supporting RHBV replication, whereas nontransmitters are not. By making crosses between individuals of these two types, the ability to support RHBV replication, and thus transmit RHBV to plants, was shown to be due to a single recessive gene. The above characteristics show that Tenuivirus transmission by planthopper vectors can be classified as circulative-propagative, and is highly specific. Furthermore, the host ranges of tenuiviruses include plants and animals, the latter being their planthopper vectors.

Geographic and Seasonal Distribution

Tenuiviruses are not universally common plant viruses, but they are generally somewhat limited in their geographical distribution, probably due in part to limits for the natural geographical ranges of their planthopper vectors. RSV and RGSV appear to be limited to rice growing areas of Asia, and RHBV and EHBV are limited to tropical and subtropical rice growing regions of the Americas. Only MSpV is found in both the New and Old Worlds. MSpV has been reported from North, Central and South America, Australia and parts of Africa. However, MSpV and its planthopper vector, *Peregrinus maidis*, are limited to subtropical and tropical maize-producing regions in these areas.

Even though they are somewhat limited in their natural incidence, the tenuiviruses are still of considerable economic importance. Their primary host plants are important food crops including rice (*Oryza sativa*) and maize (*Zea mays*), and disease incidence can be severe. For example, RHBV, which causes the only virus disease of rice in the western hemisphere, has sporadically affected rice production in many tropical and subtropical rice production areas of the Americas, and yield losses of 25–50% have been reported. When RHBV was identified in Florida, USA, in the 1950s, a quarantine on Florida rice production was implemented in hopes of preventing RHBV spread into rice producing areas of nearby states. Rice production in southern Florida did not resume until the late 1970s, and now it is a minor crop. RHBV is still of considerable importance in the rice growing regions of Latin America. In their respective geographic regions, RSV and RGSV cause similarly important diseases in rice. RSV has been reported to affect as much as 19% of the total rice acreage in Japan. MSpV causes annual losses in maize in various regions, and losses of 80% have been reported in specific maize growing regions.

Virus Structure and Composition

The name tenuivirus is derived from the nature of the slender (tenuous), filamentous ribonucleoprotein particles (RNPs) associated with the tenuiviruses. RNPs have been identified by electron microscopy in cells of tenuivirus-infected plant and insect hosts. The RNPs also have been purified from plants infected by several different tenuiviruses, including MSpV, RSV, RGSV, EHBV and RHBV. Electron microscopic analysis has shown the RNPs to be threadlike, very thin in diameter (8–10 nm; **Fig. 3**) and often without defined lengths. Frequently the RNPs appear circular, but under various conditions they can exhibit varying degrees of supercoiling.

Rate-zonal sucrose density gradient centrifugation analysis has shown that RNP preparations for specific tenuiviruses can be resolved into four or five distinct RNPs. The sedimentation coefficients of the five MSpV RNPs range from about 70 to 190 S for the slowest to fastest sedimenting RNPs, respectively. Biochemical analyses have shown that RNPs are composed of single-stranded RNA encapsidated

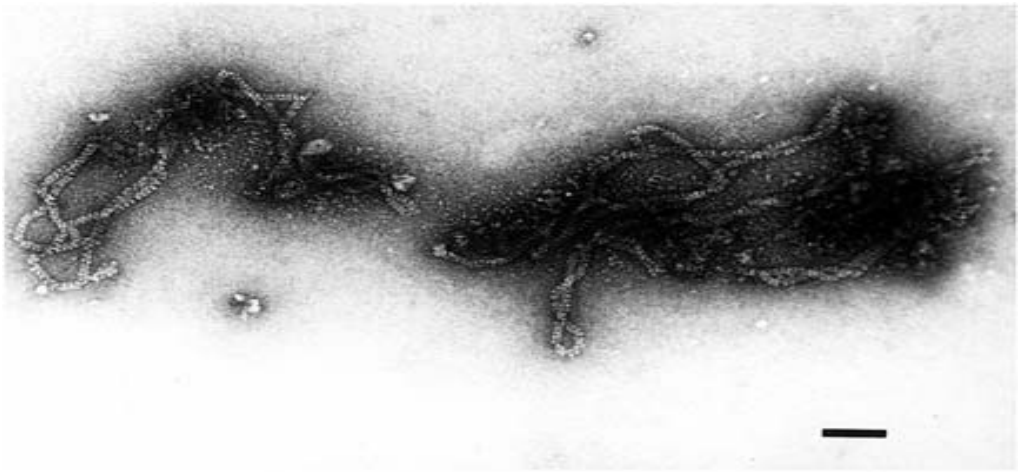


Figure 3 Transmission electron micrograph showing purified rice hoja blanca virus (RHBV) RNPs. Note circular filamentous nature of the particles. Bar = 50 nm. (Courtesy of Dra. A. M. Espinoza Esquivel, University of Costa Rica.)

essentially by a single protein species, the nucleocapsid (N) protein. The N proteins for all tenuiviruses are similar in size, about 32 000–35 000 mol.wt. In addition to the N protein, purified RNP preparations often contain small amounts of a large protein of greater than 200 000 mol.wt which is believed to be the tenuivirus RNA-dependent RNA polymerase.

Four or five distinct genomic RNAs have been isolated from purified RNPs of different tenuiviruses. The overall pattern and sizes of the RNP RNAs for different tenuiviruses, as determined by denaturing agarose gel electrophoresis, are very similar. For MSpV, sizes are estimated to be about 8.3, 3.3, 2.4, 2.2 and 1.3 kb for RNAs 1–5, respectively. Northern hybridization and nucleotide sequence analyses have shown that each of these RNAs is largely distinct, and representative of a single genome segment. Thus the multipartite genomes of tenuiviruses like MSpV are among the largest (*c.* 18 kb) genomes for the plant viruses.

Analysis of individually purified MSpV and RSV RNPs has shown that the different sized genomic RNAs are separately encapsidated in different RNPs. The smallest RNA (RNA 5) can be isolated from the slowest sedimenting RNP, with larger RNAs being found in faster sedimenting RNPs, respectively. Furthermore, careful studies with RSV have shown that the circumference of the filamentous, circular RNPs is proportional to the size of the encapsidated RNA. The five RSV RNPs are estimated to be *c.* 290 nm, 510 nm, 610 nm, 840 nm and 2110 nm in circumference for RNPs 1–5, respectively.

The RNP RNAs are believed to be single-stranded. However, under appropriate conditions, both single- and double-stranded RNAs have been detected in RNAs extracted from purified RNPs of MSpV, RGSV, EHBV and RSV. This was somewhat surprising when originally discovered, as it seemed unlikely that both single- and double-stranded RNAs would be contained within the RNPs. It is now believed that only single-stranded RNAs are encapsidated, but that for each size RNA (e.g. RNA 1), complementary molecules are separately encapsidated in different RNPs. This is supported by data which show that mostly single-stranded RNAs are detected when MSpV RNPs are disrupted with detergent and the RNAs immediately analyzed by gel electrophoresis. However, when RNP RNAs for MSpV and RSV are isolated using SDS and phenol, concentrated by ethanol precipitation, and subsequently analyzed by nondenaturing gel electrophoresis, both single- and double-stranded RNAs are detected. The double-stranded RNAs presumably arise *in vitro* by hybridization of complementary strands for each RNA.

Nucleic acid hybridization data support the above observations. Opposite polarity, complementary molecules for each of the five MSpV RNAs have been identified from purified RNPs. However, unequal amounts of the complementary molecules for all five MSpV RNAs are present. Northern hybridization studies of denatured and nondenatured MSpV RNP RNAs suggested that each of the five RNP RNAs is represented primarily as one polarity; however, smaller amounts of the complementary polarity molecules for each RNA are also present. Thus, upon annealing *in vitro*, some double-stranded RNA is formed while the RNA in excess then remains as single-stranded RNA. For a given genomic segment,

that polarity which is most abundant is referred to as vRNA, while that which is less abundant is referred to as vcRNA (viral complementary RNA).

The above characteristics are consistent and characteristic features of the tenuiviruses; however, the properties of the RNPs and the molecular biology of tenuiviruses (see below) suggest that the circular RNPs may not be true virions, but possibly they are components of a more complex virion. At this time the exact nature of tenuivirus virions remains in question.

Genome Composition and Structure

The different sized Tenuivirus RNAs are distinct genome components. Infectivity experiments demonstrating whether or not all five of the tenuivirus RNAs are necessary for competent infections have not been done. However, nucleic acid hybridization analyses have shown that for a given tenuivirus the genomic RNAs do not have a high degree of nucleotide sequence homology with each other, and nucleotide sequence analyses show that the individual genomic RNA segments have distinct organizations encoding different proteins.

The genomic RNAs of several tenuiviruses have been sequenced and characterized. While the terminal structures on the Tenuivirus genomic RNAs have not been definitively identified, the 5′ termini do not contain a VPg, nor are they capped. Genomic RNAs of both RSV and MSpV can be readily radiolabeled *in vitro* by using polynucleotide kinase. Similarly, the 3′ ends of the RSV genomic RNAs have been labeled using T4 RNA ligase, suggesting that the 3′ terminus is an OH group. Nucleotide sequence analyses also have shown that the tenuivirus genomic RNAs have conserved and complementary 5′ and 3′ termini. There are 11 nucleotides at the 5′ termini and ten nucleotides at the 3′ termini, which are largely conserved not only among the five genomic RNAs of a given tenuivirus, but also between different tenuiviruses (**Fig. 4**). The minor exceptions are that RSV RNA 1 and MSpV RNA 5 have only 9 of the 10 conserved nucleotides at their 3′ termini. It is also of interest that the terminal eight nucleotides at both the 5′ and 3′ termini are identical to those found in the vertebrate-infecting viruses of the genus *Phlebovirus* (family *Bunyaviridae*). The genomic RNA 5′ and 3′ terminal nucleotide sequences also are complementary (**Fig. 4**). The 3′ 17 terminal nucleotides are complementary to 17 of the 18 5′ terminal nucleotides. A model has been proposed for RSV which allows the 11th nucleotide from the 5′ end to bulge out so that the 17 complementary nucleotides at each terminus could base pair and form a hairpin structure.

RNA 1

5′-**ACACAAAGUCC**
3′-**UGUGUAUCAG***

RNA 2

5′-**ACACAAAGUCC**
3′-**UGUGUUUCAG***

RNA 3

5′-**ACACAAAGUCC**UGGGUAA
3′-**UGUGUUUCAG***ACCCAUU

RNA 4

5′-**ACACAAAGUCC**AGGGCAU
3′-**UGUGUUUCAG***UCCCGUA

RNA 5

5′-**ACACAAAGUCC**UUGGCAC
3′-**UGUGUAUCAG***AACCGUG

Phlebovirus

5′-**ACACAAAG**ACC
3′-**UGUGUUUCUG**

Figure 4 Terminal nucleotide sequences of *Tenuivirus* and *Phlebovirus* RNAs. For the conserved and complementary 5′ and 3′ nucleotide sequences for tenuivirus genomic RNAs, the nucleotides shown in bold are conserved among the RNA sequences determined so far. For the *Phlebovirus* sequence, the nucleotides shown in bold are those which are identical to those found for tenuiviruses. The asterisk (*) in the 3′ terminal sequences represents a space inserted so as to align 5′ and 3′ nucleotides for complementary base pairing.

Such complementarity of RNA terminal nucleotide sequences is largely unique among the plant viruses, with the exception that the genomic RNAs of tomato spotted wilt virus (genus *Tospovirus*, family *Bunyaviridae*) also exhibit this feature. The feature is common among vertebrate-infecting viruses with segmented, negative-sense RNA genomes in the families *Bunyaviridae, Arenaviridae* and *Orthomyxoviridae*.

It is of interest to note that for La Crosse virus (genus *Bunyavirus*, family *Bunyaviridae*) the complementary genomic RNA termini exist in infected cells and in RNPs as base paired, stable panhandle structures. It is believed that because the La Crosse virus RNA termini are base paired within the RNPs, this could play a role in determining the circularity of the RNPs. Interestingly, the tenuivirus circular filamentous RNPs are morphologically very similar to those of La Crosse virus, and it is tempting to speculate that the tenuivirus RNA 5′ and 3′ termini are also base paired, and that this feature, at least in part, contributes to the circular nature of the characteristic tenuivirus RNPs.

Nucleotide sequence analyses have shown that the different tenuivirus genomic RNAs exhibit different genetic organizations and encode different proteins. RSV vRNA 1 is 8970 nt and of negative polarity. The vcRNA1 potentially encodes for a protein of 336 860. The smallest tenuivirus genomic RNA, RNA 5

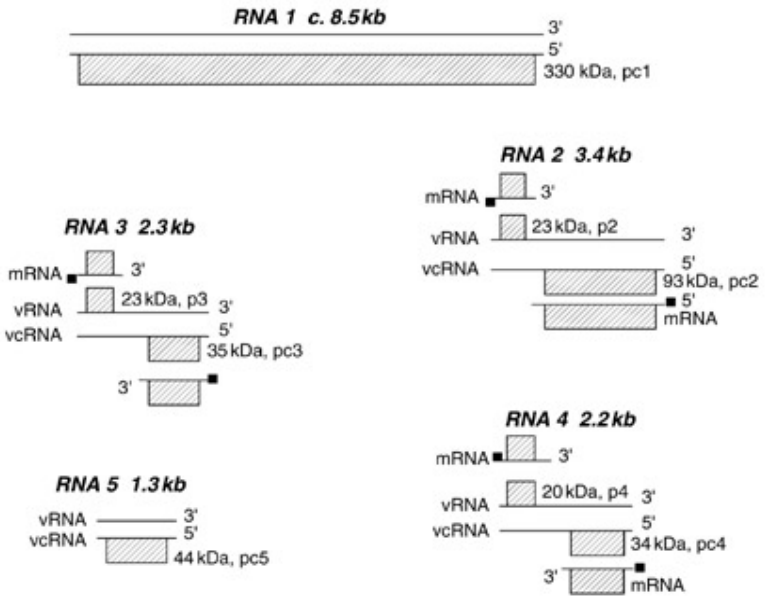


Figure 5 Generic genome map and expression strategy for viruses in the genus *Tenuivirus*. Five tenuivirus genomic RNAs and their approximate sizes (kb) are indicated. vRNA and vcRNA indicate the viral and viral complementary RNAs, respectively. Hatched boxes indicate ORFs. For the ambisense RNAs (RNAs 2, 3 and 4), mRNAs are indicated below or above the vRNA or vcRNA. Black boxes indicate capped 5′ termini on these mRNAs. No caps are shown on the monocistronic RNAs, but it is likely these mRNAs are also capped. Names and sizes of proteins (kDa) are shown.

(1317 nt) is also of negative polarity. MSpV vcRNA 5 encodes a protein, pc5, of 44 000 mol.wt. Analyses of pc5 show it to be highly basic in nature, containing 21% arginine and lysine, but as yet its function is unknown.

The organizations of tenuivirus genomic RNAs 2, 3 and 4 is more complex. Both the vRNA and vcRNAs for each genomic segment encode proteins, thus the coding strategy is ambisense (**Fig. 5**). MSpV vRNA 2 encodes a small hydrophobic protein (23 500 mol.wt) while vcRNA 2 encodes a 93 900 mol.wt protein. Because the vRNA and vcRNA each encode proteins, the terminology used to refer to the respective proteins is determined by whether they are encoded by vRNA or vcRNA. The vRNA 2-encoded protein is designated p2, while the vcRNA 2-encoded protein is pc2 (the c for a protein encoded by the vcRNA). Thus, for the remaining ambisense MSpV genomic RNAs, RNAs 3 and 4, p3 is 22 700 mol.wt, pc3 is 35 000 mol.wt, p4 is 19 800 mol.wt and pc4 is 31 900 mol.wt.

The functions of all of the tenuivirus-encoded proteins are not known. The pc1 is believed to be the RNA-dependent RNA polymerase (RDRP). Amino acid sequence analyses have shown that pc1 contains features characteristic of RDRPs, and it exhibits significant similarity to the RDRPs (L proteins) found for viruses in the genus *Phleobvirus*. The function of p2 is unknown, but computer predictions suggest it to be membrane-associated within infected host cells. The second RNA 2-encoded protein, pc2, exhibits significant similarity to the virion membrane glycoproteins found for viruses in the genus *Phlebovirus*. Interestingly, so far no membrane-bound virions have yet been identified for tenuiviruses. The pc2 is *c.* 94 000 mol.wt and for MSpV contains the proteolytic cleavage sequence conserved among the glycoproteins for Phleboviruses. VcRNA 3 encodes the *c.* 35 000 mol.wt nucleocapsid (N) protein. The vRNA 3 encodes p3, a protein of *c.* 23 000 mol.wt and for which no function is yet identified. The vRNA 4 encodes p4, a protein of *c.* 20 000 mol.wt. P4 is the most abundant protein found in tenuivirus-infected plants (see below). The vcRNA 4 encodes pc4, a protein of *c.* 32 000 mol.wt and of unknown function. Some of these proteins are discussed in more detail below, under Virus–Host Relationships or Serological and Nucleic Acid-Based Relationships.

Gene Expression Strategies

The tenuivirus full-length genomic RNAs are not mRNAs. During tenuivirus infection and replication, specific subgenomic mRNAs are generated for the ambisense genomic RNAs (RNAs 2, 3 and 4; see **Fig. 5**), and for MSpV, with the exception of that for p4,

subgenomic mRNAs have been identified both in MSpV-infected plants and planthoppers. As tenuivirus genomic RNAs 1 and 5 are monocistronic, it is likely that mRNAs for pc1 and pc5 are essentially full-length, but probably are modified from those found in RNPs.

The subgenomic mRNAs representing the RNA 2, RNA 3 and RNA 4 open reading frames (ORFs) have been detected in MSpV-infected tissues, but not in purified RNPs. The sizes of the RNA 2, RNA 3 and RNA 4 subgenomic mRNAs are *c.* 700 and 2600 nt for p2 and pc2; 650 and 1350 nt for p3 and pc3; and *c.* 950 and 1000 nt for p4 and pc4, respectively. In agreement with the polarities of the ORFs contained in each of the ambisense genomic RNAs, the subgenomic mRNAs for a given RNA segment are of opposite polarity to each other, and it is likely that subgenomic mRNAs for a given genomic RNA are not overlapping. The opposing ORFs on a specific genomic RNA are separated by relatively large A-U rich intergenic regions (>350 nt). Whether or not these intergenic regions play a role in mRNA transcription termination is not yet known.

The subgenomic mRNAs correspond in nucleotide sequence with the 5′ regions of the genomic RNA segments; however, the 5′ terminal nucleotide sequences for a given genomic RNA (i.e. vRNA 4) and the corresponding mRNA (i.e. that for the p4 ORF) are not identical. The 5′ termini for several tenuivirus mRNAs have been shown to contain short leader sequences of heterogeneous nucleotide composition, and these are immediately 5′ of the viral RNA sequence. Furthermore, and in contrast to the tenuivirus genomic RNAs, the mRNAs have a 5′ cap. It is believed that these capped leader sequences are generally of host mRNA origin and originate by 'cap snatching'.

Cap snatching is a feature which was first described for influenza virus, and which was subsequently shown to occur for other vertebrate-infecting viruses such as those in the families *Orthomyxoviridae, Arenaviridae* and *Bunyaviridae*. During mRNA transcription, host mRNAs are recruited and a virus-encoded cap-specific endonuclease cleaves the host mRNA several nucleotides downstream of the 5′ cap. The resulting 5′-capped sequence is then used as a primer for mRNA synthesis. A few of the 3′ nucleotides base pair with the mRNA template (the 3′ end of the full-length RNAs) and the short, capped ribonucleotide serves as a primer for transcription. Thus, the mRNA gains the 5′ cap and short leader sequence of the donor mRNA. The specificity of donor RNAs that can serve as mRNA primers is not yet known, but generally the capped leader sequences are short, only 10–16 nt.

Virus–Host Relationships

One of the most diagnostic features of tenuivirus infections of plants is the presence of large amounts of the tenuivirus-encoded p4 protein in plant tissues. P4 is the most abundant protein in MSpV-infected plant tissues and can be found at levels exceeding $10\,mg\,g^{-1}$. P4 can easily be detected from tenuivirus-infected plants by using serological methods, or directly by light and/or electron microscopy. Within infected plant cells, p4 aggregates into large crystalline structures or inclusion bodies which can occupy significant portions of the plant cell (**Fig. 6**). Similar p4 aggregates can be formed *in vitro*. The inclusion bodies found in the cell cytoplasm are not viroplasms or sites of tenuivirus replication, and p4 is not associated with Tenuivirus RNAs or the RNPs. As such, p4 has been referred to as the major noncapsid protein (NCP). The p4-containing inclusion bodies are detectable by light and/or electron microscopy and are of various morphological types. Light microscopic analysis has shown inclusion bodies to be needle-shaped, ring-like and even in figure-of-eight amorphous structures. Transmission electron microscopy has revealed that these structures can have two types of substructure. The amorphous, semielectron opaque inclusion bodies are the most abundant, sometimes appearing to fill almost the entire cytoplasm. Filamentous, electron-opaque bodies also can be seen but are generally less abundant. All of the above seem to be composed only of p4, and thus the various types may represent different developmental stages. Despite its abundance in tenuivirus-infected plants, p4 has not been detected in MSpV-infected *P. maidis*, the MSpV planthopper vector. The reason for the differential abundance of p4 in plant as opposed to insect hosts is not known; however, RNA hybridization analyses have shown that the p4 ORF subgenomic RNA is highly abundant in MSpV-infected plants, but has not been found in MSpV-infected *P. maidis*. In contrast, other tenuivirus-coded proteins, such as the nucleocapsid protein (N protein; pc3), and their respective subgenomic RNAs can be detected both in MSpV-infected plant and insect hosts.

Serological and Nucleic Acid-Based Relationships

Antibodies to two tenuivirus proteins, p4 (the NCP) and pc3 (N protein), have been generated for most tenuiviruses, and some serological comparisons have been done. Using stringent serological tests, antibodies to each of these proteins have generally shown positive reactions only with corresponding proteins of

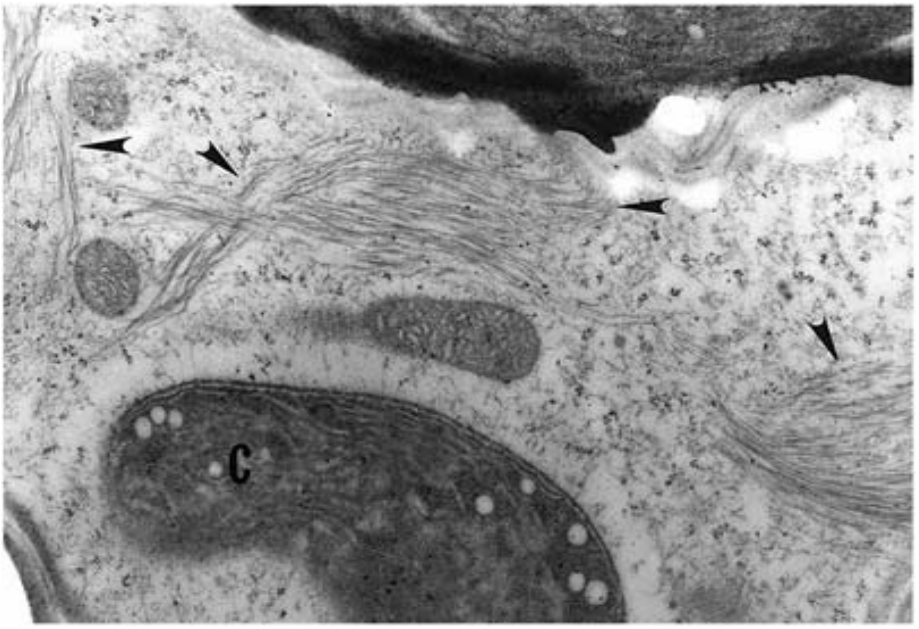


Figure 6 Transmission electron micrograph of a cell of a MSpV-infected *Zea mays* plant. C indicates a chloroplast. Arrows indicate the MSpV typical p4 (NCP) needle-shaped inclusions.

their respective tenuivirus. However, by using less stringent serological tests, slight serological cross-reactions have been obtained between similar proteins encoded by different tenuiviruses. The N proteins of RSV and MSpV are distantly serologically related, as are those of RSV and RGSV. However, the MSpV N protein does not show a serological relationship to those of RGSV or RHBV. Only slight serological crossreactions were detected when the MSpV and RHBV NCPs were compared. Thus, current data suggest that these respective Tenuivirus-encoded proteins are generally serologically distinct from each other.

More recently, computer-based comparisons of tenuivirus genomic RNA nucleotide sequences, and comparisons of deduced amino acid sequences for tenuivirus-encoded proteins have shown that the tenuiviruses are closely related, and they have some specific similarities with some vertebrate-infecting viruses. The genome organizations are similar for all tenuiviruses, and the respective encoded proteins exhibit detectable degrees of similarity. However, some of the tenuivirus-encoded proteins exhibit similarity to related proteins of other viruses. Tenuivirus pc1, pc2 and pc3 all show similarities with proteins encoded by the genomes of viruses within the genus *Phlebovirus*. The RSV pc1, the putative RDRP, shows *c.* 30% identity, between residues 493–2026, with the L protein of viruses in the genus *Phlebovirus*. RSV pc1 also contains other features found in phlebovirus L proteins, including the four conserved polymerase motifs. The MSpV pc2 shares features found for the phlebovirus glycoproteins, including a putative conserved cleavage site, and pc3 (the N protein) showed significant similarity to the N proteins for two viruses in the genus *Phlebovirus*.

Biologically the viruses in the genera *Phlebovirus* and *Tenuivirus* are distinct, but still have some general similarities. Phleboviruses infect their invertebrate vectors as well as their vertebrate hosts, while tenuiviruses infect their invertebrate vectors (planthoppers) and plants. Distinct membrane-bound spherical virions are typical for phleboviruses. Within these are three RNPs containing the three genomic RNAs. No similar virions are yet found for tenuiviruses, but RNPs similar to those of the phleboviruses are common, and these separately contain the five tenuivirus genomic RNAs. The genomic RNAs of tenuiviruses and phleboviruses have eight identical nucleotides at their 5′ and 3′ termini. Finally, viruses in these two genera exhibit somewhat similar genome organizations, coding arrangements and expression strategies, and some of their gene products show significant similarity. Thus, tenuiviruses and phleboviruses appear to have a common evolutionary lineage.

Epidemiology and Control

Tenuivirus epidemiology is determined in part by the biology and behavior of their planthopper vectors. Delphacid planthoppers are wing dimorphic, having both short- and long-winged adult forms (**Fig. 2**). Short-wing forms are capable of spreading tenui-

viruses over short distances by walking from plant to plant. Long-winged forms can disperse by active flight and as such can spread tenuiviruses over long distances (several kilometers). Planthopper vector populations fluctuate seasonally, and corresponding tenuivirus-induced diseases also fluctuate seasonally and from year to year. However, when crop incidence corresponds with high planthopper populations or activity, diseases caused by tenuiviruses often result. For example, in Florida MSpV disease in *Z. mays* occurs in fall-planted maize crops. *P. maidis* populations build up on alternate grasses during the summer months. As the fall-planted maize plants emerge, planthoppers move into the young succulent plants and severe disease can result. Later fall, or winter or spring plantings are not as severely affected. A similar situation has been reported from Japan for RSV, and when high planthopper populations coincide with young plants, resulting disease can be severe.

Tenuivirus disease control strategies based upon epidemiological data (i.e. late planting to avoid planthopper activity) may offer some help in controlling diseases caused by tenuiviruses; however at present, forecasting tenuivirus epidemics has not proven to be reliable, and thus this has hampered using tactics such as delayed planting. Both plant-derived and genetically engineered forms of resistance to tenuiviruses in plants have been described. Genetic resistance seems to be the most useful and effective strategy for attempting to control RHBV, RGSV and RSV in rice. Continued plant breeding efforts will probably be necessary to provide long-term tenuivirus disease control.>

See also: ***Bunyaviridae*: General features; Influenza viruses (*Orthomyxoviridae*): Molecular biology; Tospoviruses (*Bunyaviridae*).**

Further Reading

Falk BW and Tsai JH (1998) Biology and molecular biology of viruses in the genus *Tenuivirus*. *Annu. Rev. Phytopathol.* 36: 139–163.

Ramirez B-C and Haenni A-L (1994) Molecular biology of tenuiviruses, a remarkable group of plant viruses. *J. Gen. Virol.* 75: 467.

Toriyama S (1995) In: Singh RP, Singh US and Kohomoto K (eds) *Pathogenesis and Host Specificity in Plant Diseases*. Vol. 3: *Viruses and Viroids*. Oxford: Pergamon Press.

TETRAVIRUSES (*TETRAVIRIDAE*)

Terry N Hanzlik and **Karl HJ Gordon**, CSIRO Entomology, Canberra, Australia

Introduction and History

The *Tetraviridae* are a family of viruses isolated exclusively from lepidopteran insects (moths, butterflies) that have a positive-sense, single-stranded (ss) RNA genome encased in an icosahedral capsid. They were initially isolated and characterized in Australia and South Africa in the 1960s from cadavers of emperor moths. The extensive work on the viruses isolated from the South African *Nudaurelia capensis cyntherea* (pine emperor moth) led them to being known for a while as the *Nudaurelia* β-like group. The distinctive $T = 4$ quasi-symmetry of the capsid's icosahedral structure focused initial attention on the group and engendered the family's present name (from the Greek *tettares*, four). Tetraviruses have been difficult to study because of their limited availability, which was restricted to field-collected insect cadavers, and because of the inability to culture them in cell lines. These difficulties, combined with a perceived lack of economic importance, served to hinder work on the group until new strategies allowed new ways of looking at, and working with, tetraviruses. These studies indicate that the virology of tetraviruses has general implications and that it can be exploited in exciting new ways for agriculture and medicine.

Taxonomy and Serology

Earlier, viruses were classified as members of the family on the basis of a positive serological reaction to sera raised against a few well-characterized tetraviruses. Most of the members noted in **Table 1** have been classified on this basis. Now, however, analysis of physical characteristics is the favored approach, with serology usually used at a later stage. Once an icosahedral capsid containing an ssRNA genome has been ascertained, two strong indicators of tetraviruses

viruses over short distances by walking from plant to plant. Long-winged forms can disperse by active flight and as such can spread tenuiviruses over long distances (several kilometers). Planthopper vector populations fluctuate seasonally, and corresponding tenuivirus-induced diseases also fluctuate seasonally and from year to year. However, when crop incidence corresponds with high planthopper populations or activity, diseases caused by tenuiviruses often result. For example, in Florida MSpV disease in *Z. mays* occurs in fall-planted maize crops. *P. maidis* populations build up on alternate grasses during the summer months. As the fall-planted maize plants emerge, planthoppers move into the young succulent plants and severe disease can result. Later fall, or winter or spring plantings are not as severely affected. A similar situation has been reported from Japan for RSV, and when high planthopper populations coincide with young plants, resulting disease can be severe.

Tenuivirus disease control strategies based upon epidemiological data (i.e. late planting to avoid planthopper activity) may offer some help in controlling diseases caused by tenuiviruses; however at present, forecasting tenuivirus epidemics has not proven to be reliable, and thus this has hampered using tactics such as delayed planting. Both plant-derived and genetically engineered forms of resistance to tenuiviruses in plants have been described. Genetic resistance seems to be the most useful and effective strategy for attempting to control RHBV, RGSV and RSV in rice. Continued plant breeding efforts will probably be necessary to provide long-term tenuivirus disease control.>

See also: ***Bunyaviridae*: General features; Influenza viruses (*Orthomyxoviridae*): Molecular biology; Tospoviruses (*Bunyaviridae*).**

Further Reading

Falk BW and Tsai JH (1998) Biology and molecular biology of viruses in the genus *Tenuivirus*. *Annu. Rev. Phytopathol.* 36: 139–163.

Ramirez B-C and Haenni A-L (1994) Molecular biology of tenuiviruses, a remarkable group of plant viruses. *J. Gen. Virol.* 75: 467.

Toriyama S (1995) In: Singh RP, Singh US and Kohomoto K (eds) *Pathogenesis and Host Specificity in Plant Diseases*. Vol. 3: *Viruses and Viroids*. Oxford: Pergamon Press.

TETRAVIRUSES (*TETRAVIRIDAE*)

Terry N Hanzlik and **Karl HJ Gordon**, CSIRO Entomology, Canberra, Australia

Introduction and History

The *Tetraviridae* are a family of viruses isolated exclusively from lepidopteran insects (moths, butterflies) that have a positive-sense, single-stranded (ss) RNA genome encased in an icosahedral capsid. They were initially isolated and characterized in Australia and South Africa in the 1960s from cadavers of emperor moths. The extensive work on the viruses isolated from the South African *Nudaurelia capensis cyntherea* (pine emperor moth) led them to being known for a while as the *Nudaurelia* β-like group. The distinctive $T = 4$ quasi-symmetry of the capsid's icosahedral structure focused initial attention on the group and engendered the family's present name (from the Greek *tettares*, four). Tetraviruses have been difficult to study because of their limited availability, which was restricted to field-collected insect cadavers, and because of the inability to culture them in cell lines. These difficulties, combined with a perceived lack of economic importance, served to hinder work on the group until new strategies allowed new ways of looking at, and working with, tetraviruses. These studies indicate that the virology of tetraviruses has general implications and that it can be exploited in exciting new ways for agriculture and medicine.

Taxonomy and Serology

Earlier, viruses were classified as members of the family on the basis of a positive serological reaction to sera raised against a few well-characterized tetraviruses. Most of the members noted in **Table 1** have been classified on this basis. Now, however, analysis of physical characteristics is the favored approach, with serology usually used at a later stage. Once an icosahedral capsid containing an ssRNA genome has been ascertained, two strong indicators of tetraviruses

Table 1 Members of the *Tetraviridae*

Virus	*Acronym*	*Family of host*	*Geographic location*
Genus: Betatetravirus			
Nudaurelia capensis β virus[a]	NβV	Saturniidae	South Africa
Antheraea eucalypti virus[b]	AeV	Saturniidae	Australia
Darna trima virus	DtV	Limacodidae	Malaysia
Dasychira pudibunda virus[c]	DpV	Lymantriidae	UK
Philosamia cynthia × *ricini* virus	PxV	Saturniidae	UK
Pseudoplusia includens virus	PiV	Noctuidae	USA
Thosea asigna virus[d]	TaV	Limacodidae	Malaysia
Trichoplusia ni virus	TnV	Noctuidae	USA
Genus: Omegatetravirus			
Nudaurelia capensis ω virus[a]	NωV	Saturniidae	South Africa
Helicoverpa armigera stunt virus	HaSV	Noctuidae	Australia
Unassigned possible members[e]			
Acherontia atropas virus	AaV	Sphingidae	Canary Islands
Agraulis vanillae virus	AvV	Nymphalidae	Argentina
Callimorpha quadripuntata virus	CqV	Arctiidae	UK
Eucocytis meeki virus	EmV	Cocytiidae	Papua New Guinea
Euploea corea virus	EcV	Danadidae	Australia/Germany
Hypocritae jacobeae virus	HjV	Arctiidae	UK
Lymantria ninayi virus	LnV	Lymantriidae	Papua New Guinea
Nudaurelia ε virus[f]	NεV	Saturniidae	South Africa

[a]Type virus for genus.
[b]Serological evidence shows identity to NβV.
[c]Host renamed *Calliteara pudibunda.*
[d]Host renamed *Setothosea asigna.*
[e]Viruses showing serological relationship to a known β-like tetravirus (excluding NεV), but otherwise uncharacterized.
[f]NεV resembles the tetraviruses but is serologically unrelated to any known member.

are a particle diameter of 35–41 nm and a density in CsCl of $<1.30\ g\ mL^{-1}$. These characteristics are not definitive, however, and so the International Committee on the Taxonomy of Viruses distinguishes them in its key from similar RNA viruses, such as caliciviruses, on the basis of their being an insect virus, having a major coat protein component of M_r >60 000, and the production of a coat protein from a separate RNA.

The family is unique for positive-strand icosahedral viruses in having two genera, *Betatetravirus* (β-type viruses) and *Omegatetravirus* (ω-type viruses), distinguished by the number of ssRNAs comprising their genomes. The ω-type genus has a bipartite genome and has the Nudaurelia ω-virus (NωV) as the type virus; the β-type genus has its complete genome on a single RNA strand and has the Nudaurelia β-type (NβV) virus as its type virus. Some confusion about classifying β-type viruses can result when a second RNA is extracted from their purified virions; this proves to be a coencapsidated subgenomic RNA, corresponding to the 3′ region of the genomic RNA. Particle morphology viewed from high-resolution electron micrographs may also be used to distinguish virus particles from the two genera on a tentative basis. Reconstructed images at 32 Å resolution may show three distinct pits in planar faces of β-like particles, which are not visible in the ω-like particles (**Fig. 1**).

Serological relationships among the viruses belonging to the two groups show a contrasting pattern. The β-type viruses show a wide relationship, with each member having a positive serological reaction to at least one other member of the genus. This is despite low sequence similarity, as viewed by hybridization at low stringency. However, the two known ω-type viruses do not show a serological relationship, even though they are highly similar at the sequence level.

Physical Properties

Properties of the structural proteins

As is apparent in **Table 2**, data obtained for many tetraviruses show only one major protein, usually with an M_r of >60 000. However, these data were

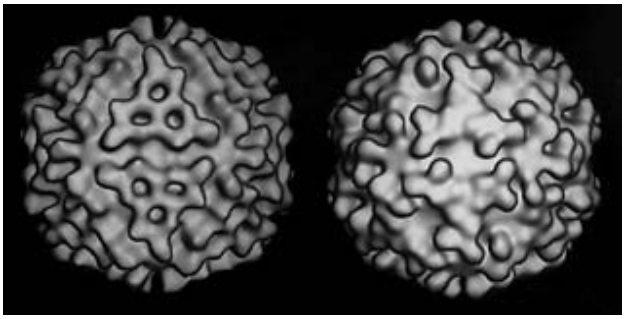


Figure 1 Image reconstructions of frozen hydrated particles of NβV (left) and NωV (right) analyzed by electron microscopy. (Courtesy of Norman Olson and Tim Baker).

obtained before more precise measurements on some tetraviruses showed the equimolar presence of a minor protein with an M_r of 7000–8000. Virions of all tetraviruses are now believed to have this protein constituent. While the minor protein is unmodified, the major protein has an N-terminal block of unknown structure. Also seen in SDS-PAGE profiles of tetravirus proteins extracted from purified virions are small amounts of a protein slightly larger than the major protein. This is a residual amount of an uncleaved protein that is the precursor to both the major and minor proteins. The cleavage takes place upon assembly of the capsid by a mechanism believed to be similar to that detailed for nodavirus capsids. The cleavage occurs towards the C-termini of the precursor proteins between Asn and Phe residues in ω-type tetraviruses and between Asn and Gly residues in β-type tetraviruses.

Properties of the genome

RNAs found encapsidated in tetravirus particles have the lengths shown in **Table 2.** As noted previously, a second species of subgenomic RNA of 2–3 kb in length may be found in minor amounts in β-type RNA, even though this group has a monopartite genome. The genomic RNA strands are capped on their 5′ ends, similarly to most mRNAs, but are not polyadenylated at the 3′ end. However, strong evidence indicates an intriguing tRNA-like secondary structure with a valine anticodon located at the 3′ terminus. The tetraviral tRNA-like structures are the only ones known for an animal virus and are distinct from the plant virus tRNA-like structures by not having a pseudoknot in the aminoacyl stem. Extensive secondary structure elsewhere on the tetraviral genomic RNAs is also likely.

Properties of the virion

Biophysical characteristics of tetravirus particles are listed in **Table 2.** The most precisely measured particle diameters are those of the type viruses, 39.7 nm for NβV and 41.0 nm for NωV. Measurements for other tetraviruses vary slightly below these values, which may be due to less precise measurements in photomicrographs. The equilibrium densities are generally at the low end of the range of densities ($<1.30\,g\,ml^{-1}$) displayed by unenveloped icosahedral viruses with ssRNA genomes. Tetraviral particles appear to be robust. They are resistant to chloroform extraction and retain stability upon exposure to a wide range of pH (3–11).

The distinctive structure of the tetraviral capsid has drawn much attention to the family, for it is the only capsid solely organized with $T = 4$ quasi-symmetry which allows 240 protein subunits to comprise the capsid. Other, more complex viruses have only substructures organized with this motif, while most other viruses with unenveloped icosahedral capsids have $T = 3$ quasi-symmetries that allow only a 180 subunit composition. Low-resolution studies from image reconstructions of electron micrographs show the 240 protein subunits are arranged in Y-shaped trimers on the surface of the particle (**Fig. 1**). Four each of the trimers compose the irregular triangles seen in **Fig. 1**, which have planar faces separated by deep grooves. The planar faces of the two β types studied in this manner (NβV and TeV) show the three distinct pits apparent in **Fig. 1**.

Further details of the unique character of the tetravirus capsid emerge from the high resolution (2.8 Å) structure of the NωV capsid determined by x-ray diffraction methods (**Fig. 2**). The protein subunit has three distinct domains located on the interior, middle and exterior of the capsid. The conformation of exterior domain is that of a c-type immunoglobulin-like (Ig-like) domain, making the tetravirus capsid the only known example of this structure found in an unenveloped icosahedral virus (one other Ig-like structure has been found on the more complex, enveloped dengue fever virus). The Ig-like structure and its association with binding, as well as its prominent location on the capsid's exterior, suggest that this domain is as least partly responsible for the tropism of NωV. The middle region of the NωV capsid protein is the canonical 'jelly-roll' possessed by all proteins forming icosahedral capsids and forms the contiguous shell of the capsid. The interior domain consists of the cleaved minor protein and the termini of the major protein. The minor protein is mostly helical in structure and appears to interact with the genomic RNAs, as does the N-terminus of the major protein. A hypothesis has been formed in which the helix of the minor protein forms a conduit at the fivefold axis for RNA to exit the capsid through a membrane during the uncoating process.

Table 2 Biophysical properties of tetraviruses

Virus	*Diameter (nm)*	*Density (g/ml^{-1})*	*Sedimentation coefficient (S)*	$e_{260/280}$	*Capsid protein (M_r)*	*RNA ($M_r \times 10^3$)*
Genus: Betatetraviruses						
NβV	39.7	1.295	210	1.45	60 572; 7975[a]	1800 (6.5 kb)[a]
AeV	32	ND	215	ND	ND	ND
TnV	35–38	1.3	200	ND	67 000–68 000	1900
DtV	35–38	1.289	199	1.44	62 000–66 000	ND
TaV	35	1.275	194	1.32	60 800	ND
PxV	35	1.275	206	1.36	62 400	ND
DpV	38	1.31	ND	1.43	66 000	1800
PiV	40	1.33	190	1.42	55 000	1900
Genus: Omegatetraviruses						
NωV	41.0	1.285	ND	ND	62 019; 7817[a]	RNA1=5300 bp RNA2=2448 bp[a]
HaSV	38	1.296	ND	1.22	63 378; 7309[a]	RNA1=5312 bp RNA2=2478 bp[a]
Unassigned possible members						
NεV	40.1	1.285	217	ND	ND	ND

ND, not determined.
[a]Data derived from sequence analysis.

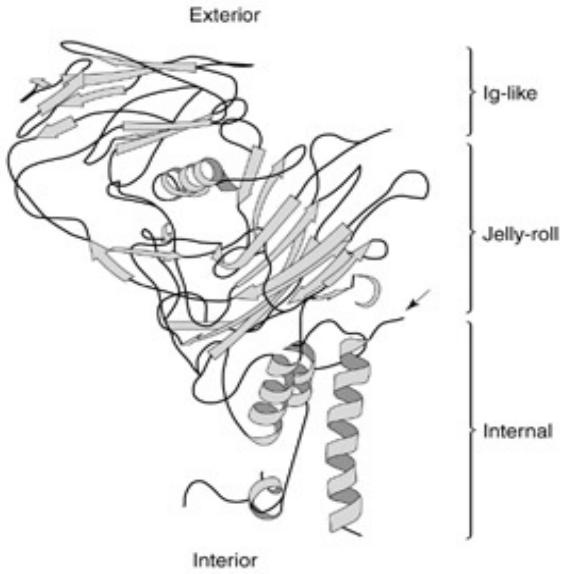


Figure 2 Schematic of the NωV coat proteins resolved to 2.8 Å. (Courtesy of Jack Johnson.) The three domains discussed in the text are noted on the right. The arrow points to the site of the assembly-dependent cleavage that produces the two coat proteins.

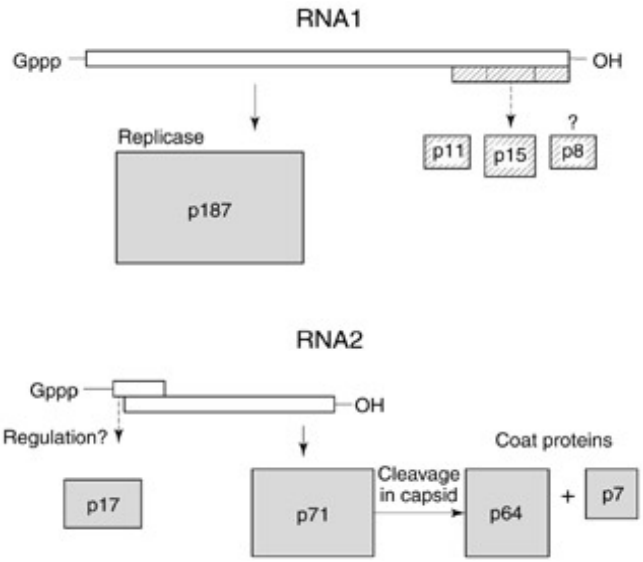


Figure 3 Genome organization and replication strategy of the ω-like tetravirus, HaSV. The genomic RNA1 and RNA2 with their ORFs are represented as open boxes above arrows leading to their functional protein products, represented as shaded rectangles enclosing their molecular weights. Dotted arrow indicates little, if any, production; question mark indicates incomplete understanding, while crosshatched rectangles indicate no evidence for expression.

Molecular Biology

Complete sequences are available for both β-type (NβV) and ω-type (HaSV) viruses. Comparison between the two sequences indicate a clear but distant relationship between the two genera. Detailed analysis of certain domains within the tetraviral sequences indicate the *Tetraviridae* belong to the α-like superfamily of RNA viruses. Analysis of the sequences give the major insights into how tetraviruses replicate, as experimental work is made difficult due to the lack of a cell culture system. However, new strategies for producing viral mutants and studying replication and coat protein assembly have given some insights into tetravirus virology.

ω-like genome organization

The complete sequence of the two RNA strands (RNA1 and RNA2) of the ω-like virus, HaSV, is available, while only RNA2 and a partial sequence of RNA1 exist for the only other known member of this genus, NωV. This information shows the gene products of the two viruses to be highly similar, except at certain key areas of the primary sequences of the coat proteins, as discussed below. Nearly the full length of HaSV RNA1 is accounted for by a single open reading frame (ORF) of 1704 codons encoding the 187 kDa replicase (**Fig. 3**). Experimental evidence exists for the function of this protein through its expression with recombinant baculoviruses (described below). It is not known whether the protein is processed into smaller fragments. Three smaller ORFs exist at the 3′ end of RNA1 out of frame with the replicase ORF which could encode proteins of 99, 140 and 73 amino acids. However, there is no evidence for a subgenomic RNA which could express them, nor is there a discernible relationship to other proteins to indicate their function, if any.

RNA2 of HaSV has two out-of-frame ORFs that overlap each other, with a 'leaky scanning' mechanism for ribosomal translation as the likely means of expressing both genes from the same RNA. The first gene has its initiating AUG at base 283 in poor context for translation initiation, which would allow most ribosomes to scan through to the second AUG in good context at base 366, which initiates the second gene. The function of the 16 522 Da product of the first gene remains unclear, although there is experimental evidence to suggest it is involved in the regulation of strand synthesis. The second gene encodes the 647 residue, 70 670 Da precursor protein for the major and minor coat proteins of HaSV. The coat protein gene for NωV encodes a slightly smaller protein than that of HaSV (644 residue) but the two are highly homologous (>67% overall identity) except for three regions of the primary sequence. The

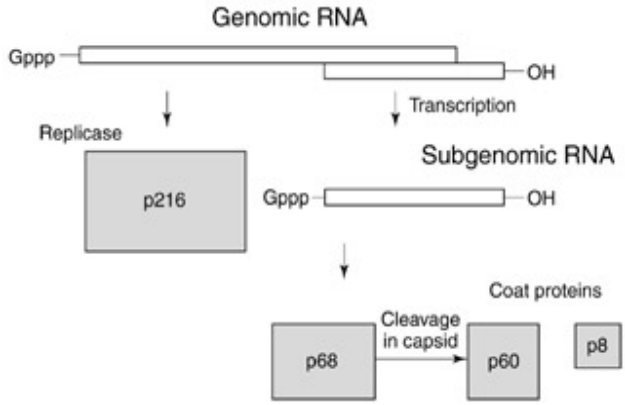


Figure 4 Genome organization of the β-like tetravirus, NβV. The NβV genomic RNA and its subgenomic RNA with their ORFs are represented as open boxes above arrows leading to their functional protein products, represented as shaded rectangles enclosing their molecular weights.

two terminal regions (amino acids 1–48 and 601–647) have 40% and 53% identity and correspond to the interior domain, while the 145 amino acid region in the middle of the primary sequence (amino acids 274–419) has only 33% identity and corresponds to the exterior Ig-like region. These dissimilar sequences in otherwise highly similar coat proteins are consistent with the putative functions of their corresponding structural domains, i.e. binding a different host cell for the exterior domain and interacting with a different RNA sequence for the interior domain.

β-like genome organization

The sequence of the NβV genomic RNA shows it to have only two genes which overlap each other (**Fig. 4**). The larger gene located at the 5′ end produces a 1925 amino acid protein with a calculated molecular weight of 216 kDa and is the RNA-dependent RNA polymerase (replicase), as determined by homologies to those of other viruses. It is not known at present whether further processing of the product of the replicase gene into smaller proteins occurs. The smaller gene located towards the 3′ end of the NβV genome encodes the 68 549 Da coat protein precursor which is cleaved upon capsid assembly to the major and minor coat proteins. This gene is able to be translated by scanning ribosomes through the production of a subgenomic RNA encoding 2.5 kb of the 3′ end of the genomic strand. This subgenomic strand is not capable of being replicated as only one species of double-stranded RNA with the length of the genomic strand is extracted from NβV infected larvae.

Genetic manipulation of tetraviruses

The lack of a cell culture system necessitated the development of a novel means of producing and examining effects of specific mutations in the genomes of tetraviruses. The strategy for producing HaSV mutants exploits its inactivity outside a host gut cell and the ability of the components of many simple icosahedral viruses like HaSV to assemble into virions in a wide variety of environments. Thus, by arranging the presence of the three main component types of HaSV (RNA1, RNA2 and coat protein) inside a plant cell where the virus is inactive, the two RNAs are able to assemble with coat protein to form a virion capable of infecting larvae. This is accomplished in a transient plant protoplast expression system (*Nicotiana plumbiganofolia*) by transfecting three plasmids, two producing the RNA strands by *in vivo* transcription off a modified plant promoter and the other expressing the coat protein precursor. The RNA-producing plasmids are constructed with cDNA copies of the viral RNAs behind a modified cauliflower mosaic virus 35S promoter designed to produce capped transcripts starting at the first viral base. An intact 3′ terminus for the RNA strand is achieved with a *cis*-acting ribozyme engineered to cleave the transcribed RNA after the last 3′ viral base. The third plasmid is engineered to express the coat protein precursor ORF behind an unmodified 35S promoter and without a ribozyme. Upon transfection of the three plasmids, the protoplasts are incubated for 3 days to allow assembly of the components and then fed to insect larvae to replicate and amplify the virus. Virus mutants are produced by site-directed mutagenesis upon the RNA-producing plasmids.

Replication

To counter the difficulty of the lack of a cell culture system to study the replication of HaSV, a system dependent on expressing tetraviral components with two different recombinant baculoviruses was developed. The first baculovirus expressed the HaSV replicase in Sf9 cells behind the polyhedrin promoter to the degree it was readily detected on an immunoblot of Sf9 cells. The high quantity of enzyme apparently compensated for a low level of activity, as replication of RNA2 templates presented to it by a second co-infected baculovirus was detected. The second baculovirus was engineered to produce precise transcripts of RNA2 using a similar strategy to the protoplast expression of tetraviral RNAs with the use of the same ribozyme and a modified *Drosophila* hsp70 promoter. Indicating replication was occurring with this system was the observation that no RNA2 transcript occurred on Northern blots unless the

RNA1 virus was present. By examining the effect of specific deletions of RNA2, this system is being used to examine which elements on RNA2 are important for its replication.

Studies of coat protein assembly

Similar to the coat proteins of many other small icosahedral viruses, assembly of virus-like particles (VLPs) occurs upon expression of only the tetraviral coat protein ORF in nonhomologous expression systems (yeast and baculoviruses). Of particular interest, however, are the observations indicating that the tetravirus coat proteins, without any other viral proteins, are able to perform all four generic functions of viral coat proteins: (1) binding each other to form a stable particle; (2) binding and incorporation of specific RNAs; (3) binding of host cells with subsequent entry; and (4) uncoating of RNA for translation. Experimentation has shown that VLPs display the assembly-dependent cleavage of the precursor, they are able to incorporate RNAs with viral sequences, and they bind specifically to a larval midgut receptor similar to native virions. Furthermore, purified VLPs can carry mRNAs of the reporter genes into gut cells of larvae where the reporter gene shows activity. VLPs carrying reporter gene mRNAs can be produced from Sf9 cells co-infected with two baculoviruses, one expressing the coat protein ORF and the other expressing a transcript having the reporter gene ORF in front of RNA2 sequences which allowed the transcript's encapsidation into the VLPs.

Pathobiology

Host range

Lepidopteran insects are the only known hosts for tetraviruses. A systematic survey of approximately a thousand diseased insects from different orders showed tetravirus hosts were confined largely to noctuid, saturniid and lymacodid moths of the Heterocera suborder of the Lepidoptera. Tetraviruses appear to differ in the breadth of the range of species they are able to infect. HaSV appears unable to infect species outside the subfamily Heliothinae, while *Tricoplusia ni* virus (TnV) and *Dasychira pudibunda* virus (DpV) are able to infect insects outside the family of their nominal host. Closely related viruses may have very distinct host ranges, as exemplified by NωV and HaSV. The former is able to infect a saturniid host, yet is unable to infect the noctuid heliothine host of the latter, despite having a high degree of relationship (>90% for the replicase).

No replication in other animals has been detected. Numerous animals injected with high titres of tetraviruses for antibody production have failed to show any abnormal response or disease symptoms. A detailed pathological study of mice injected with TaV showed no evidence that the virus was in any observable way harmful to them. Transfection of vertebrate cell lines with HaSV genomic RNA also showed no activity. However, studies on serological reactions by vertebrates (including humans) towards particles of tetraviruses, done in the 1970s, suggested that tetravirus specificity was more broad as positive reactions were noted. However, these reactions are believed to be nonspecific, owing to the type of serological test involved and indications that the reaction is not due to replication. Further work is required to consolidate these findings, especially in view of the large body of evidence showing the specificity of tetraviruses for insects and their potential widespread use in agriculture (see below).

Transmission and symptoms

Horizontal transmission via ingestion by larvae has been demonstrated for several tetraviruses. Interestingly, the range of symptoms from this means of transmission varies greatly. Few or no symptoms (only slight growth retardation at high doses) can be seen upon infection of several hosts by TnV. On the other hand, NβV displays a marked pathological effect upon larvae 7–9 days postinfection, with infected larvae in all stages ceasing to feed, becoming moribund, discolored and flaccid, and, upon death, remaining hanging by their prolegs. The cadavers are distinct from those infected with baculoviruses because an internal liquefaction occurs which leaves the integument intact. A dependence upon the larval life-stage can also be seen. HaSV is highly active against neonate larvae, with as little as 5000 particles causing them to cease feeding within a day and to die within 4 days. Surprisingly, HaSV infection of later instar larvae shows little effect. It has not been clearly demonstrated whether adults or pupae are capable of being infected by tetraviruses, although NβV was reported to be isolated from these stages.

Vertical transmission of tetraviruses is also believed to occur. It has been impossible to remove HaSV from a laboratory-bred colony of its host despite stringent sanitation procedures. Evidence from studies of HaSV pathology in early instar larvae show subpathogenic, low-level infections exist for this virus, which is highly virulent at higher doses. TnV appears to be vertically transmitted under artificial conditions and there is evidence for vertical transmission of the more virulent DpV. Symptoms of infection from this route are difficult to detect, with the most obvious being slow-growing larvae. The

evidence for vertical transmission, however, remains undefinitive and further work needs to be done to establish it. The most likely mechanism for vertical transmission, should it occur, is transovum, as tetraviruses appear to infect only midgut cells (see below).

Histopathology and tropism

All the available evidence points to the larval midgut as the exclusive site of infection of tetraviruses. Examinations by light and electron microscopy for several viruses have shown their particles only in midgut tissues. In a definitive experiment showing this phenomenon, Northern blots of RNA extracted from infected larvae showed the presence of HaSV RNA only in midgut tissue, even after infection by injection into the hemocele. This experiment also shows that the tissue tropism of HaSV is not due to the lack of exposure to the virus by other host tissues. The marked tropism of HaSV which prevents its culture in continuous cell lines appears to be a function of both the abilities of the particles to bind and enter cells and a restriction on the activity of the replicase in cells other than those of the midgut. Binding studies using the histochemical detection of HaSV particles on cross-sections of larval midguts show exclusive binding to outer cell membranes, particularly the goblet cell apical membrane. However, even when cellular uptake mechanisms for particle entry are bypassed in cultured cells (including those of the cotton bollworm host, *Helicoverpa armigera*) by transfection of HaSV genomic RNA, no replication activity is seen.

In the most detailed pathology study for a tetravirus, HaSV was seen to infect all three major cell types of the lepidopteran midgut: goblet, columnar and regenerative stem cells. Infected cells showed crystalline arrays of particles, which were associated with a massive increase in the rate of cells detaching from the basal membrane and shedding into the lumen. Cells were shed to the extent that few, if any, remained in midguts of insects with advanced infections; this phenomenon was correlated to an increased rate of apoptosis. Hence the rapid loss of infected gut cells may be a cellularly mediated defense that protects the insect against more extensive viral infection.

Ecology

Only informal reports are available to describe the broader interactions between tetraviruses and their hosts in the wild. Hence much must be surmised and inferred from laboratory studies and the limited reports of agricultural use. The broad range of disease symptoms and possible dual means of transmission suggest a complicated picture.

Tetraviruses are readily found in insect hosts with other tetraviruses and other virus types, including cytoplasmic polyhedrosis viruses, baculoviruses and other small RNA viruses. Persistence in wild populations of *Nudaurelia* for decades in South Africa has been described for NβV and NωV, with the latter succeeding the former in prevalence in recent times. This succession may have contributed to the recent decline of the host population to the point where few moths have been observed in areas formerly inundated. The persistence of tetraviruses is probably due to their ability to initiate sublethal infections and to be transmitted vertically, as well as the apparent robust nature of the particle, which would favor its vertical transmission.

At high densities of host populations, horizontal transmission is the main mode of viral spread through dispersed cadavers and frass in which tetraviruses have been shown be present. The *Nudaurelia* populations recorded large-scale epizootics at high densities, at times displaying up to 90% mortality nearly every year for at least four decades. In contrast, no HaSV epizootics have been observed in the low density *H. armigera* populations in closely scrutinized Australian cotton fields, and yet the virus clearly has the capacity for virulence. The few infected *H. armigera* larvae found in the wild are likely to be the result of vertical transmission.

Economic Use and Future Perspectives

Tetraviruses, as well other small RNA viruses of insects, have been little used in Western agriculture as biological control agents for pests. However, SaV and DtV have been used to great effect in Malaysian oil palm plantations to control limacodid caterpillar pests, which can reach very high densities. Production and dispersal of these viruses was accomplished by suspension of naturally infected cadavers in water and spraying. Laboratory tests with purified HaSV on larvae of *H. armigera* strongly indicate their potential for the control of this major pest. Furthermore, the association of NωV with the decline of *Nudaurelia* populations indicates that long-term effects on pest populations with the use of tetraviruses are possible.

There is more interest in the economic use of tetraviruses, however, when the group is looked at through the lens of biotechnology. One major obstacle to their use for insect control has been the difficulty of producing them. However, the simplicity of the tetraviral genome makes production of viral particles in nonhost cells feasible (see above, Molecular Biology). The assembly of HaSV in plant

Figure 5 Tobacco made transgenic with genes that produce a tetravirus. The genes introduced into the transgenic tobacco plant on the right were designed to produce the protein and RNA components of the tetravirus, HaSV. These components were able to assemble into infectious virions, as indicated by the stunted, HaSV infected larva encircled below the leaf on the right. The larger larvae feeding on the control leaf on the left were not infected.

protoplasts described above suggested that transgenic crop plants could also accomplish the same feat. To test this hypothesis, the three genes required for HaSV assembly were placed into the genomes of tobacco plants by *Agrobacterium*-mediated transgenesis. The plants were screened for antifeeding activity and several showed they could induce stunted larvae, which subsequently were shown to be infected with HaSV (**Fig. 5**). This experiment indicates a new approach to the control of the world's most economically important group of insect pests, the heliothine caterpillars. Assembly of HaSV is also being tested in yeast with the same three gene strategy. Production of tetraviruses by yeast fermentation would make the virus available cheaply for spraying purposes.

Biotechnology may have another use for tetraviruses that may impact on medicine as well as agriculture. Their particles have the potential to be used as versatile vehicles for the targeted delivery of nonviral RNAs to new cell types. The tetravirus virion can be viewed as a delivery vehicle for viral genes that employs an Ig-like domain to bind specifically to a particular cell surface epitope, allowing its entry into midgut cells. This suggests that its exchange for another Ig-like domain having affinity towards another surface epitope of another cell type would change the destination of the particle's RNA contents to the other cell type. This potential targeting ability, combined with the already demonstrated ability to produce functional VLPs with nonviral mRNAs (described above), makes for a potentially versatile system for delivering transient gene activities to specific cells. Possible medical uses include a novel form of gene therapy or immunotoxins where toxin mRNAs are delivered to cancer cells, allowing more specific expression of toxicity than the present forms. In agriculture, mRNAs for insect-specific toxins could be targeted more quickly to a particular insect. This approach would preclude the use of a virus for every pest insect, and such a system could be made more acceptable to consumers and regulatory authorities.

See also: **Vectors: Plant viruses; Nodaviruses (*Nodaviridae*).**

Further Reading

Gordon KHJ, Johnson KN and Hanzlik TN (1995) The larger genomic RNA of *Helicoverpa armigera* stunt tetravirus encodes the viral RNA polymerase and has a novel 3′-terminal tRNA-like structure. *Virology* 208: 84.

Hanzlik TN and Gordon KHJ (1997) The *Tetraviridae*. *Adv. Virus Res.* 48: 101.

Hendry DA, Hodgson V, Clark R and Newman J (1985)

Small RNA viruses coinfecting the pine emperor moth *Nudaurelia cytherea capensis*. *J. Gen. Virol.* 66: 627.
Moore NF (1991) The Nudaurelia β family of insect viruses. In: Kurstak E (ed.) *Viruses of Invertebrates*, p. 277. New York: Marcel Dekker.
Munshi S, Liljas L, Cavarelli J *et al* (1996) The 2.8 Å structure of a $T = 4$ animal virus and its implications for membrane translocation of RNA. *J. Mol. Biol.* 261: 1.
Service R (1996) Arming plants with a virus. *Science* 271: 145.

THEILER'S VIRUSES (*PICORNAVIRIDAE*)

Howard L Lipton, Division of Neurology, Evanston Hospital, Evanston, Illinois, USA

History, Geographic Distribution and Host Range

The mouse encephalomyelitis viruses are enteric pathogens of mice. Discovered by Max Theiler in the early 1930s and originally called murine poliovirus, these agents are frequently referred to as Theiler's murine encephalomyelitis viruses (TMEV). Theiler initially recovered isolates from mice with spontaneous paralysis housed in a research colony; subsequently the TMEV have been found in virtually all nonbarrier mouse colonies, where they cause asymptomatic intestinal infections. While the TMEV are widely distributed throughout the world in mouse colonies, their host range is quite narrow, and includes only mice and rats. Serological evidence indicates that *Mus musculus*, the feral house mouse, is the natural host, but several other species of voles and possibly rats may also serve as hosts. As is the case for other picornaviruses, following peripheral routes of infection, TMEV spreads to the central nervous system (CNS) producing encephalitis, or more commonly, spontaneous paralysis, i.e. poliomyelitis. The incidence of spontaneous paralysis is low, around one paralyzed animal per 1000–5000 mice in a colony reported in the older literature. Since the TMEV may go undetected unless appropriate serological tests for the virus are performed, these agents are a potential hazard for investigators using mice in biomedical research.

In recent years, this group of viruses has assumed additional importance because TMEV infection in mice provides one of the few available experimental animal models for multiple sclerosis. TMEV-induced demyelinating disease is a relevant animal model for multiple sclerosis because: (1) chronic pathological involvement is virtually limited to the CNS white matter; (2) myelin breakdown is accompanied by mononuclear cell inflammation; (3) demyelination results in clinical disease, e.g. spasticity, from involvement of upper motor neuron pathways; (4) myelin breakdown is in part immune-mediated; and (5) the disease is under multigenic control with a strong linkage to certain major histocompatibility complex (MHC) genes.

Classification and Serologic Relationships

Based on the complete nucleotide sequence and genome organization, TMEV have been classified in the genus *Cardiovirus* of the family *Picornaviridae* along with encephalomyocarditis virus (EMCV) and Mengo virus (**Table 1**). TMEV are a separate serological group of cardioviruses, as polyclonal antisera show no crossneutralization between TMEV and EMCV or Mengo virus. Because the coat proteins share a high level of amino acid sequence identity with the other cardioviruses, crossreactions are seen on ELISA when disrupted virions are used as antigens and on complement fixation tests.

Table 1 Classification of TMEV in the family *Picornaviridae*

Human enteroviruses: polioviruses, coxsackieviruses, echoviruses
Human rhinoviruses
Hepatitis A viruses
Aphthoviruses: foot-and-mouth disease viruses
Cardioviruses
Group A: EMCV, Mengo, MM, Columbia-SK, Maus–Elberfeld
Group B: TMEV – GDVII, FA, BeAn8386, DA, WW, TO4, TO(B15)
Group C: Vilyuisk virus[a]

[a] Vilyuisk virus is either a divergent TMEV or it belongs in a separate group as shown here.

Small RNA viruses coinfecting the pine emperor moth *Nudaurelia cytherea capensis*. *J. Gen. Virol.* 66: 627.
Moore NF (1991) The Nudaurelia β family of insect viruses. In: Kurstak E (ed.) *Viruses of Invertebrates*, p. 277. New York: Marcel Dekker.
Munshi S, Liljas L, Cavarelli J *et al* (1996) The 2.8 Å structure of a $T = 4$ animal virus and its implications for membrane translocation of RNA. *J. Mol. Biol.* 261: 1.
Service R (1996) Arming plants with a virus. *Science* 271: 145.

THEILER'S VIRUSES (*PICORNAVIRIDAE*)

Howard L Lipton, Division of Neurology, Evanston Hospital, Evanston, Illinois, USA

History, Geographic Distribution and Host Range

The mouse encephalomyelitis viruses are enteric pathogens of mice. Discovered by Max Theiler in the early 1930s and originally called murine polioviruses, these agents are frequently referred to as Theiler's murine encephalomyelitis viruses (TMEV). Theiler initially recovered isolates from mice with spontaneous paralysis housed in a research colony; subsequently the TMEV have been found in virtually all nonbarrier mouse colonies, where they cause asymptomatic intestinal infections. While the TMEV are widely distributed throughout the world in mouse colonies, their host range is quite narrow, and includes only mice and rats. Serological evidence indicates that *Mus musculus*, the feral house mouse, is the natural host, but several other species of voles and possibly rats may also serve as hosts. As is the case for other picornaviruses, following peripheral routes of infection, TMEV spreads to the central nervous system (CNS) producing encephalitis, or more commonly, spontaneous paralysis, i.e. poliomyelitis. The incidence of spontaneous paralysis is low, around one paralyzed animal per 1000–5000 mice in a colony reported in the older literature. Since the TMEV may go undetected unless appropriate serological tests for the virus are performed, these agents are a potential hazard for investigators using mice in biomedical research.

In recent years, this group of viruses has assumed additional importance because TMEV infection in mice provides one of the few available experimental animal models for multiple sclerosis. TMEV-induced demyelinating disease is a relevant animal model for multiple sclerosis because: (1) chronic pathological involvement is virtually limited to the CNS white matter; (2) myelin breakdown is accompanied by mononuclear cell inflammation; (3) demyelination results in clinical disease, e.g. spasticity, from involvement of upper motor neuron pathways; (4) myelin breakdown is in part immune-mediated; and (5) the disease is under multigenic control with a strong linkage to certain major histocompatibility complex (MHC) genes.

Classification and Serologic Relationships

Based on the complete nucleotide sequence and genome organization, TMEV have been classified in the genus *Cardiovirus* of the family *Picornaviridae* along with encephalomyocarditis virus (EMCV) and Mengo virus (**Table 1**). TMEV are a separate serological group of cardioviruses, as polyclonal antisera show no crossneutralization between TMEV and EMCV or Mengo virus. Because the coat proteins share a high level of amino acid sequence identity with the other cardioviruses, crossreactions are seen on ELISA when disrupted virions are used as antigens and on complement fixation tests.

Table 1 Classification of TMEV in the family *Picornaviridae*

Human enteroviruses: polioviruses, coxsackieviruses, echoviruses
Human rhinoviruses
Hepatitis A viruses
Aphthoviruses: foot-and-mouth disease viruses
Cardioviruses
Group A: EMCV, Mengo, MM, Columbia-SK, Maus–Elberfeld
Group B: TMEV – GDVII, FA, BeAn8386, DA, WW, TO4, TO(B15)
Group C: Vilíuisk virus[a]

[a] Vilíuisk virus is either a divergent TMEV or it belongs in a separate group as shown here.

Three-dimensional Virion Structure

Picornavirions have a relative molecular mass of $\sim 8.5 \times 10^6$ Da, of which ~30% is RNA. The spherical virus particles have an external diameter of about 30 nm. The capsid proteins are arranged in icosahedral symmetry with 60 protomers, each composed of a single copy of VP1, VP2, VP3 and VP4.

The three-dimensional structures of the GDVII, BeAn and DA strains have been determined at ~0.3 Å resolution by x-ray crystallography. The overall architecture is quite similar to that of other picornaviruses whose structures have been determined, and closely resembles that of Mengo virus. Each of the three major capsid proteins VP1, VP2 and VP3 consist of a wedge-shaped eight-stranded antiparallel β barrel, as demonstrated for other picornaviruses. The N-termini of the capsid proteins form an extensive, intertwined network on the inner surface of the protein shell. The loops connecting the β strands form the outer surface features of the protein shell and provide the surface differences with Mengo virus. The pit which has been proposed as the viral receptor is a broad depression on the virion surface at the junction between VP1 and VP2 along the twofold axis. The TMEV surface structures can be differentiated from that of Mengo virus in having: (1) a larger VP1 CD double loop, with loop I containing an extra five residues and shifted more toward the VP2 EF puff at the twofold axis, while loop II points more toward the fivefold axis; (2) an 11 residue insertion in the VP2 EF puff forming a double loop in which the inserted loop interacts with the VP1 FMDV GH loop; and (3) the tip of the VP3 knob (a loop inserted in βE) points straight outward on the rim of the pit.

Properties of the Genome

The genetic component of the virion is a positive-sense, single-strand RNA molecule that has a sedimentation coefficient of 35 S and is 8100 nucleotides long. Virion RNA has a poly(A) tract on the 3′ end and a small basic protein, VPg, covalently linked to the 5′ end. The complete genomes of the GDVII, DA and BeAn strains have been cloned and sequenced. With the notable absence of a poly(C) tract in the 5′ noncoding region, the organization and sequence of the TMEV genome is remarkably similar to that of EMCV. The polyprotein of the BeAn strain, a typical TMEV, initiates at the AUG codon at nucleotide 1065 and extends for 6909 nucleotides (or 2303 codons), ending at the single UGA termination triplet at base 7972. The polyprotein-coding region is flanked by 5′ and 3′ noncoding sequences of 1064 and 125 nucleotides, respectively. In BeAn the 5′ noncoding region contains a stretch of 11 pyrimidines interrupted by a single purine before the AUG at nucleotide 1065. In picornaviruses, the 5′ noncoding region mediates cap-independent translation and also serves as an internal ribosome entry site (IRES), e.g. when experimentally present in the intercistronic region in a bicistronic mRNA. The TMEV 5′ noncoding sequences have been predicted to form stable secondary structures, which in the 500 nucleotides upstream of the authentic AUG (at 1065) are nearly identical to those predicted for EMCV and foot-and-mouth disease virus. In BeAn, eight AUGs precede the initiator AUG, but none of them has an optimum Kozak context sequence. Hence, it could be argued that selection of the authentic initiator AUG after binding of ribosomes to TMEV RNA does not involve internal ribosome binding. However, BeAn nucleotides ~500–1065 determine a structure that serves as an IRES in bicistronic mRNAs both *in vitro* (rabbit reticulocyte lysate) and *in vivo* (BHK-21 cells). A poly(A) tail of indeterminate length is present on the 3′ end of the viral genome.

Polyprotein and Post-translational Processing

As is the case for other picornaviruses, the final TMEV gene products are the result of post-translational cleavages of the polyprotein. The 2303 amino acid polyprotein has a calculated molecular weight of 255 990. (This applies to BeAn virus; the polyprotein of GDVII virus contains no insertions or deletions; however, two VP1 amino acids are deleted in that of DA virus.) The processing scheme follows the standard L–4–3–4 picornavirus polypeptide arrangement, i.e. the leader peptide (L), four capsid polypeptides in part one (P1) of the genome, three polypeptides in P2 and four polypeptides in P3 (**Fig. 1** and see below). The coding limits of individual polypeptides have been predicted by analogy with those of EMCV, as the only confirmation to date of the deduced sequence is that of the N terminus of 1D. The eight amino acids flanking the putative cleavage sites are highly conserved for the two viruses. All of the cleavage sites in the polyprotein except for two, 1A/1B and 2A/2B, are processed by the viral protease 3C. The TMEV 3C protease therefore processes Q–C, as well as Q–S and Q–A, dipeptides and, in addition, the E–N dipeptide at the 1D/2A cleavage. However, only 6 of 8 Q–G, 2 of 13 Q–S and 1 of 7 Q–A dipeptides in the polyprotein are cleaved by 3C, indicating that involvement of secondary, tertiary, or both types of structure is also important for recognition of these particular dipeptides. The 2A/2B site is probably autocatalytically cleaved, as in EMCV.

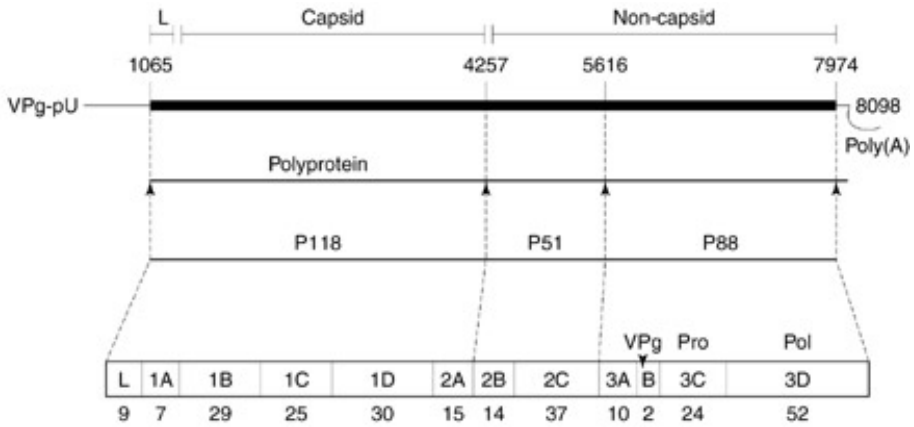


Figure 1 TMEV-specific protein cleavage scheme. The intermediate cleavage products are not shown. The numbers below the 11 final gene products are the molecular weights (in thousands) of each of the proteins as calculated from their predicted amino acid sequences.

Cleavage of the polyprotein gives rise to three primary products, the first of which (116 530 mol. wt) contains the leader protein (8593 mol. wt), the P1 capsid proteins, and the first P2 polypeptide 2A (15 353 mol. wt). Thus, the initial precursor released from the polyprotein is like that of the other cardioviruses and differs from that of other groups of picornaviruses. The capsid proteins are arranged in the following order: 1A (VP4; 7102 mol. wt), 1B (VP2; 29 433 mol. wt), 1C (VP3; 25 463 mol. wt), and 1D (VP1; 30 457 mol. wt). The second processing precursor (2BC) is 51 708 mol. wt and gives rise to 2B (14 863 mol. wt) and 2C (36 845 mol. wt). The P2 proteins 2A, 2B and 2C have not been assigned functions as yet for the cardioviruses. The third or C-terminal precursor protein is 87 950 mol. wt and is processed into the four mature proteins 3A (9934 mol. wt), 3B (2169 mol. wt), 3C (23 612 mol. wt) and 3D (52 235 mol. wt). Protein 3B, also designated VPg, is a small basic protein which is 20 amino acids in size and is found covalently linked to the 5′ end of viral RNAs. This peptide may be important in viral replication. By analogy with other picornaviruses, the 3C polypeptide is a viral protease and 3D is the viral polymerase.

Physical Properties

Since the TMEV do not have an envelope they are more resistant than lipid-containing viruses to chemicals and physical agents. They are insensitive to chloroform, ether, nonionic detergents (such as deoxycholate, NP40 and Tween-80) and the ionic detergent sodium dodecyl sulfate, but are inactivated by 0.3% formaldehyde and HCl 0.1 mol l^{-1}. TMEV are rapidly destroyed at temperatures over 50°C and lose some infectivity upon lyophilization. Purified virions can be stored for long periods of time at −70°C without loss of infectivity, but slowly lose infectivity on storage at −20°C.

As enteroviruses the TMEV require stability at low pH to pass through the acidic conditions of the stomach. The TMEV are stable over the entire pH range from 3 to 9.5. However, in contrast to the other cardioviruses, such as Mengo virus and EMCV, they are not highly thermolabile in the presence of 0.1 mol l^{-1} chloride or bromide in the pH range 5 to 7.

Theiler's virions have a sedimentation coefficient of 150 S by velocity centrifugation in sucrose and a buoyant density of 1.34 g ml^{-1} by isopycnic centrifugation in cesium salts.

Replication

The reader is referred to entries on human poliovirus and cardioviruses for the strategy of picornavirus RNA replication as no information is available on this topic for the TMEV.

Mapping Genomic Determinants Important in Pathogenesis

The existence of two distinct TMEV neurovirulence groups makes the TMEV particularly useful for molecular pathogenesis studies. The difference in virulence (LD_{50}) between the highly virulent and less virulent TMEV groups is of the order of 10^5 plaque-forming units. Further, full-length cDNA clones of the highly virulent GDVII and the less virulent DA and BeAn viruses have been assembled in different laboratories, and viral RNA transcribed from these cDNAs has been demonstrated to be infectious upon transfection of mammalian cells. To identify the

Table 2 Two TMEV neurovirulence groups

	Highly virulent	*Less virulent*
TMEV isolated	GDVII, FA, ASK1 VIE 415_{HTR}	DA, BeAn 8386, Yale, WW TO(B15), VL, TO4
Disease	Encephalitis	Polio/demyelination
Incubation period[a]	1–10 days	7–20 days/>30 days
CNS target cell[b]	Neurons	Motor neurons/macrophages and oligodendrocytes
Mean LD_{50}	10 PFU	10^6 PFU
Persistent infection	No	Yes
Temperature sensitive[c]	No	Yes

[a]Incubation period following experimental infection by the intracerebral route of inoculation.
[b]Preferential site of virus replication in the central nervous system (CNS).
[c]Inability to replicate at 39.8°C compared to 33 or 37°C.
PFU, plaque-forming units.

determinants important in pathogenesis, e.g. virulence and persistence, recombinant viruses between parental cDNAs have been assembled and analyzed *in vitro* and after inoculation of mice.

Neurovirulence and persistence have been mapped primarily to the P1 region encoding the leader and the coat proteins. These results suggest that the mechanism for the pathogenetic properties of virulence and persistence are likely to involve the exterior surface of the virus, and immunological or receptor-mediated events may be involved. Chimeric virus constructs in the coat protein region, resulting in the interaction of potentially disparate protomeric subunits of two parental viruses, have been found to be prone to assembly defects. Such chimeric viruses may exhibit compromised growth *in vitro*. Thus, the pathogenetic phenotypes of chimeric viruses need to be interpreted with caution and require analysis of their growth properties *in vitro*.

Transmission and Tissue Tropism

TMEV are transmitted by the fecal–oral route but can be separated into two biological groups based on neurovirulence (**Table 2**). The first group, consisting of three isolates, GDVII, FA and Ask-1, is highly virulent and causes a rapidly fatal encephalitis in mice. The other TMEV, some 10–20 isolates, that include viruses recovered from the CNS of spontaneously paralyzed mice and from the feces of asymptomatic mice, form a second, less virulent group. Experimentally, the less virulent viruses produce poliomyelitis (early disease) followed by demyelinating disease (late disease). When cell culture-adapted less virulent TMEV are used to inoculate mice, the poliomyelitis phase is subclinical; brain-derived stocks produce both of the less virulent disease phases.

Pathogenesis

Little information is available about the pathogenesis of TMEV infection following peripheral routes of infection, including feeding of virus. In general, isolates from either of the TMEV neurovirulence groups do not readily produce CNS disease following peripheral routes of inoculation, with the exception of one strain, TO(B15). TO(B15) is a mutant selected for its invasiveness from the intestinal tract. When mice are inoculated intracerebrally with the highly virulent strains, the virus replicates throughout the brain and spinal cord, causing encephalitis or encephalomyelitis. Thus, neurons as well as glial cells (astrocytes and probably oligodendrocytes) are infected in the cerebral cortex, hippocampus, basal ganglia, thalamus, brainstem and spinal cord. Affected mice develop a hunched posture and hind limb paralysis. The rapid demise of these animals is the result of widespread lytic infection. The following sections focus on the pathogenesis of the biphasic disease produced by the less virulent TMEV, which provides a model system for multiple sclerosis.

Clinical Features of Infection

Although TMEV are enterically transmitted, the pathogenesis of the infection has been primarily studied using the intracerebral route of inoculation which maximizes the incidence of neurological disease. Following intracerebral inoculation, the less virulent strains produce a distinct biphasic CNS disease in susceptible strains of mice, characterized by poliomyelitis during the first few weeks postinfection, followed by chronic, inflammatory demyelination that begins during the second or third week postinfection and becomes manifest clinically between 1 and 3 months postinfection. Mice with polio-

myelitis develop flaccid paralysis, usually of the hind limbs; only one limb may be affected, or paralysis may spread to involve all limbs and lead to death. In contrast to the fatal outcome of paralysis produced by the Lansing strain of human poliovirus type 2, complete recovery from TMEV-induced poliomyelitis is usual. Occasionally, residual limb deformities are seen as the result of extensive anterior horn cell infection and severe paralysis (early disease).

Gait spasticity is the clinical hallmark of the demyelinating or late disease. Late disease is first manifest by slightly unkempt fur and decreased activity, followed by an unstable, waddling gait. Subsequently, generalized tremulousness and ataxia develop, and the waddling gait evolves into overt paralysis. Incontinence of urine and priapism are commonly seen. As the disease advances, prolonged extensor spasms of the limbs can be induced followed by difficulty in righting. Weight loss is not seen until mice are severely paralyzed. The clinical manifestations of late disease are progressive and lead to an animal's demise in several to 14 months.

Pathogenesis and Histopathology

Motor neurons in the brainstem and spinal cord are the main targets of infection during poliomyelitis (early disease), but sensory neurons and astrocytes are also infected. TMEV do not replicate in endothelial and ependymal cells. A brisk microglial reaction is elicited, with the appearance of numerous microglial nodules, particularly in the anterior gray matter of the spinal cord. Examples of neuronophagia are quite frequent at this time, but very little lymphocytic response is seen. The poliomyelitis phase lasts 1–4 weeks, after which time little residual gray matter involvement is apparent other than resolving astrocytosis.

As early as 2 weeks postinfection, inflammation of the spinal leptomeninges begins to appear, followed by involvement of the white matter. Initially, the inflammatory infiltrates are almost exclusively composed of lymphocytes, but at later times plasma cells and macrophages are numerous. The influx of macrophages is in close temporal and anatomic relationship with myelin breakdown. Both light microscope and ultrastructural studies show that myelin breakdown is related to the presence of mononuclear cells, which either actively strip myelin lamellae from otherwise normal-appearing axons or are found in contact with myelin sheaths undergoing vesicular disruption. Foci of inflammation and myelin destruction extend from the perivascular spaces into the surrounding white matter, leading to sharply demarcated plaques of demyelination. The ultrastructure of oligodendrocytes during the initial phase of myelin breakdown has not shown alterations in oligodendroglial loops, which are in close apposition with naked but otherwise normal axons, suggesting that myelin injury may not be directly related to oligodendrocytopathology.

Sites of TMEV persistence

The sites of TMEV persistence are still disputed; however, TMEV persistence clearly involves active virus replication, as infectious virus can be readily isolated from the CNS of infected mice. *In situ* hybridization has revealed two populations of CNS cells positive for viral genomes. Virus replication in the majority of these cells (>90%) appears to be highly restricted, as they contain <500 viral genomes. A small percentage of CNS cells contain >1500 genomes, possibly as many as 10^4–10^5, and are probably productively infected. Highly restricted virus production has been demonstrated in macrophages isolated from the CNS of diseased mice; therefore, macrophages appear to be the primary target for persisting virus. It is also possible that some of the cells with restricted infection are astrocytes. The kinetics of virus replication in the CNS cells with restricted infection remains to be elucidated, such as the length of the replicative cycle and whether the cells are lysed or continue to produce infectious virus for longer times. *In vitro,* TMEV infection only occurs in monocytes once they have differentiated into macrophages. The infection of macrophage cell lines is highly restricted but with normal levels of viral translation, as substantial amounts of viral antigen are produced; ultimately infected macrophages undergo apoptosis. In contrast, oligodendrocytes appear to be productively infected, as an ultrastructural study has shown crystalline arrays of virions in oligodendrocytes in demyelinating lesions. Oligodendrocytes may correspond to the CNS cells containing large numbers of viral genomes by *in situ* hybridization. These data suggest that a lytic infection of oligodendrocytes contributes to demyelination along with immune-mediated mechanisms of damage.

Immune Response

During the first week, TMEV-infected mice mount a virus-specific humoral immune response that reaches a peak by 1 to 2 months postinfection and is sustained for the life of the host. Neutralizing and other virus-specific antibodies have been measured. The majority of the antiviral IgG response in persistently infected, susceptible mice is of the IgG2a subclass, with little antiviral IgM detected by day 14 postinfection, whereas IgG1 antiviral antibodies appear to pre-

dominate in resistant and immunized mice. Murine CD4+ T cells of the Th1 subset mediate delayed-type hypersensitivity (DTH) and regulate IgG2a production via interferon γ production, whereas CD4+ Th2 cells regulate IgG1 and IgE production via interleukin 4. Thus, the predominant IgG2a antiviral response in susceptible mice may be an *in vivo* measure of preferential stimulation of a Th1-like pattern of cytokine synthesis. Recently, virus-specific CD8+ cytolytic T cell responses have also been shown help in virus clearance during the acute phase of the infection, and may be responsible for the resistance of certain strains of mice to the demyelinating disease.

When infected, susceptible mice also produce substantial levels of virus-specific CD4+ T cell responses. T cell proliferation and DTH appear by 2 weeks postinfection and remain elevated for at least 6 months. Both DTH and T cell proliferation have been shown to be specific for TMEV and mediated by CD4+ class II restricted T cells. A temporal correlation has also been found between the onset of demyelination and the appearance of these virus-specific T cell responses, as well as for high levels of virus-specific DTH and the susceptibility of mice of different genetic backgrounds and mixes. DTH and T cell proliferative responses in infected and immunized SJL mice (a susceptible strain) are directed toward immunodominant regions (peptides) in each of the three major coat proteins. T cell responses to these epitopes in VP1 and VP2 are believed to participate in the immunopathology (see below).

Although mice mount virus-specific humoral and cellular immune responses early in the infection and peak virus titers fall by 100–1000-fold, TMEV somehow evade immune clearance to persist at low levels indefinitely in the CNS of the host, as described above. Extraneural persistence has not been observed. Current dogma holds that humoral immunity is more important than cellular immunity in clearing infections by nonenveloped viruses, such as picornaviruses, but this has not been established for TMEV; evidence has been presented for a role for both neutralizing antibodies and cytolytic T cells in TMEV clearance. The precise mechanism by which TMEV evade immune surveillance is not known but does not appear to involve antigenic variation. Although complement and virus-antibody deposition in the CNS parenchyma have not been detected, extracellular transport of virus as infectious virus-antibody complexes, in aggregates, or contained or enveloped within cell membranes are means whereby virus could be protected from TMEV-specific immune responses and continue to replicate. This is an area for further study to enable a better understanding of how TMEV evade immune surveillance.

Immune-mediated Mechanism of Demyelination

Appropriately timed immunosuppression can prevent the clinical signs and pathological changes of TMEV-induced demyelinating disease, indicating that the immune response participates in myelin breakdown. A number of different immunosuppressive modalities have proven to be effective, including cyclophosphamide, antilymphocyte serum, antitumor necrosis factor antibodies, and monoclonal anti-IA, CD4+ and CD8+ antibodies. If given too early in the course of early disease, the infection in neurons is potentiated and results in encephalitis and a high mortality rate. Thus, immunosuppression may be most effective when administered after the first week of infection. The incidence of demyelinating disease is increased in SJL mice infected with a dose of virus that normally produces a low incidence of disease and adoptively

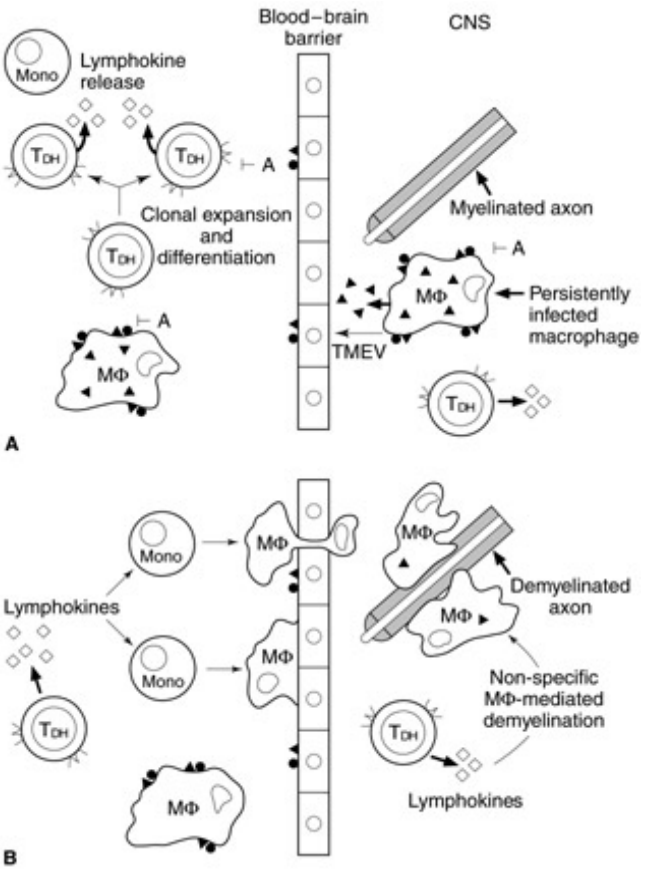


Figure 2 Proposed DTH-mediated mechanism of TMEV-induced demyelination. (**A**) Virus antigen presentation to Th1 lymphocytes, here designated T_{DH} cells, by antigen-presenting cells in either systemic lymphoid organs (left) or inside the blood–brain barrier (right). (**B**) Th1 lymphocyte response resulting in the recruitment of monocytes into the CNS and their differentiation into macrophages (MΦ) which then mediate demyelination.

immunized with TMEV VP2-specific T cell line. This observation supports a role for CD4+ T cells in mediating TMEV-induced demyelinating disease.

The effector mechanism by which a nonbudding virus, such as TMEV, might lead to immune-mediated tissue injury is unknown. Because TMEV antigens have been primarily found in macrophages, it has been proposed that myelin breakdown results from an interaction between virus-specific T cells trafficking into infected areas of the CNS and the virus. Thus, myelinated axons may be nonspecifically damaged as a consequence of a virus-specific immune response, i.e. an 'innocent bystander' response. In this circumstance, cytokines produced by MHC class II-restricted, TMEV-specific T_{DTH} cells primed by interaction with infected macrophages lead to the recruitment and activation of additional macrophages in the CNS, resulting in nonspecific macrophage-mediated demyelination (**Fig. 2**). This hypothesis is consistent with the CNS pathology observed in mice exhibiting TMEV-induced demyelinating disease and the fact that antigen-specific T cells and T cell lines have been shown to cause bystander CNS damage via macrophage activation in other model systems. Alternatively, in the case of extensive infection of oligodendrocytes, demyelination might result from immune injury to these myelin-maintaining cells expressing TMEV antigens in conjunction with H-2 class I determinants. CD8+ T cells would then be the likely T cell to kill infected oligodendrocytes; however, widespread degeneration of oligodendrocytes has not been observed.

See also: **Cardioviruses (*Picornaviridae*); Immune response: Cell mediated immune response, General features; Persistent viral infection; Polioviruses (*Picornaviridae*): General features, Molecular biology; Virus structure: Atomic structure, Principles of virus structure.**

Further Reading

Adami C, Pritchard AE, Knauf T, Luo M and Lipton HL (1998) Mapping a determinant for central nervous system persistence in the capsid of Theiler's murine encephalomyelitis virus (TMEV) with recombinant viruses. *J. Virol.* 71: 1662.

Borson ND, Paul C, Lin X *et al* (1997) Brain-infiltrating cytolytic T lymphocytes specific for Theiler's virus recognize $H2D^b$ molecules complexed with a viral VP2 peptide lacking a consensus anchor residue. *J. Virol.* 71: 5244.

Dethlefs S, Brahic M and Larsson-Sciard EL (1997) An early, abundant cytotoxic T-lymphocyte response against Theiler's virus is critical for preventing viral persistence. *J. Virol.* 71: 8875.

Grant RA, Filman DJ, Fujinami RS, Icenogle JP and Hogle JM (1992) Three-dimensional structure of Theiler virus. *Proc. Natl Acad. Sci. USA* 89: 2061.

Luo M, He C, Toth KS, Zhang CX and Lipton HL (1992) Three-dimensional structure of Theiler's murine encephalomyelitis virus (BeAn strain). *Proc. Natl Acad. Sci. USA* 89: 2409.

Monteyne P, Bureau JF and Brahic M (1997) The infection of mouse by Theiler's virus: from genetics to immunology (review). *Immunol. Rev.* 159: 163.

Penna-Rossi C, Delcroix M, Huitinga I *et al.* (1997) Role of macrophages during Theiler's virus infection. *J. Virol.* 71: 3336.

Peterson JD, Waltenbaugh C and Miller SD (1992) IgG subclass responses to Theiler's murine encephalomyelitis virus infection and immunization suggest a dominant role for Th1 cells in susceptible mouse strains. *Immunology* 75: 652.

Simas JP and Fazakerley JK (1996) The course of disease and persistence of virus in the central nervous system varies between individual CBA mice infected with the BeAn strain of Theiler's murine encephalomyelitis virus. *J. Gen. Virol.* 77: 2701.

Yauch RL, Palma JP, Yahikozawa H, Chang-Sung K and Kim BS (1998) Role of individual T-cell epitopes of Theiler's virus in the pathogenesis of demyelination correlates with the ability to induce a Th1 response. *J. Virol.* 72: 6169.

Tick-Borne Encephalitis *see* Encephalitis Viruses

TOBAMOVIRUSES

Dennis J. Lewandowski and **William O. Dawson**, Department of Plant Pathology, Citrus Research and Education Center, University of Florida, Lake Alfred, Florida, USA

Introduction

Early research in the late 1800s on the causal agent of the mosaic disease of tobacco led to the discovery of the phenomenon of viruses. Thus tobacco mosaic virus (TMV), the type member of the tobamovirus group, became the first virus to be discovered, and since then has had a central role in many fundamental discoveries in virology. The first quantitative biological assay for plant viruses was the use of *Nicotiana glutinosa*, which produces necrotic local lesions when inoculated with TMV. TMV was the first virus to be purified and crystallized, which led to the discovery of the nucleoprotein nature of viruses and determination of the atomic structure of the coat protein and the virion. TMV was the first virus to be visualized in the electron microscope, confirming the predicted rigid rod shape. The genetic material of TMV was shown to be RNA, a property previously thought to be restricted to DNA. TMV was the first virus to be mutagenized. The first viral protein for which an amino acid sequence was determined was the coat protein of TMV. Subsequent determination of coat protein sequences from a number of strains and mutants helped to establish the universality of the genetic code. Methods of infecting plant protoplasts with viruses were developed with the tobacco–TMV combination, creating a synchronous system to study events in the infection cycle.

Taxonomy and Classification

Tobamoviruses are in the genus *Tobamovirus*; they have not been assigned to a family. There are 15 members within the *Tobamovirus* genus (**Table 1**). Although tobamoviruses are one of the most intensively studied groups of viruses, the taxonomy is often confused. Historically, plant viruses with rigid virions of approximately 18 × 300 nm were classified as a strain of TMV. Many viruses originally referred to as strains of TMV are now recognized as distinct tobamoviruses. For example, the tobamovirus that has been referred to as the tomato strain of TMV, and which is approximately 80% similar to TMV at the nucleotide sequence level, is actually tomato mosaic tobamovirus. Different tobamoviruses for which the entire nucleotide sequence is known are less than 90% identical similar to TMV at the nucleotide level.

Virus Structure and Composition

Tobamovirus virions are straight tubes of approximately 18 × 300 nm with a central hollow core 4 nm in diameter. Virion composition is approximately 95% protein and 5% RNA. Approximately 2100 individual subunits of a single coat protein are arranged in a right-handed helix around a single RNA molecule, with each subunit associated with three nucleotides.

Purified coat protein and viral RNA assemble into infectious particles *in vitro*. Protein–protein associations are the essential first event of virion assembly. Coat protein subunits assemble into several types of aggregates. Coat protein monomers and small heterogeneous aggregates of a few subunits are collectively referred to as 'A-protein'. The equilibrium between A-protein and larger aggregates is primarily dependent upon pH and ionic strength. The larger aggregates that have been characterized are disks, which are composed of two individual stacked rings of coat protein subunits, and protohelices. Protohelices contain approximately 40 coat protein subunits arranged in a spiral around a central hollow core, similar to the arrangement within the virion.

A sequence-specific stem-loop structure in the RNA, the 'origin of assembly', initiates encapsidation and prevents defective packaging which could result from multiple initiation events on a single RNA

Table 1 Definitive tobamoviruses

TMV	Tobacco mosaic virus
ToMV	Tomato mosaic tobamovirus
TMGMV	Tobacco mild green mosaic virus
ORSV	Odontoglossum ringspot virus
PMMV	Pepper mild mottle virus
CGMMV	Cucumber green mottle mosaic virus
SHMV	Sunn-hemp mosaic virus
RMV	Ribgrass mosaic virus
Ob	Tobamovirus Ob
TVCV	Turnip vein clearing virus
YMV	Youcai mosaic virus
FrMV	Frangipani mosaic virus
MaMV	Maracuja mosaic virus
SOV	Sammon's Opuntia virus
UMMV	Ullucus mild mosaic virus

molecule. The origin of assembly is located within the open reading frame (ORF) for the movement protein of most tobamoviruses and within the coat protein ORF of sunn-hemp mosaic (SHMV) and cucumber green mottle mosaic (CGMMV) tobamoviruses. Subgenomic mRNAs containing the origin of assembly are encapsidated into shorter virions that are not required for infectivity. The level of expression of a particular subgenomic mRNA containing the origin of assembly determines the relative proportion of that particular virion species. Consequently, in most tobamoviruses, in which the origin of assembly is located within the movement protein ORF, subgenomic mRNAs account for only a minor fraction of the total virion population. In contrast, where the origin of assembly is located within the highly expressed coat protein subgenomic mRNA, as in SHMV and CGMMV, a significant proportion of smaller virions are produced. Hybrid nonviral RNAs containing an origin of assembly will also assemble with coat protein into virus-like particles of length proportional to that of the RNA.

Virion assembly initiates as the primary loop of the origin of assembly is threaded through a coat protein disk or protohelix with both ends of the RNA trailing from one side. The conformation of the coat protein protohelix changes as the RNA becomes embedded within the groove between the two layers of subunits. Elongation is bidirectional, proceeding rapidly towards the 5′ end of the RNA as the RNA loop is extruded through the elongating virion and additional coat protein disks are added. There is disagreement about the mechanism of elongation towards the 3′ end of the RNA, but it appears that this slower process involves the addition of smaller protein aggregates.

Genome Organization

The tobamovirus genome consists of one single-stranded (ss) positive-strand RNA of approximately 6400–6600 nucleotides (**Fig. 1A**). There is a methylguanosine cap at the 5′ terminus, followed by an AU-rich leader approximately 70 nt in length. The 3′ nontranslated end of the RNA consists of sequences that can be folded into a series of pseudoknot structures, followed by a tRNA-like terminus. The tRNA-like terminus can be aminoacylated *in vitro*, and in most cases specifically accepts histidine. The exception is SHMV, which accepts valine and appears to have arisen by a recombination event between a tobamovirus and a tymovirus.

Four ORFs that are contained within the tobamovirus genome (**Fig. 1A**) correspond to the proteins found in infected tissue. Two overlapping ORFs begin

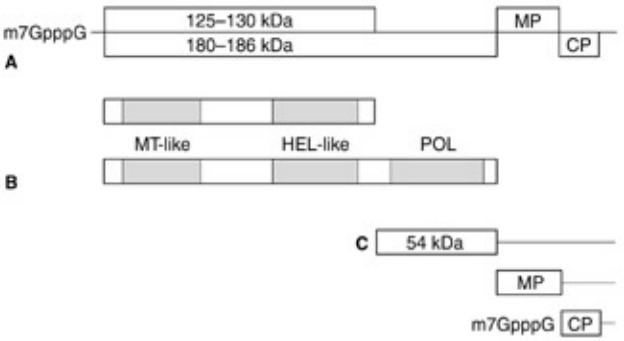


Figure 1 Tobamovirus genome organization and gene expression strategy. (**A**) Tobamovirus genome organization. ORFs designated as open boxes. Untranslated regions designated as solid lines. (**B**) Nonstructural proteins involved in tobamovirus replication. Domains of amino acid sequence similarity to viruses within 'alphavirus supergroup' are designated as hatched boxes. (**C**) Subgenomic mRNAs with 5′ proximal ORF labelled. MP, movement protein; CP, coat protein; MT, methyltransferase; HEL, helicase; POL, polymerase.

at the 5′ proximal start codon. Termination at the first inframe stop codon produces a 125–130 kDa protein. A 180–190 kDa protein is produced by readthrough of this termination codon approximately 5–10% of the time. Both proteins are necessary for efficient replication.

The remaining proteins are expressed from individual 3′ coterminal subgenomic mRNAs, from which only the 5′ proximal ORF is expressed (**Fig. 1C**; **Fig. 2**). The next ORF encodes the movement protein, which has RNA binding activity and is required for cell-to-cell movement of the virus. The 3′ most ORF encodes the coat protein (17–18 kDa). A subgenomic mRNA containing an ORF for a 54 kDa protein that encompasses the readthrough domain of the 180–190 kDa ORF has been isolated from infected tissue, although no protein has been detected.

Within the protein-coding regions of the genome, there are nucleotide sequences that have additional functions as *cis*-acting elements, such as subgenomic mRNA promoters and the origin of assembly. The promoter elements for subgenomic mRNA synthesis are located on the genomic length complementary RNA, presumably upstream from the respective initiation sites. Gene expression from subgenomic mRNAs is regulated both temporally and quantitatively. The movement protein is produced early and accumulates to low levels, whereas the coat protein is produced late and in massive quantities. Several factors are probably involved in regulation of gene expression from the various subgenomic mRNAs. The subgenomic mRNA for the movement protein is not capped and has a long 5′ leader, whereas the coat protein

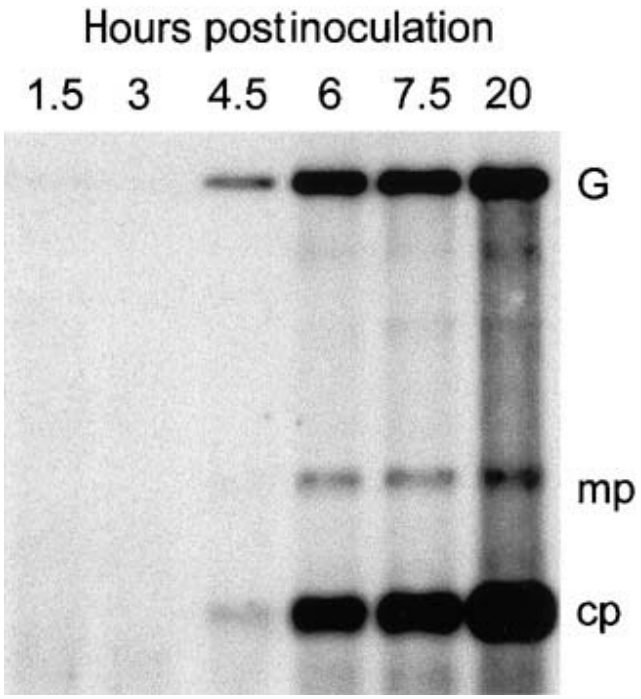


Figure 2 Northern blot of accumulation of TMV positive-stranded RNAs in tobacco protoplasts. Total RNA was extracted from tobacco protoplasts transfected with *in vitro* RNA transcripts of an infectious TMV cDNA clone at the times indicated. G, genomic RNA; mp, subgenomic mRNA for movement protein; cp, subgenomic mRNA for coat protein.

subgenomic mRNA is capped, has a short AU-rich leader, and is an efficient mRNA. Also, the levels of movement protein and coat protein production in TMV mutants are directly related to distance from the 3′ terminus. There are no obvious sequence similarities between the promoters for the movement and coat protein subgenomic mRNAs, suggesting that secondary structure may be important for recognition.

Viral Proteins

The TMV 126/183 kDa proteins are involved in efficient viral replication. Both are contained in crude replicase preparations, and temperature-sensitive replication-deficient mutants map to these ORFs. There are three domains of amino acid sequence similarity shared with replicase proteins from other ssRNA plant and animal viruses (**Fig. 1B**). The N-terminal domain of the 126/183 kDa proteins has sequence similarity with a domain having methyltransferase activity associated with viral RNA capping. The C-terminal domain of the 126 kDa protein (also shared with the 183 kDa protein), is proposed to have helicase activity, based upon conserved sequence motifs. The third domain, which has a signature sequence for RNA polymerase function, is located within the readthrough region of the 183 kDa protein. Additionally, the 126/183 kDa proteins are symptom determinants, as mutations in mild strains map to these ORFs.

The movement protein has a plasmodesmatal binding function associated with its C-terminus and a single-stranded nucleic acid-binding domain associated with the N-terminus. The movement protein–host interaction determines whether the virus can systemically infect some plant species.

Although principally a structural protein, the coat protein is also involved in other host interactions. Coat protein is required for efficient long-distance movement of the virus. Coat protein is also a symptom determinant in some susceptible plant species and an elicitor of plant defense mechanisms in other plant species.

Interactions between Viral and Host Proteins

Available evidence suggests that the interactions of viral proteins with host factors are important determinants of viral movement and host ranges. Amino acid substitutions in the movement protein can alter the movement function in different hosts. Some viruses, including tobamoviruses, can assist movement of other viruses that are incapable of movement in a particular plant species. These interactions suggest that there are more precise associations of viral proteins with host factor(s) than with viral RNA. Additionally, precise coat protein–plant interactions are required for movement to distal positions within the plant.

Virus Replication

Virions or free viral RNA will infect plants or protoplasts. Since tobamoviruses have a genome consisting of messenger-sense RNA that is infectious, one of the first events is translation of the 5′ proximal ORFs to produce the proteins required for replication of the genomic RNA and transcription of subgenomic mRNAs. When virions are the infecting agent, the first event is thought to be cotranslational disassembly, in which the coat protein subunits at the end of the virion surrounding the 5′ end of the RNA loosen, making the RNA available for translation. Ribosomes then associate with the RNA, and translation of the 126/183 kDa ORFs is thought to displace coat protein subunits from the viral RNA.

After the formation of an active replicase complex, a complementary minus-strand RNA is synthesized from the genomic positive-stranded RNA template. Minus-strand RNA serves as template for both

genomic and subgenomic mRNAs. Negative-stranded RNA synthesis ceases early in infection, while positive-stranded RNA synthesis continues. This results in an asymmetric positive- to negative-stranded RNA ratio. Early in infection, genomic RNA functions as template for minus-strand RNA synthesis and as mRNA for production of the 126/183 kDa proteins. Later in the infection cycle, most of the newly synthesized genomic RNA is encapsidated into virions. Subgenomic mRNAs transcribed during infection function as mRNA for the 3′ ORFs that are not translated directly from the genomic RNA.

Within an infected leaf, replication proceeds rapidly between approximately 16 and 96 h postinfection within a cell, after which replication ceases. Even though the infected cells become packed with virions, these cells remain metabolically active for long periods of time. During the early stages of infection of an individual cell, the infection spreads through plasmodesmatal connections to adjacent cells. This event requires the viral movement protein that modifies plasmodesmata to accommodate larger molecules. Movement through plasmodesmata does not require the coat protein. A second function of the movement protein appears to be binding to the viral RNA to assist its movement through the small plasmodesmatal openings. The movement protein also appears to associate with the cytoskeleton. As the virus spreads from cell to cell throughout a leaf, it enters the phloem for rapid long-distance movement to other leaves and organs of the plant. This process requires the coat protein.

Cis-acting Sequences

The 5′ nontranslated region contains sequences that are required for replication. This region has also been shown to be an efficient translational leader. The 3′ nontranslated region contains *cis*-acting sequences that are involved in replication. Certain deletions within the pseudoknots are not lethal, but result in reduced levels of replication. Exchanges of 3′ nontranslated regions between heterologous tobamoviruses result in viable viruses, suggesting that these are structural elements. The 3′ nontranslated region appears to be a translational enhancer, both in the viral genome and when fused to heterologous reporter mRNAs. Sequences encoding the internal ORFs for the movement and coat proteins are dispensable for replication.

The coat protein subgenomic mRNA promoter is better defined than the movement protein subgenomic promoter. Duplication of the coat protein subgenomic promoter results in transcription of an additional new subgenomic mRNA. Subgenomic promoters from heterologous tobamoviruses are recognized and are active. Foreign sequences inserted behind tobamovirus subgenomic mRNA promoters can be expressed in plants and protoplasts.

Satellite Tobacco Mosaic Virus

Satellite tobacco mosaic virus (STMV), a tobamovirus-dependent satellite virus, has been isolated from *Nicotiana glauca* infected with the tobacco mild green mosaic tobamovirus (TMGMV). The genome consists of one plus-sense ssRNA of 1059 nucleotides. The 240 3′ nucleotides share approximately 65% sequence similarity with TMGMV and TMV, contain two pseudoknot structures and have a tRNA-like terminus. No sequence similarity to tobamoviruses exists over the remainder of the genome. Two overlapping ORFs that are expressed in *in vitro* translation reactions are present in the genomic RNA of most STMV isolates. The 5′ proximal ORF encodes a 6.8 kDa protein that has not been detected *in vivo*. The second ORF encodes the 17.5 kDa coat protein, which is not serologically related to any tobamovirus coat protein. The 17 nm icosahedral virions are composed of a single STMV genomic RNA encapsidated within 60 STMV coat protein subunits. Replication of natural populations of STMV is supported by other tobamoviruses, but at lower levels than with the natural helper virus, TMGMV. The host range of STMV parallels that of the helper virus. No effects on symptom expression by any of the helper tobamoviruses have been observed.

See also: **Plant virus disease – economic aspects; Satellite RNAs and Satellite viruses.**

Further Reading

Buck KW (1996) Comparison of the replication of positive-stranded RNA viruses of plants and animals. *Adv. Virus Res.* 47: 159.

Dawson WO (1992) Tobamovirus–plant interactions. *Virology* 186: 359.

Dawson WO and Lehto KM (1990) Regulation of tobamovirus gene expression. *Adv. Virus Res.* 38: 307.

Gibbs AJ (1977) Tobamovirus group. *CMI/AAB Descriptions of Plant Viruses,* No. 184.

Matthews REF (1991) *Plant Virology,* 3rd edn. New York: Academic Press.

Turpen TH and Dawson WO (1992) Amplification, movement and expression of genes in plants by viral-based vectors. In: Hiatt A (ed.) *Transgenic Plants: Fundamentals and Applications.* New York: Marcel Dekker.

van Regenmortal MHV and Fraenkel-Conrat H (eds) (1986) *The Plant Viruses.* Vol. 2: *The Rod-Shaped Plant Viruses.* New York: Plenum Press.

TOBRAVIRUSES

Peter B Visser, Alexander Mathis and **Huub JM Linthorst**, Institute of Molecular Plant Sciences, Gorlaeus Laboratories, Leiden, The Netherlands

Taxonomy and Classification

The tobraviruses (type virus: tobacco rattle virus) are bipartite plant viruses which are members of the genus *Tobravirus* (a family has not been assigned). These are (1) tobacco rattle viruses (TRV), (2) pea early browning viruses (PEBV) and (3) pepper ringspot virus (PRV), which was formerly known as the CAM strain of TRV. The first two viruses contain several to numerous distinguishable strains or isolates, whereas only one PRV has yet been identified. Their classification into one genus of tobraviruses was originally based on common properties such as particle morphology, transmission vector and the unusual ability to cause a systemic infection in plants with only part of the genome. Recently this grouping has been supported by molecular studies. Comparisons based on molecular characteristics have also indicated that the tobraviruses are members of the supercluster of Sindbis-like viruses. Another plant virus member of this superfamily is the tobamovirus tobacco mosaic virus (TMV), which, both in morphology and genome organization, closely resembles the tobraviruses.

Virus Structure

The tobravirus genome is composed of two single-stranded RNA molecules of positive polarity, which are capped at the 5′ termini and which fold into a tRNA-like tertiary structure at the 3′ termini. However, this tRNA-like structure cannot be aminoacylated, as is the case in other viruses showing such a structure at the 3′ terminus.

The RNAs, designated RNA1 and RNA2, are separately encapsidated by coat protein subunits. The structure of the virions is determined by a helical array of coat protein molecules surrounding the RNA helix, which is very similar to that of tobacco mosaic virus. The virions are rigid rod-shaped particles with similar diameter (20–23 nm) but with a different length. The L (large) particles of TRV, PEBV and PRV have a similar size (180–210 nm); they encapsidate RNA1. The length of the S (small) particles varies considerably (50–110 nm), even between different strains in one subgroup. This difference in size of the S particles reflects the difference in length of the encapsidated RNA2.

During a normal infection cycle, both L particles and S particles are generated, and homogenates of infected plants are highly infectious (M-type infections, where 'M' stands for 'multiplying'). However, sometimes a second type of natural infection is apparent in which no particles are formed (NM-type infections, 'non-multiplying'). While homogenates of plants with NM-type infections are not or hardly infectious, infectious RNA is produced, as is evident after phenol extraction. The recent advancement of our knowledge of plant viruses has made it possible to explain this unique property of the tobraviruses in molecular genetic terms and to relate it to the specific way in which the genes are separated over the two genome segments.

Genome Organization and Molecular Biology

RNA1 molecules are highly homologous between isolates within the same subgroup, but little homology was identified between TRV, PEBV and PRV. RNA2 molecules are very variable in size. Generally, RNA2 of different isolates shows little sequence homology, except for the 3′-terminal region, which is also homologous to the 3′ terminus of RNA1 (**Fig. 1**).

For each of the three tobraviral subgroups, cDNA clones corresponding to the two genomic segments have been characterized. Both RNA1 of TRV strain SYM and RNA1 of PEBV strain SP5 were completely sequenced and shown to encode four open reading frames (ORFs). The first ORF of TRV strain SYM terminates at a UGA stop codon and encodes a protein of 134 kDa. Suppression of this UGA termination codon results in a readthrough protein of 194 kDa. Also, the first two ORFs of PEBV strain SP5 are fused by a UGA termination codon, which, upon suppression, allows the translation of two partially overlapping polypeptides. *In vitro* translation studies have indeed shown that both TRV RNA1 and PEBV RNA1 code for two polypeptides with sizes corresponding to the ones deduced from the nucleotide sequences. Similar mechanisms of genome expression via readthrough translation have been found with viruses belonging to several groups, among which are plant viruses from the tobamo-, luteo-, tymo- and carmovirus groups. The 3′-terminal one-third of

tobraviral RNA1 contains two ORFs encoding smaller proteins (29 kDa and 16 kDa for TRV, and 30 kDa and 12 kDa for PEBV), which are probably expressed via subgenomic mRNAs.

These data, supplemented with partial sequence data from other tobraviruses, have indicated that extended similarities exist between the various proteins encoded by the different members of the tobravirus group and even with viruses from other plant virus groups. For instance, the 134 and 194 kDa TRV-SYM proteins contain regions with high homology with similar regions in the TMV 126 and 183 kDa proteins and in the proteins encoded by RNA1 and RNA2 of the tricornaviruses. Based on these similarities, functions have been inferred for the different encoded proteins. The second half of the 134 kDa TRV RNA1-encoded peptide contains a nucleotide-binding motif present in helicases, whereas the readthrough portion of the 194 kDa protein accommodates a so-called 'GDD box', which is present in the catalytic subunit of RNA-dependent RNA polymerases. The presence of these motifs is a strong argument in favor of the involvement in genome replication of the large tobravirus RNA1-encoded proteins.

The 29 kDa protein encoded by TRV RNA1 has homology with the TMV 30 kDa movement protein. The functional homology of these two proteins was proven by mutation analysis. A TRV mutant with a defect in the gene for the 29 kDa protein could not infect whole tobacco plants completely. This defectiveness can be complemented by transgenic expression of the TMV 30 kDa protein. The TRV 29 kDa protein may also play a role in symptom induction as this same mutant, complemented with TMV 30 kDa protein, did not produce necrotic spots on test plants, as does the wild-type TRV.

The small proteins encoded by the 3′-terminal ORF in RNA1 of PEBV (12 kDa) and TRV (16 kDa) are homologous. Both polypeptides contain a cysteine-rich putative zinc-finger structure but lack homology with proteins encoded by other plant viruses. TRV infected protoplasts accumulate large amounts of the 16 kDa protein. Infectious transcripts of TRV RNA1 containing mutated 16 kDa ORFs indicated that this protein is not required for replication. While combinations of infectious transcripts of PEBV RNA1 and RNA2, lacking an intact 12 kDa ORF, were highly infectious and the resulting virus accumulated to high levels in leaves and pods, virus could not be detected in pollen grains and ovules, and seed transmission of the mutant was less than 1% of that of the wild-type virus.

Thus, the combination of replication and movement functions allows RNA1 to replicate and spread on its own to give rise to the NM-type infections.

In contrast to similarity in size of RNA1, RNA2 is more variable in both size and nucleotide sequence. However, in all instances the 5′-proximal gene encodes the coat protein. The coat protein of TRV strain TCM is over 90% similar to the coat protein of known isolates of PEBV at the amino acid sequence level. Likewise, coat protein genes of TRV strains PLB, PSG and PpK20 share over 90% homology, whereas the similarity between coat proteins of TCM and PLB is about 40%. The PRV coat protein cistron has only 40% homology with coat proteins of both TCM and PLB.

The variability in length of RNA2 is due both to the presence of additional genes on the larger molecules and to a variable sized 3′-terminal region homologous with that of the corresponding RNA1 (**Fig. 1**). Northern blot hybridization using probes complementary to different regions in RNA2 of TRV strain PpK20 showed that various subgenomic RNAs are produced which enable separate translation of the three RNA2-derived proteins, including the 5′-proximal coat protein. Similarly, ORFs of PEBV strain SP5 also appear to be produced from subgenomic RNAs.

Computer database analysis revealed that the 29.6 kDa product of PEBV RNA2 has 35% overall

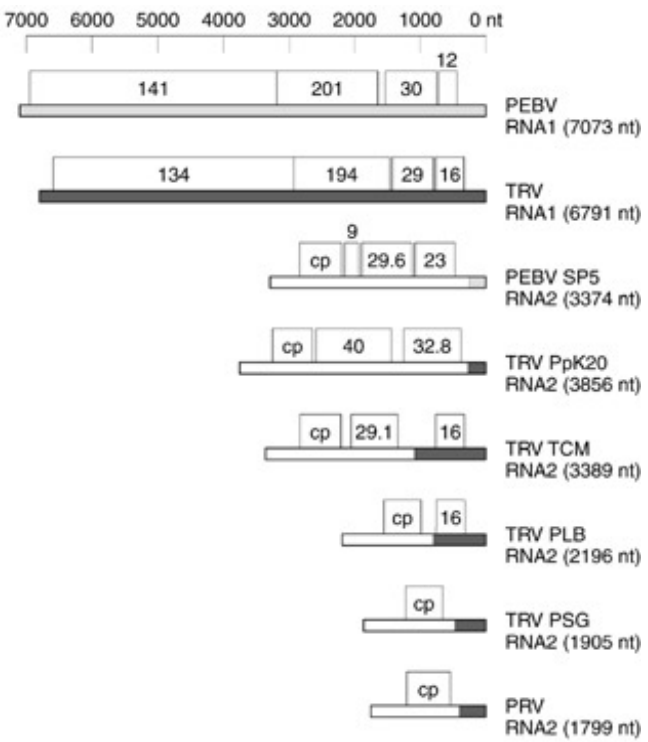


Figure 1 Genomic organization of TRV and PEBV RNA 1 and six tobraviral RNA2 molecules. Relative positions are given of the coat protein and other ORFs (with numbers indicating the molecular weight of their potential products). Shaded bars at the 5′ termini of RNA2 correspond with nucleotide sequences homologous with RNA1.

amino acid sequence homology with the 29.1 kDa putative protein of TRV strain TCM. Apart from that, no other homologies with previously described proteins were found for the other putative proteins encoded by RNA2.

Since TRV strain PSG is infectious by mechanical inoculation and its RNA2 does not contain any ORF in addition to that of the coat protein, the extra genes present on RNA2 of other strains probably have no function in replication. However, strain PSG (but also PLB and TCM which do contain extra ORFs in RNA2) lack the ability to be transmitted by any known nematode vector. Recently, infectious cDNA clones of nematode transmissible isolates became available: PEBV TPA56 and TRV PpK20. Sequencing PEBV TPA56 revealed that this isolate is nearly homologous with the nontransmissible PEBV SP5 strain, with only 11 differences out of 3374 nucleotides. Deletions or frameshifts in the nonstructural genes of PEBV TPA56 RNA2 abolished the transmission of this virus by its associated vector nematode *Trichodorus primitivus*. Also, mutations that affected the 40K gene of TRV strain PpK20 abolished nematode transmission by *Paratrichodorus pachydermus*, whereas a large deletion in the 32.8K gene had no effect on transmission. None of the mutations in the nonstructural genes interfered with encapsidation or replication of the virus. From these experiments it could be concluded that at least some of the RNA2 encoded nonstructural proteins of the tobraviruses are involved in transmission by their vector nematodes. Functions of the other nonstructural proteins encoded by RNA2 remain unknown, but it was suggested that they might play a role in the transmission of tobraviral strains by as yet unidentified species of nematode vectors.

Virus Genetics

In all tobraviruses sequenced up till now, very high homology at the 3′ terminus between both genomic RNAs of each isolate was found. The homologous sequence varies between about 500 and 1100 nucleotides (TRV isolates) and was found to be 266 nucleotides in PEBV-SP5 and 459 in PRV. Whereas these sequences are identical in the RNAs of the TRV strains and in PRV, there are nine differences in the PEBV-SP5 homologous stretch. In tobraviruses, the acquisition of the 3′ terminus of RNA1 by RNA2 is thought to have occurred by recombination based on a copy-choice mechanism of the viral replicase. The junction between RNA2- and RNA1-specific sequences shows a sequence that closely resembles the one that occurs at 5′ termini of subgenomic TRV RNAs.

The nature of the selection pressure that leads to perfect or almost perfect homologous 3′-terminal sequences between RNA1 and RNA2 is not known. Earlier work with pseudorecombinants, which are the experimental combination of RNA1 (or RNA1-containing L particles) from one strain with RNA2 (or S particles) from another, suggested that identical 3′-terminal sequences of the two genomic RNAs are essential for virus stability, maybe by playing a role in template recognition of the viral replicase. It was found that the ability to form pseudorecombinants is independent of serological relationship between the strains used, but is only possible within one subgroup, e.g. between different TRV strains but not between a TRV strain and a PEBV strain. However, replication studies on recombinant tobraviruses showed that only the 5′-noncoding region of TRV RNA2 or PEBV RNA2 is sufficient in hybrid RNAs to permit their replication by, respectively, TRV RNA1 or PEBV RNA1, regardless of the origin of the 3′-terminal region. Thus, the specificity of template recognition is determined by the 5′-noncoding, but not by the 3′-noncoding region and, at least under laboratory conditions, nonhomologous 3′ termini between RNA1 and RNA2 do not affect virus stability.

The susceptibility of TRV RNA2 for recombination was demonstrated by analyzing the so-called 'anomalous' isolates. These isolates combine properties of TRV (symptom expression, ability to create pseudorecombinants with other TRV isolates) and PEBV (serological relationship). The genome of the 'anomalous' isolates was shown to consist of an RNA1 molecule with extensive similarity to that of other TRV isolates, whereas RNA2 appeared to contain PEBV-like coding regions with 3′- and/or 5′-terminal sequences homologous with TRV RNAs.

Thus, by maintaining the terminal homology with TRV RNA1 and capturing internal (coding) sequences of PEBV RNA2 by recombination, it seems that TRV has evolved a flexible mechanism to adapt itself to environmental changes. Laboratory experiments confirm TRV RNA2's proneness to recombination. When the RNA of strain PpK20 was serially passed in tobacco plants by using phenol-extracted RNA as the inoculum in each transfer, several defective interfering (DI) RNAs rapidly accumulated. All DI RNAs were found to have deletions in the coding sequences of the RNA2. In most cases the coat protein gene and the 40K gene was lost completely, whereas part of the 32.8K gene remained translatable, sometimes as a fusion protein with coat protein sequences (**Fig. 2**). Two DI RNAs were found to be recombinants containing a 5′ sequence derived from RNA2 and a 3′ sequence derived from RNA1, which emphasizes the importance of the noncoding terminal

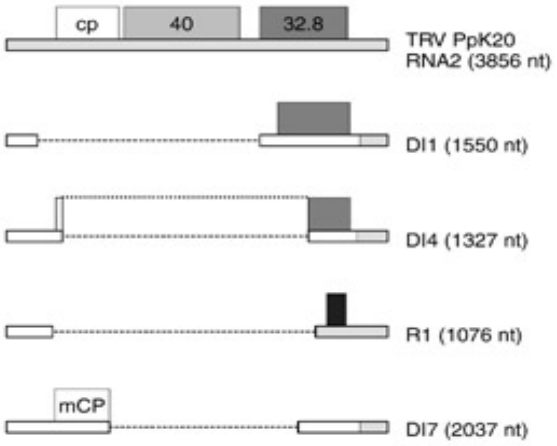


Figure 2 PpK20 defective interfering RNAs and wild-type PpK20 genomic RNA2. The relative positions of the ORFs are shown. Shaded bars at the 5′ termini of the RNAs correspond with nucleotide sequences homologous with RNA1. The dashed lines represent the deleted sequences. The dotted line in DI4 joins the N-terminal and C-terminal regions of a potentially encoded fusion protein. The mutated coat protein encoded by DI7 has been designated mCP (Hernández *et al*, 1996).

sequences for virus stability. When serial passage of TRV strain PpK20 was carried out using leaf homogenates as inocula in each transfer, accumulation of a single DI RNA with a truncated but functional coat protein gene was observed (DI 7). This DI RNA rapidly outcompeted the full-length RNA2, resulting in loss of nematode transmissibility. RNA recombination may explain why most laboratory strains, which have been maintained through the years by mechanical passages, have lost their nematode transmissibility and contain RNA2 molecules of widely varying lengths.

Diseases Caused

Tobraviruses have wide host ranges, infecting herbaceous and a few woody plant species. They cause several types of disease. One of the diseases led to the name of this virus group: when the wind blows through a heavily infected tobacco field a rattle-like sound is produced by the TRV-infected dried-out leaves.

Among tobraviruses, TRV has by far the widest host range, probably the widest of any plant virus. TRV infects more than 100 species in nature, including several common weed species (e.g. *Stellaria media, Capsella bursa-pastoris, Senecio vulgaris*). Experimentally, it was shown that more than 400 plant species in over 50 families can be infected with TRV by inoculation with sap. In about half of these infected plant species, the virus can spread systemically.

In many naturally infected species the virus remains localized at the initial site of infection. Other species are invaded systemically but remain symptomless (as *Stellaria media*) or may develop a wide variety of symptoms. On leaves, symptoms range from necrosis to all kinds of yellow markings (blotching, mottling, mosaic, ringspot), often accompanied by a variable degree of distortion. Of economic importance is the damage caused in bulbous ornamental crops such as tulip, narcissus, lily and crocus, in which leaves become mottled, and gladiolus, which develops notched leaves. Symptoms on underground plant parts include corky arcs in potato tubers (spraing), which lowers the value of the crops, and necrotic spots (malaria) in hyacinth bulbs. Furthermore, vigor and yield are decreased in tomato, sugarbeet, spinach, tobacco, artichoke, celery, pepper and lettuce.

The PEBV subgroup includes the tobravirus isolates that systemically infect leguminous plants. Only four crop species (pea, bean, broad bean and lucerne) are reported to become naturally infected. Symptoms caused by PEBV include large necrotic spots on pea leaflets, leaf distortion with chlorotic V-shaped markings in lucerne and mosaic in bean leaves. Only early browning of pea is known to be a widespread disease. PEBV probably also has hosts among weed species, but these have not been well studied.

No extensive data are available for PRV. This virus is of local concern in Brazil and was reported to cause leaf markings in artichoke and tomato. In addition, wild plants are known to be hosts.

Tobraviruses, especially TRV and PEBV, exist as many serological variants and are known to be very variable in type and severity of symptoms produced. This variability in symptoms may be the result of the specific transmission of tobraviruses by trichodorid nematodes (see below), with different viral strains being transmitted by different nematode species. Furthermore, variation in symptom expression on one host species is well known to occur independently of antigenic variation of the viruses. Experimentally, it was shown that 14 TRV isolates of the same serotype which are naturally transmitted by the same nematode species produced symptoms ranging from severe systemic malformation to mild chlorotic spots on the mechanically inoculated leaves only.

Detection and identification of tobraviruses is further complicated by the existence of NM-type isolates (in which only RNA1 replicates without RNA2). Such infections rapidly spread from cell to cell but only slowly systemically; the symptoms caused are usually more severe than those induced

by M-type isolates (in which both RNA1 and RNA2 are present).

Geographic Distribution

TRV is the most widespread of the tobraviruses and has been recorded throughout Europe, in North America, New Zealand and in Japan. PEBV is known to occur in Europe and North Africa, whereas PRV has only been described in South America (Brazil).

Virus Transmission

Nematode transmission

Tobraviruses are naturally transmitted by root-feeding nematodes of the genera *Trichodorus* and *Paratrichodorus*. At present, seven species within the genus *Paratrichodorus* and four within *Trichodorus* are known to be vectors of tobraviruses.

Acquisition of virus particles takes place together with ingestion of the cytoplasm when the vector nematode is feeding on root cells of infected plants. Nematodes remain viruliferous for long periods of time, for example, more than 1 year in *Trichodorus* spp., but not after molting. There is no evidence for multiplication of tobraviruses in the nematodes, nor for passage to progeny nematodes.

Release of the virus particles from the nematode feeding apparatus is thought to occur by a change in pH caused by saliva flow produced when the nematode starts feeding on a new plant.

In nematodes, TRV particles have been observed throughout the length of the esophageal lumen adsorbed to the cuticular lining of the lumen. This particular region has been shown to stain for carbohydrate, suggesting that lectin-like structures are involved in binding the virus particles to the nematode pharynx. Nuclear magnetic resonance studies on tobraviral particles revealed that protruding, flexible elements are present at the particle surface. Further studies indicated that these flexible elements are formed by a number of amino acid residues at the extreme C-terminus of each coat protein subunit, and that the length and amino acid composition of these flexible ends is strain-specific. Therefore, it is assumed that differences between the C-terminal sequences may reflect differences in transmissibility between virus strains. However, there is genetic evidence that, in addition to the coat protein, other viral proteins are involved in the transmission process. Deletions and frameshifts in the reading frames of RNA2 coding for the non-structural proteins of the transmissible isolate PEBV TPA56 showed that the 29 kDa and 23 kDa proteins, and possibly the 9 kDa protein, are necessary for virus transmission by *Trichodorus primitivus*. Similar experiments with TRV isolate PpK 20 showed that the 40 kDa non-structural protein, but not the 32.8 kDa protein plays a role in the transmission of this virus by *Paratrichodorus pachydermus*. It is suggested that these nonstructural proteins might link the nematode surface to virus particles, perhaps by bridging between the carbohydrate-containing material and the protruding C-terminal part of the virus coat protein. These nonstructural proteins could then be considered as helper components, functionally similar to the helper component of potyviruses or the aphid transmission factor of caulimoviruses.

The factors that determine the specificity of viral transmission have hardly been elucidated. A highly specific relationship has been found between TRV strains and the nematode species involved in their transmission: individual species of *(Para)Trichodorus* transmit serologically distinct isolates of tobraviruses. In addition, certain species are able to transmit more than one virus within the tobravirus group.

Seed transmission

Seed transmission of TRV is not known to occur in crop plants but is possible in several weed species. PEBV and PRV are reported to be seed-borne in pea and tomato, respectively. As described above, there is evidence that the RNA1 encoded 3′-terminal ORF of PEBV is involved in seed transmission, perhaps by allowing passage of the virus over the border between nongenerative and generative tissue.

Virus Epidemiology and Control

The occurrence of tobraviruses depends on the distribution of their nematode vectors, which tend to be prevalent on lighter, sandy or loamy soils. Tobraviruses can survive at sites in three main ways: they can persist in the nematode vector, in perennial plants and in infected seeds.

Viral spread at a site depends on the number, activity and transmitting efficiency of vector nematodes. The number of trichodorids depends mainly on the type of previous crops and on the degree and type of weed infestation (as nematodes multiply differently on different host plants). Weed infestation is also of importance in determining the proportion of nematodes that carry viral particles. A wide range of weed species (e.g. *Stellaria media*) can harbor TRV, with a high incidence of systemic infection. Such plants are a constant source of viral particles to transmitting nematodes. Tests on naturally occurring *Stellaria media* plants are a reliable indication of whether or not TRV occurs at a site.

The activity of nematodes is mainly determined by

soil water content as nematodes need a water film on soil particles to be able to move through the soil. Therefore, incidence of tobravirus-caused diseases is increased after wet periods or in irrigated crops. Optimum temperature for transmission was found to be 15–20°C with little transmission occurring at 4°C.

In Scotland it was shown that 80% of the trichodorid populations in arable land were carrying TRV. The rate of viral transmission by populations of viruliferous nematodes was found to be rather low. This probably reflects the small proportion of virus-carrying individuals. Experiments with single nematodes, however, revealed that, once a nematode has acquired viruses, subsequent transmission to healthy plants can be very efficient.

Another factor affecting the spread of tobraviruses is the vertical and horizontal distribution of vector nematodes. Infected plants typically are patchily distributed in crops. These patches do not necessarily represent the horizontal distribution: as trichodorid nematodes seem to occur in considerable numbers in somewhat deeper soil layers (below the depth of cultivation) but above hard layers, such patches may occur where the topsoil is shallow.

As nematodes move only small distances laterally (probably less than 50 cm per year), spread of virus-carrying nematodes to new sites occurs by agricultural activities. Transport of vector nematodes in wind-blown soil is probably inefficient, as the nematodes are very susceptible to desiccation. Virus-infected seed and vegetative plant material can also be carried for long distances to sites with previously virus-free populations of vector nematodes. Thus it could be observed that trichodorids appeared soon after colonization of a sand dune by grasses but TRV was not recorded until the flora included *Viola tricolor*, in which the virus is seed-borne.

The control of tobraviruses depends largely on the use of tolerant or resistant cultivars (potato, pea) and on the application of expensive nematode-controlling chemicals to vector-infested land. Weeds are both a virus source and a vehicle for virus spread (via seeds). Rigorous control of weeds in order to eliminate virus sources, however, can actually increase TRV infections because virus-carrying trichodorids that may prefer to feed on weed roots are then obliged to feed on crop plants.

Virus dissemination can be minimized by using virus-free planting material (pea, flower bulbs).

See also: **Sindbis and Semliki Forest viruses (*Togaviridae*); Potyviruses (*Potyviridae*); Plant pararetroviruses (*Caulimoviridae*): Caulimoviruses: general features, Caulimoviruses: molecular biology, Legume caulimoviruses; Tobamoviruses.**

Further Reading

Brown DJF, Robertson WM and Trudgill DL (1995) Transmission of viruses by plant nematodes. *Annu. Rev. Phytopathol.* 33: 223.

Harrison BD and Robinson DJ (1986) Tobraviruses. In: Van Regenmortel MHV and Fraenkel-Conrat H (eds) *The Plant Viruses*. Vol. 2: *The Rod-Shaped Plant Viruses*, p. 339. London: Plenum Press.

Hernández C, Carette JE, Brown DJF and Bol JF (1996) Serial passage of tobacco rattle virus under different selection conditions results in deletion of structural and nonstructural genes in RNA 2. *J. Virol.* 70: 4933.

MacFarlane SA (1997) Natural recombination among plant virus genomes: evidence from tobraviruses. *Semin. Virol.* 8: 25.

Wang D, MacFarlane SA and Maule AJ (1997) Viral determinants of pea early browning virus seed transmission in pea. *Virology* 234: 112.

TOMBUSVIRUSES

DM Rochon, Agriculture and Agri-Food Canada, Pacific Agri-Food Research Centre, Summerland, British Columbia, Canada

Taxonomy and Classification

Tombusviruses are 30 nm nonenveloped isometric plant viruses which consist of a monopartite positive-sense RNA genome of about 4.7 kb and 180 copies of an approximate 41 kDa coat protein. The *Tombusvirus* genus presently consists of 13 type species. These are listed in **Table 1** along with a brief description of the geographic distribution and transmission of each member.

soil water content as nematodes need a water film on soil particles to be able to move through the soil. Therefore, incidence of tobravirus-caused diseases is increased after wet periods or in irrigated crops. Optimum temperature for transmission was found to be 15–20°C with little transmission occurring at 4°C.

In Scotland it was shown that 80% of the trichodorid populations in arable land were carrying TRV. The rate of viral transmission by populations of viruliferous nematodes was found to be rather low. This probably reflects the small proportion of virus-carrying individuals. Experiments with single nematodes, however, revealed that, once a nematode has acquired viruses, subsequent transmission to healthy plants can be very efficient.

Another factor affecting the spread of tobraviruses is the vertical and horizontal distribution of vector nematodes. Infected plants typically are patchily distributed in crops. These patches do not necessarily represent the horizontal distribution: as trichodorid nematodes seem to occur in considerable numbers in somewhat deeper soil layers (below the depth of cultivation) but above hard layers, such patches may occur where the topsoil is shallow.

As nematodes move only small distances laterally (probably less than 50 cm per year), spread of virus-carrying nematodes to new sites occurs by agricultural activities. Transport of vector nematodes in wind-blown soil is probably inefficient, as the nematodes are very susceptible to desiccation. Virus-infected seed and vegetative plant material can also be carried for long distances to sites with previously virus-free populations of vector nematodes. Thus it could be observed that trichodorids appeared soon after colonization of a sand dune by grasses but TRV was not recorded until the flora included *Viola tricolor*, in which the virus is seed-borne.

The control of tobraviruses depends largely on the use of tolerant or resistant cultivars (potato, pea) and on the application of expensive nematode-controlling chemicals to vector-infested land. Weeds are both a virus source and a vehicle for virus spread (via seeds). Rigorous control of weeds in order to eliminate virus sources, however, can actually increase TRV infections because virus-carrying trichodorids that may prefer to feed on weed roots are then obliged to feed on crop plants.

Virus dissemination can be minimized by using virus-free planting material (pea, flower bulbs).

See also: **Sindbis and Semliki Forest viruses (*Togaviridae*); Potyviruses (*Potyviridae*); Plant pararetroviruses (*Caulimoviridae*): Caulimoviruses: general features, Caulimoviruses: molecular biology, Legume caulimoviruses; Tobamoviruses.**

Further Reading

Brown DJF, Robertson WM and Trudgill DL (1995) Transmission of viruses by plant nematodes. *Annu. Rev. Phytopathol.* 33: 223.

Harrison BD and Robinson DJ (1986) Tobraviruses. In: Van Regenmortel MHV and Fraenkel-Conrat H (eds) *The Plant Viruses*. Vol. 2: *The Rod-Shaped Plant Viruses*, p. 339. London: Plenum Press.

Hernández C, Carette JE, Brown DJF and Bol JF (1996) Serial passage of tobacco rattle virus under different selection conditions results in deletion of structural and nonstructural genes in RNA 2. *J. Virol.* 70: 4933.

MacFarlane SA (1997) Natural recombination among plant virus genomes: evidence from tobraviruses. *Semin. Virol.* 8: 25.

Wang D, MacFarlane SA and Maule AJ (1997) Viral determinants of pea early browning virus seed transmission in pea. *Virology* 234: 112.

TOMBUSVIRUSES

DM Rochon, Agriculture and Agri-Food Canada, Pacific Agri-Food Research Centre, Summerland, British Columbia, Canada

Taxonomy and Classification

Tombusviruses are 30 nm nonenveloped isometric plant viruses which consist of a monopartite positive-sense RNA genome of about 4.7 kb and 180 copies of an approximate 41 kDa coat protein. The *Tombusvirus* genus presently consists of 13 type species. These are listed in **Table 1** along with a brief description of the geographic distribution and transmission of each member.

Table 1 Natural hosts and geographic distribution of definitive and tentative tombusvirus species[a]

Virus	*Acronym*	*Natural host(s)*	*Geographical distribution*	*Transmission*[b]
Definitive species				
Artichoke mottled crinkle virus	AMCV	Artichoke	Mediterranean	+M
Carnation Italian ringspot virus	CIRV	Carnation, sweet cherry	UK, Italy, USA, Germany	+G, +M
Cucumber necrosis virus	CNV	Cucumber	Canada	+C, +F (*Olpidium bornovanus*), +M, +So
Cymbidium ringspot virus	CyRSV	Cymbidium, white clover	UK	+C, +M, −Se
Eggplant mottled crinkle virus	EMCV	Eggplant, *Solanum capsicastrum*	Lebanon, India	+G, +M
Grapevine Algerian latent virus	GALV	Grapevine, pear, plum	Algeria, Italy, Germany	+M, found in river water in Germany and Sicily
Lato river virus	LRV	Unknown	Unknown	+M, found in water in Lato River, Italy
Moroccan pepper virus	MPV	Pepper, tomato, eggplant, pelargonium	Morocco, Germany	+G, +M,
Neckar river virus	NRV	Unknown	Unknown	+M, found in water in River Neckar, Germany
Pelargonium leaf curl virus	PLCV	Pelargonium	Eurasia, Mediterranean, N. America	+G, +M, −S
Petunia asteroid mosaic virus (=TBSV-Ch)	PAMV	Petunia, cherry, spinach, grapevine, hop, pepper, plum, privet, tomato	Eurasia, Czechoslovakia, Germany, Spain, Switzerland, UK, former Yugoslavia, Korea	+M,+P, +Se (some hosts)
Sikte waterborne virus	SWBV	Unknown	Unknown	From water from River Sitke, Germany
Tomato bushy stunt virus	TBSV	Tomato, pepper, eggplant, lettuce, spinach, tulip, apple, pear, fringe tree	Europe, Mediterranean, N. and S. America	+M, +G, +Se (some hosts)
Tentative species				
Cucumber leaf spot virus	CLSV	Cucumber	Germany, Greece, Jordan, UK	−C,+F (*O. bornovanus*), +M, +Se
Pothos latent virus	PoLV	Pothos(?)	Southern Italy	+M, found in hydroponic solution

[a]Represents a summary of information from original papers, Martelli *et al* (1988) and the VIDE database on plant viruses (*http://biology.anu.edu.au/research-groups/MES/VIDE/refs.htm*)

[b]Abbreviations for modes of transmission: C, contact between plants; F, fungus transmitted; G, graph transmission; M, mechanical transmission; P, pollen transmitted; Se, seed transmission; (+) indicates known means of transmission; (−) indicates that this means of transmission has been ruled out. Note that most tombusviruses are assumed to be soil-transmitted (see text).

Complete nucleotide sequences have been obtained for the genomic RNAs of artichoke mottled crinkle virus (AMCV), carnation Italian ringspot virus (CIRV), cucumber necrosis virus (CNV), Cymbidium ringspot virus (CyRSV) and the cherry strain of tomato bushy stunt virus (TBSV-Ch). The genomes of these viruses have highly similar structures (see **Fig. 1**) and considerable sequence identity exists between comparable regions at both the nucleic acid and protein level (see **Table 2**). The high level of similarity has prompted the suggestion that some of the tombusviruses currently classified as separate species should instead be classified as strains.

Recently, genome sequences of two other potential tombusviruses, cucumber leaf spot (CLSV) and pothos latent virus (PoLV), have been determined. The predicted genome structures are similar to the genome structures of the definitive species (**Fig. 1**). A high level of sequence identity exists between the protein products of these two viruses, however, three of the five viral encoded proteins share only limited similarity with the corresponding proteins of definitive tombusviruses (**Table 2**).

The family *Tombusviridae* encompasses the genus *Tombusvirus* and seven other distinct genera including *Carmovirus*, *Dianthovirus*, the monotypic *Machlomovirus* and *Necrovirus*. The oat chlorotic stunt virus is in the genus *Avenavirus* and panicum mosaic

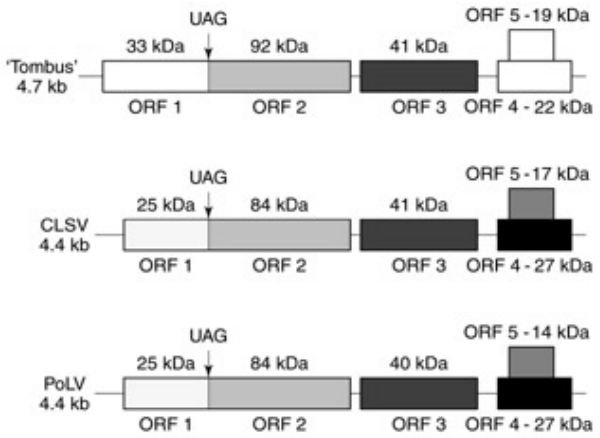


Figure 1 Comparison of the genomic structures of tombusviruses, CLSV and PoLV. The typified 'tombusvirus' genome structure is described in **Fig. 5** legend. The CLSV and PoLV genomic structures are shown individually. Similarly shaded ORFs indicate that the encoded proteins have greater than 30% amino acid sequence identity.

virus in the genus *Panicovirus*. Shared features of the *Tombusviridae* include similar particle morphology, similar genome organizations and expression strategies and also a significant level of amino acid sequence identity in the RNA-dependent RNA polymerase (RdRp) and coat proteins. **Figure 2** shows a dendogram depicting relationships among the RdRps of several members of the *Tombusviridae*. Phylogenetic trees derived from RdRp sequence alignments of *Tombusviridae* are generally reliable indicators of the taxonomic positions of individual members as defined by other criteria. This is in contrast to phylogenetic trees obtained using aligned coat protein (CP) sequences (**Fig. 3**). *Tombusvirus* CP sequences (and notably the P domains, *see also* **Virus Structure**) are the most variable in the genome. This hypervariability likely accounts for antigenic distinctions observed between otherwise very closely related viruses.

A phylogenetic study of the RdRp sequences encoded by several positive-strand RNA viruses has shown that tombusviruses (and other *Tombusviridae* genera) group together with members of the animal pesti- and flaviviruses and the bacterial leviviruses forming one of three major supergroups of RNA viruses.

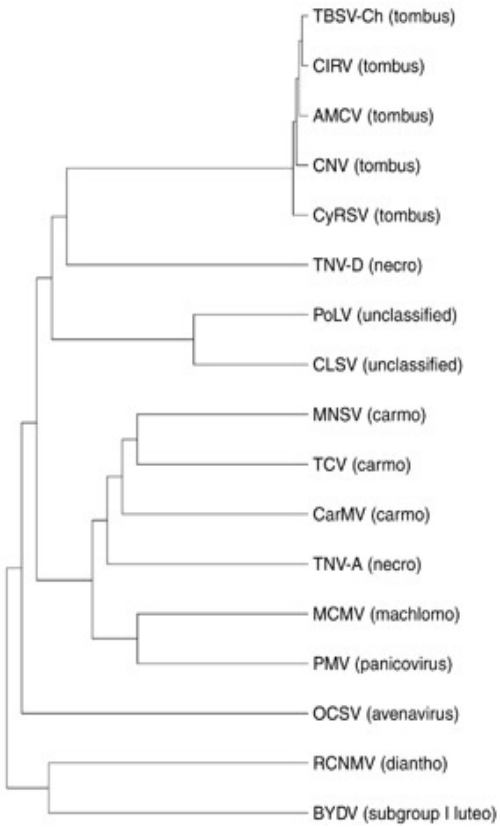


Figure 2 Dendrogram depicting relationships among the RdRps of several definitive and tentative *Tombusviridae* members. Only the readthrough or frameshifted region is aligned. The genus to which the virus belongs is indicated in parentheses. PoLV and CLSV are unclassified. Abbreviations: TBSV-Ch, tomato bushy stunt virus; CIRV, carnation Italian ringspot virus; AMCV, artichoke mottled crinkle virus; CNV, cucumber necrosis virus; CyRSV, Cymbidium ringspot virus; TNV-D, tobacco necrosis virus strain D; PoLV, pothos latent virus; CLSV, cucumber leaf spot virus; MNSV, melon necrotic spot virus; TCV, turnip crinkle virus; CarMV, carnation mottle virus; TNV-A, tobacco necrosis virus strain A; MCMV, maize chlorotic mottle virus; PMV, panicum mosaic virus; OCSV, oat chlorotic stunt virus; RCNMV, red clover necrotic mosaic virus; BYDV, barley yellow dwarf virus.

Table 2 Percentage of identical amino acid residues in pairwise comparisons of proteins encoded by ORFs 1–5 of several tombusviruses, CLSV and PoLV

	ORF 1[a]	*ORF 2*	*ORF 3*[b]	*ORF 4*	*ORF 5*
Tombus/Tombus[c]	53–94	93–97	34–87	84–98	72–92
Tombus/CLSV or PoLV[d]	21–25	43–45	30–53	19–20	12–20
CLSV/PoLV[e]	60	80	33	71	61

[a]The CIRV alignments gave unusually low identity scores. All other alignments gave values between 80 and 94%.
[b]The PLCV CP sequence is included in pairwise alignments.
[c]Shows the range of values obtained in all pairwise comparisons of AMCV, CIRV, CNV, CyRSV, and TBSV-Ch.
[d]Shows the range of values obtained in all pairwise comparisons of AMCV, CIRV, CNV, CyRSV, TBSV-Ch and either CLSV or PoLV.
[e]Shows the values obtained in pairwise comparisons of CLSV and PoLV.

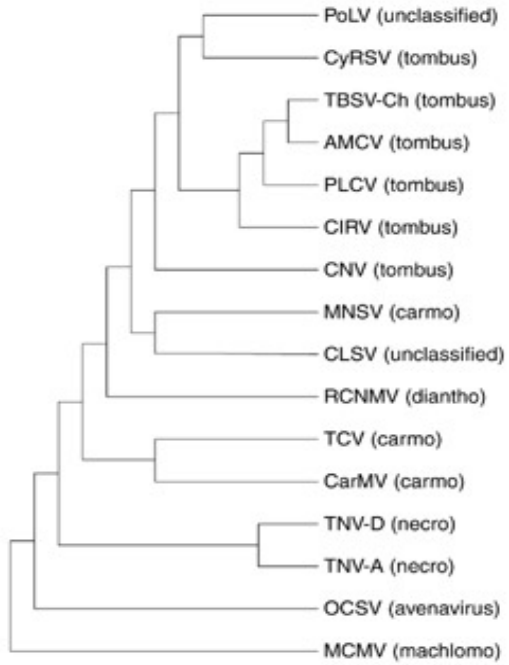


Figure 3 Dendrogram depicting relationships among the coat proteins of several definitive and tentative *Tombusviridae* members. See **Fig. 2** legend for details and abbreviations.

Symptomatology, Host Range and Geographic Distribution

Table 1 summarizes the natural host range, geographic distribution and modes of transmission of the definitive tombusviruses and also PoLV, CLSV and Sikte waterborne virus (SWBV). Tombusviruses as a whole share a wide geographic distribution having been reported in North and South America, several locations in Europe and the Mediterranean and also in Algeria. Individual members of the tombusvirus group, however, generally have a restricted geographic distribution with the exception of TBSV and pelargonium leaf curl (PLCV) which was likely spread internationally through infected pelargoniums. Most tombusviruses naturally infect only a single or a few hosts. This is in contrast to their experimental host range which is wide and diversified. Tombusviruses systemically invade naturally infected plants but remain localized in the majority of experimentally infected plants; common exceptions to this rule are the experimental hosts *Nicotiana clevelandii* and *N. benthamiana*. Tombusvirus-infected crops generally show stunting and diffuse mottling and malformation of leaves. Characteristic lesions associated with the indicator plants basil, globe amaranth and *Chenopodium quinoa* can be used to identify tombusviruses. Further verification can be obtained using antisera or cloned cDNA probes which are widely available. Several tombusviruses have been found associated with defective interfering (DI) RNAs in laboratory infections raising the possibility that DI RNAs can modulate symptoms in natural infections. Finally, many tombusviruses can be inactivated *in vivo* by growing plants at elevated temperatures (ca. 36 °C).

Recent studies have shown that the protein products of open reading frames (ORFs) 4 and 5 are important symptom determinants (see below, Molecular Biology).

Serologic Relationships

Most tombusvirus members are serologically interrelated with cross reactivities ranging from strong to nearly undetectable. CNV and SWBV are the only members which show no detectable serological reaction with other tombusviruses. The tentative species, CLSV, is also serologically unrelated to all tested tombusviruses.

Transmission

Tombusvirus particles are very stable and reach high concentrations in infected tissues. In addition, tombusviruses are efficiently spread and can become established in diverse environments. Different tombusviruses have been shown to be spread in nature by a number of means (see **Table 2**) including seed and pollen transmission, transmission through propagation material and possibly mechanical transmission. Experimentally, tombusviruses are readily sap transmissible and infected leaf extracts retain infectivity after freezing for several years. It is very likely that several tombusviruses are spread through the soil and probably also through irrigation water but attempts to transmit them (except CNV, see below) by soil-borne vectors have not been successful. In addition, attempts to transmit different tombusviruses using aphids, whiteflies or mites have also been unsuccessful.

Several reports have identified tombusviruses in association with rivers and lakes throughout the world and there is evidence suggesting that tombusviruses can enter a field through irrigation water. TBSV is infectious after passage through the human alimentary tract suggesting that tombusviruses may possibly enter rivers through sewage treatment of infective feces.

CNV is the only definitive member of the *Tombusvirus* genus which has been demonstrated to have a specific soil-borne vector. The tentative member, CLSV, is similarly transmitted. Transmission of these viruses is facilitated by zoospores of the Chytrid fungus, *Olpidium bornovanus* (formerly *Olpidium*

radicale). Transmission is mechanistically similar to that demonstrated for the transmission of tobacco necrosis necrovirus by *Olpidium brassicae*. Virus particles and *Olpidium* zoospores are released independently into the soil from roots of infected plants and virus particles adsorb to the axonemal sheath of the zoospore flagellum. Virus gains entry into plants following zoospore infection of plant roots. Different *O. bornovanus* isolates transmit CNV and other viruses in a highly specific manner suggesting that a specific recognition mechanism exists between particles and zoospores. It is possible that this high specificity has precluded the identification of fungal vectors for other tombusviruses.

Molecular studies of CNV transmission by *O. bornovanus* have shown that the CNV coat protein contains determinants which specify transmission. Further studies have shown that a single amino acid change in the coat protein shell domain significantly reduces transmission without altering infectivity, particle stability or viral RNA accumulation. This mutation also affects the level of binding of virus particles to zoospores *in vitro* suggesting that the reduction in transmissibility is at least partly due to less efficient recognition between virus particles and a putative zoospore receptor.

Cytopathology

Cytopathic inclusions known as multivesicular bodies (MVB) and virus-containing bleb-like evaginations of the tonoplast are associated with infection by tombusviruses (**Fig. 4**). MVB are composed of vesicles intermingled with or surrounding granular electron-dense material and are believed to originate from either mitochondria, peroxisomes and/or chloroplasts depending on the virus/host combination examined. Clumps of densely staining amorphous material found scattered or loosely aggregated in the cytoplasm of systemically infected cells (dense granules) are also found in tombusvirus-infected cells but not in all virus–host combinations tested. MVB are believed to be sites of RNA replication and dense granules to be accumulations of excess CP subunit.

Experiments using hybrid genomes created between CyRSV and CIRV, which induce MVB from peroxisomes and mitochondria, respectively, have delineated the MVB-inducing region to a portion of the protein encoded by ORF 1.

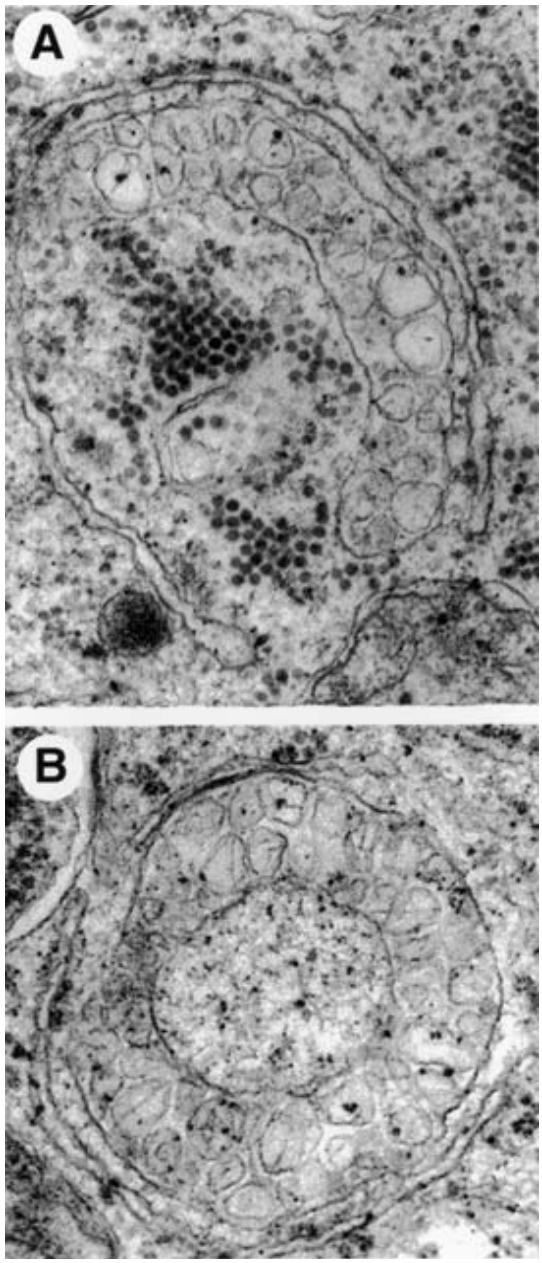


Figure 4 Electron micrographs showing multivesicular bodies formed in CNV-infected *N. clevelandii*. Magnification (**A**) 65 000×; (**B**) 75 000×.

Particle Structure

The structure of TBSV has been determined by x-ray crystallography and is diagrammatically represented in **Fig. 5**. The particle is a $T = 3$ icosahedron approximately 30 nm in diameter which consists of 180 identical CP subunits. Each of the subunits folds into three major domains: an N-terminal highly basic inward-facing RNA binding domain (R); a tightly bonded globular shell domain (S) and a C-terminal outward facing protruding domain (P). The R domain is connected to the S domain by the arm (a) and a five amino acid hinge connects the S and P domains. The P domain projects from the surface of the particle in 90

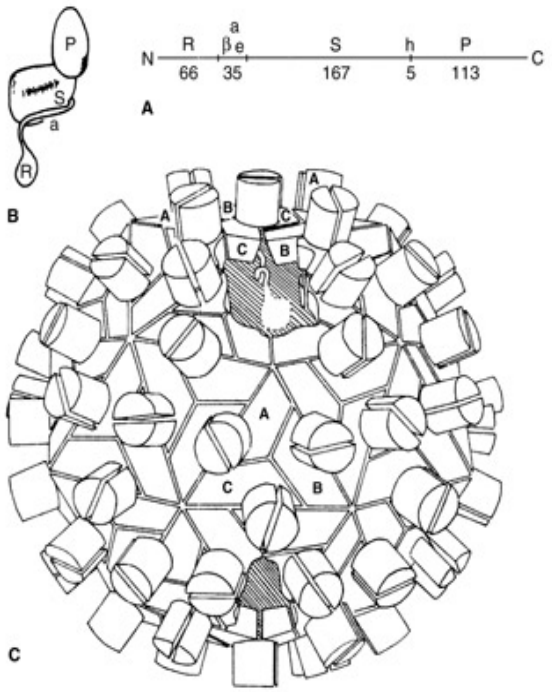


Figure 5 Architecture of the TBSV particle. (**A**) linear arrangement of domains (designated R, a, S, h and P) in the subunit polypeptide. The number of amino acid residues comprising each domain is indicated. (**B**) Schematic diagram of the folding of the polypeptide chain. (**C**) Arrangement of subunits in the virus particle. A, B and C denote the three packing environments of the subunit. (Reprinted with permission from Olson AJ, Bricogne C and Harrison SC (1983) Structure of tomato bushy stunt virus IV. *J. Mol. Biol* 171: 61–93.).

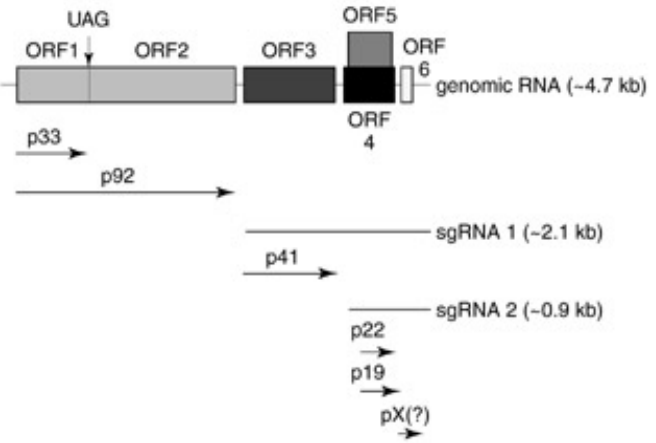


Figure 6 Structure and expression of the tombusvirus genome. The similar structure and expression strategies adopted by tombusviruses is shown. The five major ORFs are indicated by open boxes and the proteins encoded by each ORF by horizontal arrows beneath the ORF. Subgenomic RNA is indicated by a bar beneath the portion of the genome it is derived from. The approximate sizes, in kb, for genomic and sgRNA is indicated. Sizes of proteins may be approximate since they vary slightly from tombusvirus to tombusvirus (i.e. the CIRV 'p33' is actually p36, the CIRV 'p92' is p95, the CNV 'p22' is p21 and the CNV 'p19' is p20).

pairwise clusters. The subunit adopts three conformational states (A, B, C) in the particle due to flexion of the hinge and either an ordered or disordered arm.

TBSV swells at alkaline pH in the absence of calcium ions due to repulsive forces of adjacent negatively charged carboxylate groups. Calcium ions may be important in the release of virion nucleic acid in an infected cell. P domain dimer contacts may also contribute to tombusvirus particle stability. *In vitro* mutagenesis studies show that viral particles do not accumulate when the CNV P domain is deleted. These observations are consistent with a role for the tombusvirus P domain in particle stability. The P domain may also be involved in aggregate formation during particle assembly.

The tombusvirus CP subunit and particle structure shares features in common with several small spherical plant and animal virus particles including those of picornaviruses, black beetle virus, southern bean sobemovirus, turnip crinkle carmovirus and cowpea mosaic and bean-pod mottle comoviruses. Similarities include overall subunit architecture (jelly-roll topology), subunit packing, role of arm domain in coordinating assembly and transition to an expanded virion. The P domain, however, is a feature unique to tombusviruses, carmoviruses and dianthoviruses.

Tombusvirus Molecular Biology

Genome structure

The *Tombusvirus* genus represents one of the most extensively characterized plant virus genera. The genome is a monopartite, plus sense, single-stranded RNA of ~4.7 kb. The genomic sequences of five tombusviruses (AMCV, CIRV, CNV, CyRSV, TBSV-Ch) have been completed and highly infectious transcripts are available for AMCV, CNV, CyRSV and TBSV-Ch. The genomic structure and expression strategy utilized by tombusviruses is highly similar and is summarized in **Fig. 6**. The genome contains five long ORFs (ORFs 1–5) and encodes proteins with approximate molecular weights of 33, 92, 41, 22 and 19 kDa (designated p33, p92, etc.). The sizes of these proteins vary slightly from tombusvirus to tombusvirus (see **Fig. 6** legend) but these designations will apply hereafter. An additional small ORF (ORF 6) located at the 3′ terminus of the genome has been suggested as a possible protein-encoding region. Short

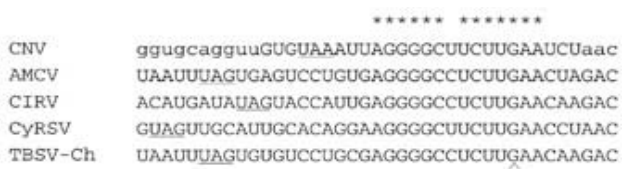


Figure 7 Sequences surrounding the CNV sgRNA2 promoter and comparison to the putative promoters of other tombusviruses. Sequences comprising the core CNV sgRNA promoter are shown in capital letters. Asterisks indicate identity in all five tombusviruses. The sgRNA start site is indicated with a caret and the underlined sequences correspond to the upstream coat protein terminator codon.

intercistronic regions separate ORFs 2 and 3 and ORFs 3 and 4. The 5′ noncoding region ranges in size from 77 to 180 nucleotides (nt) and the 3′ noncoding region (which includes ORF 6) from 338 to 351 nt. The 3′ terminus of the tombusvirus genome does not contain a poly(A) tail and it is presently uncertain as to whether the 5′ end contains a cap structure or some other modification. It is noted that highly infectious transcripts of the tombusvirus genome can be obtained *in vitro* without capping.

Genome expression

ORF 1 initiates with the first AUG codon at the 5′ terminus and terminates with an amber codon to produce p33. The amber codon can be readthrough to produce p92. ORF 2 encodes the CP (p41) and is expressed from the first AUG codon at the 5′ terminus of a ~2.1 kb subgenomic mRNA (sgRNA 1). ORF 5 is nested within ORF 4 in a different reading frame. The respective p19 and p22 translation products are both produced from a single bifunctional ~0.9 kb sgRNA (sgRNA 2; see below).

Genomic plus and minus strand synthesis Most of the data on *cis*-acting sequences required for genomic RNA synthesis rely on studies using defective interfering RNAs (see below, Defective Interfering RNAs).

sgRNA synthesis Subgenomic RNAs accumulate to high levels during tombusvirus infection and are assumed to arise through internal initiation on (−) sense genomic RNA. In CNV and AMCV, synthesis of sgRNA 2 occurs before that of sgRNA 1. A sgRNA which could encode pX is easily detected in CNV-infected plants and protoplasts. Sequences comprising the core promoter for CNV sgRNA 2 have been determined. The promoter lies within a region located 20 nucleotides upstream and six nucleotides downstream of the sgRNA start site. Comparison of sequences within the CNV promoter with the analogous region of other tombusviruses shows strict conservation of 13 of 26 nucleotides (**Fig. 7**). There is little similarity between the sgRNA 2 promoter and the 5′ terminus of the genome or the region surrounding the sgRNA 1 start site. In addition, sequences similar to the ICR2-like motifs found in the promoters of several alphavirus-like (supergroup III) plant and animal viruses are not apparent.

Leaky scanning sgRNA 2 is a bifunctional mRNA capable of encoding distinct proteins *in vitro* and *in vivo* from nested ORFs (ORFs 4 and 5). In CNV, ORF 4 initiates with the first AUG codon on sgRNA 2 16 nt from the 5′ end. ORF 5 initiates at the second AUG codon 29 nt downstream of the ORF 4 AUG. Mutations which increase or decrease the ability of ribosomes to recognize the ORF 4 AUG codon have an inverse effect on ORF 4 expression suggesting that its AUG codon is accessed by leaky ribosomal scanning. Further studies showed that longer sgRNA 2 leader lengths increased expression of ORF 4 relative to ORF 5.

Protein function

p33 and p92 The readthrough portion of p92 contains the canonical 'GDD' motif and surrounding amino acids present in the polymerase domains of positive-strand RNA viruses. Although direct biochemical evidence that these proteins form part of the replicase is absent, genetic evidence is convincing. Methyltransferase, nucleotide binding or helicase motifs have not been identified in either of these two proteins or in any other tombusvirus-encoded protein. The absence of a helicase motif in tombusviruses suggests that a host component may fulfill this function during viral RNA replication.

Immunological analyses of CyRSV- and TBSV-Ch-infected cells have shown that both p33 and p92 accumulate during infection with p33 being approximately 20-fold more abundant than p92. Both proteins are associated with the membrane fraction consistent with a proposed role in viral RNA replication. Mutational analyses of p33 and p92 suggest that both proteins are required for RNA replication but strict evidence for the involvement of p33 is still lacking. Protoplasts from transgenic plants expressing CyRSV p33 and p92 support replication of CyRSV-defective interfering RNAs. In addition, CyRSV deletion mutants lacking ORFs 3, 4 and 5 can replicate in protoplasts. These results provide evidence that ORFs 1 and 2 are the only virus-encoded proteins necessary for viral RNA replication. Tom-

busviruses are believed to replicate in the membranous structures of MVBs formed during virus infection. Evidence exists that p33 is involved in determining the origin of MVB (see Cytopathology).

CP Mutational analyses of the CNV, CyRSV and TBSV-Ch *CP* genes indicate that the *CP* gene is dispensable for viral cell-to-cell and systemic movement. However, systemic spread of coat-protein mutants is not as efficient as wild-type virus as exhibited by delays in the onset of systemic symptoms, a lower percentage of systemically infected plants, or a reduction in the severity of systemic invasion. It is possible that virion formation is required for efficient entry into the vascular system.

The CP P domain (see Virus Structure) is a feature unique to tombusviruses, carmoviruses and dianthoviruses. Deletion of all or part of the P domain coding sequence in CNV (see Particle Structure) results in the absence of particle formation suggesting a role for the P domain in virus particle stability and/or in the formation of particle assembly intermediates. Interestingly, CNV P domain deletion mutants (but not shell domain mutants) rapidly further delete most of the remaining viral CP sequences during infection. The deletion derivatives likely arise through RNA recombination. It is possible that the deletion derivatives have a higher capacity for cell-to-cell movement due to increased production of the cell-to-cell movement protein.

The CNV CP has been found to contain elements involved in the specificity of fungus transmission (see Transmission for additional details).

p22 Several lines of evidence show that the tombusvirus ORF 4 product (p22) encodes the cell-to-cell movement protein. p22 is not required for RNA replication but is required for accumulation of viral RNA in inoculated plants. In addition, histochemical assays of plants inoculated with TBSV-Ch p22 mutants which contain the GUS gene in place of the CP gene show that infection is limited to single cells. Amino acid sequence comparisons of tombusvirus p22 proteins with known and putative viral movement proteins has shown limited but significant similarity. Finally, TBSV-Ch p22 has been found associated with the membrane fraction of plant cells, consistent with its role in viral cell-to-cell movement.

Potato virus X (PVX) derivatives which express the TBSV-Ch p22 gene produce local lesions in *Nicotiana glutinosa* and *N. edwardsonii* rather than the mild mosaic symptoms typical of PVX. These results indicate that p22 is the primary determinant responsible for local lesion formation in these two host plants. As described below, TBSV-Ch p19 and its analogue in other tombusviruses is the primary symptom determinant in other hosts.

p19 Mutations which affect ORF 5 expression in CNV, CyRSV and TBSV-Ch cDNA result in an attenuated phenotype without affecting viral accumulation in plants. In addition, expression of p19 using a heterologous viral vector, PVX, showed that p19 induced a generalized necrosis on infection of *N. benthamiana* and *N. clevelandii* and local necrotic lesions in *N. tabacum*. These symptoms occurred in place of the mild mosaic associated with PVX infection. These results provide evidence that p19 is a major symptom determinant in tombusviruses and further suggest that p19 may interact with host-specific resistance genes present in related *Nicotiana* species.

Start and stop codon mutations which abolish production of the p19 analogue in CNV (p20) lead to loss of necrotic symptoms on inoculated leaves as well as a highly accelerated rate of defective interfering RNA production in plants. The basis for the enhanced rate of *de novo* DI RNA production requires further investigation.

pX Computer assisted analyses of the 3′ terminal regions of several tombusviruses has suggested the existence of a possible sixth small ORF (ORF 6) in tombusviruses leading to production of a small protein (pX) which is between 3.5 and 7.6 kDa. Mutational analyses of the CyRSV ORF 6 have shown that if pX is produced during infection it is not essential for viral infectivity. However, certain mutations in ORF 6 result in nonviable mutants, suggesting that the pX ORF may harbor important *cis*-acting replication signals. A role for pX in viral infection remains to be established.

Defective Interfering (DI) RNAs

DI RNAs

DI RNAs have been found in association with CIRV, CNV, CyRSV and TBSV-Ch and have been extensively characterized in terms of structure, mechanism of formation and mode of interference. DI RNAs occur naturally in plants infected under laboratory conditions and are also generated *de novo* from genomic RNA. DI RNAs are associated with a marked attenuation of the severe symptoms typical of tombusvirus infections.

DI RNA structure DI RNAs correspond to conserved, noncontiguous portions of the tombusvirus genome and range in size from approximately 400 to

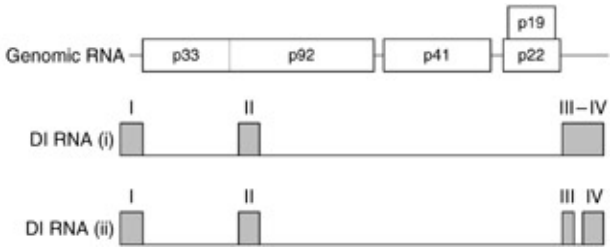


Figure 8 Structure of a prototypical tombusvirus DI RNA. The tombusvirus genome structure is at the top and the two major types of DI RNAs are shown below. Genomic RNA sequences retained in the DIs are indicated by boxes in the DIs whereas horizontal lines correspond to deleted sequences. The four major regions (I–IV) found in DI RNAs are indicated. (Adapted from White (1996).)

800 nt. A prototypical tombusvirus DI RNA consists of four segments of the viral genome designated regions I through IV (**Fig. 8**). Region I corresponds to the 5′ terminal noncoding region and regions III and IV correspond to contiguous or noncontiguous segments at the 3′ terminus of the genome. Region II corresponds to an internal portion of the genome. DI RNAs differ from each other in the amount of sequence deleted between different regions, in the extent of internal deletions within regions, or through duplications of regions or portions of regions. In CNV and CyRSV, head-to-tail dimers of complete monomeric DI RNA units have been observed in plants. The significance of the dimers is not known but dimers can be formed from monomers and it is possible that dimers are templates for the formation of monomers during DI RNA replication.

DI RNA formation A stepwise deletion model for the formation of DI RNAs from genomic RNA has been proposed. In this model, DI RNAs are formed following a series of deletion events as a result of a copy choice mechanism of RNA recombination. Both primary and secondary sequence elements as well as selection for replication competence contribute to the final structure.

Sequences/structures involved in DI replication The smallest DI RNA molecules (~400 nt) tolerate little sequence or structural change suggesting that the retained sequences contain the minimal essential elements for replication/accumulation. A putative stem–loop structure contained within the 3′ terminal 77 nucleotides of CyRSV RNA has been suggested to be important in negative strand synthesis of DI RNA as well as genomic RNA.

Interference DI RNAs are associated with reductions in virus-induced symptoms and virus accumulation. At least two mechanisms account for the observed symptom attenuation. One is that DI RNAs compete with genomic RNA for the viral replicase resulting in reduced viral RNA synthesis. The other is that the presence of DI RNA results in preferential suppression of sgRNA 2 levels relative to genomic RNA. This suppression leads to lower levels of p22 and p19 and consequently reduces symptoms.

Satellite RNAs

A satellite RNA associated with CyRSV has been characterized and found to be composed of 621 nucleotides. Two regions of the satellite RNA show homology to genomic RNA: 9 of the first 14 nt are shared and 49 of 53 nt of the satellite RNA are similar to a segment in the 5′ noncoding region of genomic RNA. These conserved regions are also present in tombusvirus DI RNAs suggesting an important role of these particular sequences in the replication process.

Virus-Resistant Transgenic Plants

Plants transformed with portions of viral genomes often show resistance to viral infection in a specific manner. Transgenic *N. benthamiana* plants expressing the CyRSV CP are resistant to infection but only at very low virus inoculum concentrations. No resistance occurs at higher inoculum concentrations or when plants are inoculated with RNA. Selected transgenic plant lines expressing CyRSV p92 show resistance when inoculated with RNA or virus. Resistance is specific and inversely correlated with the level of expression of transgene mRNA suggesting resistance is RNA-mediated. Transgenic plants expressing CyRSV DI RNAs were found to be protected from the apical necrosis and subsequent plant death normally associated with infection by this virus. Interestingly, transgenic plants expressing CyRSV satellite RNAs actually increased symptom severity. It was suggested that the expressed satellite RNA sequences may interfere with the ameliorating effects of naturally occurring DI RNAs.

See also: **Carmoviruses (*Tombusviridae*); Defective interfering viruses; Plant virus disease – economic aspects; Satellite RNAs and Satellite viruses; Virus structure: Atomic structure, Principles of virus structure.**

Further Reading

Harrison SC (1983) Virus structure: high resolution perspectives. *Adv. Virus Res.* 28: 175.

Martelli GP, Gallitelli D and Russo M (1988) Tombus-

viruses. In: Koenig R (ed.) *The Plant Viruses*, vol. 3, p. 13. New York: Plenum.
Russo M, Burgyan J and Martelli GP (1994) Molecular biology of Tombusviridae. *Adv. Virus Res.* 44: 381.
Scholthof HB, Scholthof K-BG and Jackson AO (1995) Identification of tomato bushy stunt virus host-specific determinants by expression of individual genes from a potato virus X vector. *Plant Cell* 7: 1157.
White KA (1996) Formation and evolution of tombusvirus defective interfering RNAs. *Semin. Virol.* 7: 409.

TOROVIRUSES (CORONAVIRIDAE)

Marian C Horzinek, Virology Unit, Veterinary Faculty, Utrecht, The Netherlands

History

Toroviruses were defined in the early 1980s as a result of a trilateral, partly collaborative study between groups in Berne, Switzerland (isolation in cell culture), Ames/Iowa, USA (discovery of Breda virus and propagation in calves) and Utrecht, The Netherlands (biologic and molecular characterization).

An equine torovirus (ETV), originally referred to as Berne virus, was accidentally isolated in equine kidney cells in 1972 from a rectal swab taken from a horse with diarrhea. Upon post-mortem examination pseudomembranous enteritis and miliary granulomas and necrosis in the liver were diagnosed; Salmonella Lille (O, 6, 7, Z_{38}) was considered to be the causative agent. ETV can be propagated in lines of equine dermis or embryonic mule skin cells, where it causes a cytopathic effect that results in cell lysis. The virus was not neutralized by antisera against known equine viruses. Serologic crossreactions were observed in neutralization and ELISA with sera from calves that had been experimentally infected with morphologically similar particles, the Breda viruses.

A bovine torovirus (BTV), first described as Breda virus was discovered in 1979 during investigations in a dairy herd in Breda (Iowa), in which severe neonatal calf diarrhea had been a problem for three consecutive years. Despite repeated attempts, BTV has not been adapted to growth in cell or tissue culture, which has hampered its biochemical, biophysical and molecular characterization. The pathogenesis and pathology of BTV infections have been studied in gnotobiotic calves.

Torovirus-like particles have been seen in EM preparations from fecal samples of pigs and humans (children and adults); proof that the observed structures are not artefacts was obtained when toroviral RNA sequences were found in the feces of piglets and in stools from humans with diarrhea.

Taxonomy and Classification

The name of the viruses assembled in this genus is derived from the term *torus* (Latin) which designates the lowest convex molding in the base of a column or pilaster. It refers to the biconcave disk or doughnut shape of the virion that is determined by a tubular capsid of helicoidal symmetry, which is surrounded by a peplomer-bearing envelope. In addition to the unique toroid form, virions may also appear as straight or bent rod-shaped particles.

Analysis of the genetic information and replication mode of the prototype ETV showed that toroviruses are evolutionarily related to the *Coronaviridae* and, to a lesser extent, to the *Arteriviridae*. However, the lack of sequence homology in the structural genes and the absence of antigenic relatedness with coronaviruses justify their separate taxonomic position as a genus, *Torovirus*, in the *Coronaviridae* family.

The families *Coronaviridae* and *Arteriviridae* form the Order *Nidovirales* (from *nidus*, Latin for 'the nest' – alluding to the nested set of subgenomic RNAs transcribed during replication), the second order recognized in animal virus taxonomy so far.

Virion Properties

Extracellular ETV particles possess a helical nucleocapsid coiled into a hollow tube (diameter 23 nm, average length 104 nm, periodicity 4.5 nm) which is either straight or bent into an open torus. A tightly fitting envelope, 11 nm thick, surrounds this structure. Consequently, the virion may assume an erythrocyte-like or a kidney shape, depending on whether the envelope follows the small curvature of the nucleocapsid or not. The largest diameter of ETV is estimated at 120–140 nm. Club-shaped projections (average length 20 nm) are present on the particle surface.

viruses. In: Koenig R (ed.) *The Plant Viruses*, vol. 3, p. 13. New York: Plenum.
Russo M, Burgyan J and Martelli GP (1994) Molecular biology of Tombusviridae. *Adv. Virus Res.* 44: 381.
Scholthof HB, Scholthof K-BG and Jackson AO (1995) Identification of tomato bushy stunt virus host-specific determinants by expression of individual genes from a potato virus X vector. *Plant Cell* 7: 1157.
White KA (1996) Formation and evolution of tombusvirus defective interfering RNAs. *Semin. Virol.* 7: 409.

TOROVIRUSES (CORONAVIRIDAE)

Marian C Horzinek, Virology Unit, Veterinary Faculty, Utrecht, The Netherlands

History

Toroviruses were defined in the early 1980s as a result of a trilateral, partly collaborative study between groups in Berne, Switzerland (isolation in cell culture), Ames/Iowa, USA (discovery of Breda virus and propagation in calves) and Utrecht, The Netherlands (biologic and molecular characterization).

An equine torovirus (ETV), originally referred to as Berne virus, was accidentally isolated in equine kidney cells in 1972 from a rectal swab taken from a horse with diarrhea. Upon post-mortem examination pseudomembranous enteritis and miliary granulomas and necrosis in the liver were diagnosed; Salmonella Lille (O, 6, 7, Z_{38}) was considered to be the causative agent. ETV can be propagated in lines of equine dermis or embryonic mule skin cells, where it causes a cytopathic effect that results in cell lysis. The virus was not neutralized by antisera against known equine viruses. Serologic crossreactions were observed in neutralization and ELISA with sera from calves that had been experimentally infected with morphologically similar particles, the Breda viruses.

A bovine torovirus (BTV), first described as Breda virus was discovered in 1979 during investigations in a dairy herd in Breda (Iowa), in which severe neonatal calf diarrhea had been a problem for three consecutive years. Despite repeated attempts, BTV has not been adapted to growth in cell or tissue culture, which has hampered its biochemical, biophysical and molecular characterization. The pathogenesis and pathology of BTV infections have been studied in gnotobiotic calves.

Torovirus-like particles have been seen in EM preparations from fecal samples of pigs and humans (children and adults); proof that the observed structures are not artefacts was obtained when toroviral RNA sequences were found in the feces of piglets and in stools from humans with diarrhea.

Taxonomy and Classification

The name of the viruses assembled in this genus is derived from the term *torus* (Latin) which designates the lowest convex molding in the base of a column or pilaster. It refers to the biconcave disk or doughnut shape of the virion that is determined by a tubular capsid of helicoidal symmetry, which is surrounded by a peplomer-bearing envelope. In addition to the unique toroid form, virions may also appear as straight or bent rod-shaped particles.

Analysis of the genetic information and replication mode of the prototype ETV showed that toroviruses are evolutionarily related to the *Coronaviridae* and, to a lesser extent, to the *Arteriviridae*. However, the lack of sequence homology in the structural genes and the absence of antigenic relatedness with coronaviruses justify their separate taxonomic position as a genus, *Torovirus*, in the *Coronaviridae* family.

The families *Coronaviridae* and *Arteriviridae* form the Order *Nidovirales* (from *nidus*, Latin for 'the nest' – alluding to the nested set of subgenomic RNAs transcribed during replication), the second order recognized in animal virus taxonomy so far.

Virion Properties

Extracellular ETV particles possess a helical nucleocapsid coiled into a hollow tube (diameter 23 nm, average length 104 nm, periodicity 4.5 nm) which is either straight or bent into an open torus. A tightly fitting envelope, 11 nm thick, surrounds this structure. Consequently, the virion may assume an erythrocyte-like or a kidney shape, depending on whether the envelope follows the small curvature of the nucleocapsid or not. The largest diameter of ETV is estimated at 120–140 nm. Club-shaped projections (average length 20 nm) are present on the particle surface.

In the cell, predominantly rod-shaped particles are encountered. Cross-sections through tubular virions appear as three concentric circles of high electron density with an electron-lucent center.

Negatively stained BTV virions appear to be either kidney- or C-shaped, measuring 30–120 nm, or approximately circular, measuring 75–90 nm in diameter. Their envelope bears prominent drumstick-shaped peplomers (17–24 nm), and a fringe of shorter spikes 8–10 nm in length. The short projections represent the hemagglutinin esterase protein, which is present on the surface of bovine torovirions. In intestinal cells of calves killed 48–96 h after infection, tubules of 21 nm diameter and indeterminate length were found both in the cytoplasm and in nuclei.

In thin-sectioned preparations, intracellular torovirions show a bacilliform morphology (rods with both ends rounded – in contrast to the circular outline of coronavirions); extracellular particles may reveal twin circular structures resulting from cross-sections through both limbs of the C-shaped tubular nucleocapsid.

A model of a torovirion is given in **Fig. 1**.

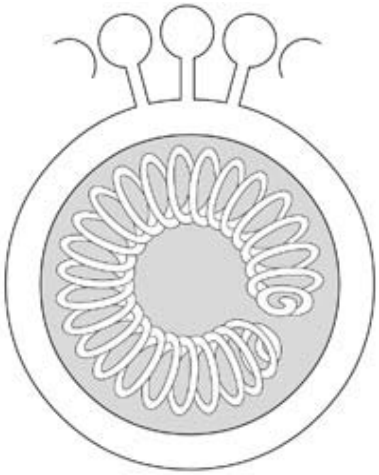


Figure 1 A schematic model of a torovirion. Illustration by Ank Klein-Willink.

Genome

ETV virions (sedimentation coefficient 380S) contain one species of polyadenylated RNA of positive polarity; in agarose gel electrophoresis its length appears as $\geq$20 kb. When assayed under hypertonic transfection conditions genomic RNA is found infectious and RNase-sensitive.

Proteins

Proteins with molecular weights of 20 kDa, 22 kDa, 37 kDa, and 80–100 kDa are identified in labeled ETV virions. Detergent treatment releases the 22, 37 and 80–100 kDa species from the virion, which indicates their association with the envelope. Only the 20 kDa protein is present in purified ETV nucleocapsids and was accordingly named nucleocapsid (N) protein. The heterogeneous, N-glycosylated 80–100 kDa protein is recognized by both neutralizing and hemagglutination-inhibiting monoclonal antibodies and is therefore identified as the peplomer (P) protein. Another membrane-associated polypeptide is the non-glycosylated envelope protein (E; 22 kDa); the 37 kDa molecule also occurs in close association with the viral membrane, but its virus specificity could not be established.

From the deduced amino acid sequence of the nucleocapsid (N) protein gene a basic protein of 18.3 kDa is predicted. *In vitro* transcription and translation, followed by immunoprecipitation, were used to identify the gene. Identification was confirmed by metabolic labeling, using the knowledge that cysteine residues are absent from the amino acid sequence of the N protein. Smaller N-related polypeptides encountered in ETV-infected cell lysates are products of aberrant translation, due to initiation on AUG codons further downstream in the N protein gene.

The 26.5 kDa product of the ETV membrane (M) protein gene was identified by *in vitro* transcription and translation. Computer analysis revealed the characteristics of a class III membrane protein lacking a cleaved signal sequence but containing three successive transmembrane α-helices in the N-terminal half. Only small portions of either end of the polypeptide are exposed on opposite sides of the vesicle membranes; the C-terminus protrudes at the cytoplasmic side of the membrane. The M protein accumulates in intracellular membranes, predominantly those of the endoplasmic reticulum.

The nucleotide sequence of the peplomer or spike (S) protein gene encodes an apoprotein of 1581 amino acids with an M_r of about 178 kDa. The deduced amino acid sequence contains domains typical for type I membrane glycoproteins: an N-terminal signal sequence, a putative C-terminal transmembrane anchor, and a cytoplasmic tail.

Since BTV has not been labeled in cultured cells, its protein composition was studied by means of surface radioiodination of purified virus. Polypeptide species of 105, 85, 37, and 20 kDa were identified, of which the former two probably represent the BTV peplomeric surface structures. Rabbit antisera raised against purified BTV recognize the ETV S protein in immunoprecipitation experiments.

Physical Properties

Buoyant densities of 1.16, 1.18 and 1.14 g ml^{-1} are reported for ETV, BTV serotype 2 and human toroviruses, respectively.

Under experimental conditions ETV is remarkably stable, even to the action of phospholipase C or deoxycholate; Triton X-100 and organic solvents destroy its infectivity. BTV1 appears to be less stable than ETV, as changes in its sedimentation behavior and density have been observed after prolonged storage at −70°C. The infectivity of a fecal preparation containing BTV1 was lost completely after 3 weeks at 4°C whereas ETV in cell-free supernatant remained stable for 92 days. Two cycles of freeze–thawing of purified BTV2 resulted in loss of peplomers.

Replication

Equine torovirus (strain Berne) replication occurs in the cytoplasm via a 3′-coterminal nested set of five subgenomic mRNAs. Preformed tubular capsids bud through membranes of the Golgi stack and of the endoplasmic reticulum. A host cell nuclear function seems to be required since UV preirradiation of cells, actinomycin D and α-amanitin reduce virus yields.

Transcription

In ETV-infected cells five virus-specific polyadenylated RNA species are found with >20.0, 7.5, 2.1, 1.4, 0.8 kb.

Northern (RNA) blot hybridizations with restriction fragments from cDNA clones showed that the five ETV mRNAs form a 3′-coterminal nested set. Sequence analysis revealed the presence of four complete open reading frames of 4743, 699, 426 and 480 nucleotides, with initiation codons coinciding with the 5′ ends of ETV RNAs 2 through 5, respectively; RNA 5 is contiguous on the consensus sequence. ETV RNAs 1, 2 and 3 are transcribed independently, as shown by UV transcription mapping. Upstream of the AUG codon of each open reading frame a conserved sequence pattern is encountered, probably a core promoter sequence in subgenomic RNA transcription. In the area surrounding the core promoter region of the two most abundant subgenomic ETV RNAs, a number of homologous sequence motifs occur.

Translation

The 7.5, 2.1 and 0.8 kb RNAs encode a 151 kDa product (possibly the precursor to the S protein), the M protein, and the N protein, respectively, as shown by *in vitro* translation.

The 3′ part (8 kb) of the polymerase gene of ETV contains at least two open reading frames (designated ORF 1a and ORF 1b) which overlap by 12 nucleotides. The complete sequence of ORF 1b (6873 nucleotides) is known. Like corona- and arteriviruses, ETV expresses its ORF 1b by ribosomal frameshifting during translation of the genomic RNA; also the predicted tertiary RNA structure (a pseudoknot) in the frameshift-directing region is similar. The amino acid sequence of the predicted ETV ORF 1b translation product contains homologies with the ORF 1b product of coronaviruses. Four conserved domains are present: the putative polymerase domain, an area containing conserved cystein and histidine residues, a putative helicase motif, and a domain apparently unique for toro- and coronaviruses.

Post-translational Processing

The *N*-glycosylated peplomer protein is derived from processing of a 200 kDa precursor present in infected cells but not in virions. Eighteen potential *N*-glycosylation sites, two heptad repeat domains, and a possible 'trypsin-like' cleavage site exist in the peplomer gene. The mature S protein consists of two subunits and their electrophoretic mobility on endoglycosidase F treatment suggests that the predicted cleavage site is functional *in vivo*. The heptad repeat domains are probably involved in the generation of an intra-chain coiled-coil secondary structure; similar interchain interactions can play a role in the formation of the observed S protein dimers. The intra- and interchain coiled-coil interactions may stabilize the elongated ETV peplomers.

Assembly

About 10 h after infection ETV particles are seen within parts of the unaltered Golgi apparatus and extracellularly. At that time, tubular structures of variable length, diameter and electron-density appear in the cytoplasm and in the nucleus of infected cells, probably representing preformed nucleocapsids. It is unknown whether the accumulation of nucleocapsids in the nucleus reflects a nuclear phase in the replication of ETV or some sort of defective assembly.

Viruses predominantly bud into the lumen of Golgi cisternae. The preformed nucleocapsid tubules approach the Golgi membrane with one of both rounded ends and attach to it along one side. During budding the nucleocapsid is apparently stabilized, leading to a higher electron density and a constant diameter (23 nm).

Intracellular BTV virions are rod-shaped with rounded ends; they measure 35–40 nm in diameter and are 80–100 nm long.

Defective Interfering Virus

Defective interfering (DI) genomes of ETV can be generated by serial undiluted passages. Isokinetic sucrose gradient analysis showed that they are packaged into virus-like particles with lower S values than standard virions. DI RNAs contain sequences from the presumed 5′ end and the proven 3′ end of the ETV genome. Using probes from the 5′ end, a consensus nucleotide sequence of about 800 nt and the 5′ end of the putative ETV polymerase gene were identified. A conserved sequence motif, probably involved in subgenomic RNA transcription, is situated immediately downstream of the 5′ end of the DI RNAs. There is no evidence for the presence of a common leader sequence in ETV RNAs.

In the gut of a BTV-infected calf, a wave of simultaneous infections progresses through a population of susceptible cells. In view of the immense particle numbers encountered in the feces, enteroabsorptive epithelial cells are probably infected at high multiplicities; DI particles may also be generated *in vivo* and may play a role in modulating the pathogenesis of torovirus infections.

Geographic and Seasonal Distribution

Toroviruses in cattle have been evidenced by ELISA serology in Europe, North America and Asia. Seasonal patterns of infection have been described in calves in relation to herd management (pasture/stable). Most adult horses in Switzerland possess neutralizing antibodies to ETV. Possible human toroviruses have been found in France, Great Britain, The Netherlands and the USA.

Host Range and Virus Propagation

By using the neutralization assay, antibodies to ETV were found in sera from horses, cattle, goats, sheep, pigs, rabbits and feral mice, but not in humans or in carnivores. Torovirus-like particles have been seen in fecal samples of cats with a transmissible diarrhea, but neither serologic nor molecular identification was obtained.

By electron microscopy, pleomorphic virions have been observed in the feces of children and adults with diarrhea; the particles were coated and aggregated after the addition of anti-BTV calf sera. The stool specimen reacted in an ELISA for the detection of BTV antigen in calves, and possessed a low titer of hemagglutinin for rat erythrocytes, which was blocked by antisera to BTV.

Toroviral RNA sequences have been evidenced in the stools from humans with diarrhea. The use of fresh material (avoiding freeze/thawing) is essential for obtaining unequivocal results. Sequence analysis of RNA extracted from specimens of pediatric patients and amplified by reverse transcriptase–polymerase chain reaction (RT-PCR) showed a high degree of identity with the corresponding ETV sequence. This is surprising in view of the observation that the divergence between porcine and bovine/equine torovirus sequences is greater than between human and bovine/equine torovirus sequences. If confirmed, this would indicate occasional spillover of the infection from ungulates to humans, rather than human-to-human transmission.

Cultured cells of equid origin (horse, mule) can be infected with ETV; no other cell species tested supports viral growth. Bovine toroviruses could not be propagated in any culture of primary cells or permanent lines and had to be passaged in gnotobiotic calves. Putative torovirus isolations from young calves with respiratory symptoms (pneumonia) in MDBK cells have been challenged, and a bovine coronavirus was identified in the culture.

Genetics

No information is available.

Evolution

Toroviruses and coronaviruses are ancestrally related by divergence of their polymerase and envelope proteins from common ancestors. In addition, their genome organization and expression strategy, which involves the synthesis of a 3′-coterminal nested set of mRNAs, are comparable. Four domains of amino acid sequence homology exist in the product of ORF 1b of the *POL* gene, which underlines the existence of an evolutionary relationship. In view of these findings, toro- and coronaviruses have been classified as separate genera in the family *Coronaviridae,* which, together with the *Arteriviridae* forms the order *Nidovirales.*

Nucleotide sequence analysis of the ETV genome has revealed the results of two independent non-homologous RNA recombinations: ORF 4 encodes a protein with significant sequence similarity (30–35% identical residues) to a part of the hemagglutinin esterase proteins of coronaviruses and of influenza virus C. Although this gene is truncated in ETV, it is intact and translated into a functional, enzymatically active protein in BTV; this product is visible as a

second fringe of (short) projections on the viral envelope.

The sequence of the C-terminal part of the predicted ETV polymerase ORF 1a product contains 31–36% identical amino acids when compared with the sequence of a nonstructural 30/32 kDa coronavirus protein. The cluster of coronaviruses which contains this nonstructural gene does not express it as a part of their polymerase, but by synthesizing an additional subgenomic mRNA.

Serologic Relationships and Variability

One strain of ETV has been isolated (and re-isolated from the same material), but all attempts to obtain a second equine isolate were fruitless.

Two strains of BTV have been reported in addition to the original isolate by Woode and colleagues: one had been detected in feces from a 5-month-old diarrheal calf in Ohio, and a second Iowa strain was recovered from a 2-day-old experimental animal. On the basis of their reactivity in ELISA, immune electron microscopy and hemagglutination/hemagglutination inhibition assays using rat erythrocytes the three isolates were assigned to two serotypes: BTV1, represented by the Iowa 1 isolate, and BTV2 comprising the Ohio and the second Iowa isolate.

The occurrence of antigenically different toroviruses is not unlikely. Two serotypes of BTV have been described and more probably exist. It is anticipated that serologically unrelated toroviruses will be identified with the aid of nucleic acid probes.

Epidemiology

The high prevalence of BTV antibodies in cows cannot be explained by the few infections found in calves and cows with diarrhea. The viruses may circulate in herds through inapparently or chronically infected animals, as described for rota- and coronaviruses. The level of maternal BTV-specific antibodies influences the clinical outcome of the infection, as differences in the severity of diarrhea were observed between colostrum-fed and colostrum-deprived animals.

With the aid of solid-phase immune-electron microscopy, torovirions can be identified in fecal material; without this selection, virion pleomorphism makes diagnosis by electron microscopy ambiguous.

Transmission and Tissue Tropism

Toroviruses probably spread through feco-oral contact. In feces samples from experimentally BTV-infected calves HA titers in excess of 10^7 units ml^{-1} are measured which would correspond to particle concentrations of $>10^{11}$. Therefore, once an outbreak is under way the infection spreads rapidly, especially when highly susceptible animals are on the premises (e.g. in the calving season).

Using RT-PCR, torovirus sequences were obtained from fecal samples of weaning piglets; an association with weaning diarrhea has not been established.

Pathogenicity

Torovirus infections play a role in diarrhea of breeding calves up to two months of age, and in winter dysentery of adult cattle. Torovirus was detected four times more often in diarrheal calves than in healthy animals. Torovirus-associated diarrhea of calves started later (average 12.7 days of age) than enteritis due to rota- or coronaviruses (average 7.7 and 8.3 days, respectively). Seroconversion was found significantly more often after winter dysentery outbreaks than on farms without a disease history; coronavirus seroconversion was less common.

Clinical Features of Infection

Seroconversions to ETV occurred in all horses between 10 and 12 months of age, but without symptoms. Experimentally infected animals (intravenous route) seroconverted without clinical signs. To the author's knowledge oral infection experiments in horses have not been reported so far.

All BTV strains are pathogenic – although with varying virulence – for newborn gnotobiotic and nonimmune conventional calves after oral infection. Most calves develop anorexia, a watery, yellow–green diarrhea that lasts 4–6 days, and shed virus for 3–4 days. In some calves the diarrhea is preceded by a mild temperature reaction (40°C). In the calves with a normal intestinal flora the diarrhea is generally more severe than in gnotobiotic calves. Reduction of D-xylose resorption may reach 65% in severely affected calves. In some animals depression and dehydration is observed, occasionally with shivering, hyperpnea and watery eye discharge. Mortality in experimental infections approaches 25%.

Pathology and Histopathology

Target organs of BTV in calves are the lower half or two-thirds of the small intestine and the entire large intestine, particularly the spiral colon. There is little macroscopic evidence of the infection. Histological examination shows villous atrophy and epithelial desquamation from the mid-jejunum to the lower small intestine, and areas of necrosis in the large intestine. Both crypt and villus epithelial cells contain antigen as shown by immunofluorescence. The

watery diarrhea is probably a result of loss of resorptive capacity of the colonic mucosa, combined with malabsorption in the small intestine. Infection of crypt epithelium may affect the duration of diarrhea, as regeneration of villus epithelium starts in the crypts. The germinal centers of the Peyer's patches are depleted of lymphocytes and may occasionally show fresh hemorrhage. The dome epithelial cells, including the M cells, display the same cytopathic changes as seen in the absorptive cells of villi. Virions are found in cells of both the small and large intestine. Extracellular virus is closely associated with microvilli of absorptive cells and in the coated pits between microvilli, indicating receptor-mediated endocytosis. In addition, virions are found between enterocytes at the basal and lateral plasma membranes. Virions in various stages of degradation are found within macrophages in the lamina propria.

Antigen is detected as early as 48 h after infection in epithelial cells of the lower half of the villus and of the crypts of the affected areas, as well as in dome epithelium. Fluorescence is cytoplasmic (although a few nuclei may be faintly stained) and generally most pronounced in the intestines with the least tissue damage. The mid-jejunum is infected first, the infection eventually reaching the large intestine. Diagnosis by immunofluorescence test (IFT) should be performed preferentially on sections of the large intestine from calves killed after the onset of diarrhea (i.e. several days after the infection of epithelium).

Immune Response

Up to the age of 4 months, all calves in a sentinel experiment regularly excreted BTV in the feces. They showed early serum IgM responses despite the presence of IgG1 isotype maternal antibodies, but no IgA seroconversion. Antibody titers then decreased below detection, persistent IgG1 titers developed in only a few animals. After introduction into the dairy herd at 10 months of age, all calves developed diarrhea and shed virus. Seroconversion for all antibody isotypes was observed at this stage, indicating lack of mucosal memory. In contrast, coronavirus infection in the presence of maternal antibodies leads to isotype switch and a memory response.

Prevention and Control

No control strategies have been implemented.

Future

The pathogenic significance of toroviruses for animals and humans, also as agents of nonenteric infections needs to be established. Diagnostic procedures for the discovery of more distantly related viruses of this cluster will have to include procedures for the recognition of nucleotide sequence motives.

See also: **Arteriviruses (*Arteriviridae*); Defective interfering viruses.**

Further Reading

Cavanagh D and Horzinek MC (1993) Genus Torovirus assigned to the *Coronaviridae*. *Arch. Virol.* 128: 395.

Cornelissen LA, Wierda CM, van der Meer FJ *et al* (1997) Hemagglutinin-esterase, a novel structural protein of torovirus. *J. Virol.* 71: 5277.

Koopmans M and Horzinek MC (1994) Toroviruses of animals and humans: a review. *Adv. Virus Res.* 43: 233.

Snijder E and Horzinek MC (1993) Toroviruses: replication, evolution and comparison with other members of the coronavirus-like superfamily. *J. Gen. Virol.* 74: 2305.

Snijder E and Horzinek MC (1995) The molecular biology of toroviruses. In: Siddell S and ter Meulen V (eds) *Coronaviruses*. New York: Plenum Press.

Vries AAF de, Horzinek MC, Rottier PJM *et al* (1997) The genome organization of the Nidovirales: similarities and differences between arteri-, toro-, and coronaviruses. *Semin Virol.* 8: 33.

TOSPOVIRUSES (*BUNYAVIRIDAE*)

James W. Moyer, Department of Plant Pathology, North Carolina State University, Raleigh, North Carolina, USA

History

Diseases incited by tomato spotted wilt virus (TSWV) were first reported in 1915 and were considered to be of viral etiology by 1930. This taxon of plant viruses was categorized as a monotypic virus group consisting of a single virus (tomato spotted wilt virus; TSWV) until the report of Impatiens necrotic spot

watery diarrhea is probably a result of loss of resorptive capacity of the colonic mucosa, combined with malabsorption in the small intestine. Infection of crypt epithelium may affect the duration of diarrhea, as regeneration of villus epithelium starts in the crypts. The germinal centers of the Peyer's patches are depleted of lymphocytes and may occasionally show fresh hemorrhage. The dome epithelial cells, including the M cells, display the same cytopathic changes as seen in the absorptive cells of villi. Virions are found in cells of both the small and large intestine. Extracellular virus is closely associated with microvilli of absorptive cells and in the coated pits between microvilli, indicating receptor-mediated endocytosis. In addition, virions are found between enterocytes at the basal and lateral plasma membranes. Virions in various stages of degradation are found within macrophages in the lamina propria.

Antigen is detected as early as 48 h after infection in epithelial cells of the lower half of the villus and of the crypts of the affected areas, as well as in dome epithelium. Fluorescence is cytoplasmic (although a few nuclei may be faintly stained) and generally most pronounced in the intestines with the least tissue damage. The mid-jejunum is infected first, the infection eventually reaching the large intestine. Diagnosis by immunofluorescence test (IFT) should be performed preferentially on sections of the large intestine from calves killed after the onset of diarrhea (i.e. several days after the infection of epithelium).

Immune Response

Up to the age of 4 months, all calves in a sentinel experiment regularly excreted BTV in the feces. They showed early serum IgM responses despite the presence of IgG1 isotype maternal antibodies, but no IgA seroconversion. Antibody titers then decreased below detection, persistent IgG1 titers developed in only a few animals. After introduction into the dairy herd at 10 months of age, all calves developed diarrhea and shed virus. Seroconversion for all antibody isotypes was observed at this stage, indicating lack of mucosal memory. In contrast, coronavirus infection in the presence of maternal antibodies leads to isotype switch and a memory response.

Prevention and Control

No control strategies have been implemented.

Future

The pathogenic significance of toroviruses for animals and humans, also as agents of nonenteric infections needs to be established. Diagnostic procedures for the discovery of more distantly related viruses of this cluster will have to include procedures for the recognition of nucleotide sequence motives.

See also: **Arteriviruses (*Arteriviridae*); Defective interfering viruses.**

Further Reading

Cavanagh D and Horzinek MC (1993) Genus Torovirus assigned to the *Coronaviridae*. *Arch. Virol.* 128: 395.

Cornelissen LA, Wierda CM, van der Meer FJ *et al* (1997) Hemagglutinin-esterase, a novel structural protein of torovirus. *J. Virol.* 71: 5277.

Koopmans M and Horzinek MC (1994) Toroviruses of animals and humans: a review. *Adv. Virus Res.* 43: 233.

Snijder E and Horzinek MC (1993) Toroviruses: replication, evolution and comparison with other members of the coronavirus-like superfamily. *J. Gen. Virol.* 74: 2305.

Snijder E and Horzinek MC (1995) The molecular biology of toroviruses. In: Siddell S and ter Meulen V (eds) *Coronaviruses*. New York: Plenum Press.

Vries AAF de, Horzinek MC, Rottier PJM *et al* (1997) The genome organization of the Nidovirales: similarities and differences between arteri-, toro-, and coronaviruses. *Semin Virol.* 8: 33.

TOSPOVIRUSES (*BUNYAVIRIDAE*)

James W. Moyer, Department of Plant Pathology, North Carolina State University, Raleigh, North Carolina, USA

History

Diseases incited by tomato spotted wilt virus (TSWV) were first reported in 1915 and were considered to be of viral etiology by 1930. This taxon of plant viruses was categorized as a monotypic virus group consisting of a single virus (tomato spotted wilt virus; TSWV) until the report of Impatiens necrotic spot

virus (INSV) in 1990. Thus, most of the characteristics on which the *Tospovirus* genus is defined were obtained through investigations of TSWV. Although TSWV was originally associated with tomato (*Lycopersicon esculentum*), by the 1950s it had one of the largest known host ranges of any plant virus. By 1998 the known natural and experimental host range exceeded 900 plant species and spanned both monocotyledonous and dicotyledonous plant species. TSWV was known by 1930 to be insect vectored by thrips. It was not until the mid 1960s that the enveloped virion morphology was revealed and the molecular characterization and genome organization did not occur until after 1990. In addition, understanding of the virus–vector biology, cytopathology of both the plant and insect hosts and virus genetics have developed rapidly since 1990. However, efficient *in vitro* cell culture systems for either plant or thrips systems are not available nor is there a system for reverse genetics.

Table 1 List of tospoviruses and serogroups

Tospovirus species	*Abbreviation*
TSWV serogroup:	
Tomato spotted wilt virus	TSWV
Groundnut ringspot virus	GRSV
Tomato chlorotic spot virus	TCSV
WMSV serogroup:	
Watermelon silver mottle virus	WSMV
Watermelon bud necrosis virus	WBNV
Groundnut bud necrosis virus	GBNV
Serologically unrelated	
Chrysanthemum stem necrosis virus	CNSV
Impatiens necrotic spot virus	INSV
Iris yellow spot virus	IYSV
Peanut yellow spot virus	PYSV
Peanut chlorotic fan-spot virus	FCFV
Physalis severe mottle virus	
PSMV Zucchini lethal chlorosis virus	ZLCV

Taxonomy, Classification and Serological Relationships

Tospovirus constitutes the only plant-infecting genus in the *Bunyaviridae*. Tospoviruses are characterized by a quasi-spherical, enveloped virion containing a tripartite RNA genome typical of the family. A single open reading frame (ORF) in the viral complementary-sense (negative) is located on the large RNA (L) and two ORFs in an ambisense (see Molecular Biology) orientation are on both the middle (M) and small (S) segments. The presence of two ambisense RNAs is the defining molecular characteristic that distinguishes this genus from the other genera in the *Bunyaviridae*. Biologically, the tospoviruses multiply in plants and in their insect (thrips) vectors. However, their individual host ranges are highly variable. Some tospoviruses have an extremely large host range (e.g. TSWV) whereas others such as iris yellow spot virus (IYSV) are known to infect only a small number of plant species.

The discovery of INSV led to the current scheme for classifying viruses within the *Tospovirus* genus that is based on nucleic acid sequence homology between nucleocapsid genes. New viruses are named consistent with rules of the International Committee on Taxonomy of Viruses with each virus equivalent to a species. Newly discovered tospoviruses were classified in serogroups, e.g. serogroup I, II etc., and this system was used in the literature. However, the proliferation of viruses and number of monotypic serogroups prompted the abandonment of the numerical system. Distinct viruses are not classified as a group until more than one virus is identified in that group. The serogroup is then identified by the type virus, e.g. tomato spotted wilt virus serogroup (**Table 1**). This system currently distinguishes at least 12 different viruses (**Table 1**). Although the sequence homology between tospoviruses varies with the gene, sequence differences between the nucleocapsid genes are accepted as a measure of overall relatedness. The nucleocapsid protein was chosen not only because it is abundant, but also because of its putative role in regulating the switch from transcription to replication, function in the replication complex and encapsidation of the genome based on viruses with negative strand genome organization. Serological relatedness based on ELISA or Western blot analysis of the nucleocapsid protein is used to classify viruses within serogroups and, as expected, is generally consistent with homology of the nucleocapsid genes. This is distinct from the serological relatedness in other genera of the *Bunyaviridae* that have been measured by neutralization or hemagglutination assays that are indicative of functions mediated by viral glycoproteins which reside in the envelope.

Isolates in the *Tospovirus* genus with greater than 90% nucleocapsid sequence homology are classified as strains of the same virus. Serologically related isolates with 80–90% sequence homology are subjectively classified as strains or as distinct viruses depending on additional criteria. Isolates with less than 80% homology to all of the other known viruses are classified as distinct viruses or species. Those viruses exhibiting serological relatedness are classified in the same serogroup. For example, groundnut chlorotic spot virus is a distinct virus with 78%

homology to TSWV and a distant serological relationship, was formerly classified in serogroup II, but is now classified as a member of the TSWV serogroup. Homology with TSWV for distinct viruses range from about 60% for INSV to less than 40% for watermelon silver mottle virus (WSMV).

Properties of Virion

Tospovirus virions are quasi-spherical, enveloped particles 80–120 nm in diameter. Two viral-coded glycoproteins, G1 and G2, are embedded in the viral envelope. Ribonucleoprotein (RNP) particles consisting of the viral RNA encapsidated in the nucleoprotein and a small number of polymerase molecules are contained within the envelope. Intact virions as well as carefully prepared RNPs retrieved from sucrose or $CsSO_4$ gradients are infectious. There are several reports of TSWV and INSV isolates that are defective for virion formation, but remain infectious.

Properties of Genome

Tospoviruses have a single-stranded, tripartite RNA genome with segments designated as L, M and S in order of decreasing length. The termini of each of the RNA segments consist of an eight nucleotide sequence (5′ AGAGCAAU 3′) that is conserved throughout the genus on each segment of the viral genome. The first 12 to 15 nucleotides at the termini of each segment are complementary with a high degree of complementarity extending throughout the terminal untranslated regions. The base pairing at the termini results in a panhandle structure that may initiate encapsidation and regulation of transcription. The L RNA is approximately 8.9 kb and codes for the RNA-dependent RNA-polymerase (RdRp) that is translated from the viral complementary sense RNA. The M RNA is approximately 4.8 kb with two ORFs in an ambisense arrangement. The ORF nearer the 5′ terminus codes for the 33.6 kDa nonstructural protein (NSm) in the viral sense and is followed by an A-U rich, 200–250 nucleotide intergenic region. The second ORF codes for the G1/G2 precursor protein in the viral complementary sense. The S RNA is 2.9–3.0 kb with two ORFs in a similar ambisense arrangement. A nonstructural protein (NSs) is coded from the ORF nearer the 5′ terminus in the viral sense and the nucleocapsid gene from the ORF in the viral complementary sense located near the 3′ terminus. The two ORFs are separated by an A-U rich intergenic region of variable length. The S segment from isolates of TSWV may vary in length by as much as 100 nucleotides and is due entirely to insertions and deletions in the intergenic region. In addition, highly conserved sequences are embedded within the intergenic region. In one group of isolates, a 33 nucleotide duplicate sequence was associated with reduced competitiveness of the S RNA from those isolates.

Full-length molecules of the M and S ambisense RNA segments are found in infected tissue and purified virions in both the viral and viral complementary sense (approximate ratio of 10:1) which is consistent with ambisense segments from other viruses. Defective interfering RNAs (DIs) associated with attenuated symptoms are also frequently observed. DIs in TSWV infected tissue are the result of a single deletion event in the L RNA. The formation of DIs is favored by repeated passage in certain hosts, high inoculum concentration and low temperatures. Available evidence supports the hypothesis that secondary structure rather than sequence is the primary determinant of the site of deletion as is viral RNA recombination. There is also a high frequency of DIs that maintain the original reading frame resulting in translation of truncated proteins whose existence was confirmed in nucleocapsid preparations.

Properties of Proteins

A 330 kDa protein encoded by the L RNA has been identified as the putative RdRp through sequence homology. RdRp activity has been associated with TSWV virions. Full length and truncated RdRp molecules have been associated with virions containing DIs. The 33.6 kDa NSm protein encoded by the M RNA has been shown to induce tubule structures from protoplasts and cell surfaces from plants and insects, respectively. Induction of tubules and association with the plasmodesmata in plant cells is evidence that this protein is involved in cell-to-cell movement. The G1/G2 precursor protein is also coded for by the M RNA. These proteins occur in the viral envelope and have been associated with recognition by the thrips. The NSs protein (54 kDa) encoded on the S RNA accumulates to high levels as loose aggregates or paracrystalline arrays of filaments, but has not been linked with any function. The nucleocapsid (N) protein, also encoded by the S RNA, ranges in size from 29 to 31 kDa depending on the virus. This protein encapsidates the viral RNA segments and has been demonstrated to have RNA binding properties. It is highly abundant and is the predominant protein detected in serological assays.

Replication

The RNA of this virus, similar to other negative strand viruses is not infectious and recombinant DNA

techniques have not been applied to elucidate genome function. As yet there are no efficient systems for detailed molecular investigations of tospoviruses, although there are reports of infection of plant protoplasts and insect cells. It is known that replication of viral RNA and assembly of virions occurs in the cytoplasm. The RNP containing the L RNA is hypothesized to replicate similar to other negative-strand viruses. The viral RNA is the template for replication and the viral complementary strand serves as the template for translation of the RdRp protein. The S and M ambisense RNAs also replicate similar to negative-strand RNAs. However, translation of these proteins probably occurs from subgenomic mRNAs. Subgenomic mRNAs of appropriate size for each of the ORFs on these two RNAs, have been detected in infected plants. The subgenomic RNAs are capped at the 5′ terminus with 12–20 nucleotides of nonviral origin indicating that tospoviruses utilize a cap-snatching mechanism to regulate transcription. Virions are assembled in the cisternae of the endoplasmic reticulum. Initially they are double-membraned particles that soon coalesce to become groups of virions with a single membrane surrounded by another membrane.

Geographic Distribution

Tospoviruses are found worldwide. However, individual viruses may have limited distribution. In general, they are found in temperate regions colonized by their thrips vector. Exceptions are the viruses (e.g. watermelon silver mottle group) transmitted by *Thrips palmi*, that is found in subtropical regions. TSWV has the most extensive host range and is found worldwide. Conversely, INSV has been of greatest concern in greenhouse-grown floral crops in North America, Central America and Europe. Viruses in the watermelon silver mottle serogroup have been identified in India, Japan and Taiwan, although their vector is found in other parts of the world. IYSV has been reported in Israel, Brazil and western North America. Collectively, tospoviruses have been included among the 'emerging viruses' due to their wide and rapid distribution and the severity of the diseases they incite.

Host Range and Virus Propagation

TSWV has one of the most diverse host ranges of any plant-infecting virus. The virus infects over 900 plant species which include both monocotyledonous and dicotyledonous plants. Other tospoviruses have much narrower host ranges and thus the broad host range of TSWV is not characteristic of the genus. Tospoviruses also replicate in their thrips vectors but distinct from viruses in other genera of this family, are not transmitted transovarially or by plant seeds. These viruses can be transmitted mechanically for experimentation or by their thrips vector. There are no robust plant or insect culture systems for these viruses. However, plant protoplasts have been successfully inoculated.

Genetics and Evolution

The knowledge base for genetics and evolution of tospoviruses has been derived almost exclusively from TSWV. TSWV is unusually adept at adapting to new hosts as well as losing phenotypic characters following repeated passage in experimental hosts, especially *Nicotiana benthamiana*. The virus occurs in plants as a heterogeneous mixture of isolates that have been distinguished by symptom phenotype and more recently with molecular markers. As many as five stable variants have been isolated from a single thrips inoculation site. TSWV has also been shown to form new phenotypes from the mixture. These complex populations provide a reservoir of genetic information that could account for the rapid adaptation. Recombination (sensu reassortment) was shown via classical genetics to be a possible mechanism for reassembling the genetic information among isolates in 1961. TSWV has now been shown to use reassortment of genome segments as a mechanism for adaptation to resistant hosts. The determinants of adaptation to resistance in tomato and pepper have been mapped to the M and S segments, respectively.

Transmission and Epidemiology

Tospoviruses are transmitted from plant to plant by a small number of thrips species. Among the more common vectors are *Frankliniella occidentalis*, *F. fusca*, *F. schultzei*, *Scirtothrips dorsalis*, *Thrips palmi*, *T. setosus* and *T. tabaci*. Thrips feed on the cytoplasm of plant cells. The contents of infected cells are ingested and the virus is transported along the lumen of the digestive tract to the midgut. The virus is then transported by endocytosis across the membrane of endothelial cells which line the midgut of the thrips. This process of acquisition only occurs in thrips in the larval stage of development. Evidence for replication of the virus in the insect vector is based on the accumulation of nonstructural protein (NSs) and the visualization of other inclusions in endothelial cells, muscle cells and the salivary glands. Viral inoculum is introduced into plants in the insect saliva coincident with feeding on the plant. Although the virus is maintained trans-stagially throughout the life

of the insect, there is no evidence for transovarial transmission. Thus, each generation of thrips must acquire the virus during the larval stage of development.

The primary mechanisms for virus increase in plant populations is by the thrips vector and dissemination of infected somatic tissue in vegetatively propagated crops. Transmission of the virus through plant seed is not recognized as a significant factor in the epidemiology of tospoviruses. These viruses are thought to move long distances in thrips carried by wind currents. They may also survive in commercial agricultural systems in weeds that serve as a bridge between crops. Secondary spread within a crop can only occur in crops that support virus infection and reproduction of the vector as only the larval stage can acquire the virus for transmission. The recent emergence of these viruses as serious pathogens in crops has been attributed to the increased prevalence of thrips as agricultural pests on a worldwide basis. For example, *F. occidentalis*, the western flower thrips, is a highly efficient vector of several tospoviruses. Its emergence in the floral crop industry was closely followed by the emergence of INSV in the late 1980s.

Pathogenicity and Histopathology

Tospoviruses are noted for the severity of the diseases they cause in plants. Symptoms include chlorotic or necrotic lesions or line patterns on inoculated and systemically infected leaves. Systemic invasion of plants is frequently nonuniform. Many plants exhibit chlorotic, concentric rings as a characteristic symptom. Stems and petioles may exhibit necrotic lesions. Symptoms are sufficiently severe and atypical that many syndromes observed on many plants mimic disease and injury caused by other stresses such as bacterial or fungal pathogens or chemical injury. Infection of younger plants result in severely stunted plants and are frequently lethal. The pathology of infected thrips has not been thoroughly examined, however, preliminary studies indicate that infected thrips have reduced reproductive rates and longevity.

Although tospoviruses also induce characteristic cytopathic structures, it should be noted that the occurrence of these structures, even virions, vary from plant to plant and virus to virus. In addition to virions, inclusions or viroplasms consisting of the nonstructural protein (NSs) and nucleocapsid proteins may be abundant in the cytoplasm. NSs accumulates as a filamentous structure that may aggregate in loose bundles (e.g. TSWV) or in highly ordered paracrystalline arrays (e.g. INSV). Excess nucleocapsid protein occurs in granular electron-dense masses. NSs and nucleocapsid protein inclusions have been observed in infected plant and insect cells.

Prevention and Control

Tospoviruses can be effectively managed in well-defined cropping systems such as glasshouses by obtaining plant propagules known to be free of the virus, implementing a preventative thrips control program in high risk areas together with constant monitoring of production areas for infected plants and the vector. However, these strategies are costly and require intensive management. Control of the viruses in field crops is problematic due to the array of external sources of inoculum. Vector control is generally ineffective against the introduction of virus from external sources, however, it may be more effective against secondary spread within the field. Deployment of resistant cultivars has provided temporary benefits. Although little is known about the benefits of host resistance against most of the tospoviruses, TSWV defeated nearly every resistance gene deployed against it in many crops. Pathogen-derived resistance utilizing the nucleocapsid and NSm genes has been effective in some greenhouse and field tests. However, isolates have recently been obtained that overcome nucleocapsid-mediated resistance. The impact of these viruses on agricultural production is in large part due to the absence of highly effective control measures.

***See also: Bunyaviridae*: General features; Replication; Plant virus disease – economic aspects.**

Further Reading

Best RJ (1968) Tomato spotted wilt virus. *Adv. Virus Res.* 13: 65.

De Avila AC, de Haan P, Kormelink R *et al* (1993) Classification of tospoviruses based on phylogeny of nucleoprotein gene sequences. *J. Gen. Virol.* 74: 153.

German TL, Ullman DE and Moyer JW (1992) Tospoviruses: diagnosis, molecular biology, phylogeny, and vector relationships. *Annu. Rev. Phytopathol.* 30: 315.

Prins M and Goldbach R (1998) The emerging problem of tospovirus infection and nonconventional methods of control. *Trends Microbiol.* 6: 31.

Ullman DE, Sherwood JL and German TG (1997) Thrips as vectors of plant pathogens. Lewis TL (ed.) *Thrips As Crop Pests*, p. 539. Wallingford: CAB International.

TOTIVIRUSES (*TOTIVIRIDAE*)

Contents

General Features

Said A Ghabrial, Department of Plant Pathology, University of Kentucky College of Agriculture, Lexington, Kentucky, USA

Jean L Patterson, Department of Virology and Immunology, Southwest Foundation for Biomedical Research, San Antonio, Texas, USA

Introduction

The discovery of the killer phenomenon in the 1960s in the yeast (*Saccharomyces cerevisiae*) and in the smut fungus (*Ustilago maydis*) eventually led to the discovery of the isometric double-stranded (ds) RNA mycoviruses with undivided genomes, presently classified in the family *Totiviridae*. Yeast or smut killer strains secrete a protein toxin to which they are immune, but which is lethal to sensitive cells. The killer toxin is encoded by a satellite dsRNA which is dependent on a helper virus with undivided dsRNA genome (totivirus) for encapsidation and replication. Unlike the helper totiviruses associated with the yeast and smut killer systems, the member viruses in the family *Totiviridae* that infect filamentous fungi and parasitic protozoa are not known to be associated with killer phenotypes. However, purified preparations of some of these viruses are often associated with dsRNA species suspected of being satellite or defective dsRNAs. Viruses with nonsegmented dsRNA genomes of approximately 5200 bp in length have been identified in over 13 strains of the new world parasitic protozoa, *Leishmania braziliensis* and in one strain of the old world parasite, *L. major*. These viruses have recently been placed in the genus *Leishmaniavirus* in the family *Totiviridae*.

The yeast and smut totiviruses and associated killer systems, as well as the totiviruses infecting the parasitic protozoa *Giardia lamblia*, are considered in separate entries in this text. This entry will focus on the totiviruses infecting filamentous fungi and parasitic protozoa, with special emphasis on *Helminthosporium victoriae* 190S virus (Hv190SV) and the totiviruses infecting the protozoa *Leishmania* spp.

Taxonomy and Classification

The family *Totiviridae* comprises three genera, *Totivirus*, *Giardiavirus* and *Leishmaniavirus* (**Table 1**). Viruses in the genus *Totivirus* infect fungi, whereas those belonging to the genera *Giardiavirus* and *Leishmaniavirus* infect parasitic protozoa. The isometric dsRNA totiviruses are unique among dsRNA viruses in that their genomes are undivided, whereas the genomes of all other dsRNA viruses are segmented.

Virion Properties

The sedimentation coefficients $s_{20,w}$ (in Svedberg units) for members of the totivirus genus are in the range of 160–190 S. Particles lacking nucleic acid sediment at the rate of $s_{20,w}$ = 98–113 S. Buoyant density in CsCl = 1.40–1.43 g ml^{-1}. Isolates of ScV-L-A and UmV-H1 may have additional components, containing satellite or defective dsRNAs, with different sedimentation coefficients and buoyant densities.

Virion Structure and Composition

The totiviruses have isometric particles, approximately 40 nm in diameter, with icosahedral symmetry. The capsids are single-shelled and comprised of a single major polypeptide. The capsids consists of 120 capsid protein (CP) subunits of molecular mass in the range of 69–88 kDa, arranged in $T = 1$ lattices.

Although the capsid of Hv190SV, like other totiviruses, is encoded by a single gene, it is comprised of two closely related major CPs, either p88 and p83 or p88 and p78. The capsids of all other totiviruses so far characterized appear to contain only a single major CP. Purified Hv190S virion preparations contain two types of particles, 190S-1 and 190S-2, that differ in sedimentation rates and capsid composition. The 190S-1 and 190S-2 virions are believed to represent different stages in the virus life cycle. The 190S-1 capsids contain p88 and p83, occurring in approximately equimolar amounts, and the 190S-2 capsids are comprised of similar amounts of p88 and p78. p88 and p83 are phosphoproteins, whereas p78 is nonphosphorylated.

The virions of totiviruses encapsidate a single mol-

Table 1 Virus members in the family *Totiviridae*

Genus	*Virus (Alternative name)*	*Abbreviation*	*Accession number*
Totivirus	Saccharomyces cerevisiae virus L-A	ScV-L-A	J04692
	(Saccharomyces cerevisiae virus L1)	ScVL1	X13426
	Saccharomyces cerevisiae virus La	ScV-La	U01060
	(Saccharomyces cerevisiae virus L-BC)	ScV-L-BC	
	Ustilago maydis virus H1	UmV-H1	V01059
	Helminthosporium victoriae 190S virus	Hv190SV	U41345
	Aspergillus foetidus virus S[a]	AfV-S	
	Aspergillus niger virus S[a]	AnV-S	
	Gaeumannomyces graminis virus 87-1-H[a]	GgV-87-1-H	
	Mycogone perniciosa virus[a]	MpV	
Giardiavirus	Giardia lamblia virus	GLV	L13218
	Trichomonas vaginalis virus[a]	TVV	U08999
Leishmaniavirus	Leishmania RNA virus 1-1	LRV1-1	M92355
	Leishmania RNA virus 1-4	LRV1-4	U01899
	Leishmania RNA virus 2-1	LRV2-1	U32108

[a]Tentative member.

ecule of dsRNA 4.7–6.7 kbp in size. Some totiviruses may additionally contain satellite dsRNAs or defective dsRNAs, which are encapsidated separately in capsids encoded by the totivirus genome. The complete nucleotide sequences of eight totiviruses belonging to three genera have been published and the GenBank accession numbers are listed in **Table 1**.

Genome Organization and Expression

The genome organization and expression strategy of the totiviruses infecting fungi and protozoa are similar: each virus genome contains two large open reading frames (ORFs); the 5′ proximal ORF encodes a CP and the 3′ ORF encodes an RNA-dependent RNA polymerase (RDRP). Except for LRV2-1, the RDRP ORF overlaps the CP ORF and is in the −1 frame (ScV-L-A, ScV-La, Hv190SV and GLV) or in the +1 frame (LRV1-1, LRV1-4 and TVV) with respect to the CP ORF. The RDRP ORF of LRV2-1 does not overlap the CP ORF, and is separated from it by a stop codon.

Expression of RDRP as CP-RDRP (*gag-pol*-like) fusion protein via −1 ribosomal frameshifting has been well documented only for ScV-L-A. Virion-associated CP-RDRP has been detected as a minor protein in the case of ScV-L-A, ScV-La (L-BC) and GLV. The RDRP of Hv190SV (and possibly that of LRV1-4) is present as a nonfused separate virion-associated minor protein. Although CP-RDRP fusion proteins were not detected *in vivo*, nor associated with virions of TVV, LRV1-1, LRV1-4 or LRV2-1, expression of RDRP as a fusion protein by +1 ribosomal frameshifting (TVV, LRV1-1 and LRV1-4) or ribosomal hopping (LRV2-1) has been proposed.

The overlap region (16 nt) between the two ORFs of Hv190SV dsRNA (**Fig. 1**) is considerably smaller than that in ScV-L-A (130 nt), LRV1-1 and LRV1-4 (71 nt), and GLV (122 nt). The overlap region of these totiviruses contain sufficient information (structures necessary for ribosomal frameshifting, including a slippery site and a pseudoknot structure involving a predicted stem–loop structure) to promote fusion of ORF1 and ORF2 *in vivo*. The overlap region in the Hv190SV dsRNA genome, on the other hand, lacks a heptamer slippery site and a potential pseudoknot structure cannot be predicted from the secondary structure of the sequences flanking the 3′-terminal region of the CP gene. These observations suggest that expression of RDRP occurs by a mechanism different from translational frameshifting. The finding that the termination codon of the CP ORF (nucleotide position 2605-AUG-2607) overlaps with the predicted

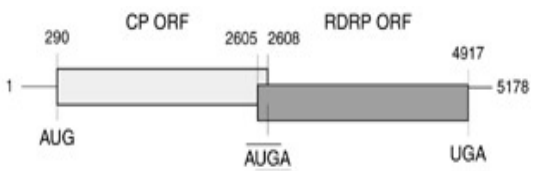


Figure 1 Genome organization of Helminthosporium victoriae 190S virus (Hv190SV) dsRNA. Two large overlapping open reading frames (ORFs) with the 5′ ORF encoding a capsid protein (CP) and the 3′ ORF encoding an RNA-dependent RNA polymerase (RDRP). Note that the termination codon of the CP ORF overlaps the initiation codon of the RDRP ORF in the tetranucleotide sequence AUGA.

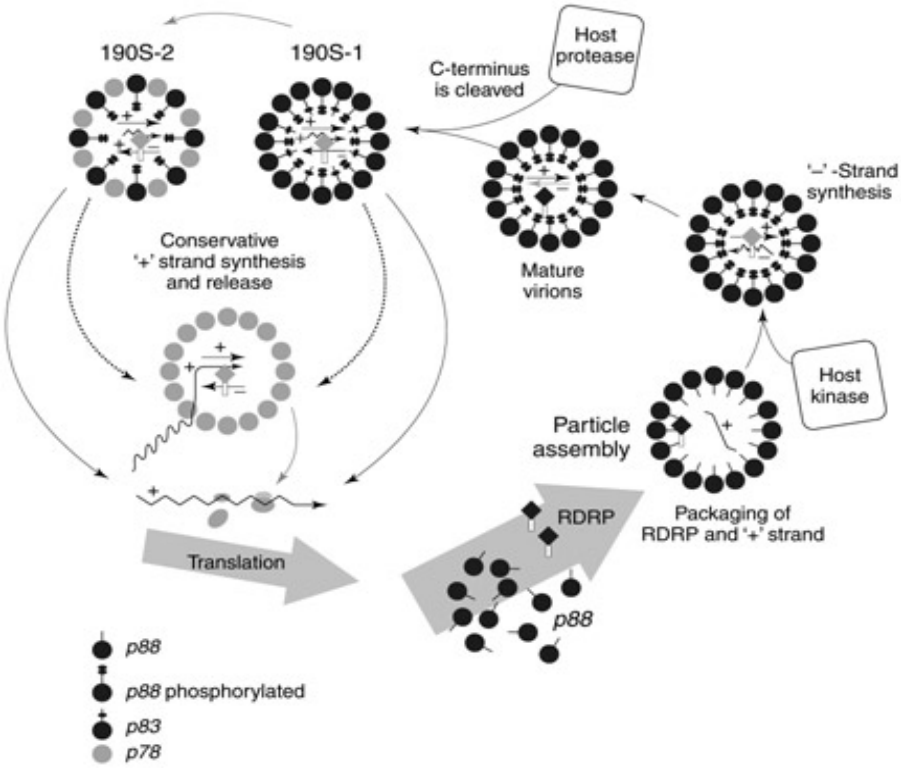


Figure 2 Life cycle of Hv190SV. Mature virions contain a single dsRNA molecule and their capsids are composed entirely or primarily of the capsid protein (CP) p88. Virions representing different stages of the virus life cycle can be purified from the infected fungal host *Helminthosporium victoriae* including the well-characterized 190S-1 and 190S-2 virions. These two types of virions differ in sedimentation coefficient, phosphorylation state and CP composition; 190S-1 capsids contain p88 and p83, whereas the 190S-2 capsids contain p88 and p78 (p88 is the primary translation product of the *CP* gene; p83 and p78 represent post-translational proteolytic processing products of p88 at the C-terminus). p88 and p83 are phosphorylated, whereas p78 is nonphosphorylated. The virions with phosphorylated CPs (p88 + p83) have significantly higher transcriptase activity *in vitro* than those containing the nonphosphorylated p78. Transcription occurs conservatively and the newly synthesized (+)-strand RNA is released from the virions. Phosphorylation of CP is catalyzed by a host kinase, and is proposed to play a regulatory role in transcription/replication. A host-encoded protease catalyzes the proteolytic processing of phosphorylated p88; this occurs in two steps, leading first to p83 (the generation of the 190S-1 virions) and then to p78 (190S-2 virions). The conversion of p88 → p83 → p78 is proposed to play a role in the release of the (+)-strand RNA transcripts from virions. The released (+)-strand RNA is the RNA that is translated into CP and RNA-dependent RNA polymerase (RDRP) and packaged in capsids assembled from the primary translation product p88. It is not known whether p88 is phosphorylated before or after assembly. Synthesis of (−)-strand RNA occurs on the (+)-strand RNA template inside the virion; phosphorylation may be involved in turning on the replicase activity.

start codon for the RDRP ORF (2606-UGA-2608) in the sequence AUGA (**Fig. 1**) suggests that RDRP is translated by an internal initiation mechanism.

Double-stranded RNA Replication

Information on the replication cycle of totivirus dsRNA has mainly been derived from *in vitro* studies of virion-associated RNA polymerase activity and the isolation of particles representing various stages in the replication cycle. In *in vitro* reactions, the RNA polymerase activity associated with virions of the fungal totiviruses ScV-L-A, UmV-H1 and HvV-190S, isolated from lag phase cultures, catalyzes end-to-end transcription of dsRNA, by a conservative mechanism, to produce mRNA for capsid polypeptide, which is released from the particles. Purified ScV-L-A virions, isolated from log phase cells, contain a less dense class of particles which package only (+)-strand RNA. In *in vitro* reactions, these particles exhibit a replicase activity that catalyzes the synthesis of (−)-strand RNA to form dsRNA. The resultant mature particles, which attain the same density as that of the dsRNA-containing virions isolated from the cells, are capable of synthesizing and releasing (+)-strand RNA.

A proposed life cycle of Hv190SV is depicted in **Fig. 2**. Host-encoded protein kinase and protease have been shown to be involved in post-translational modification of CP. Phosphorylation and proteolytic

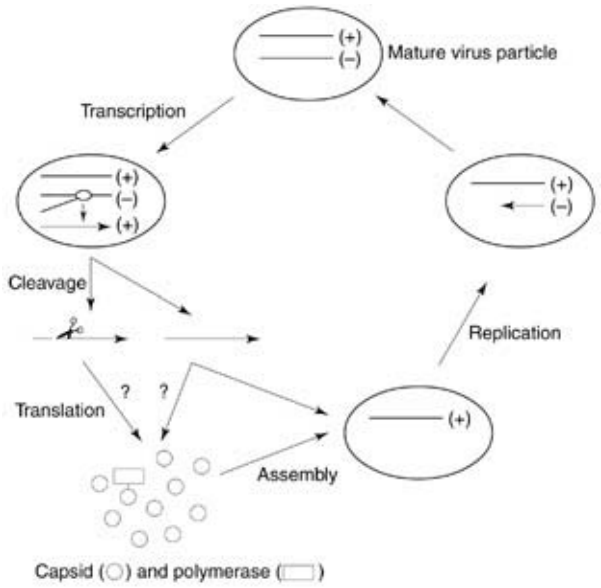


Figure 3 Replication cycle of leishmaniaviruses.

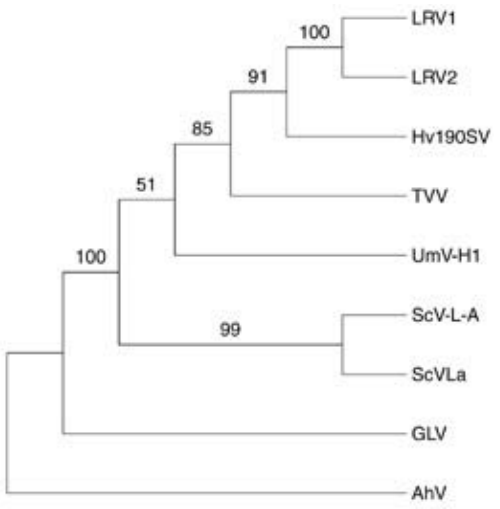


Figure 4 Phylogeny estimates of totiviruses derived from aligned deduced amino acid sequences of RDRP. The resulting consensus tree of 100 bootstrap replicates is shown; the number above each node indicates the percent of bootstrap replicates in which that node was recovered. Tree was outgroup-rooted to the partitivirus Atkinsonella hypoxylon virus (AhV). See **Table 1** for abbreviations of totivirus names.

processing are proposed to play a role in the virus life cycle; phosphorylation of CP may be necessary for its interaction with viral nucleic acid and for subsequent assembly into virions, and/or phosphorylation may regulate dsRNA transcription/replication. Proteolytic processing and cleavage of a C-terminal peptide, which leads to dephosphorylation and the conversion of p88 to p78, may play a role in the release of the (+)-strand RNA transcripts from virions (**Fig. 2**).

Leishmaniaviruses have a slight variation in their life cycle from the other totiviruses. The CP has endoribonuclease activity which is responsible for cleaving 320 nt from the 5′ end of the full-length single-stranded (+)-sense RNA. It has been hypothesized that the cleavage activity generates the functional message by removing translation inhibitory sequences. The proposed life cycle is depicted in **Fig. 3**.

Evolutionary Relationships among Totiviruses

Sequence comparison analysis of the predicted amino acid sequences of totivirus RDRPs indicated that they share significant sequence similarity and characteristically contain eight conserved motifs. This sequence similarity was common to the totiviruses that infect the yeast and smut fungi as well as those infecting parasitic protozoa. The RDRP of Hv190SV, a recently characterized totivirus that infects a filamentous ascomycetous fungus, was also found to contain the same eight conserved motifs. The sequence similarity among the RDRPs of these viruses infecting simple eucaryotes extends beyond the highly conserved eight motifs. Of the 70 amino acid positions contained in these conserved motifs, the Hv190SV RDRP is identical in 48, 47, 46, 40, 38 and 21 positions, respectively, to the RDRPs of LRV1, TVV, ScV-L-A, UmVH1, ScV-La and GLV.

Phylogeny estimates derived from multiple alignments of totivirus RDRP ORFs (**Fig. 4**) indicated that the two leishmaniaviruses LRV1 and LRV2 (percent sequence identities of 46%) are most closely related to each other. This is also true for the two yeast viruses ScV-L-A and ScV-La (identity of 32%). It is of considerable interest that Hv190SV, a mycovirus infecting a filamentous fungus, was found to be a sister clade to the leishmaniaviruses and not to the yeast viruses (**Fig. 4**).

Transmission and Host Range

There are no known natural vectors for the transmission of the fungal totiviruses. They are transmitted intracellularly during cell division, sporogenesis and cell fusion. Although the yeast viruses are effectively transmitted via ascospores, the totiviruses infecting the ascomycetous filamentous fungi, e.g. GgV-87-1-H, are essentially eliminated during ascospore formation. The leishmaniaviruses are not infectious and are propagated during cell division. Like other totiviruses, the leishmaniaviruses have no extracellular phase to their life cycles. Successful transfection of the

protozoa *Giardia lamblia*, however, has been accomplished via electroporation with (+)-strand RNA transcribed *in vitro* from GLV dsRNA.

There are no known experimental host ranges for the fungal totiviruses because of the lack of suitable infectivity assays. As a consequence of their intracellular modes of transmission, the natural host ranges of totiviruses are limited to individuals within the same or closely related vegetative compatibility groups. Furthermore, mixed infections with two or more unrelated viruses are common, probably as a consequence of the ways by which fungal viruses are transmitted in nature. Dual infection of yeast with ScV-L-A and ScV-L-BC is an example of mixed infection involving totiviruses. Leishmaniaviruses have been found in *L. braziliensis*, *L. guyanensis* and *L. major*.

Virus–Host Relationships

The yeast killer system, comprised of a helper totivirus (ScV-L-A) and associated satellite dsRNA (M-dsRNA), is one of a very few known examples where virus infection is beneficial to the host. The ability to produce killer toxins by immune yeast strains confers an ecological advantage over sensitive strains. The use of killer strains in the brewing industry provides protection against contamination with adventitious sensitive strains.

Totiviruses maintain only the genes that are essential for their survival (*RDRP* and *CP*), but make efficient use of host proteins. The host cells have evolved to support only a defined level of virus replication, beyond which virus infection may become pathogenic. Because of amenability to genetic studies, the yeast–virus system has provided significant information on the host genes required to prevent viral cytopathology. A system of six chromosomal genes, designated superkiller (or *SKI*) *SKI2*, *SKI3*, *SKI4*, *SKI6*, *SKI7* and *SKI8*, negatively control the copy number of the ScV-L-A totivirus and its satellite M-dsRNAs. The only essential function of these genes is to block virus multiplication. Mutations in any of these *SKI* genes lead to the development of the superkiller phenotype as a result of the increased copy number of M-dsRNA. The *SKI* genes affect primarily the initiation of translation rather than the stability of mRNA, and are thus part of a cellular system that specifically blocks translation of nonpolyadenylated mRNAs (like the (+)-strand transcripts of totiviruses). About 30 chromosomal genes, termed *MAK* genes (for maintenance of killer), are required for stable replication of the satellite M-dsRNA. Only three of these *MAK* genes are necessary for the helper virus (ScV-L-A) multiplication. Mutants defective in any of 20 *MAK* genes show a decreased level of free 60S ribosomal subunits. Since the *mak* mutations affecting 60S subunit levels are known to be suppressed by *ski* mutations, and since the latter are now known to act by blocking translation of nonpolyadenylated mRNAs, the level of 60S ribosomal subunits is believed to be also critical for translation of nonpolyadenylated mRNAs.

The Hv190S totivirus that infects the plant pathogenic fungus *Helminthosporium victoriae* utilizes host-encoded proteins (a protein kinase and a protease) for post-translational modification of its CP. Phosphorylation and proteolytic processing of CP may play a role in regulating transcription and the release of (+)-strand transcripts from virions (**Fig. 2**).

See also: **Cryptoviruses (*Cryptoviridae*); Hypoviruses (*Hypoviridae*); Partitiviruses – fungal (*Partitiviridae*); Giardiaviruses (*Totiviridae*); *Ustilago maydis* viruses; Yeast RNA viruses (*Totiviridae*).**

Further Reading

Ghabrial SA (1994) New developments in fungal virology. *Adv. Virus Res.* 43: 303.

Ghabrial SA (1998) Origin, adaptation and evolutionary pathways of fungal viruses. *Virus Genes* 16: 119.

Ghabrial SA, Bruenn JA, Buck KW *et al* (1995) *Totiviridae*. In: Murphy F *et al* (eds) *Virus Taxonomy: Sixth Report of the International Committee on Taxonomy of Viruses*. p. 245. New York: Springer.

Patterson JL (1990) Viruses of protozoan parasites. *Exp. Parasitol.* 70: 111.

Patterson JL (1993) The current status of leishmania RNA virus 1. *Parasitol. Today* 9: 135.

Ustilago Maydis Viruses

Jeremy Bruenn, Department of Biological Sciences, SUNY/Buffalo, Buffalo, USA

Taxonomy

The *Totiviridae* is a remarkable family of fungal and protozoan viruses. These viruses have a single essential double-stranded RNA (dsRNA) and are recognizably related to each other as viewed by comparison of their RNA-dependent RNA polymerases (RDRPs). They have been discovered in at least four genera of protozoans (*Leishmania*, *Eimeria*, *Giardiavirus* and *Trichomonas*) and nine genera of fungi (*Saccharomyces*, *Ustilago*, *Helminthosporium*, *Gaeumannomyces*, *Mycogone*, *Yarrowia*, *Aspergil-*

protozoa *Giardia lamblia*, however, has been accomplished via electroporation with (+)-strand RNA transcribed *in vitro* from GLV dsRNA.

There are no known experimental host ranges for the fungal totiviruses because of the lack of suitable infectivity assays. As a consequence of their intracellular modes of transmission, the natural host ranges of totiviruses are limited to individuals within the same or closely related vegetative compatibility groups. Furthermore, mixed infections with two or more unrelated viruses are common, probably as a consequence of the ways by which fungal viruses are transmitted in nature. Dual infection of yeast with ScV-L-A and ScV-L-BC is an example of mixed infection involving totiviruses. Leishmaniaviruses have been found in *L. braziliensis*, *L. guyanensis* and *L. major*.

Virus–Host Relationships

The yeast killer system, comprised of a helper totivirus (ScV-L-A) and associated satellite dsRNA (M-dsRNA), is one of a very few known examples where virus infection is beneficial to the host. The ability to produce killer toxins by immune yeast strains confers an ecological advantage over sensitive strains. The use of killer strains in the brewing industry provides protection against contamination with adventitious sensitive strains.

Totiviruses maintain only the genes that are essential for their survival (*RDRP* and *CP*), but make efficient use of host proteins. The host cells have evolved to support only a defined level of virus replication, beyond which virus infection may become pathogenic. Because of amenability to genetic studies, the yeast–virus system has provided significant information on the host genes required to prevent viral cytopathology. A system of six chromosomal genes, designated superkiller (or *SKI*) *SKI2*, *SKI3*, *SKI4*, *SKI6*, *SKI7* and *SKI8*, negatively control the copy number of the ScV-L-A totivirus and its satellite M-dsRNAs. The only essential function of these genes is to block virus multiplication. Mutations in any of these *SKI* genes lead to the development of the superkiller phenotype as a result of the increased copy number of M-dsRNA. The *SKI* genes affect primarily the initiation of translation rather than the stability of mRNA, and are thus part of a cellular system that specifically blocks translation of nonpolyadenylated mRNAs (like the (+)-strand transcripts of totiviruses). About 30 chromosomal genes, termed *MAK* genes (for maintenance of killer), are required for stable replication of the satellite M-dsRNA. Only three of these *MAK* genes are necessary for the helper virus (ScV-L-A) multiplication. Mutants defective in any of 20 *MAK* genes show a decreased level of free 60S ribosomal subunits. Since the *mak* mutations affecting 60S subunit levels are known to be suppressed by *ski* mutations, and since the latter are now known to act by blocking translation of nonpolyadenylated mRNAs, the level of 60S ribosomal subunits is believed to be also critical for translation of nonpolyadenylated mRNAs.

The Hv190S totivirus that infects the plant pathogenic fungus *Helminthosporium victoriae* utilizes host-encoded proteins (a protein kinase and a protease) for post-translational modification of its CP. Phosphorylation and proteolytic processing of CP may play a role in regulating transcription and the release of (+)-strand transcripts from virions (**Fig. 2**).

See also: **Cryptoviruses (*Cryptoviridae*); Hypoviruses (*Hypoviridae*); Partitiviruses – fungal (*Partitiviridae*); Giardiaviruses (*Totiviridae*); *Ustilago maydis* viruses; Yeast RNA viruses (*Totiviridae*).**

Further Reading

Ghabrial SA (1994) New developments in fungal virology. *Adv. Virus Res.* 43: 303.

Ghabrial SA (1998) Origin, adaptation and evolutionary pathways of fungal viruses. *Virus Genes* 16: 119.

Ghabrial SA, Bruenn JA, Buck KW *et al* (1995) *Totiviridae*. In: Murphy F *et al* (eds) *Virus Taxonomy: Sixth Report of the International Committee on Taxonomy of Viruses*. p. 245. New York: Springer.

Patterson JL (1990) Viruses of protozoan parasites. *Exp. Parasitol.* 70: 111.

Patterson JL (1993) The current status of leishmania RNA virus 1. *Parasitol. Today* 9: 135.

Ustilago Maydis Viruses

Jeremy Bruenn, Department of Biological Sciences, SUNY/Buffalo, Buffalo, USA

Taxonomy

The *Totiviridae* is a remarkable family of fungal and protozoan viruses. These viruses have a single essential double-stranded RNA (dsRNA) and are recognizably related to each other as viewed by comparison of their RNA-dependent RNA polymerases (RDRPs). They have been discovered in at least four genera of protozoans (*Leishmania*, *Eimeria*, *Giardiavirus* and *Trichomonas*) and nine genera of fungi (*Saccharomyces*, *Ustilago*, *Helminthosporium*, *Gaeumannomyces*, *Mycogone*, *Yarrowia*, *Aspergil-*

lus, Thielaviopsis, and probably *Agaricus*). *Ustilago maydis* viruses are in the *Totivirus* genus of the *Totiviridae* family.

Characteristics of the Host Fungus

The fungus *Ustilago maydis* belongs to the class Basidiomycetes and the family Ustilaginaceae. Its life cycle has three phases: diploid, haploid and dikaryon. While dikaryon formation and development is restricted to host tissue, the diploid and haploid mycelia may grow rapidly in a yeast-like manner in laboratory media. A transient dikaryon (or heterokaryon) may be formed between compatible strains on laboratory medium. *U. maydis* is heterothallic. Sexual reproduction between two haploid strains depends on two unlinked genes. The *a* gene has two alleles and the *b* gene has multiple alleles. Cells with opposite *a* alleles will fuse under appropriate conditions to form a heterokaryon which will infect the host and allow the completion of the life cycle only if the *b* alleles are also different. The *a* gene can, therefore, be regarded as the mating type locus homologous to the simple two-allele mating systems of most yeasts, whereas the *b* gene controls the development of the dikaryon in infected host cells, which is the only place that sexual development can proceed.

Virion Structure and Composition

All the dsRNA viruses are now known to have at least one capsid polypeptide present in 120 copies per virion. This is unique among icosohedral viruses and superficially appears to violate the Caspar and Klug rules for assembly of isometic virus particles. However, in the one case where the x-ray crystal structure of the virion has been determined (Bluetongue virus, or BTV), this apparent $T = 2$ symmetry is really a twofold $T = 1$ symmetry, in which 60 of the subunits adopt one configuration and 60 another. Hence, even though there are 120 copies of the virion protein, half of them are in one conformation and half in the other.

The totiviruses are small icosohedral viruses. *Ustilago maydis* virus (UmV) is about 41 nM in diameter, sedimenting at 172 S and with a buoyant density in CsCl of 1.42 g ml^{-1}. In UmV the capsid polypeptide is about 75 kDa, but no accurate estimate of its size is available. In the one case where the sequence of its coding RNA is known (P1H1), the C-terminus of the protein appears to be generated by cleavage, and the cleavage site has not been determined. Electron microscopy, in the case of UmV and *Saccharomyces cerevisiae* virus (ScV), has demonstrated that there are 120 copies of a single polypeptide in their capsids, arranged as 12 pentamers of dimers of the capsid polypeptide. Presumably, as in BTV, these adopt two configurations in equal numbers in the capsid. In ScV, two of the capsid monomers are thought to be the N-terminal portions of a capsid polypeptide–RDRP fusion protein. Since the P1H1 capsid polypeptide is initially synthesized as such a fusion prior to (presumed) cleavage, this is likely for UmV as well.

Ustilago Maydis Viruses

Most fungal dsRNA viruses do not confer on their host cells any characteristic phenotype. They are persistent, segmented dsRNA viruses whose segments are separately encapsidated and whose only means of transmission is mitosis or meiosis. However, ScV and UmV are known to encode killer toxins, which are thought to provide a selective advantage to cells harboring the responsible viruses.

Interstrain inhibition (the killing phenomenon) in *U. maydis* was discovered by Puhalla during heterokaryon experiments. The inhibitory factor (killer toxin) was shown to be a secreted protein or proteins. Crosses and heterokaryon transfer experiments showed that the inhibitory effect is caused by cytoplasmically inherited factors, which are now known to be single dsRNA segments present in some multisegmented dsRNA viral genomes (see below). A small proportion of *U. maydis* cells (about 1%) can produce killer toxins, to which they are resistant; sensitive cells are the majority in wild populations. There are also some cells that do not have killing ability but are resistant to one toxin. Killer toxins are known in eight genera of yeasts, but the killer toxins of *Ustilago* are the only ones known in a filamentous fungus. The *U. maydis* killer toxins are effective against species in the family Ustilaginaceae, including those that are known as pathogens to wheat, oats and barley.

There are three killer types: KP1, KP4 and KP6, which secrete KP1, KP4 and KP6 toxins, respectively. Correspondingly, there are three groups of resistant cells, in which the resistance is determined by three independent recessive nuclear genes: *p1r, p4r* and *p6r*. In KP1, cytoplasmically inherited factors also confer immunity to the KP1 killer toxin. KP4 probably has no cytoplasmically determined immunity, and recent experiments show that KP6 has no cytoplasmically determined immunity. Cells cannot produce the KP6 toxin unless they have the p6r gene, and the same is probably true of KP4. The *p1r, p4r* and *p6r* genes are thought to encode cellular (membrane, or membrane–cell wall) receptors for the toxin. Since these putative receptors are different in each case, there is no crossresistance to the toxins. The KP6 receptor is

apparently recognized by cytoplasmic toxin polypeptides as well as by toxin in the medium. There are no single resistance alleles that confer simultaneous resistance to all three toxins.

Structure and Function of the Killer Toxins

KP6

The three-dimensional structures of two of the *Ustilago* toxin polypeptides have been determined. The crystal structure of KP6α (space group $P6_322$, a = 48.3 Å, c = 124.8 Å) was determined by use of heavy atom isomorphous techniques and by selenomethionine substitution (R-factor = 0.169, resolution 1.8 Å). The monomer has a three finger structure similar to neurotoxins and cardiotoxins and is composed of a four stranded antiparallel β sheet with two α helices on one side, an additional β strand, and a small α-helix segment at the N-terminus. Three intermolecular salt bridges link monomers of KP6α into trimers organized around a threefold segment axis. The trimer has a funnel shape that is wide across the mouth and narrows to a 3.2 Å opening just large enough to permit an ion to pass.

The trimer has many structural features that have been postulated to be critical elements in pore domains of mammalian ion channels, including a solvent-filled funnel lined with charged amino acid residues that could be a selectivity screen for specific ions and a network of salt bridges that stabilize the timer. The 3.2 Å funnel bore is lined by six phenyl rings. These phenyl rings arise in part from a GFG sequence in KP6α that may correspond to a G(Y/F)G sequence that is conserved in K^+ channels. These phenyl groups in KP6α form a cage similar to one postulated to be responsible for K^+ selectivity relative to Na^+ on the basis of *ab initio* calculations. In crystals two trimers are related by a twofold axis perpendicular to the funnel axis, generating a symmetric pair of funnels. The hexamer has dimensions and characteristics that suggest it is a self-assembling ion pore closely resembling the pore (H5) commonly found in voltage-gated and inwardly-rectifying ion channels. The open ends of the two funnels would lie on the bilayer surface and the narrow bore would be at the center of the bilayer. The salt bridges that hold the trimers together are separated by 30 Å and portions of the funnel would extend well out of the membrane.

KP6β may be a functional analogue of the transmembrane strands that are thought to flank the pore in mammalian ion channels and facilitate entry of the pore into the membrane. The structure of KP6β has been modeled upon that of KP6α by examining sequence similarities. Examination of the stereochemical topology of the KP6α hexamer and the KP6β monomer and the charge distribution on their surfaces suggested a model for a dodecamer composed of six molecules of KP6α (as observed in the crystal structure) surrounded by six molecules of KP6β. The resultant KP6$\alpha_6\beta_6$ dodecamer has an outer surface that is essentially neutral and compatible with membrane insertion. The interface between the observed hexamer of KP6α and the modeled hexamer of KP6β exhibits excellent complementarity in surface charge and stereochemistry. Preliminary experiments with planar lipid bilayers are consistent with the KP6 toxin functioning as a channel-former.

KP4

The structure of KP4 was determined to 1.9 Å resolution and is now refined to 1.4 Å using a single isomorphous derivative, with phases improved using real space averaging. KP4 belongs to the α/β sandwich family of proteins and has a double split β-α-β motif. The toxin has a total of seven β strands and three α helices, with the major secondary structure elements consisting of five antiparallel stranded β sheets (β1, β3, β4, β6, β7) with two antiparallel α helices (α2, α3) lying at approximately 45° to the strands in the β sheet. The *U. maydis* KP4 killer toxin and the *Pichia farinosa* KK1 toxin have no evident primary sequence similarity, but their tertiary structures are essentially identical even though KP4 is a single polypeptide and KK1 is two polypeptides. Thus KP4 appears from its structure to be a tandem duplication of one sequence which has subsequently diverged, and it could easily have arisen from two polypeptides processed by Kex2p by a single deletion of the genomic sequence encoding the intervening sequence and the cleavage sites in the prepropolypeptide. This would conveniently explain the absence of Kex2p processing of KP4.

The scorpion toxins are a family of neurotoxins with some interesting similarities to KP4. Albeit about half the size of KP4, the long toxins are similar to KP4 in that they are all highly basic proteins that are stabilized by an extensive network of disulfide bonds. The core motif of the α and β neurotoxins are quite similar and the functional differences between them may lie in the length and orientation of protruding loops extending from this core region. In light of this tenuous similarity to the scorpion toxins, KP4 killing efficacy was tested on sensitive *Ustilago* cells. Cells were rescued by very small concentrations of Ca^{2+} but not with similar concentrations of monovalent cations.

These rescue experiment results strongly suggested

that KP4 affects Ca^{2+} channels. Therefore, the effect of KP4 was tested on mammalian cells lines where more is known about the types and kinetics of cationic channels. Standard whole-cell patch clamp techniques were used to examine the effects of KP4 on voltage-activated Na^+, K^+, Ca^{2+} currents in PC12, GH_3 and adrenal chromaffin cells, and the prevalent action of KP4 was inhibition of voltage-activated Ca^{2+}-channel currents.

Genome Structure and Translation

The UmV viral genomes consist of three distinct size groups of dsRNAs. They are heavy (H), medium (M) and light (L). Three original isolates (named after the killer toxins they encoded) were named P1, P4 and P6. Cells may have as few as three viral dsRNAs (some isolates of P6 strains) or as many as seven viral dsRNAs (some isolates of P4 strains). The H segments appear to encode the essential viral polypeptides: a capsid polypeptide and an RDRP. One of the H1 segments has been sequenced: P1H1 is 6099 bp, and encodes on one open reading frame (540–5999) a polypeptide of 1820 amino acids with a putative capsid polypeptide in the N-terminal region and a typical RDRP sequence in the C-terminal thousand amino acids. P1H1 and P6H1 are nearly identical in sequence, while P1H1 and P1H2 share about 31% amino acid sequence identity in the RDRP region. This is consistent with there being at least two totiviruses in *Ustilago* (H1 and H2), which is similar to the ScVL1 (ScVL-A) and ScVLa (ScVL-BC) viruses in *S. cerevisiae*. Processing of the P1H1 polypeptide is proposed to occur by self-cleavage.

One or more of the M segments in each subtype encode the toxin. By sequence analysis of both the toxin polypeptides and P6M2 and synthesis of toxin in heterologous (yeast and tobacco) and homologous cDNA expression vectors, as well as immunological evidence, the KP6 coding region in P6M2 has been completely defined. P6M2 is 1234 bp, encoding a preprotoxin of 219 amino acids, which is subsequently processed by signal peptide cleavage after residue 19, Kex2p cleavage after residues 27, 108 and 138, and Kex1p cleavage after residue 137 to yield two polypeptides, α of 79 amino acids (8.6 kDa), and β of 81 amino acids (9.1 kDa).

Similarly, P4M2, of 1006 bp, encodes the KP4 preprotoxin of 127 amino acids, which is processed solely by signal peptidase cleavage after residue 22, yielding a toxin of 105 amino acids (11.1 kDa). The KP4 toxin has been expressed from cDNA clones in yeast, tobacco and *Pichia pastoris*.

P1M2, of 1034 bp, encodes the KP1 toxin, which is synthesized as a preprotoxin of 291 amino acids. The KP1 preprotoxin is processed by signal peptidase (24), Kex2p (142 and 173), and Kex1p (141), resulting in an α peptide of 117 amino acids and a β peptide of 118 amino acids (13.4 kDa). Only β is necessary for activity.

All the *Ustilago* toxin peptides are small, highly stable proteins in which all the cysteines appear to be in disulfides. KP6α has four disulfides, KP6β three, KP4 five, and KP1β three. None of the *Ustilago* toxin polypeptides is glycosylated, although KP6α and KP1β have possible *N*-linked glycosylation sites, and the KP6α site is utilized in *S. cerevisiae*. None of the M segments (including P1M1) have detectable sequence similarities at either the nucleotide level or the protein level, except in the 3′ region of the plus strand, from which the L segments are derived.

The L segments (355 bp) are derived from the 3′ ends of the plus strands of M segments: P6L from P6M2, P1L from P1M1, and P4L from P4M2. Neither the mode of synthesis of these segments nor their function is understood. All the L segments share a great deal of sequence identity (50–95% at the nucleotide level). In general, homologous P1 and P4 segments are very close in sequence (the L segments are 95% identical), while the homologous P6 segments are more divergent. The highest conservation of sequence among P6M2, P1M1 and P4M2 is within 20 bases on either side of the region of M encoding the 5′ end of the L plus strand. This suggests that the L segments are derived by replication of cleavage products of M plus strands (see below).

Complete sequences are known for the following *Ustilago* dsRNAs: P1H1 (6099 bp), P6M2 (1234 bp), P6L (355 bp), P4M2 (1006 bp), P4L (354 bp), P1M1 (1504 bp), P1M2 (1034 bp) and P1L (354 bp). Partial sequences are available for P6H1 and P4H1. P1M1 could encode two secreted polypeptides of 81 amino acids and 143 amino acids after signal peptidase and Kex2 cleavage, but neither peptide has been detected as a component of the P1 toxin (**Fig. 1**).

Replication and Transcription

All dsRNA viruses separate transcription from replication by packaging. Viral plus strands are synthesized in viral particles and extruded into the cell cytoplasm. These have two fates: translation and packaging. Viral plus strands that are packaged into nascent particles are subsequently replicated (by synthesis of the minus strand) after packaging is complete. In ScV, the packaging signal is a small (20 base) sequence. The identity of the packaging signal in UmV is unknown. However, all sequenced UmV

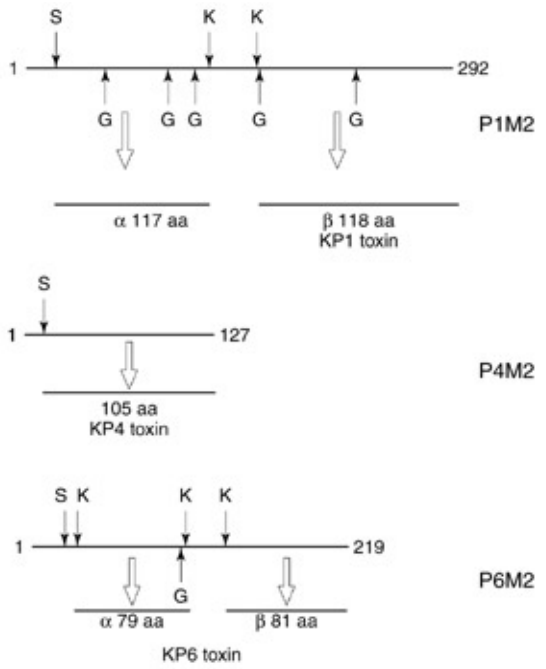


Figure 1 Processing of the KP1, KP4 and KP6 killer toxins, encoded by P1M2, P4M2 and P6M2 respectively. Processing by signal peptidase is indicated by S and processing by Kex2 by K. Possible sites of *N*-linked glycosylation are shown by G, but none of these sites is glycosylated in the mature peptides.

RNAs have the sequence GAAAAA at the 5′ end and $AUGCA_{OH}$ at the 3′ end of the plus strand, except for the L segments. These have the sequence UCCG at the 5′ end of the plus strand. Since the 5′ sequence GAAAAA is common among the fungal dsRNA viruses, and since the complementary region of the minus strand is required for transcriptase activity at least in ScV, this has been taken to be the required start for transcription.

The absence of this sequence in the L segments and their highly conserved sequences around the position of the 5′ end of the L plus strand within M probably indicate that the L segments are generated by cleavage of the M plus strand followed by replication. This hypothesis is further supported by the presence of a long putative double-stranded region (a stem) immediately adjacent to the putative site of cleavage. In P4M2, this stem is a remarkable 23 uninterrupted base pairs in length.

Replication and transcription in dsRNA viruses may be either conservative or semiconservative. That is, the plus strand extruded during transcription may be either the parental strand (semiconservative) or the newly synthesized strand (conservative). Replication is conservative in ScV, but for at least one UmV segment, the M2 dsRNA, replication is semiconservative.

Evolution

Due to the extant similarities among totiviruses present in divergent phyla, ranging from protozoans parasitic to animals (*Leishmania*) to fungi parasitic to plants (*Ustilago*), and the fact that these viruses are not infectious but have only vertical transmission, they are thought to have originated very early in evolution, prior to the differentiation of multicellular from single-celled eucaryotes. Sequence relationships among the totivirus RDRPs support this model. For instance, the closest relative of UmVP1H2 is ScVLa, even though UmVP1H1 and UmVP1H2 are present in the same *Ustilago* cells while ScVLa and ScVL1 are present in the same *S. cerevisiae* cells. Similarly, another fungal totivirus (from *Helminthosporium*) is more closely related to the protozoan totiviruses than to the other fungal totiviruses. Although the totiviruses are noninfectious, the closely related *Giardiavirus* is infectious, and both ScV and UmV have been shown to be infectious, albeit very inefficiently, to spheroplasts. These experiments have selected for transformation by *S. cerevisiae* or *U. maydis* DNA plasmids in the presence of purified virus, using recipient cells lacking the viruses. A small percentage of the selected transformants carry the virus. Hence the cell wall (in fungi) or the cell membrane (in protozoans) seems to limit infection.

Geographic Distribution

The same UmV isolates are present in *Ustilago* from Mexico, the USA, the UK, Poland and China. So far only three toxin specificities have been found. Protein purification and sequencing has demonstrated a toxin with the same N-terminus as KP4 in Korea.

Economic Significance

Ustilago is a pathogen on a number of grain crops, the most important of which is maize. It does cause major crop losses of sweet corn. Although UmV has no apparent effect on the virulence of its host, it may be possible to introduce the viral killer toxin genes into the plants, making them resistant to the fungus. The UmV toxins of *U. maydis* are particularly attractive as biological control agents because they have no known effects when ingested. The UmV toxins can be effective against a wide range of *Ustilago* species known to be crop pathogens, including pathogens of maize, wheat, oats and barley. The UmV toxins should therefore be very useful as a means of introducing novel resistance to the ustilaginales into plants, provided the toxins can be expressed at high levels, are correctly processed, and end up in cellular

or extracellular compartments where they can inhibit growth of the fungus.

The KP6 and KP4 toxins have been successfully expressed in transgenic tobacco plants, so this may be a possible strategy. Tobacco plants are capable of correctly synthesizing, processing and secreting KP6 toxin polypeptides with the same activity and specificity as the authentic KP6 toxin produced by *U. maydis*. The activity of toxin produced is rather low (about 200-fold lower than in *Ustilago*), possibly due to incorrect processing of the C-terminus of α. Tobacco plants produce about 500 times as much KP4 activity from the same expression vector as that used for KP6. Toxin activity is easily detected at the surface of leaves of transgenic KP4 tobacco plants. Transgenic maize plants expressing genes for both KP4 and KP6 toxins could be resistant to all but a fraction of a percent of the natural *U. maydis* population. Since ingested UmV toxins have no toxic effects on human cells, they would be safe for consumption and have less environmental impact than the fungicides currently used to deter fungal pathogens.

See also: **Cypoviruses (*Reoviridae*); Giardiaviruses (*Totiviridae*); Totiviruses (*Totiviridae*): General features; Partitiviruses – fungal (*Partitiviridae*); Phage ϕ6 (*Cystoviridae*); Reoviruses (*Reoviridae*): General features, Molecular biology, Plant reoviruses; Virus structure: Atomic structure; Yeast RNA viruses (*Totiviridae*).**

Further Reading

Bruenn JA (1993) A closely related group of RNA-dependent RNA polymerases from double-stranded RNA viruses. *Nucleic Acids Res.* 21: 5667.

Gu F, Khimani A, Rane S *et al* (1995) Structure and function of a virally encoded fungal toxin from *Ustilago maydis:* a fungal and mammalian Ca^{2+} channel inhibitor. *Structure* 3: 805.

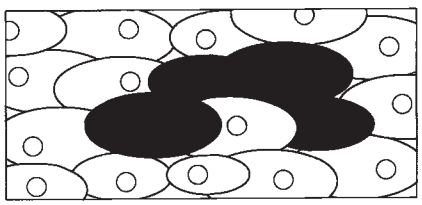
Kinal H, Park C-M, Berry JO, Koltin Y and Bruenn JA (1995) Processing and secretion of a virally encoded antifungal toxin in transgenic plants: evidence for a Kex2p pathway in plants. *Plant Cell* 7: 677.

Park C-M, Bruenn JA, Ganesa C *et al* (1994) Structure and heterologous expression of the *Ustilago maydis* viral toxin KP4. Mol. Microbiol. 11: 155.

Park C-M, Banerjee N, Koltin Y and Bruenn JA (1996) The *Ustilago maydis* virally encoded KP1 killer toxin. *Mol. Microbiol.* 20: 957.

Tao J, Ginsberg I, Banerjee N *et al* (1990) The *Ustilago maydis* KP6 killer toxin: structure, expression in *Saccharomyces cerevisiae* and relationship to other cellular toxins. *Mol. Cell. Biol.* 10: 1373.

TRANSFORMATION – ANIMAL VIRUSES



Ron Wisdom, Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

Inder M Verma, The Salk Institute, San Diego, California, USA

Introduction

The observation that certain viruses rapidly and reproducibly induce tumors in the host was first made nearly a century ago. The ability to accurately duplicate this process in tissue culture has made possible the investigation of neoplastic transformation *in vitro*. This, in turn, has allowed the techniques of molecular biology and genetics to be applied to the problem of cancer, especially cancer induced by viruses. Much of our current knowledge of the molecular mechanisms of cancer today is derived from the study of virally induced tumors. Virus-induced tumors are not only important as laboratory tools for investigation of the fundamental mechanisms of tumor formation; some human cancers, including hepatocellular carcinoma, cervical carcinoma, Burkitt's lymphoma and acute T-cell leukemia, appear to have a viral etiology.

The types of viruses that are capable of inducing tumors are remarkably diverse, and include viruses with RNA and DNA genomes. Nonetheless, certain common features have emerged from the study of neoplastic transformation by different viruses. The first is that tumor formation induced by viruses is the result of the acquisition by the host cell of the viral genome. In all cases of virally induced tumors so far described, at least a part of the viral genome is

or extracellular compartments where they can inhibit growth of the fungus.

The KP6 and KP4 toxins have been successfully expressed in transgenic tobacco plants, so this may be a possible strategy. Tobacco plants are capable of correctly synthesizing, processing and secreting KP6 toxin polypeptides with the same activity and specificity as the authentic KP6 toxin produced by *U. maydis*. The activity of toxin produced is rather low (about 200-fold lower than in *Ustilago*), possibly due to incorrect processing of the C-terminus of α. Tobacco plants produce about 500 times as much KP4 activity from the same expression vector as that used for KP6. Toxin activity is easily detected at the surface of leaves of transgenic KP4 tobacco plants. Transgenic maize plants expressing genes for both KP4 and KP6 toxins could be resistant to all but a fraction of a percent of the natural *U. maydis* population. Since ingested UmV toxins have no toxic effects on human cells, they would be safe for consumption and have less environmental impact than the fungicides currently used to deter fungal pathogens.

See also: **Cypoviruses (*Reoviridae*); Giardiaviruses (*Totiviridae*); Totiviruses (*Totiviridae*): General features; Partitiviruses – fungal (*Partitiviridae*); Phage ϕ6 (*Cystoviridae*); Reoviruses (*Reoviridae*): General features, Molecular biology, Plant reoviruses; Virus structure: Atomic structure; Yeast RNA viruses (*Totiviridae*).**

Further Reading

Bruenn JA (1993) A closely related group of RNA-dependent RNA polymerases from double-stranded RNA viruses. *Nucleic Acids Res.* 21: 5667.

Gu F, Khimani A, Rane S *et al* (1995) Structure and function of a virally encoded fungal toxin from *Ustilago maydis:* a fungal and mammalian Ca^{2+} channel inhibitor. *Structure* 3: 805.

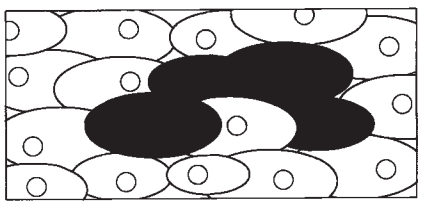
Kinal H, Park C-M, Berry JO, Koltin Y and Bruenn JA (1995) Processing and secretion of a virally encoded antifungal toxin in transgenic plants: evidence for a Kex2p pathway in plants. *Plant Cell* 7: 677.

Park C-M, Bruenn JA, Ganesa C *et al* (1994) Structure and heterologous expression of the *Ustilago maydis* viral toxin KP4. Mol. Microbiol. 11: 155.

Park C-M, Banerjee N, Koltin Y and Bruenn JA (1996) The *Ustilago maydis* virally encoded KP1 killer toxin. *Mol. Microbiol.* 20: 957.

Tao J, Ginsberg I, Banerjee N *et al* (1990) The *Ustilago maydis* KP6 killer toxin: structure, expression in *Saccharomyces cerevisiae* and relationship to other cellular toxins. *Mol. Cell. Biol.* 10: 1373.

TRANSFORMATION – ANIMAL VIRUSES



Ron Wisdom, Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

Inder M Verma, The Salk Institute, San Diego, California, USA

Introduction

The observation that certain viruses rapidly and reproducibly induce tumors in the host was first made nearly a century ago. The ability to accurately duplicate this process in tissue culture has made possible the investigation of neoplastic transformation *in vitro*. This, in turn, has allowed the techniques of molecular biology and genetics to be applied to the problem of cancer, especially cancer induced by viruses. Much of our current knowledge of the molecular mechanisms of cancer today is derived from the study of virally induced tumors. Virus-induced tumors are not only important as laboratory tools for investigation of the fundamental mechanisms of tumor formation; some human cancers, including hepatocellular carcinoma, cervical carcinoma, Burkitt's lymphoma and acute T-cell leukemia, appear to have a viral etiology.

The types of viruses that are capable of inducing tumors are remarkably diverse, and include viruses with RNA and DNA genomes. Nonetheless, certain common features have emerged from the study of neoplastic transformation by different viruses. The first is that tumor formation induced by viruses is the result of the acquisition by the host cell of the viral genome. In all cases of virally induced tumors so far described, at least a part of the viral genome is

present. Furthermore, the continued expression of at least a part of the viral genome is required for the transformed phenotype to be present. In the case of the transforming RNA viruses, the viral genome is present in the form of a DNA provirus integrated into the host genetic material. An important consequence of the presence of the viral genetic material is the fact that the transformed phenotype induced by the virus is heritable.

Cancer cells show many properties that distinguish them from normal cells. The hallmark of malignant cells is their ability to grow in the absence of appropriate signals from the extracellular environment. While the extracellular signals that are either not sensed or not required may vary from tumor to tumor, the property of malignancy is cell autonomous, that is, it is a property of the transformed cell and not the surrounding normal tissue. Genetic events that give rise to cancer, then, alter the cellular response to external stimuli. The investigation of cancer over the last decade has served to outline a cell signaling pathway that allows cells to respond to changes in the external environment. Support for the idea that this signaling pathway is the direct target for cancer-inducing mutations has been found in a wide range of both viral and nonviral tumors.

Retroviruses

The study of retroviruses has contributed more to our understanding of cancer than the study of any other group of viruses. The relevant features of the retroviral life cycle involve reverse transcription of the RNA genome into double-stranded proviral DNA, integration of the proviral DNA in the host genome, and expression of the viral genome from the chromosomal site of integration. The retroviruses have been of particular importance for several reasons. First, the small size of the retroviral genome and the small number of proteins expressed from the retroviral genome (a transforming retrovirus frequently encodes only one protein) has made them experimentally favorable for molecular analysis. Second, the proteins that produce neoplastic transformation are not required for the virus to complete its life cycle. Third, the retroviral transforming genes are mutated forms of normal cellular genes, giving their study direct relevance to nonviral forms of tumor formation.

Retroviruses can be divided into those that induce transformation acutely (in days to weeks) and form polyclonal tumors, and those that induce transformation over long periods of time (months) and form monoclonal or oligoclonal tumors. The distinction is important because the mechanism of tumorigenesis is fundamentally different in the two cases. Acutely transforming retroviruses consist of a mixture of replication-defective viruses that contain the transforming activity, and replication-competent nontransforming (helper) viruses that serve to propagate the transforming virus. The transforming virus in these cases often encodes only a single protein, and it is this protein that has the oncogenic activity. These transforming viruses are potent carcinogens, in the sense that the infection of a cell is sufficient to induce transformation with a very short latency. The transforming viral genes are transduced mutated copies of cellular genes, and the transforming proteins correspond to mutant versions of the corresponding cellular proteins. Structural and functional analyses of the viral and cellular forms of transforming proteins allow elucidation of the features that convert a normal cellular protein into an oncogenic one.

In contrast to the acutely transforming retroviruses, the retroviruses that induce tumors only after long latency have several important distinctions. They are replication-competent viruses that have not transduced cellular genes and so do not contain a transforming gene. Tumor formation by these viruses is often the result of the mutation of cellular genes during the integration process. Expression of cellular proto-oncogenes may be transcriptionally activated by the occasional nearby integration of retroviral promoter and enhancer elements, as in the case with c-*myc* activation by avian leukosis virus (ALV) and murine leukemia virus (MLV). As retroviruses integrate into a wide range of sites in the host DNA, only rare integration events will give rise to the activation of a protooncogene, explaining the long latency and the monoclonal nature of the tumors. In addition to being potentially tumorigenic, the juxtaposition of the retroviral transcriptional control signals with cellular protooncogenes may be of importance for a second reason. It is likely that production of a fusion virus–oncogene mRNA is the first step in the viral transduction event that results in the generation of an acutely transforming retrovirus through an aberrant viral recombination event.

Cell signaling

The ability of cells to respond to their external environment is mediated by the interaction of factors outside the cell (peptide and nonpeptide hormones and growth factors, cell matrix and other cells) with specific receptors located on the cell surface. These cell surface receptors contain at least two distinct functions. First, they contain a ligand-binding function which allows sensing of the presence or absence of the appropriate factor in the external environment,

and second, they contain or control some type of enzymatic activity that generates an intracellular change in response to the presence of the ligand. Often, this signal is in the form of reversible phosphorylation in response to ligand. The initial signal frequently generates a cascade of intracellular events, which include the generation of small molecule second messengers, such as cyclic nucleotides, calcium ions, and phospholipids, the activation of signals generated by the small GTP-binding proteins, and the activation of other protein kinases. Any long-lasting change in cell behavior, such as growth, requires new gene expression, and an important consequence of the signaling cascade is the activation of a set of transcription factors that directly influence the expression of genes required to alter cell behavior. Theoretically, it should be possible to generate mutant cells defective in the response to extracellular changes by alteration or mutation in any part of the signaling pathway, and the last ten years has witnessed the experimental confirmation of this idea. Thus, neoplastic transformation can be due to mutations in proteins that function as autocrine growth factors, growth factor receptors, membrane-bound and cytoplasmic components of the cell signaling apparatus, and transcription factors, all the normal activities of which are subject to control by changes in the external environment.

Growth factors and receptors

The simian sarcoma virus (SSV) contains a single open reading frame and encodes a single protein (v-Sis). This protein is a fusion protein between the retroviral *env* gene product and the product of a gene derived from the c-*sis* gene of the SSV host, the woolly monkey. The *env* sequences comprise little besides the signal sequence of the Env protein, and the remainder of the protein is the product of the transduced c-*sis* gene. Nucleotide sequence analysis has shown that c-*sis* is the platelet-derived growth factor (PDGF) B chain gene. The v-Sis protein is therefore a form of PDGF, a peptide mitogen for a number of cell types. That the v-*sis* gene product functions as an autocrine growth factor to induce transformation is supported by several lines of evidence. Transformation by v-Sis is limited to cells that express the PDGF receptor. The PDGF receptor is a protein tyrosine kinase the activity of which is dependent on the presence of ligand. In v-Sis-transformed cells, there is increased activity of the PDGF receptor and an increase in total cellular phosphotyrosine-containing proteins when compared to nontransformed cells. As both the v-Sis protein and the PDGF receptor are present in the endoplasmic reticulum, binding and activation of the receptor may occur at sites other than the cell surface. That this is the case is suggested by the observation that activated intracellular forms of the receptor can be found in v-Sis-transformed cells, but not in cells exposed to PDGF. Therefore, the v-*sis* gene product functions as an autocrine growth factor in susceptible cells.

A second class of oncogenic mutations predicted from the cell signaling model outlined above are mutations that separate activation of growth factor receptors from the binding of ligand. The first described example of such a mutation was the v-*erbB* gene of avian erythroblastosis virus (AEV). The v-*erbB* gene is a virally transduced mutant form of the cellular epidermal growth factor (EGF) receptor. AEV infection of chickens results in the rapid induction of erythroblastosis, and although AEV carries a second gene (v-*erbA*), expression of v-*erbB* is necessary and sufficient for the induction of erythroblastosis. The EGF receptor is typical of many growth factor receptors in its molecular architecture. It contains an extracellular domain involved in ligand (EGF) binding, a hydrophobic membrane-spanning domain, a cytoplasmic domain that contains protein tyrosine kinase activity and a C-terminal region involved in regulation of the kinase activity. Several observations suggest that the v-ErbB protein is a constitutively active form of the EGF receptor. In comparison to the EGF receptor, v-ErbB has suffered several structural changes. These include deletion of the extracellular ligand-binding domain and alterations of the C-terminal regulatory region. Molecular analysis has shown that both of these alterations are important for the transforming activity of the protein. Consistent with its proposed function, v-ErbB shows protein tyrosine kinase activity in the absence of EGF, and AEV-transformed cells show increased phosphotyrosine-containing proteins when compared to nontransformed cells. All mutations that impair the kinase activity of v-ErbB impair the transforming activity of the virus in a coordinate manner. Thus, the v-*erbB* oncogene product is a ligand-independent form of the EGF receptor.

Intracellular signal transduction molecules

A number of retroviral oncogenes have been identified that function as intracellular effectors of changes in the external environment. The v-Src protein of Rous sarcoma virus (RSV) is a cytoplasmic tyrosine kinase anchored to the inner surface of the plasma membrane by an N-terminal modification by myristic acid addition. The v-Src protein contains several mutations when compared to c-Src. Important among these is mutation of a C-terminal tyrosine in c-Src that serves as a negative regulator of the c-Src tyrosine

kinase activity. Although it is not known what the physiologic mechanisms are for activating c-Src tyrosine kinase activity, v-Src functions as a constitutively activated enzyme, and RSV-transformed cells contain increased amounts of phosphotyrosine-containing proteins. An additional important feature in the Src protein is the presence of regulatory sequences known as SH2 and SH3 domains. These domains were initially recognized as conserved motifs in other proteins, first in tyrosine kinases and then in other molecules, including the v-*crk* oncogene and a number of substrates for tyrosine kinase enzymes, such as phospholipase C, phosphatidylinositol 3′-kinase (PI3 kinase), and the *ras* GTPase-activating protein (GAP). The investigation of the v-*crk* oncogene has been particularly instructive. The transforming protein, v-Crk, is composed only of SH2 and SH3 sequences, without any tyrosine kinase catalytic domain. However, v-Crk-transformed cells contain elevated levels of phosphotyrosine-containing proteins. Investigation of this phenomenon has led to the realization that SH2 domains function as ligands for proteins containing phosphotyrosine. As all active tyrosine kinase enzymes are themselves phosphorylated on tyrosine, potential substrates may bind through their SH2 domains to the active phosphotyrosine-containing forms of the enzymes. This may be a way of controlling availability of substrates for these enzymes, and the presence of SH2 domains on the substrates of tyrosine kinases is likely to accelerate the identification of substrates, a process that until now has been frustratingly slow.

A second class of cytoplasmic signaling molecule that can be rendered oncogenic by mutation is the Ras family of proteins. Initially identified as v-Ras, the transforming proteins of Harvey murine sarcoma virus (Ha MuSV), the Ras proteins have been conserved as essential functions from yeast to man, with very little sequence variation. The Ras proteins are small GTP-binding proteins with GTP-hydrolyzing activity. The GTPase activity is essentially a way to control the signaling activity, as only the GTP-bound form is active. Recent genetic and biochemical evidence suggests that at least one function of Ras proteins is to mediate the signals generated by protein tyrosine kinases. In contrast to the normal cellular form of Ras, the oncogenic v-Ras contains two point mutations that impair the GTPase activity and so trap the molecule in an 'on' or signal-generating state. Unfortunately, the nature of the signals generated by active Ras remains elusive.

Studies of the regulation of Ras GTPase activity have revealed a second mechanism of oncogenic activation through the Ras signaling pathway. The intrinsic GTPase activity of Ras proteins is augmented by GTPase-activating proteins, or GAPs. The GAP molecules increase the hydrolysis of GTP, and so convert active Ras to inactive Ras. An important observation has been the realization that the gene that confers predisposition to neurofibromatosis (NF1), an inherited form of cancer, encodes a GAP. Inactivating mutations in NF1 GAP are associated with a decrease in GAP activity, a consequent increase in the amount of Ras protein in the active (GTP-bound) state and the formation of tumors.

Cell signaling through transcription factors

The generation of second messengers and cytoplasmic signals by external factors eventually results in changes in gene expression. Several retroviral oncogenes are transduced forms of cellular transcription factors involved in regulating the response to external factors. The transcription factor AP1 is a heterodimeric transcription factor with sequence-specific DNA-binding activity composed of peptides derived from the Fos and Jun families of proteins. The *fos* family of genes was identified as the cellular homologues of v-*fos*, the transforming gene of FBJ MuSV, while the *jun* family was identified by homology to v-*jun*, the transforming gene of avian sarcoma virus (ASV) 17. AP1 activity is tightly regulated, and is rapidly and transiently induced by a wide variety of mitogens, including the peptide mitogens PDGF and EGF. The induction of AP1 activity requires *de novo* protein synthesis, and is a consequence of the early induction of the *fos* and *jun* genes in response to mitogens. Several lines of evidence suggest that transformation by v-Fos is due to overexpression of AP1 activity. Overexpression of either c-Fos or v-Fos protein is sufficient to result in neoplastic transformation, and mutant proteins that are unable to bind AP1 target DNA sequences are nontransforming. Cells transformed by Fos protein show increased levels of AP1 activity compared to nontransformed cells, consistent with the idea that transformation is the result of activation of a specific set of genes that contain AP1 regulatory sequences in their upstream regions. The identification of the relevant genes the activation of which is important for transformation has proven to be difficult.

A somewhat different situation is represented by the transforming gene, v-*rel*, of the reticuloendotheliosis virus (REV-T). The Rel family of proteins constitute a large family of proteins with NF-KB activity. NF-KB is a transcription factor that is involved in the response of lymphoid cells to a variety of external stimuli. NF-KB activity is regulated by a number of complex mechanisms, but an important form of regulation appears to be retention in the

cytoplasm through its interaction with specific inhibitor proteins. External stimuli then result in the dissociation of the inhibitor and allows translocation of NF-KB to the cytoplasm. An usual feature of transformation by v-Rel is that mutant v-Rel proteins that are localized in the cytoplasm can induce transformation. This suggests that direct transcriptional activation by v-Rel is not the mechanism of neoplastic transformation, and suggests that v-*rel* may function as a dominant negative allele of c-*rel*, and this idea has received some experimental support. According to this model, NF-KB activity would function to promote cellular differentiation and inhibit cell growth, and oncogenesis would be the consequence of decreasing NF-KB activity.

Oncogene cooperation

Spontaneously occurring tumors are recognized to be the result of a multistage process in which multiple mutations arising at different times conspire to produce a tumor. On the other hand, neoplastic transformation due to infection with the acutely transforming retroviruses usually occurs as the result of the introduction of a single gene. Yet even the acutely transforming retroviruses contain examples of cooperativity among oncogenes. AEV contains two genes, v-*erbA*, a transduced mutant form of the thyroid hormone receptor gene, and v-*erbB*, a constitutively active form of the EGF receptor gene. Although v-*erbB* is sufficient to direct neoplastic growth of infected erythroid cells, the expression of v-*erbA* is required for growth of the erythroid cells under some culture conditions. Thus, the fully transformed phenotype is the product of a cooperative interaction between the two genes. This theme of cooperation between various oncogenic proteins has been extensively documented in the process of neoplastic transformation of primary cells.

Transformation by human retroviruses

Retroviruses may acquire transforming activity by transduction of cellular proto-oncogenes, they may activate cellular proto-oncogenes by insertional mutagenesis, or they may transform through the action of regulatory genes required for the viral life cycle to proceed. The causative agent of acute T-cell leukemia, human T-cell leukemia virus (HTLV-1), appears to operate through the last type of mechanism. The viral gene *tax* is a regulator of transcription from the viral long terminal repeat, as well as from certain cellular promoters. Interestingly, Tax activates both the interleukin 2 (IL-2) promoter and the IL-2 receptor promoter, generating a loop with potential autocrine activity in T cells. The mechanism of Tax activation of transcription remains unclear. It is able to activate transcription directed by NF-KB and cyclic AMP-responsive sites, although there is no evidence that Tax itself binds DNA. The mechanism of transformation induced by HTLV-1 is likewise unclear; the tumors arise only after a long latency, sometimes 20 years, and are monoclonal.

DNA Tumor Viruses

Several classes of DNA viruses are able to induce neoplastic transformation in culture. In contrast to the situation with transforming retroviruses, in which transformation is the result of the activation of a gene that is not required for the completion of the viral life cycle, transformation by DNA viruses is the result of expression of proteins intimately involved in the replication of the viral genome.

Simian virus 40 (SV40)

Transformation by SV40 is associated with nonproductive infection and is the result of the expression of a single viral protein, the SV40 large T antigen (T Ag). In contrast to productively infected cells in which the SV40 genome is episomal, in transformed cells the viral genome is integrated into the host DNA in a manner which allows for expression of T Ag. T Ag is a multifunctional protein that forms noncovalent interactions with a number of cellular proteins. Key among these are the products of the cellular tumor suppressor genes p53 and Rb. Oncogenic activation of the p53 and Rb genes is associated with loss, rather than gain of function. p53 was initially identified as a protein stably associated with T Ag in transformed cells. The normally rapid turnover of p53 in uninfected cells is greatly reduced by virtue of complex-formation with T Ag, giving rise to increased levels of the protein in transformed cells. The net result of this interaction is not to increase p53 function, however, but rather to sequester it in an inactive complex. The protein product of the retinoblastoma susceptibility gene, pRb, is also noncovalently associated with T Ag, and this complex results in loss of pRb function. Transformation by T Ag has therefore served as the mechanism of identification of one tumor suppressor gene (p53), and has been the entry point for biochemical investigation of another (pRb).

Adenovirus

Transformation by the human adenoviruses has been observed only *in vitro*. As is the case with transformation by SV40, transformation by adenovirus is the result of a nonproductive infection. DNA transfection experiments reveal that the expression of two different viral genes, E1a and E1b, is required for full

transformation. E1a is a multifunctional protein with a bewildering array of activities. The important feature for transformation appears to be the ability to interact with pRB. Expression of the E1a gene alone is sufficient for immortalization of primary cells, but not for full transformation, which requires expression of the E1b gene. The product of the E1b gene is complexed physically with p53. Thus, the combined activities of E1a and E1b are reminiscent of the activities of SV40 T Ag.

Human papilloma virus

Investigation of the transforming activities of the human papillomaviruses (HPV) is of interest for reasons of medical importance as well as scientific interest. Nearly all human cervical carcinomas are associated with the presence of DNA from either HPV 16 or HPV 18. Completion of the life cycle of the HPVs is unusual in that the early and late stages of the life cycle appear to take place in different stages of epithelial cell differentiation, and complete viral replication takes place only in benign papillomas, or warts. This feature has so far made it impossible to grow the HPVs in culture, a problem that has slowed analysis. However, molecular analysis has been informative. Cervical carcinomas are associated with the presence of the viral genome integrated into the host DNA in a manner that allows for expression of the early region of the viral genome. This region is quite complex, with at least eight open reading frames; there is evidence that at least five are involved in generating the transformed phenotype. In a scenario reminiscent of adenovirus, the products of the E6 and E7 open reading frames are physically associated with p53 and pRB respectively. These interactions result in the functional inactivation of p53 by accelerated proteolysis, and pRb by sequestration.

Hepatitis B virus

The hepadnaviruses should be mentioned because of their potential role in human cancers. The hepatitis B virus, especially when acquired by vertical transmission, is associated with the development of hepatocellular carcinoma. At this point, there is no decisive evidence that this association is causal, but strong circumstantial data are present. Experimental evidence suggests two potential mechanisms: activation of cellular oncogenes by integration in a manner similar to insertional mutagenesis by retroviruses, and repetititive mitogenic stimulation due to the toxic effects of chronic exposure to the surface antigen proteins.

Epstein–Barr virus

Nonproductive infection with Epstein–Barr virus (EBV) is associated with Burkitt's lymphoma, a malignancy of B lymphocytes. Once again, there is no decisive evidence for a causal role, but the ability of the virus to immortalize human B lymphocytes suggests a potential mechanism. Infection with EBV may result in the generation of a large pool of preneoplastic cells that are predisposed to further oncogenic mutations.

Future Perspectives

Much of what we understand about the molecular mechanisms of cancer is the result of investigation of virus-induced tumors. This area of investigation has demonstrated, clearly and convincingly, that cancer is a disease caused by mutations in specific genes. We have learned the identity of many of these genes, whether they are carried by the virus, activated by viral insertion, or inactivated by the physical association of their products with viral proteins. The products of these genes form a network of signaling elements that allows cells to respond to the external environment. Where understood, oncogenic mutations result in the production of proteins that propagate mitogenic signals in the absence of appropriate monitoring of the extracellular environment, a result consistent with the proposed normal functions of these proteins. Although many oncogenes were initially identified by virtue of their presence in viral genomes, it is now clear that these genes are also the targets of oncogenic mutations in tumors of nonviral origin. It seems certain that future investigations of virus-induced transformation will further contribute to our understanding of cancer, an understanding that does not appear to be as elusive as a decade ago.

See also: **Adenoviruses (*Adenoviridae*): General features; Avian type C retroviruses (*Retroviridae*); Epstein-Barr virus (*Herpesviridae*): General features; Hepatitis E virus; Human T-cell leukemia viruses (*Retroviridae*): HTLV-1; Murine leukemia viruses (*Retroviridae*); Retroviral Oncogenes; Papillomaviruses – human (*Papovaviridae*): General features; Reticuloendotheliosis viruses (*Retroviridae*); Simian virus 40 (*Papovaviridae*); Tumor viruses – human.**

Further Reading

Hoppe-Seyler F and Butz K (1995) Molecular mechanisms of virus-induced carcinogenesis: the interaction of viral factors with cellular tumor suppressor proteins. *J. Mol. Med.* 73(11): 529.

[This article is reproduced from the 1st edn (1994).]

TRANSPLANTATION AND VIRUS INFECTIONS

Robert A Krance, **Helen E Heslop** and **Malcolm K Brenner**, Stem Cell Transplantation Program, Shell Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, Texas, USA

Introduction

Organ transplantation is being used to correct an ever broader range of medical abnormalities. In large part the expanding application and success of transplantation has been based on improved methods of producing immunosuppression in the recipient, which allow acceptance of a genetically disparate graft. This is particularly true following bone marrow transplantation (BMT), where immunosuppression must be especially profound, since the aim is not only to prevent rejection of the graft by the host but to prevent the incoming immune system from 'rejecting' the recipient to produce graft-versus-host disease (GVHD).

Unfortunately, the immunosuppression required by transplant recipients has a number of adverse consequences. Among the most important of these is the suppression of host immune responses directed against endogenous and exogenous microorganisms. Suppression of immune surveillance allows invasion by opportunist fungi and by protozoa and permits recrudescence of those endogenous viruses which are usually maintained in the latent state. Immunosuppression may also increase the severity of infection after exposure to exogenous viruses. All transplant recipients are vulnerable to virus infections, but bone marrow transplant recipients who are the most intensely immunosuppressed, are the most vulnerable of all. Notwithstanding, the introduction of a number of different therapeutic agents, viral disease remains one of the leading causes of morbidity and mortality after transplantation.

Herpesviruses

The herpes viruses, *Cytomegalovirus*, Epstein–Barr virus, herpes simplex and varicella/zoster represent the greatest potential threat to transplant recipients. All four are present in the latent state in a high proportion of individuals, and all four can cause serious or fatal disease in the immunocompromised host.

Cytomegalovirus (CMV)

Background In a normal individual, defense against CMV is mediated by specific cytotoxic T lymphocytes, by production of specific antibody and perhaps also by MHC unrestricted cellular effector mechanisms. In the transplant recipient, immunosuppression-mediated impairment of these defense mechanisms places the recipient at risk of CMV disease. Depending on location and socioeconomic status, 40–90% of transplant recipients have a past history of primary CMV infection and are at risk of viral reactivation. The remaining seronegative recipients are at risk of acquiring primary infection, either from the donor tissue or from blood products required during the post-transplant period. CMV reactivation or infection does not always lead to CMV disease in the transplant recipient and it is important to distinguish between these phenomena.

Although detection of CMV is not always predictive of disease, there may be a high probability that disease will subsequently occur in patients receiving intensive immunosuppression. Following solid organ grafts, the risk of CMV disease rises with the intensity of the immunosuppression administered and is especially increased by the use of monoclonal anti-T cell antibodies, such as OKT3. Bone marrow recipients who have had their own immune system ablated as part of the conditioning regimen and who have not yet regenerated a donor-derived system are particularly likely to develop CMV disease.

Incidence of CMV infection/reactivation and CMV disease *From blood product support* The risk of a seronegative recipient of seronegative donor tissue acquiring CMV infection from blood product support was previously significant, but the incidence of this mode of transmission has been substantially reduced by the use of CMV seronegative or, more commonly, leukocyte poor blood products and is now less than 5%.

From solid organ grafts Seropositive recipients have a 50–80% chance of developing evidence of viral reactivation, and 10–25% of these individuals will develop evidence of CMV disease. The donor organ is also a source of infection and 50–100% of seronegative recipients will develop infection if they receive a

transplant from a seropositive donor. Disease occurs in up to one-half of such patients.

From BMT About 50–60% of both allograft and autograft recipients who are seropositive or have a seropositive donor will have evidence of either primary infection or reactivation, with a peak incidence in the first three months post-transplant. Patients receiving autologous marrow have a low incidence of disease with less than 5% of seropositive recipients developing pneumonitis. By contrast, half of the allograft recipients who develop a primary infection or reactivation will, in the absence of treatment, develop pneumonitis which is fatal in 80–90%. The presence of moderate to severe graft-versus-host disease markedly increases the risk of CMV disease, especially pneumonitis and some studies have shown a reduced incidence of CMV in allograft recipients who receive more effective graft-versus-host disease prophylaxis.

Pathogenesis CMV initially infects a variety of cells including fibroblasts, marrow stromal cells and hemopoietic progenitors. The mechanism of latency is not well understood, but reactivation results in viral shedding. In some situations, such as in AIDS patients, viral replication alone results in disease, but in the transplant patient an immunopathological component is implicated, and both host-versus-graft and graft-versus-host reactions correlate with CMV disease. For example, a host-versus-graft relationship is seen in recipients of liver transplants, who are particularly likely to develop CMV hepatitis. In allogeneic bone marrow transplant recipients, there is evidence that damage to the lungs in CMV pneumonitis results from an abnormal response to antigen, and that this is exacerbated by graft-versus-host disease. The activated immune system may also damage or destroy uninfected 'bystander cells', since antiviral agents which eliminate viral shedding and prevent antigen expression have limited effect on disease progression (see below under Treatment).

Diagnosis CMV infection may be diagnosed either by serological means or by direct isolation of the virus or its DNA. The virus is generally cultured from blood, throat, urine or tissues. CMV is cultured on human fibroblasts and it usually takes 1–4 weeks for the characteristic cytopathic effect to become evident. The use of monoclonal antibodies directed at early or intermediate antigens in conjunction with centrifugation of the specimen on to monolayers of fibroblasts – the shell vial technique – allows diagnosis within 24–48 h. The presence of the virus can be diagnosed even more rapidly by the use of the polymerase chain reaction (PCR) to amplify CMV DNA and RNA, but how accurately detection of viral infection/reaction by such sensitive methodology predicts subsequent CMV disease remains in dispute.

Serological evidence of infection is usually obtained using ELISAs or indirect fluorescence. Diagnosis requires a fourfold rise in titer and may be problematic after BMT when patients are so severely immunocompromised that antibody production is impaired.

Clinical aspects CMV infection in the immunosuppressed host ranges from an asymptomatic illness similar to that in normal individuals to disseminated disease. However, there is a higher incidence of diffuse organ involvement which may present with a variety of clinical manifestations including hepatitis, enteritis, pneumonitis, fever and bone marrow suppression. Disease presentation may also vary according to the organ transplanted. In recipients of renal transplants, pneumonitis is rare and patients are more likely to present with mononucleosis-like symptoms or hepatitis. Recipients of lung, heart or liver transplant have a higher risk of more severe CMV infections such as pneumonitis. Liver transplant recipients are particularly at risk of CMV hepatitis which is more common after primary infection and may be difficult to distinguish from rejection. Finally, following allogeneic bone marrow transplantation, pneumonitis is the most common presentation of CMV disease and will occur in 40–50% of recipients with evidence of infection on blood culture. Pneumonitis presents with fever, cough dyspnea and chest radiograph shows an interstitial pattern. About 80–90% of BMT recipients who develop CMV pneumonitis will die of this complication.

Treatment A number of measures have been used to treat established CMV disease. All are of most benefit after solid organ grafting, and of least value after BMT. Interferon has antiviral activity and may reduce morbidity in renal transplant recipients, but has little effect in BMT patients. Similarly, acyclovir and gancyclovir may reduce viral shedding and may be effective in CMV disease in solid organ recipients, but given alone they do not significantly modify morbidity and mortality from CMV disease in allogeneic BMT recipients.

Intravenous immunoglobulin (IVIg) as a single agent is also ineffective in most studies. However, the combined use of gancyclovir and IVIg in allogeneic BMT recipients with CMV pneumonitis results in disease resolution in around 50–70%. This response rate appears significantly better than the 10% response rate of historical controls, though no

Table 1 Effect of prophylaxis in preventing CMV reactivation/disease

	Effect on		
Prophylactic regimen	*Reactivation/Infection*	*Disease*	*Mortality*
CMV-negative blood products	+	+	+
Acyclovir (low dose)	+/−	+/−	−
Acyclovir (high dose)	+	+	+/−
Gancyclovir	− (see text)	+	+
Intravenous immunoglobulin	−	+	+/−

randomized trials have been performed and the improved survival may in part reflect changes in diagnostic criteria and techniques. Indeed, subsequent studies where the drug combination was administered to patients who were ventilated yielded lower response rates.

Prophylaxis (Table 1) The most effective preventative measure is to use CMV-negative blood products for seronegative recipients who receive seronegative organ grafts. Several studies have convincingly shown that this policy can almost completely eliminate CMV disease. Seronegative recipients of seropositive allogeneic marrow also benefit from seronegative blood products. It has been suggested that seropositive recipients should also receive CMV-negative products to reduce exposure to new strains. In practice most blood banks do not have enough CMV-negative products to supply all these populations and leukocyte-poor products – prepared by filtration of blood – are used instead. It appears that leukocyte-poor products are just as effective – at least in low risk patients.

Antiviral agents have also been explored as prophylactic agents. Acyclovir in renal transplant recipients reduces the rate of CMV infection and disease but did not improve survival. Neither α-interferon nor low-does acyclovir have any convincing effect in BMT recipients. Some studies suggest that acyclovir may reduce the incidence of both CMV infection and invasive disease, but these results are not confirmed in other studies. Gancyclovir effectively reduces the incidence of CMV disease in allograft recipients with CMV infection detected by shell viral technique or by PCR, and more recent studies using prophylactic or pre-emptive gancyclovir have shown that the drug may be used to prevent viral reactivation. Unfortunately, resistant strains of CMV are now beginning to be detected, and CMV disease may also occur once gancyclovir prophylaxis has been stopped. Foscarnet may be an acceptable, non-cross-resistant, substitute for gancyclovir, although the relative efficacy of this drug in BMT patients remains uncertain.

IVIg administered after BMT, resulting in passive transfer of specific antibody, does not reduce the rate of viral reactivation or infection but does decrease the incidence of pneumonia and death. Such treatment may therefore be justified in seropositive recipients. The expense of IVIg does not appear to be justified in seronegative recipients receiving seronegative blood products, in whom risk of CMV infection and disease is negligible. Similarly, in seronegative renal recipients receiving a seropositive graft, IVIg reduces the incidence of CMV disease though not the rate of viral isolation or seroconversion. Neutralizing human monoclonal antibodies are currently being evaluated in phase III trials.

Immunomodulation Several studies have correlated the pattern of CMV infection in allogeneic BMT recipients with the ability to generate CMV specific $CD8^+$ cytotoxic T lymphocytes, which may allow the host to control CMV infection. These observations suggested that adoptive transfer of specific cytotoxic T lymphocytes (CTLs) (expanded *in vitro*) to high risk patients may allow long term protection from overt disease, without the risk of developing chemoresistant strains of virus. Initial studies of this approach have produced promising results.

Epstein–Barr virus (EBV)

Background Even though more than 90% of adults have serological evidence of EBV infection, disease produced by reactivation of this herpesvirus has, until recently, been less prevalent in transplant recipients than disease produced by CMV. However, the development of more aggressive and effective techniques for immunosuppression has not only increased the success of tissue transplantation, but has also substantially increased the incidence of the lymphoproliferative syndrome associated with uncontrolled EBV reactivation in the immunocompromised host.

This complication is particularly common in patients receiving mismatched or unrelated donor BMT, in seronegative neonatal recipients of seropositive liver and in patients receiving small bowel transplants.

Incidence Following solid organ grafting, up to 60% of patients may show evidence of EBV reactivation or infection, assessed on the basis of rising antibody titers to viral proteins. Less than one-third of these patients will develop the clinical features of a viral illness. The incidence of lymphoproliferative syndrome is between 1 and 13%, and is lowest in patients receiving renal allografts and highest in those receiving heart–lung double transplants. Within each group, patients receiving the most intensive immunosuppression, with CSA, antilymphocyte globulin or anti-T cell monoclonal antibody (e.g. OKT3), have the highest incidence.

After BMT the incidence is even more variable. At one extreme, adult patients receiving major histocompatibility complex (MHC) identical sibling allografts as treatment for leukemia have an incidence of 0.25%. At the other extreme, children transplanted for congenital immunodeficiency syndromes who receive marrow from an MHC nonidentical or unrelated donor have a 100-fold greater incidence of lymphoproliferative disease, which occurs in up to 25% of such recipients. This wide variation is in part attributable to the increased immunosuppression required by recipients of mismatched/unrelated marrow, but probably also reflects the long delay in immune reconstitution exhibited by patients receiving a genetically disparate immune system. Because the numbers of mismatched/unrelated donor BMT are steadily rising, some recent BMT series show that death from EBV-induced lymphoproliferation now exceeds that from CMV disease.

Pathogenesis and diagnosis The primary pathologic process is uncontrolled proliferation of mature B lymphocytes. Analysis of proliferating lymphoblasts following solid organ transplant using restriction fragment length polymorphisms (RFLPs) or minisatellite probes, has shown that EBV lymphoproliferation in these patients almost always arises from recipient B cells. In contrast, lymphoproliferation after BMT commonly arises from cells of donor origin. In all cases, the transformed B cells closely resemble morphologically and phenotypically, the lymphoblastoid cell lines generated *in vitro* when human B cells are exposed to EBV in the absence of T cells. The infected lymphocytes may have an immunoblastic or plasmacytoid appearance and are usually CD19, CD20, CD21 and CD24 positive (see below under Treatment). Like Burkitt lymphoma cells, the lymphoblasts are positive for EBV nuclear antigens 1 (EBNA1) but unlike Burkitt lymphoma cells they usually express all of the five other virus-encoded latent cycle nuclear antigens and are positive for most latent membrane proteins. Again in contrast to Burkitt lymphoma cells, lymphoblasts in transplant patients express a number of cell adhesion molecules and ligands. All these phenotypic features would make them intensely vulnerable to cytotoxic T cell killing in a normal individual, and it is only the profound suppression of the immune system after transplantation which permits their outgrowth.

The B cell proliferation may be oligoclonal or monoclonal, and may be associated with production of oligoclonal or monoclonal peaks of immunoglobulin which can be detected by serum electrophoresis. Within the B cells, the virus may be present in linear (replicative) or circular (latent) form. Analysis of these attributes may predict the response to therapy.

Clinical manifestations Early in the illness, fever, malaise and circulating atypical lymphocytes are usually seen. The proliferating lymphoblasts may then produce a number of disease patterns. They may diffusely infiltrate a number of different organ systems, including lungs, liver, kidney, gut, bone marrow and central nervous system. Infiltration may be so extensive that organ failure results. Alternatively, a classical 'lymphoma' pattern is observed, with lymphadenopathy, hepatosplenomegaly, and a biopsy appearance of a diffuse immunoblastic lymphoma. Rarely, a predominantly leukemic picture occurs. Although these distinctive clinical patterns may be seen in isolation, a combination of features often evolves (**Fig. 1**).

Clinical course Initial reactivation of EBV, associated with an 'infectious mononucleosis'-like illness, may resolve spontaneously. But once viral induced-lymphoprolipherative syndrome (LPS) has occurred, the course is generally rapidly progressive. Death may result from renal, hepatic or pulmonary failure, from hemorrhage due to ulceration of bowel tumor, or from central nervous system (CNS) involvement.

Treatment Withdrawal of immunosuppression is associated with spontaneous remission of the tumors in up to 50% of recipients of solid organ grafts; regression is more likely to occur in oligoclonal then in monoclonal tumors. Withdrawal of immunosuppression alone is rarely effective when the LPS has occurred after BMT.

Acyclovir may disrupt the replicative lytic cycle of the (linear) virus and interferon may prevent infection of fresh lymphocytes, but neither approach can signi-

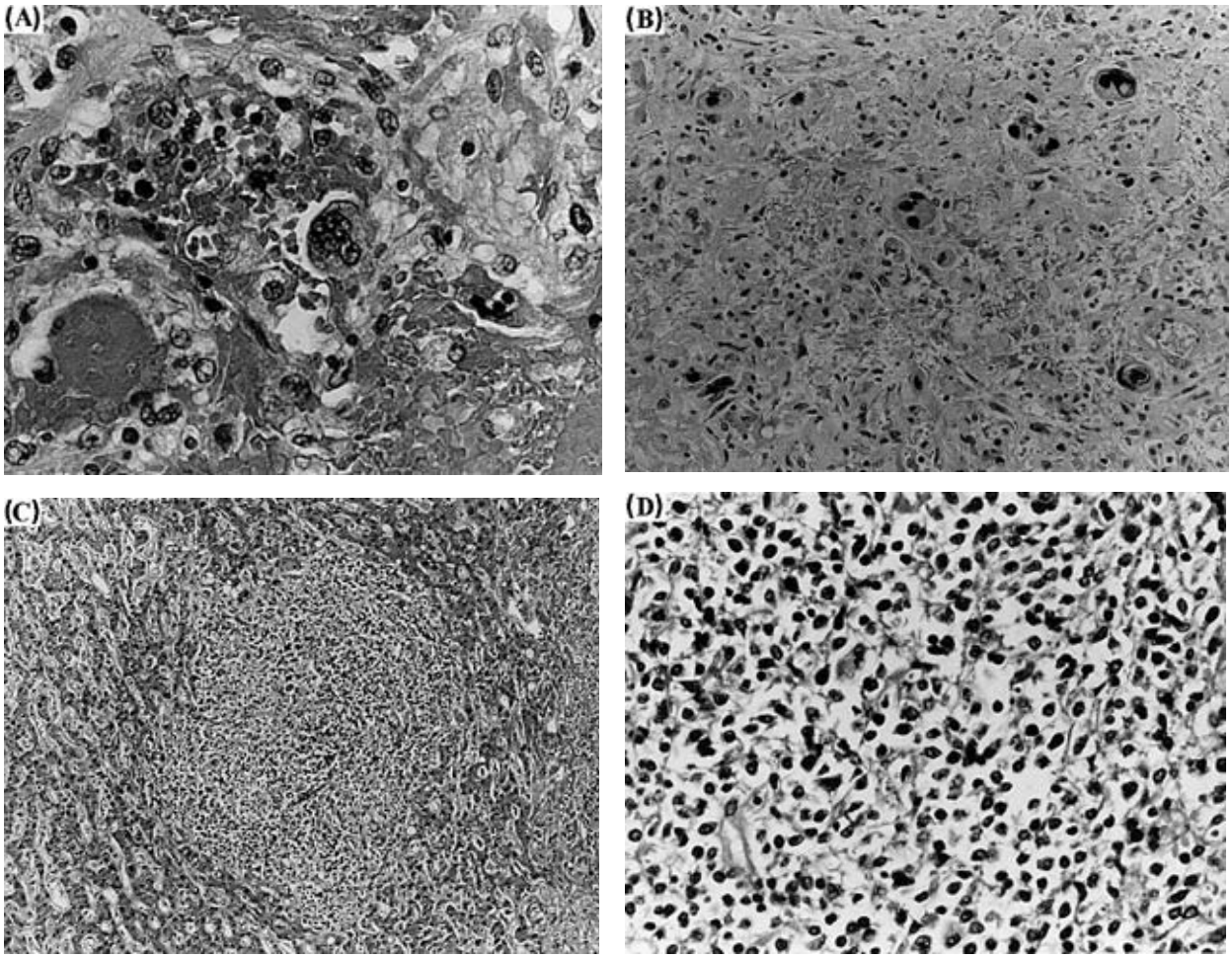


Figure 1 (**A**) Section of lung, infected with CMV, containing intraalveolar hemorrhage and multinucleated giant cell. This is a histologically atypical infection producing only scattered cells with relatively small intranuclear and intracytoplasmic inclusions and no large cells with 'owls-eye' nuclei (hematoxylin and eosin stain; ×315). (**B**) Same infection as in (A) demonstrating numerous cells positive for CMV antigen using monoclonal antibody and immunoperoxidase technique (avidin–biotin complex technique; ×315). (**C**) (EBV low power) Section of liver with lymphoid infiltrate (EBV infection) causing portal expansion (hematoxylin and eosin stain; ×80). (**D**) Same infection as in (C). The portal infiltrate is polymorphous and contains cells ranging from small mature lymphocytes to larger immunoblasts (hematoxylin and eosin stain; ×315).

ficantly modify the growth of already transformed B cells which contain nonreplicating (circular) virus. Nonetheless, remissions have been reported in response to both drugs, although these are more likely in recipients of solid organ grafts and if the tumor is oligoclonal. More recent reports suggest that infusion of monoclonal antibodies to CD21 and CD24 – molecules present on lymphoblasts – may rid the patient of infected B lymphocytes. As with other treatments, this approach seems most effective in solid organ recipients with oligoclonal LPS.

At present the most satisfactory approach to prophylaxis and treatment in BMT recipients is to attempt to restore immunocompetence to EBV by the adoptive transfer of immune T cells. The majority of individuals are EBV seropositive, and between 1 in 1000 and 1 in 10 000 of their circulating CTLS are EBV specific. Transfer of marrow donor T cells therefore regularly produces disease response in patients with uncontrolled lymphoproliferation. Unfortunately, because donor T cells also contain significant numbers of alloreactive CTLs, response may be associated with the development of severe graft-versus-host disease. More recently, it has proved possible successfully to prevent and treat EBV disease by transferring virus-specific CTLs, without the development of graft-versus-host-disease. Alternative approaches have used bulk T cell populations genetically modified to include a thymidine kinase suicide gene. Should graft-versus-host disease develop, these modified cells may be destroyed by administration of gancyclovir.

Finally, the development of mouse models in which proliferating human lymphoblasts can be studied may help in the development and assessment of novel therapeutic strategies.

Varicella Zoster virus

Incidence Reactivation of herpes zoster virus (VZV) infection occurs in 5–25% of solid organ transplant recipients and in as many as 50% of bone marrow transplant patients. Primary infection develops only rarely, and usually in children.

Epidemiology and pathogenesis Like other herpesviruses, the probability of VZV reactivation is increased when cell-mediated immunity is compromised. In solid organ transplant recipients, the eruption of clinical shingles often follows the initiation or increase of medications to prevent graft rejection. The risk of VZV reaction in bone marrow transplant patients is even greater because the attendant immune deficiency is more severe and prolonged than that of solid organ transplant patients. Unlike herpes simplex virus (HSV) reactivation where patients with high HSV antibody titer are more likely to develop reactivation, pretransplant VZV antibody titer does not predict post-transplant infection.

Although VZV infection may develop in the first weeks after transplantation, the median time to reactivation is four to five months for patients not receiving prophylactic acyclovir. Late graft rejection requiring aggressive immunosuppressive measures is frequently accompanied by VZV reactivation in solid organ transplant patients. In allogenic bone marrow transplantation, VZV infection may be more common in patients with graft-versus-host disease, whereas for autologous transplantation VZV infection is more common in patients with Hodgkin's disease or non-Hodgkin's lymphoma.

Manifestations and treatment Classical cutaneous herpes zoster (shingles) is the most common manifestation of VZV infection among transplant patients. Depending on the dermatome affected, VZV reactivation may cause post-herpetic neuralgia, cutaneous scarring or corneal opacification. Solid organ transplant recipients rarely develop disseminated or invasive VZV infection.

In bone marrow transplant patients, VZV infection may present as typical dermatomal herpes zoster, as herpes zoster with cutaneous dissemination, or even as varicella. Serology in these patients indicates past VZV infection. Prior to the availability of acyclovir, cutaneous zoster infection progressed to invasive disease in up 50% of patients, and 10% died. The consequences of visceral dissemination included hepatitis, pancreatitis, and encephalitis; however, VZV pneumonia was the usual cause of death.

Intravenous acyclovir is the most effective and least toxic therapy for VZV infection in transplant patients. In bone marrow transplant patients, prophylactic administration of acyclovir can prevent VZV reactivation, but reactivation is common upon discontinuing the drug. Consequently, prophylactic administration of acyclovir to prevent VZV infection is not recommended. Vidarabine and α-interferon are effective against VZV infections but they are secondary treatments since they are more toxic and less efficacious. Their use is limited to situations when VZV resistance to acyclovir is suspected.

In those patients without prior VZV infection, varicella zoster immune globulin administered within 72 h of VZV exposure may prevent or modify illness. Varicella vaccine is a promising therapy for children who may require transplantation but have not yet acquired their primary immunity.

Herpes Simplex virus

Incidence Reactivation of latent herpes simplex virus (HSV) infection has occurred in 30–90% of seropositive transplant patients. The majority have suffered a mild, usually self-limited illness, with fever accompanied by lesions of the oral or genital mucosa. However, in patients undergoing bone marrow transplant HSV disease has been more severe.

Epidemiology and pathogenesis Less than 5% of HSV illness following transplantation has been due to primary HSV infection. There are two possible sources of primary HSV exposure: intimate contact with an individual shedding HSV or transfer of HSV through the transplanted organ. Transfer of HSV by liver, pancreas, heart and kidney transplantation has been documented. Presumably, the virus, latent in neural tissue within the organ, became reactivated and initiated primary HSV illness in the host.

Between 30% and 100% of adults are HSV seropositive, and 15–30% of these experience recurrent vesicular eruptions. Although the events leading to HSV reactivation are incompletely understood, the combination of high prevalence of HSV seropositivity and iatrogenic immunosuppression have undoubtedly promoted HSV illness among transplant patients. Interestingly, a high antibody titer to HSV pretransplant has been linked to a high risk of viral reactivation.

Clinical manifestations and treatment The incidence of HSV infection peaks during the first weeks after transplantation when cell-mediated immunity is

most suppressed. The classic perioral vesicular eruption is the typical manifestation of HSV infection in solid organ transplant recipients. Genital HSV infection is also common. In bone marrow transplant patients, the diagnosis of HSV infection may be problematic because the classic lesions are often superimposed on mucositis caused by the pretransplant chemotherapy and/or radiotherapy.

Visceral dissemination of HSV has been rare following kidney and liver transplantation, more frequent following heart, lung and heart–lung transplantation, and most common following bone marrow transplantation. Manifestations of visceral dissemination include esophagitis, pneumonia, hepatitis and encephalitis; HSV esophagitis and pneumonia are thought to develop from contiguous spread of oral/perioral disease. HSV has been isolated from 5% of bone marrow transplant patients with pneumonia. Often a co-isolate with cytomegalovirus (CMV), the role of HSV in the pathogenesis of pneumonia is unclear.

The incidence of hepatitis or encephalitis due to HSV has been less than 0.5%. Unlike HSV pneumonia or esophagitis, the onset of hepatitis has not always followed oral/perioral lesions. In the first weeks after liver transplantation, immediate liver biopsy is recommended for patients who develop hepatic dysfunction, since prompt initiation of acyclovir has arrested the progression of HSV hepatitis to fulminant and usually fatal liver failure.

Treatment Acyclovir is the principal treatment for HSV infection. It is effective for prophylaxis as well as treatment of established infection. Because the likelihood of disseminated HSV disease is enhanced by immunosuppression, acyclovir prophylaxis may be used routinely in seropositive recipients of bone marrow transplantation, although gancyclovir (given primarily for prophylaxis of CMV as described above) may be substituted. In solid organ transplantation, where immunosuppression is neither as intense nor as prolonged, opinion is divided on the necessity for prophylaxis. If prophylaxis is used, HSV reactivation often occurs once acyclovir is discontinued. In addition, approximately 10% of patients develop HSV reactivation even while receiving prophylaxis.

Disconcerting reports of acyclovir-resistant HSV, particularly among human immunodeficiency virus (HIV)-infected patients, have been confirmed. Vidarabine and foscarnet, an investigational agent, may be effective alternatives.

Other Herpesviruses

In the last few years several new herpesviruses, HHV-6, HHV-7 and HHV-8, have been identified. Only HHV-6 has so far been recognized as a pathogen in transplant patients. HHV-6 is an endemic virus with over 90% of healthy adults seropositive. It infects CD4 cells and therefore also predisposes to the reactivation of other viruses. Infection occurs in 30–60% of transplant recipients usually in the early post-transplant period. Many patients are asymptomatic and marrow suppression, pneumonitis and encephalitis are the most common disease manifestations. The antiviral susceptibility of HHV-6 is similar to CMV and the organism is sensitive to foscarnet and gancyclovir.

Hepatitis Viruses

Hepatitis A virus or hepatitis delta and epsilon viruses have been uncommon sources of hepatitis in transplant patients. In contrast, infections with hepatitis B virus (HBV) or hepatitis C virus (HCV) have been frequent. Transplant patients may be more susceptible to the complications of HBV and HCV infection, but the uncertainty regarding epidemiology and pathogenesis of these infections complicates decisions regarding the suitability of patients with hepatitis for transplant.

There have been many impediments to an understanding of the consequence of hepatitis. Until the recent development of the anti-HCV assay, the course of patients with HCV infection could not be distinguished from the course of patients with other hepatic abnormalities. Even though patients with HCV can now be identified, the current assay is insensitive. Meanwhile, assays for HBV infection, long considered sensitive and specific, do not adequately detail the potential for HBV reactivation and infectivity. Thus, the outcome of patients previously infected with HBV or HCV and reexposed to one or both of these viruses during transplantation is presently hard to predict on an individual basis, although it is evident that their overall risk of continued and progressive liver dysfunction is increased fourfold or more over uninfected patients. Transplant patients may also have serologic evidence of infection by both viruses and distinguishing the active agent by serologic assays is difficult. Finally, liver dysfunction in the post-transplant patient is a common event and other sources of hepatic injury are often present concurrently.

Incidence

The incidence of hepatitis B surface antigen (HBsAg) positivity varies widely. In Taiwan where 15–20% of the population are HBsAg positive, 93% of bone marrow recipients were positive prior to transplant. In the USA and Italy, 0.1% and 6% of bone marrow

transplant patients, respectively, were antigen positive. Among renal dialysis patients, 3% in the USA and 46% in France were chronically infected. The prevalence of HBsAg seropositivity among liver transplant recipients is skewed by the particular admission requirements to transplant centers. Because of the high rate of HBV reactivation and subsequent hepatic failure, HBsAg-positive patients are not accepted for liver transplantation in some programs. Nonetheless, HBV chronic active hepatitis with hepatic failure is among the most common indications for liver transplantation.

Non-A, non-B chronic active hepatitis with cirrhosis is the most commonly diagnosed condition in patients undergoing liver transplantation. Anti-HCV has been identified in half of these patients. In addition, 37% and 27% of patients undergoing transplant for Laennec's cirrhosis and chronic HBV infection, respectively, were seropositive for HCV. Among kidney transplant patients, up to 26% tested positive for anti-HCV before transplantation. The incidence of HCV infection in bone marrow recipients is uncertain. The widespread use of intravenous immunoglobulin preparations has been blamed for the prevalence (up to 100% of bone marrow transplant patients) of anti-HCV seropositivity; however, immunoglobulin concentrates have not been implicated as a source of HCV transmission.

Epidemiology and pathogenesis

HBV or HCV infection during transplantation is either due to transmission through infected blood or transplanted organ in a previously uninfected patient or more commonly to reactivation or progression of illness in a previously infected patient. There is little evidence that prognosis differs following either primary or secondary infection. Virus reactivation is fomented by the immunosuppression required to establish the allograft. Reactivation does not immediately lead to changes in liver function, and indeed laboratory evidence of hepatocellular injury may not emerge until withdrawal or reduction of immunosuppression.

Within the HBV-infected group, patients at greatest risk for reactivation are asymptomatic HBsAg-positive carriers. Anti-HBs-positive but HBsAg-negative patients are considered immune. However, the intensive immunosuppression administered for bone marrow transplantation, has promoted HBV reactivation in 'immune' patients.

Transmission of HCV by allograft has also been documented, but the frequency of this event has not yet been established. Transfusion of contaminated blood has been assumed to be a major source of HCV infection in transplant patients. Infection by other routes is possible.

Details of HCV pathogenesis are largely unknown. The assay for anti-HCV antibody has not proved enlightening in that regard, since anti-HCV does not appear for weeks to months after infection, and seropositivity may persist for years without evidence of clinical disease. Alternative assays may provide the missing details. For example, serum HCV RNA can now be measured and its presence correlates with infectivity. Although prolonged viremia is common in patients with chronic hepatitis, it remains to be determined whether clearance of HCV viremia indicates permanent immunity or merely a subclinical state capable of reactivation.

Clinical manifestations and treatment

Hepatitis B virus The reported incidence of HBV reactivation following bone marrow transplantation has been 5–12%. However, these figures undoubtedly underestimate the true incidence of reactivation.

Most HBsAg-positive bone marrow transplant recipients develop transient abnormalities in liver function tests, but clinically significant hepatic dysfunction is uncommon. Patients who were HBsAg positive pretransplant rarely cleared antigenemia post-transplant, whereas antigenemia appearing post-transplant persisted for months but almost always resolved. Regardless of whether HBsAg was cleared, intermittent elevation of liver enzymes persisted in over half these patients. The long-term prognosis for these patients is presently unknown. Currently, HBsAg seropositivity is not an absolute contraindication to bone marrow transplantation.

By eight years after kidney transplant, 25–40% of HBsAg-positive patients have developed chronic active hepatitis, cirrhosis, or hepatocellular carcinoma. Data from one study in which HBV reactivation was documented by PCR showed that HBV DNA was detected post-transplant in the 20% of HBV immune patients. Although these patients remained HBsAg negative, their incidence of chronic hepatitis was equivalent to HBsAg-seropositive patients.

Following orthotopic liver transplantation, more than 80% of HBsAg-positive patients will develop reinfection. Overall survival in these patients was significantly reduced compared to other liver transplant patients. HBV-immune patients have not developed reinfection, and this has led to therapies designed to eradicate HBsAg antigenemia: passive immunization with anti-HBs immunoglobulin, active immunization with HBV vaccine, combined active and passive immunization, and α interferon. It appears that HBV reinfection can be delayed by

passive and combined immunization, but late reinfection has occurred. Non-hepatic reservoirs of HBV have been implicated as the cause of reinfection.

Hepatitis C virus There are few reports that document the course of HCV infection in transplant patients. In one, 22% of renal transplant recipients tested repeatedly positive for anti-HCV; 50% of these developed chronic hepatitis. Cirrhosis would be the expected outcome in half of the patients developing chronic hepatitis. Transplantation of solid organs from HCV-seropositive donors has infected over half the recipients. Typically, liver disease progressed to subfulminant hepatic failure or became chronic. At present, transplantation of organs from anti-HCV-positive donors is contraindicated.

Therapy

Some patients with chronic hepatitis secondary to HBV or HCV infection have responded to α-interferon therapy, and this response was occasionally sustained once therapy was completed. Whether α-interferon will improve survival is unknown. Vidarabine and acyclovir have also been effective in a proportion of patients with chronic HBV although their activity was less predictable and vidarabine was more toxic than α-interferon. α-Interferon may enhance graft-versus-host mechanisms in bone marrow transplant recipients. Limited use of α-interferon in patients undergoing orthotopic liver transplantation has failed to prevent HBV reinfection. More recently, the antiretroviral agent lamivudine has been used successfully in small series of transplant patients, where it reduced antigen load and appeared to prevent progression of liver disease due to HBV.

Prevention

Prevention of HBV and HCV infections is the single most effective means of controlling post-transplant hepatitis. In France, immunization against HBV infection has not been the common practice; 46% of chronic dialysis patients are HBsAg positive and 71% of the HBsAg-negative patients are anti-HBs positive. In the USA, measures to prevent HBV infection including isolation of infected patients and HBV immunization have reduced the prevalence to 2.7% and 12% for HBsAg and anti-HBs, respectively. The ability to test blood for anti-HCV will further reduce the incidence of transmitted hepatitis.

Polyomavirus

Primary and usually clinically undetected infections with BK virus (BKV) and JC virus (JCV) occur during childhood, since measurable antibodies to JCV and BKV are present in 70% and 90% of adults, respectively. Such persistence of seropositivity suggests equal persistence of infection, and autopsy studies have identified the viral genome – presumably in a latent state – in various tissues including the kidney. Thus most infections with JCV or BKV in transplant patients are attributed to viral reactivation. However, seronegative renal transplant recipients have acquired primary infection from the transplanted kidney.

Situations in which host immunity, especially cell-mediated immunity, are compromised favor the reactivation of BKV and JCV. Four to eight weeks following kidney transplantation, 26–44% of patients develop viruria. The incidence is higher in patients treated with antilymphocyte serum to prevent graft rejection. Up to half of bone marrow transplant patients also develop viruria with BKV or JCV. Viruria onset has been noted between weeks 2 and 8 and typically persisted for 3–4 weeks. JCV was isolated from 6.7% of seropositive patients, and 55% of BKV seropositive patients excreted the virus. This disparity between the frequency of JCV viruria and BKV viruria is unexplained and contrasts with the experience in renal transplant patients where the incidence of viruria is similar.

BKV or JCV reactivation has been associated with stenosis of the ureteral anastomosis in kidney transplant patients, since virus has been identified in the tissue at the stenosis site. In bone marrow transplant patients, BKV has been linked to hemorrhagic cystitis, with more than 80% of viruric patients developing this complication, an incidence four times that of patients who were not viruric. Compared to autologous or syngeneic transplants, the likelihood of hemorrhagic cystitis was markedly increased among viruric patients undergoing allogeneic bone marrow transplantation. The explanation for this is not apparent. Presumably the more profound immunosuppression that occurs with allogeneic transplantation contributes to the virulence of BKV reactivation.

BKV/JCV have also been identified in the lesions of multifocal leukoencephalopathy, a progressive encephalitis which occurs in a minority of patients on long-term immunosuppression. The contribution of these viruses to the disease state is unclear, but responses to intrathecal cytosine arabinoside have been reported. In general, however, there is no effective therapy identified for BKV or JCV.

Respiratory Viruses

Respiratory syncytial viruses

Infection with parainfluenza and respiratory syncytial virus (RSV) can lead to severe, even fatal, pulmonary

disease in transplant patients. Although RSV is a common pathogen among children, infection confers only transient resistance; most adults will be susceptible at the time of transplant. About 20–50% of patients exposed to RSV in the hospital will be nosocomially infected and RSV infection in transplant patients has coincided with community outbreaks of RSV disease. The communicability of RSV emphasizes the need for stringent isolation measures.

Pneumonia is the most serious consequence of RSV infection. RSV pneumonia has seldom been fatal in solid organ transplant recipients, but the mortality rate among bone marrow transplant patients has been 50%. The importance of RSV as a pathogen appears to be increasing. In one transplant center, RSV was identified in 27% of patients with pulmonary disease.

After BMT, the onset of pneumonia is early in the post-transplant course, usually before bone marrow engraftment. Sinusitis and otitis media often precede the pulmonary symptoms, an important diagnostic clue, since other viral pneumonias are not accompanied by these upper respiratory tract findings. Diagnosis of infection before the onset of pneumonia has been made by finding RSV antigen in upper airway secretions. RSV infections that occur after 100 days post-transplant have not been fatal.

Identification of RSV prior to the onset of pulmonary symptoms and treatment with nebulized ribavirin has arrested disease progression. Once the lower respiratory tract is clinically involved, ribavirin treatment has been less effective. Improved methods of drug delivery, including intravenous ribavirin, may improve the outcome in this latter group.

Adenovirus

Incidence

Adenovirus has been isolated in 5–10% of patients after solid organ transplantation and in 5–25% of patients undergoing allogeneic bone marrow transplantation. Less than half the patients in whom adenovirus was isolated had symptoms of viral infection and in some that were ill, cytomegalovirus or herpes simplex virus was isolated concurrently, confusing the assignment of cause. Nonetheless there is undeniable evidence that adenovirus has caused invasive and fatal disease in transplant recipients.

Pathogenesis and epidemiology

Most patients are seropositive for adenovirus prior to transplant. Tonsils, adenoids, other lymphoid tissue and kidneys are recognized as latent virus reservoirs. This plus the absence of a contagious source suggest that reactivation of latent virus may often be the cause of adenovirus infection following transplantation. Rarely latent virus residing in the transplanted tissue has been implicated as an infectious source.

Although many adenovirus serotypes have been isolated post-transplant, not all isolates have been associated with invasive disease. Several serotypes, notably types 11, 12, 34 and 35, which rarely cause community outbreaks, have been isolated in immunosuppressed patients with pneumonia. Adenovirus type 5, implicated as a cause of intussusception and sporadic cases of hepatitis, has commonly been recovered from liver transplant recipients with adenovirus-associated hepatitis. The disproportionate representation of certain serotypes in transplant patients with invasive disease is unexplained.

Immunosuppression promotes adenovirus infection. In liver transplant patients, invasive disease has been most common when additional therapy was needed to prevent graft rejection. Severe graft-versus-host disease, which is immunosuppressive in itself and is managed by immunosuppressive agents, has been noted to promote adenovirus infection in bone marrow transplant patients. The frequency of adenovirus infection has also increased with increasing numbers of alternative donor stem cell transplants.

Clinical manifestations and treatment

Adenovirus infections have occurred as early as the second week or as late as several months after transplantation. The virus has been isolated from the throat, stool, urine, blood and from tissue parenchyma. Manifestations include gastroenteritis, hemorrhagic cystitis, hepatitis, pneumonia, meningoencephalitis and hemophagocytic syndrome. In some circumstances, infections have been mild and recovery complete. Renal dysfunction suggestive of kidney allograft rejection has followed adenovirus viruria and hemorrhagic cystitis. Renal failure requiring dialysis has complicated the course of bone marrow transplant patients, and in these patients adenovirus was isolated from the renal parenchyma. Adenovirus hepatitis following liver transplantation and pneumonia following bone marrow transplantation have been almost uniformly fatal.

At present there is no specific therapy for adenovirus infection. Treatment consists in aggressive support. There are anecdotal reports of successful management of disseminated adenovirus infection with immunoglobulin, ribavirin, gancyclovir or with adoptive transfer of immune lymphocytes (see EBV disease above). One patient, after undergoing liver transplant, developed adenovirus hepatitis and liver failure and was successfully retransplanted. Undoubt-

edly measures such as these will prevail until adenovirus specific treatment is available.

Immunization

Vaccination

Although viral illnesses occur with high frequency and often devastating effect in transplant recipients, vaccination presently has only a limited role in disease prevention. There are a number of reasons why this should be so. Transplant patients are heavily immunosuppressed, and make poor antibody or T cell responses to killed/subunit vaccines. Administration of live attenuated vaccines, which are potentially more immunogenic, is fraught with peril, since the immune response may be so feeble that even attenuated viruses may produce fatal disease. Finally, effective vaccines to herpes viruses in humans are simply not available. Since it is precisely that virus group which is responsible for most viral-related morbidity and mortality after transplantation, vaccination can at best have a limited impact on outcome. For example, several studies have examined the effect of attenuated Towne strain in renal transplant recipients. Unfortunately, the vaccine induces minimal specific cytotoxic T lymphocytes in seronegative renal transplant recipients, many of whom also fail to make antibody. In clinical trials there was no benefit to seropositive recipients, and no change in the incidence of infection in seronegative recipients. Morbidity was, however, decreased in seronegative recipients.

Despite these limitations, vaccination should still be considered for all transplant patients. In particular, killed or subunit vaccines to poliomyelitis and hepatitis B are readily justified and often produce protective levels of antibody. After BMT, antibody responses to vaccines may be greatly enhanced by immunizing both donor and recipient pre-BMT to allow adoptive transfer of high titer responses. In contrast, live vaccines should be withheld while patients are receiving immunosuppression. After BMT especially, such vaccines should not be given for at least two years; in the presence of chronic graft-versus-host disease this time period may need to be extended indefinitely.

Passive immunization

Although vaccination has a limited role after transplantation, it has been repeatedly suggested that passive immunization with pooled immunoglobulins reduces the incidence or severity of disease associated with viral infection or reactivation after transplantation in general and after BMT in particular (see section on CMV above). These claims have yet to be fully substantiated.

See also: **Adenoviruses (*Adenoviridae*): Animal viruses, General features, Malignant transformation and oncology, Molecular biology; Antivirals; Cytomegaloviruses (*Herpesviridae*): Animal cytomegaloviruses, General features (human), Molecular biology (human), Murine cytomegaloviruses; Epstein-Barr virus (*Herpesviridae*): General features, Molecular biology; Hepadnaviruses (*Hepadnaviridae*): Hepatitis B Virus: General features, Molecular biology; Hepatitis C virus (*Flaviviridae*); Herpes simplex viruses (*Herpesviridae*): General features, Molecular biology; Immune response: Cell mediated immune response, General features; Polyomaviruses – murine (*Papovaviridae*): General features, Molecular biology; Respiratory viruses; Varicella-Zoster virus (*Herpesviridae*): General features, Molecular biology.**

Further Reading

Heslop HE, Rooney CM (1997) Adoptive cellular immunotherapy for EBV lymphoproliferative disease. *Immunol. Rev.* 157: 217.

Hibberd PL, Snyder DR (1995) Cytomegalovirus infection in organ transplant recipients. *Infect. Dis. Clin. North Am.* 9: 863.

Hierholzer JL (1992) Adenovirus infection in the immunocompromised host. *Clin. Microbiol. Rev.* 5: 262.

Riddell SR (1995) Pathogenesis of cytomegalovirus pneumonia in immunocompromised hosts. *Semin. Respir. Infect.* 10: 199.

Shelhamer JH, Gill VJ, Quinn TC *et al* (1996) The laboratory evaluation of opportunistic pulmonary infections. *Ann. Intern. Med.* 124: 585.

Singh N, Carrigan DR (1996) Human herpesvirus-6 in transplantation – an emerging pathogen. *Ann. Intern. Med.* 124: 1065.

Terrault NA, Wright TL, Pereira BJ (1995) Hepatitic C infection in the transplant recipient. *Infect. Dis. Clin. North Am.* 9: 943.

Walter EA, Bowden RA (1995) Infection in the bone marrow transplant recipient. *Infect. Dis. Clin. North Am.* 9: 823–847

Transportable Bacteriophages *see* **Mu-like Phages (Myoviridae)**

TREE SHREW HERPESVIRUSES (*HERPESVIRIDAE*)

Christian A Tidona and **Gholamreza Darai**, Institute for Medical Virology, University of Heidelberg, Heidelberg, Germany

History

The tree shrews (*Tupaia* sp., family Tupaiidae) belong to a group of primitive higher mammals (*Proteutheria*) and are classified as a separate order (*Scandentia*). The first discovery of herpesvirus-like particles in the tree shrew was reported by Mirkovic in 1970. This *Tupaia* herpesvirus (THV-1), which was isolated from a spontaneously degenerating lung tissue culture from an apparently healthy animal, was characterized by McCombs in 1971 by electron microscopy. Subsequently six additional THV isolates (THV-2 to -7) were obtained and characterized. THV-2 was isolated in 1979 from a degenerating lymphoma cell culture which had been established from a moribund 8-year-old tree shrew. Similarly, THV-3 was isolated from a degenerating cell culture of a Hodgkin's sarcoma (Hodgkin's disease, lymphocytic depletion type) from a moribund 9-year-old tree shrew. The other four THVs (THV-4 to -7) were isolated from spleen tissues of moribund animals aged 4–11 years.

Taxonomy and Classification

THVs are still-unclassified members of the family *Herpesviridae*; however, the recent analysis of the primary structure of the THV-2 genome clearly indicates that these viruses belong to the beta-subfamily of herpesviruses.

Properties of the Virion

Naked viral capsids have a diameter of about 100 nm. Extracellular herpesvirus particles comprise an envelope studded with small surface projections (**Fig. 1A**). Beneath this envelope, an electron dense area is detectable. Some of the enveloped particles contain several viral capsids. The diameter of the envelope usually ranges from 200 to 350 nm (**Fig. 1B**).

Properties of the Genome

THV genomes have attracted attention because they consist of a unique linear double-stranded DNA molecule without any detectable long inverted repeat sequences greater than 40 bp within or at either end of the viral genome. The DNAs of the THV isolates 1–4 were subjected to analytical ultracentrifugation and a specific buoyant density of 1.724 g ml^{-1} was determined. From the UV-absorbance/temperature profile of the viral DNA in 15 mmol l^{-1} NaCl, 1.5 mmol L^{-1} sodium citrate a melting temperature (T_m) of 81.2 ± 0.8°C was calculated. This corresponds to an overall G+C content of 64.5 ± 1.9%, which is in agreement with results obtained from DNA nucleotide sequence analysis.

Measurement of the contour length of viral DNA molecules by electron microscopy revealed molecular sizes of about 194.8 ± 5, 200.8 ± 3, 196.3 ± 3 and 196.3 ± 5 kbp for THV isolates 1, 2, 3 and 4, respec-

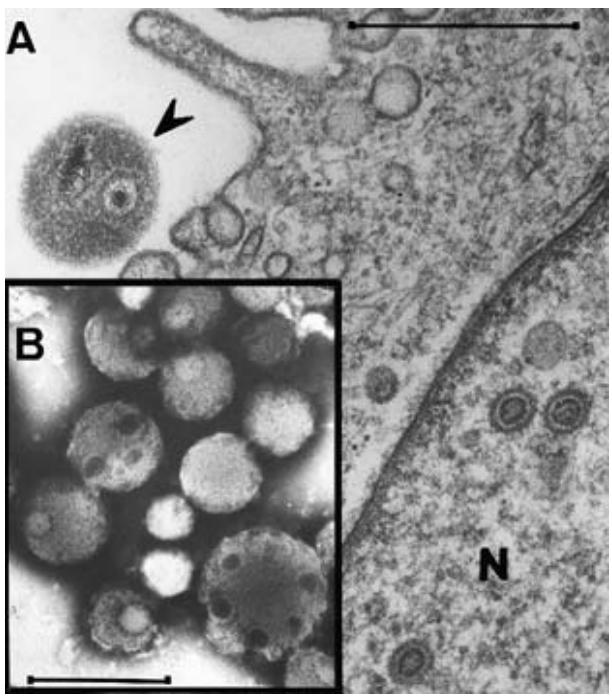


Figure 1 Electron micrographs of *Tupaia* herpesvirus 2 particles. Bars = 500 nm. (**A**) Ultrathin section of a *Tupaia* embryonic fibroblast cell 5 days after infection with THV-2. In the nucleus (N) three virus capsids are present and the mature extracellular virion (arrow) contains two capsids. (**B**) Negative staining of pellets of THV-2 infected tissue culture supernatants using 2% phosphotungstic acid at pH 7.2 reveals the relatively high purity of such materials. Envelope structures are remarkably stable and contain different numbers of capsids.

tively. Physical maps of the THV-2 genome have been constructed.

THV-2 DNA synthesis reaches a maximum between 24 and 36 h post infection, preceded by a transient stimulation of host cell DNA synthesis. Linear concatemeric and circular viral DNA molecules are found during DNA synthesis. The nucleotide sequence of terminal DNA regions of the molecularly cloned THV-2 genome is characterized by a relatively high G+C content of about 75%. Furthermore, the termini contain numerous short repeat elements. A sequence ($A_3C_8AAAGGCAC_6G_5$), postulated to be a consensus signal for site-specific endonucleolytic cleavage in terminal regions of the genomes of herpes simplex virus 1 and 2 (HSV-1, HSV-2), Epstein–Barr virus (EBV) and varicella-zoster virus (VZV), is present at the terminal region of the THV-2 genome. It was found that the signal sequences of HSV and THV-2 are located at similar distances from the genomic termini (at nucleotide positions 432 and 470 bp). This consensus sequence, in combination with several short repeats, which are asymmetrically bracketed by GC-rich arrays, may play a role in forming the mature ends of the THV DNA. Alternatively, this consensus sequence could be the target site for the processing of concatemeric viral DNA molecules into unit-length segments and for the packaging of the processed unit-length molecules into viral capsids.

It was found that THV-1 to -4 genomic DNAs cause infections in tree shrew embryonic fibroblasts.

Viral Proteins

The polypeptide patterns of THV-1 to -3 are remarkably similar, each consisting of at least 35 polypeptides with similar apparent molecular masses ranging from 120 to 230 kDa. While the majority of analogous polypeptides of the three viruses are of indistinguishable electrophoretic mobility, some (e.g. 82–86 kDa polypeptides) showed small differences in apparent molecular mass which were characteristic of the viral strain. By comparative sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) it is possible to distinguish the THV isolates from each other. At least five glycoproteins are found in purified THV virions. Two-dimensional electropherograms reveal at least 47 discernible protein spots, some of which are specific for a given THV isolate and which are detectable in lysates of THV-infected cells.

Viral Enzymes

A protein kinase activity is associated with THV. Divalent cations such as Mg^{2+} or Mn^{2+} are necessary as well as ATP. The predominant sites of phosphorylation are the free hydroxyl groups of serine and threonine residues. Distinct THV polypeptides (molecular masses of 100, 82, and 53 kDa) were found to be targets of phosphorylation in the presence of 5 mmol l^{-1} Mg^{2+}. At a higher Mg^{2+} concentration (20 mmol l^{-1}), additional viral proteins (220, 71, 31 and 20 kDa) were phosphorylated. The gene encoding the viral DNA polymerase was identified by nucleotide sequence analysis.

Geographic and Seasonal Distribution

THV infection of tree shrews is endemic only to south and southeast Asia, corresponding to the geographic distribution of the animal. Seasonal dependence for viral isolation has not been documented.

Host Range and Virus Propagation

The host range of the different THV strains *in vitro* clearly shows that tree shrew embryonic fibroblasts are the most susceptible cells for viral replication. Primary rabbit kidney cells, human foreskin fibroblasts and marmoset skin fibroblasts are less susceptible than tree shrew cells, whereas THVs do not replicate in rodent cell lines. Tree shrew embryonic fibroblasts are the cells of choice for propagation of THVs.

Genetics

A high degree of DNA sequence homology between the different THV isolates has been detected using DNA/DNA hybridization, heteroduplex mapping of the viral DNA molecules, and generation of intratypic recombinant viruses. This indicates the close genetic relationship between the individual THV strains. The seven isolates of THV are genetically grouped into five strains according to DNA fragmentation patterns. THV strains 1, 2, 3 and 5 comprise isolates 1, 2, 3 and 7 respectively, whereas isolates 4, 5 and 6 together represent THV strain 4. However, minor differences between the individual members of THV strain 4 are detectable in respect of slight size variations of specific restriction fragments.

Evolution

The tree shrew, which is the original host of THVs, is thought to have diverged at the basis of the evolutionary tree of mammals. THVs may therefore be fundamental representatives of mammalian herpesviruses. This is supported by the fact that the THV

genome does not contain complex terminal repeat elements and does not occur in different isomeric forms such as modern herpesviruses (e.g. herpes simplex virus).

Serologic Relations and Variability

Serological crossreaction between different THV strains can usually be demonstrated. Crossreaction is sufficiently strong and can be detected with antisera against individual viral strains; however, it is possible to distinguish THV strains from each other in neutralization tests by significant differences in the resulting titers.

Epidemiology

THV epidemiology has not been the subject of intensive studies but analysis of the sera of a limited number of apparently healthy animals in captivity reveals that less than 1% of tree shrews have neutralizing antibodies against THVs.

Transmission and Tissue Tropism

The natural route of transmission of THVs is probably the same as for other herpesviruses. Most infections occur as a result of contact between open lesions and/or moist surfaces; however, spontaneous reactivation of latent virus should also be considered. The target organ for latent THVs is the spleen, as this is the only organ from which infectious viruses can be recovered even after a long period of chronic infection of tree shrews (24 months) and rabbits (14 months). Analysis of the genomes of recovered viruses by different restriction enzymes shows the same DNA fragment patterns when compared to the DNA of the originally inoculated viruses.

Pathogenicity

The pathogenicity of THV-1 to -4 for tree shrews as their indigenous host was studied using juvenile, young adult and adult animals inoculated intravenously or intraperitoneally. *In vivo* pathogenicity studies show that the tree shrew is highly susceptible to these viruses. Intravenous inoculation is always lethal (lethality 100%). In contrast, the majority of intraperitoneally inoculated animals survive the infection (lethality 25%). The major pathology is inflammatory hemorrhagic necrosis of the lungs. High virus titers are detectable in the tissues and whole blood of the infected animals.

It is remarkable that two of the five known THV strains were directly isolated from metastasizing tumors, in one case from a malignant lymphoma and in the other case from a Hodgkin's-like disease. However, only one malignant lymphoma has been induced experimentally in a tree shrew using THV-2. The tumor developed 3.5 years after intraperitoneal administration of the virus. Infectious virus that was genetically identical to the original inoculum was recovered from cultured tumor cells. The failure to routinely induce malignant lymphomas in the tree shrew after experimental inoculation with THV-2 and -3 is probably due to the short period of observation, as tree shrews can reach an age of 14 years.

The response of rabbits to THV-1, -2, -3 and -4 infections is well documented. THV-2 and -3 induce hyperplasia of the thymus in newborn New Zealand rabbits (80%), which in 8% of all cases develop malignant thymomas. Infectious virus can be recovered only from established spleen cultures of the infected rabbits. The spontaneous degeneration of the rabbit splenocyte cultures always develops by the first or second tissue culture passage. The genomes of the recovered viruses are identical when compared with the genome of the originally inoculated viruses.

Clinical Features of Infection

Clinical illness usually appears in juvenile animals infected with THV-1 to -4 on the second day after inoculation. The general symptoms worsen. Intravenous inoculation of these viruses leads to death of the infected animals. Death occurs from day 5 to day 18 post infection as a result of inflammatory hemorrhagic necrosis of the lungs. High virus titers are detectable in the tissues and whole blood of infected animals.

Pathology and Histopathology

It is of particular interest that THV-2 and -3 were isolated from degenerating cultured tumor cells from a high-grade malignant lymphoma and a Hodgkin's-like sarcoma, respectively. The lymphoma was detected in an adult (8-year-old) female tree shrew. Histopathological investigations revealed a discordant hyperplasia of lymphoreticular tissues with formation of nodal follicle-like proliferates. Two types of cells were observed. The first was small with polymorphic nuclei of clear appearance and small nucleoli; the second was of medium to large size with large rounded nuclei. The chromatin showed marginal aggregations with marginal nucleoli. Mitosis was relatively frequent. Several thick reticulin fibers were sparsely distributed after silver staining. Tumor cell infiltration was observed in the parenchyma of the pancreas, the peribronchial lymph follicles, the connective tissue of the pelvis of the kidney, the subserous

membrane of the duodenum, and the cerebral meninges.

A generalized lymphoproliferative disorder resembling human Hodgkin's disease was observed in a female tree shrew of 9 years of age. Histological examination revealed hyperplasia of the lymphoreticular tissue with a polymorphic cellular population consisting of immunoblasts, numerous Hodgkin-like cells and Reed–Sternberg-like cells, eosinophilic granulocytes, and a marked lymphocytic depletion. The spleen showed a diffuse infiltration pattern; liver and peripelvic tissue of kidneys were likewise infiltrated.

Future Perspectives

Many interesting aspects of THV replication, such as transcription, post-transcriptional and post-translational processing, viral enzyme activity, etc., are still not understood and deserve intensive study in the future. Furthermore, genetic analysis of other tree shrew viruses, such as tree shrew retro-, rhabdo-, paramyxo- and adenovirus, is necessary for the final determination of their evolutionary roles.

See also: **Cytomegaloviruses (*Herpesviridae*): Animal cytomegaloviruses, General features (human), Molecular biology (human), Murine cytomegaloviruses; Epstein-Barr virus (*Herpesviridae*): General features, Molecular biology; Herpes simplex viruses (*Herpesviridae*): General features, Molecular biology.**

Further Reading

Darai G, Flügel RM, Matz B and Delius H (1981) DNA of *Tupaia* herpesviruses. In: Becker Y (ed.) *Herpesvirus DNA*, p. 345. The Hague: Nijhoff.

Darai G, Zöller L, Matz B *et al* (1982) Tupaia herpesviruses: characterization and biological properties. *Microbiologica* 5: 185.

Koch H-G, Delius H, Matz B (1985) Molecular cloning and physical mapping of the *Tupaia* herpesvirus genome. *J. Virol.* 55: 86.

TRICHOVIRUSES

Sylvie German-Retana and **Thierry Candresse**, Station de Pathologie Végétale, INRA, Villenave d'Ornon, France

History

The genus *Trichovirus* was established for plant viruses characterized by an elongated very flexuous and open particle morphology and a small genome size (<8.5 kb) as compared to other members of the *Closteroviridae* family which share an essentially similar particle morphology but have much larger genomes, in general in excess of 15 kb. The name 'tricho' is derived from the Greek 'thrix' meaning hair. The genus was approved by the International Committee of Taxonomy of Viruses in 1993, at the 9th International Congress of Virology, in Glasgow and has recently been placed in the family *Closteroviridae*.

Apple chlorotic leaf spot virus (ACLSV), the type member of the *Trichovirus* genus, had been previously classified in the subgroup A of closteroviruses, according to the morphology of its flexuous and filamentous viral particle. ACLSV was the first clostero-like virus whose genome was completely sequenced and genomic organization determined. When molecular information became available on other closteroviruses, it became evident that there were very significant differences in genome properties and structure between ACLSV and beet yellows virus (BYV) the type member of the *Closterovirus* genus. These molecular differences, when added to the differences in particle and genome length, vector transmission, tissue tropism and cytopathic effects, led to the splitting of the *Closterovirus* genus and to the establishment of a new viral genus typified by ACLSV and for which the name *Trichovirus* was coined.

Taxonomy and Classification

The genus *Trichovirus* contains nine viral species (including seven tentative members) with similar biological, morphological, and ultrastructural properties. ACLSV and potato virus T (PVT) are definitive members of the genus, whereas grapevine virus A (GVA), grapevine virus B (GVB), grapevine virus C

membrane of the duodenum, and the cerebral meninges.

A generalized lymphoproliferative disorder resembling human Hodgkin's disease was observed in a female tree shrew of 9 years of age. Histological examination revealed hyperplasia of the lymphoreticular tissue with a polymorphic cellular population consisting of immunoblasts, numerous Hodgkin-like cells and Reed–Sternberg-like cells, eosinophilic granulocytes, and a marked lymphocytic depletion. The spleen showed a diffuse infiltration pattern; liver and peripelvic tissue of kidneys were likewise infiltrated.

Future Perspectives

Many interesting aspects of THV replication, such as transcription, post-transcriptional and post-translational processing, viral enzyme activity, etc., are still not understood and deserve intensive study in the future. Furthermore, genetic analysis of other tree shrew viruses, such as tree shrew retro-, rhabdo-, paramyxo- and adenovirus, is necessary for the final determination of their evolutionary roles.

See also: **Cytomegaloviruses (*Herpesviridae*): Animal cytomegaloviruses, General features (human), Molecular biology (human), Murine cytomegaloviruses; Epstein-Barr virus (*Herpesviridae*): General features, Molecular biology; Herpes simplex viruses (*Herpesviridae*): General features, Molecular biology.**

Further Reading

Darai G, Flügel RM, Matz B and Delius H (1981) DNA of *Tupaia* herpesviruses. In: Becker Y (ed.) *Herpesvirus DNA*, p. 345. The Hague: Nijhoff.

Darai G, Zöller L, Matz B *et al* (1982) Tupaia herpesviruses: characterization and biological properties. *Microbiologica* 5: 185.

Koch H-G, Delius H, Matz B (1985) Molecular cloning and physical mapping of the *Tupaia* herpesvirus genome. *J. Virol.* 55: 86.

TRICHOVIRUSES

Sylvie German-Retana and **Thierry Candresse**, Station de Pathologie Végétale, INRA, Villenave d'Ornon, France

History

The genus *Trichovirus* was established for plant viruses characterized by an elongated very flexuous and open particle morphology and a small genome size (<8.5 kb) as compared to other members of the *Closteroviridae* family which share an essentially similar particle morphology but have much larger genomes, in general in excess of 15 kb. The name 'tricho' is derived from the Greek 'thrix' meaning hair. The genus was approved by the International Committee of Taxonomy of Viruses in 1993, at the 9th International Congress of Virology, in Glasgow and has recently been placed in the family *Closteroviridae*.

Apple chlorotic leaf spot virus (ACLSV), the type member of the *Trichovirus* genus, had been previously classified in the subgroup A of closteroviruses, according to the morphology of its flexuous and filamentous viral particle. ACLSV was the first clostero-like virus whose genome was completely sequenced and genomic organization determined. When molecular information became available on other closteroviruses, it became evident that there were very significant differences in genome properties and structure between ACLSV and beet yellows virus (BYV) the type member of the *Closterovirus* genus. These molecular differences, when added to the differences in particle and genome length, vector transmission, tissue tropism and cytopathic effects, led to the splitting of the *Closterovirus* genus and to the establishment of a new viral genus typified by ACLSV and for which the name *Trichovirus* was coined.

Taxonomy and Classification

The genus *Trichovirus* contains nine viral species (including seven tentative members) with similar biological, morphological, and ultrastructural properties. ACLSV and potato virus T (PVT) are definitive members of the genus, whereas grapevine virus A (GVA), grapevine virus B (GVB), grapevine virus C

Table 1 Properties of definitive and tentative trichoviruses

Virus	Particle length (nm)	Coat protein (kDa)	Genome (nt)	Mechanical transmissibility from natural host	Tissue localization in natural host	Vector
Definitive species						
ACLSV	720	22	7555	Easy	Parenchyma	Unknown
PVT	640	27	c. 7600	Easy	Parenchyma	Unknown
Tentative species						
GVA	800	22.5	7349	Difficult	Phloem	Mealybugs
GVB	800	23	7599	Difficult	Phloem	Mealybugs
GVC	725	25.7	nd	Difficult	Phloem	Mealybugs
GVD	825	20.5	c. 7600	Difficult	Phloem	Mealybugs
GBINV	740	22	c. 7600	Easy	Parenchyma	Unknown
HLV	730	22	nd	Easy	nd	Aphids
CMLV	760	20.5	c. 8200	Easy	nd	Mites

nd = not determined.

(GVC), grapevine virus D (GVD), grapevine berry inner necrosis virus (GBINV), cherry mottle leaf virus (CMLV) and heracleum latent virus (HLV) have properties that qualify them as tentative members of the genus (**Table 1** and see below).

Note added in proof: GVA, GVB, GVC, GVD, CMLV and HLV have recently been split from trichoviruses to create a new genus, Vitivirus. ACLSV, PVT and GBINV thus remain as the only members of the trichovirus genus (see below).

Geographic Distribution and Host Range

Trichoviruses differ in the extent of their geographical distribution. ACLSV, GVA and GVB are found worldwide, whereas PVT has been reported only in the Andean region of South America, GBINV in Japan and HLV in Scotland. CMLV appears to be distributed over North America, but the precise identity of European isolates of CMLV remains to be evaluated. Information is still lacking on the extent of the geographical distribution of GVC and GVD. The natural host range of trichoviruses is restricted to either a single host (PVT, HLV, GVA, GVB, GVC, GVD, GBINV), or to a somewhat wider host range in the case of ACLSV which naturally infects most temperate rosaceous fruit crops.

The experimental host range for ACLSV includes dicotyledonous hosts from a few families and includes 'general purpose' hosts such as *Chenopodium quinoa, C. amaranticolor, Phaseolus vulgaris* and *Nicotiana occidentalis*. Experimental host reactions to GBINV inoculation are quite similar to those of ACLSV except that *C. amaranticolor* is not infected by GBINV. PVT shows a similarly rather restricted experimental host range. The experimental host range for HLV contains hosts corresponding to over nine families and includes also one monocotyledonous host, *Zea mays*. GVA, GVB and GVD and CMLV have a very narrow experimental host range including only *Vitis* and a few *Nicotiana* in the case of the grapevine viruses and *Prunus* and *Chenopodium* for CMLV.

Particle Structure and Composition

Although resembling that of some other flexuous elongated plant viruses such as potex- and carlaviruses, the very flexuous and open particle structure is the most conspicuous characteristic of trichoviruses. This unusual morphology is, however, shared with members of the *Closterovirus* and *Capillovirus* genera. Particles have, in general, a diameter of about 12 nm, with a length of 640–825 nm, depending on the virus considered. The RNA content is usually 5–6% and a single type of coat protein subunit is observed with a molecular weight of 20.5 kDa (CMLV)–27 kDa (PVT), although the calculated weight has been found to be as low as 17.6 kDa in the case of GVD.

The pitch of the primary helix, which can usually be measured from the obvious crossbanding of the particle, has been found to be in the range 3.4–3.8 nm, giving estimated ratios of four nucleotides per protein subunit and of about 10 protein monomers per turn of the helix. However, PVT particles differ slightly from those of ACLSV in having a slightly larger

diameter (13–14 nm) with a pitch of the helix (3.4 nm) at the lower end of the range for trichoviruses. Correlated with the very open structure of the particle, the genomic RNA of several of these viruses, such as ACLSV and HLV, has been found to be sensitive to RNases in their encapsidated forms. Genome size has been found to be directly correlated with particle length and is reported to vary from 7.3 kb to about 8.2 kb.

An unusual property of the coat proteins of ACLSV, HLV, GBINV, GVA, GVB and GVD, is that they are devoid of tryptophan. This probably explains the high (1.4–1.8) A_{260}/A_{280} absorbance ratios of the corresponding viruses.

Genome Structure

In all cases examined, the genome of trichoviruses has been found to consist of a single molecule of single-stranded (ss) RNA. The genomic RNA of ACLSV is infectious and those of ACLSV, GVA and GVB have messenger activity *in vitro*. Thus, trichoviruses should be regarded as having monopartite ssRNA genomes of positive polarity. The genomic RNAs of the type member ACLSV (in fact, four strains of ACLSV differing in their original host and geographical origin), and two tentative members, GVA and GVB, have been totally sequenced, and the sequences of the 3′ terminal regions of PVT, GVD and GBINV genomes have been determined. Relevant sequence database accession numbers are: ACLSV-P863 (M31714), ACLSV-Bal1 (X99752), ACLSV-P205 (D14996), GVA (X75433), GVB (X75448), PVT (D10172), GVD (Y07764) and GBINV (D88448). The genomic RNAs have a polyadenylated 3′ terminus and are capped at their 5′ end.

Genome Organization and Expression: Affinities with other Virus Groups

Genomic organization of the trichoviruses for which sequence information is available is shown in **Fig. 1.** The ACLSV RNA (7.5 kb) contains three open reading frames (ORFs) encoding proteins with approximate molecular weights of 216 kDa, 50 kDa and 22–28 kDa. The partial sequences available for PVT and GBINV fit the same pattern: the extremity of the large replication-associated protein is followed by two smaller ORFs of 39 kDa (GBINV) or 40 kDa (PVT), and 22 kDa (GBINV) or 27 kDa (PVT).

The large, 5′-located ORF 1 codes for a protein that contains three signature sequences, typical of replicase-associated proteins of the 'Alpha-like' super group of plant viruses: the methyl transferase (MT), the nucleotide binding site of the helicase (HEL) and the RNA-dependent RNA polymerase (RdPd) signatures. Homologies with putative papain-related proteinases of positive-strand RNA viruses such as tymo- and furoviruses, are also found in a block of amino acids preceding the HEL domain (**Fig. 1**).

The ORF 2 of ACLSV shares distant similarities with the cauliflower mosaic virus gene I and TMV 30K movement proteins. The ORF 2-encoded protein has been included in the proposed family I of movement proteins (MP), which also includes the MPs of tobamoviruses, tobraviruses, comoviruses, caulimoviruses and geminiviruses. Furthermore, it has been shown that the ORF 2 protein of ACLSV can be detected in both cell wall and cell membrane fractions prepared from infected *Chenopodium quinoa* tissues, and is phosphorylated *in vivo*. All these properties fit with the assignment of this protein as the movement protein of the virus.

The capsid protein (CP) ORF is located at the 3′ terminus of the genomic RNA. The CP contains amino acids that are highly conserved in the CP of filamentous virus, and hypothesized to be involved in the formation of a salt bridge, assumed to play a key role in structure formation.

As shown in **Fig. 1**, the complete sequences of GVA and GVB contain five ORFs, including two that are absent from ACLSV, PVT and GBINV. ORF 1 which is the large replication-associated protein shows clear homologies and a similar arrangement of the conserved signature sequences with the ORF 1 of ACLSV. ORF 2 codes for a 20 kDa polypeptide with unknown function and for which no significant homology has been found in protein databases. ORF 3 encodes a 36.5 kDa (GVB) or 31 kDa (GVA) protein possessing the conserved motifs of the family I of movement proteins, and recent results show that

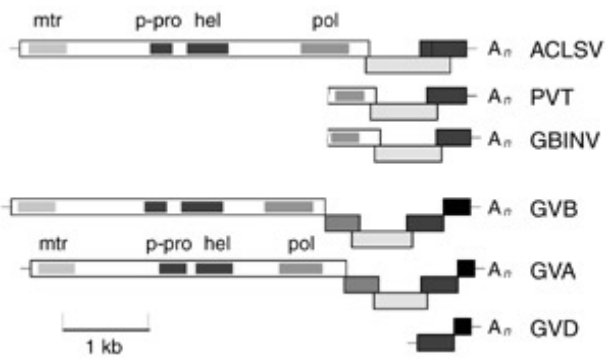


Figure 1 Genomic organization of trichoviruses. ACLSV, apple chlorotic leaf spot virus; PVT, potato virus T; GBINV, grapevine berry inner necrosis virus; GVA, grapevine virus A; GVB, grapevine virus B; GVD, grapevine virus D. mtr, methyl transferase domain; p-pro, papain-like proteinase domain; hel, helicase domain; pol, RNA-dependent RNA polymerase domain.

these proteins are associated with the cell wall, sometimes at the level of plasmodesmata, supporting the hypothesis of their role in the cell to cell movement of the virus. ORF 4 (21.5–21.6 kDa) is the CP cistron showing again low but clear homologies with the CPs of ACLSV, PVT and GBINV. ORF 5 codes for a small (10–14 kDa) protein homologous to the small cystein-rich nucleic acid binding-proteins found in the genome of other plant viruses, such as hordeiviruses and carlaviruses. In the 3′ end of the GVD genome, two ORFs were identified, encoding a cystein-rich 10 kDa protein and the 17.6 kDa CP, respectively; the genome of GVD is thus assumed to follow the same organization as that of GVA and GVB.

Subgenomic messenger RNAs (sgRNAs) have been observed for ACLSV and allow the expression of the ORFs located downstream of the 5′-most ORF. Although there is conflicting evidence for proteolytic processing of the 216 kDa protein of ACLSV, a possible proteolytic maturation of the 216 kDa protein is hypothesized, from the presence of the conserved papain-like protease signature sequence. *In vitro* translation experiments using the genomic GVB RNA as a template have tentatively shown that the 195 kDa viral replicase could undergo proteolytic cleavage.

Comparative analysis of proteins of definitive and tentative trichoviruses showed that they are all phylogenetically related. However, distinct clusters can be observed, in particular, when analyzing the movement and capsid proteins. In particular, GVA, GVB and GVD are much closer to each other than to ACLSV, PVT and GBINV.

Virus Host Relations: Cytopathic Effects

All trichoviruses can be experimentally transmitted by sap inoculation and by grafting. Mechanical transmission from natural host is easy for ACLSV, PVT, HLV, GBINV and CMLV, whereas sap inoculation is difficult for GVA, GVB, GVC and GVD. It is not surprising that the viruses that are easiest to transmit mechanically are also those that do not appear to be phloem-restricted. GVA, GVB, GVC and GVD are phloem-restricted in their natural host, grapevine, whereas ACLSV, PVT and GBINV seem to multiply primarily in parenchyma cells. However, GVA and GVB can invade the parenchyma of artificially infected herbaceous hosts and become mechanically transmissible under these circumstances. Most trichoviruses are not seed-borne, but PVT has been reported to be transmitted rather efficiently in this manner from a number of hosts and ACLSV may be seed-transmitted at a low frequency in apricot.

The cells of plants infected by GVA, GVB and GVD show conspicuous alterations. Virions accumulate in large bundles or paracrystalline aggregates in infected cells. The vascular bundles of systemically infected leaves of *N. occidentalis* have a strongly affected cytology with some cells in stage of necrosis. The cytoplasm shows membrane proliferation, vesiculation, and secondary vacuolation. Vesicular evaginations of the tonoplast contain finely fibrillar material. Mitochondria are damaged and chloroplasts are rounded and swollen, with a disrupted lamellar system and enlarged starch grains.

GBINV particles are found in vascular parenchyma and mesophyll cells. Particles exist as aggregated masses in the cytoplasm, but not in the nucleus or vacuole. No virus-specific inclusion bodies are observed. These modifications are comparable to those observed with ACLSV, except that ACLSV particles are sometimes found in the nucleus.

Viral Transmission

The mode of natural transmission differs substantially between the species of *Trichovirus*. No natural vectors are known for ACLSV, PVT and GBINV, natural dissemination probably being mediated by vegetative multiplication of propagation material.

In the case of HLV, aphid-transmission in a semipersistent manner is assisted by a helper closterovirus [Heracleum virus 6 (HV6)]. Cherry mottle leaf virus (CMLV) is transmitted by the scale mite *Eriophyes inaequalis*. Grapevine trichoviruses appear to be transmitted by several species of mealybugs in a nonspecies-specific manner. Both GVA, GVB and GVD are transmitted in nature by several species of the pseudococcid mealybug genera *Pseudococcus* and *Planococcus* (*Planococcus ficus*, *Pseudococcus longispinus*, *Pseudococcus citri* and *Pseudococcus affinis*). Studies of the acquisition and transmission of GVA by *Ps. longispinus*, showed that *Ps. longispinus* can acquire GVA in a short time (15 min) when feeding on *N. clevelandii*. The virus can be retained for up to 48 h when the insect is fasting. There is no latent period for the inoculation of GVA to *N. clevelandii* after a 30 min feeding. Such properties are consistent with a semipersistent mode of transmission.

Diseases and their Economic Significance

The economic importance of ACLSV is largely due to its worldwide distribution. Although it is more or less symptomless in pome fruits, it is responsible for serious diseases in stone fruit trees such as pseudopox (false plum pox) and bark split of plum, butteratura

and viruella of apricot. In addition ACLSV includes incompatibility problems and bud failure following grafting of infected material, which can result in important losses in nurseries.

Infection by PVT or HLV induces little or no symptoms. The main disease for PVT has been reported only for potato (*Solanum tuberosum*) in which it is usually latent, but can produce a mild leaf mottle. HLV occurs commonly in Scotland in wild *Heracleum sphondylium* (hogweed) plants, without causing any symptoms.

Cherry mottle leaf virus (CMLV) is associated with a disease of both economic and quarantine importance in sweet cherry. The symptoms are irregular chlorotic mottling and distortion of the terminal leaves. In some cultivars, both the quality and the quantity of fruits are affected, the fruits being tasteless, and showing delayed ripening.

GVA, GVB, GVC and GVD are currently thought to be involved in the etiology of the rugose wood complex of diseases, an economically important problem in grapevine. At least four different disorders participate (corky bark, Rupestris stem pitting, Kober stem grooving and LN33 stem grooving). Individual disorders can be distinguished by indexing on specific *Vitis* indicators, but not in the field, due to the absence of specific symptoms. Rugose wood-affected vines are less vigorous, show a more or less pronounced swelling at the bud union and a marked difference between the diameter of scion and rootstock. The affected vines may decline and die within a few years. The major characteristic features of the disease are symptoms of pitting and grooving in the woody cylinders of the scion, rootstock, or both. There are cases in which the scion also shows an atypical production of corky tissues, just above the graft union, called 'corky rugose wood'. Unraveling the etiology of rugose wood appears to be a very complex problem that has not yet been solved. Circumstantial evidence points to GVA and GVB being the probable agents of, respectively, Kober stem grooving and corky bark diseases.

Grapevine berry inner necrosis, reported in Japan, and originally called mosaic disease, is one the most economically important virus diseases of grapevines in Japan. The diseased grapevines grow less vigorously, sprout late in spring, and show inner necrosis in shoots, shortened internodes and various mosaic patterns on leaves. Berries are small, show external discoloration and interior necrosis.

Serological Relationships and Molecular Variability

Very limited serological relationships have been detected between the trichoviruses. It has been recently reported that GVA, GVB and GVD are distantly serologically related, the shared antigenic determinant between GVA and GVD corresponding probably to a cryptotope. GVA and HLV have also a distant serological relationship. No other serological relationship has been reported between other members of the genus.

The type member of the genus, ACLSV, is characterized by a large biological diversity. Polymerase chain reaction (PCR) has been used to study the variability of ACLSV in the region of overlap between the 50 kDa movement protein and the coat protein genes. Extensive variability is observed between isolates at the nucleotide sequence level, most isolates evaluated showing divergence rates in the 10–20% range from each other. However, at the protein level, the CP is highly conserved between the isolates and variations in the 50 kDa movement protein explain most of the variations in the nucleotide sequence. These results are in keeping with the large biological diversity and the low serological diversity observed between ACLSV isolates. No information on the molecular variability of other trichoviruses has been reported so far.

Virus Epidemiology and Control

In most cases, natural transmission by vectors is not the main cause of long-distance dispersion of the viruses, which is mostly due to human distribution of contaminated material. In regions from which a given virus is absent, quarantine measures are the best protective measure. In affected regions, viral infection is usually controlled through the production and use of virus-free propagation material, along with control of insect vector populations and destruction of virus reservoirs. The most frequently used detection techniques for certification of propagation and planting material include bioassays on susceptible hosts and enzyme-linked immunosorbent assays (ELISA). In addition, detection of dsRNAs associated with viral infection (ACLSV, GVA, GVB, GVD), immunoelectron microscopy (IEM), molecular hybridization and PCR detection methods have been reported for several of these viruses. In the case of ACLSV, it has been possible to obtain virus-free plants through the use of meristem tip culture, thermotherapy or a combination of both, eventually coupled with chemotherapy using agents such as virazole (ribavirin, a cap structure analogue). For GVA and GVB, it is possible to eliminate the viruses from grapevine with the use of meristem tip culture, although with great difficulty, as

these viruses are among the hardest to eliminate from the grapevine.

Future Perspectives

Differences in molecular organization, biological and epidemiologic behavior, between definitive (ACLSV, PVT) and tentative (GVA, GVB, GVC, GVD) trichoviruses, seem to be wide enough to warrant the splitting of the *Trichovirus* genus. The creation of a new genus named *Vitivirus*, which would contain GVA, GVB, GVC, GVD, HLV and CMLV, has recently been approved by the Executive Committee of the ICTV.

See also: **Capilloviruses; Closteroviruses (*Closteroviridae*); Plant virus disease – economic aspects.**

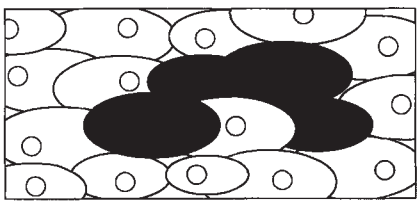
Further Reading

Bar-Joseph M and Murant AF (1982) Closterovirus group. *CMI/AAB Description of Plant Viruses*, no. 260.

Brunt A, Crabtree K, Dallwitz M, Gibbs A and Watson L (eds) (1996) *Viruses of plants, Descriptions and Lists from the VIDE Database*. Wallingford: CAB International.

Martelli GP, Candresse T and Namba S (1994) *Trichovirus*, a new genus of plant viruses. *Arch. Virol.* 134: 451.

TUMOR VIRUSES – HUMAN



Herbert Pfister, Institute for Virology, University of Koln, Koln, Germany

Bernhard Fleckenstein, Institute for Clinical and Molecular Virology, Friedrich-Alexander University, Erlangen, Germany

Criteria for Causal Relationship between Virus and Cancer

Although there are numerous animal models for viral oncogenesis, only in rare cases is the virus sufficient by itself for tumor induction. In those systems, experimental infection may lead to sizeable tumors within a few weeks, thus leaving no doubt about the etiologic role of the particular pathogen. In humans, none of the viruses incriminated as being oncogenic appear to be independent of other factors. Many years, and often several decades, pass by before tumors develop from primary infection in a small number of infected individuals. Monoclonal tumors arise from the pool of virus-infected cells. This suggests that the virus is, at best, one of several factors that together increase the probability of a cell undergoing malignant transformation. Possible cofactors are chemical or physical carcinogens. Furthermore, the oncogenic activity of the virus may be restricted to a specific genetic background of the patient or to a susceptible stage of cell development. These complex interrelations and the ethical ban on experiments in humans render it extremely difficult to prove the causative role for the virus.

Viruses may contribute to tumor development at various stages of the multistep carcinogenesis process and by different mechanisms. If viral activity is necessary, the virus should be found at some stage in every case in which the tumor occurs. Viral footprints do not, however, have to appear in cancer cells. Human immunodeficiency virus (HIV), for example, significantly increases the risk of development of Kaposi sarcomas and B-cell lymphomas, probably because of its suppressive effect on the immune system. For some herpesviruses and papillomaviruses, a 'hit and run' mechanism has been proposed, which implies virally induced irreversible damage at some point early in tumorigenesis, but no role later on in the maintenance of the malignant phenotype; the virus may therefore completely disappear from the cancer. Viruses may act as mutagens or, even more indirectly, by increasing the risk of mutations via induction of cell proliferation in the course of chronic inflammatory reactions. The etiologic significance of a virus is very difficult to prove in this case. Large-scale and long-term prospective epidemiological studies are the only way to define the risk of a given infection. As the virus may be present for short periods only, the infection is best monitored by the detection of specific antibodies. Previous studies focused on anticapsid antibodies, but with many DNA viruses the production of particles and oncogenic transformation appear to be mutually exclusive,

these viruses are among the hardest to eliminate from the grapevine.

Future Perspectives

Differences in molecular organization, biological and epidemiologic behavior, between definitive (ACLSV, PVT) and tentative (GVA, GVB, GVC, GVD) trichoviruses, seem to be wide enough to warrant the splitting of the *Trichovirus* genus. The creation of a new genus named *Vitivirus*, which would contain GVA, GVB, GVC, GVD, HLV and CMLV, has recently been approved by the Executive Committee of the ICTV.

See also: **Capilloviruses; Closteroviruses (*Closteroviridae*); Plant virus disease – economic aspects.**

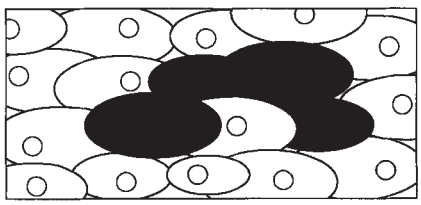
Further Reading

Bar-Joseph M and Murant AF (1982) Closterovirus group. *CMI/AAB Description of Plant Viruses*, no. 260.

Brunt A, Crabtree K, Dallwitz M, Gibbs A and Watson L (eds) (1996) *Viruses of plants, Descriptions and Lists from the VIDE Database*. Wallingford: CAB International.

Martelli GP, Candresse T and Namba S (1994) *Trichovirus*, a new genus of plant viruses. *Arch. Virol.* 134: 451.

TUMOR VIRUSES – HUMAN



Herbert Pfister, Institute for Virology, University of Koln, Koln, Germany

Bernhard Fleckenstein, Institute for Clinical and Molecular Virology, Friedrich-Alexander University, Erlangen, Germany

Criteria for Causal Relationship between Virus and Cancer

Although there are numerous animal models for viral oncogenesis, only in rare cases is the virus sufficient by itself for tumor induction. In those systems, experimental infection may lead to sizeable tumors within a few weeks, thus leaving no doubt about the etiologic role of the particular pathogen. In humans, none of the viruses incriminated as being oncogenic appear to be independent of other factors. Many years, and often several decades, pass by before tumors develop from primary infection in a small number of infected individuals. Monoclonal tumors arise from the pool of virus-infected cells. This suggests that the virus is, at best, one of several factors that together increase the probability of a cell undergoing malignant transformation. Possible cofactors are chemical or physical carcinogens. Furthermore, the oncogenic activity of the virus may be restricted to a specific genetic background of the patient or to a susceptible stage of cell development. These complex interrelations and the ethical ban on experiments in humans render it extremely difficult to prove the causative role for the virus.

Viruses may contribute to tumor development at various stages of the multistep carcinogenesis process and by different mechanisms. If viral activity is necessary, the virus should be found at some stage in every case in which the tumor occurs. Viral footprints do not, however, have to appear in cancer cells. Human immunodeficiency virus (HIV), for example, significantly increases the risk of development of Kaposi sarcomas and B-cell lymphomas, probably because of its suppressive effect on the immune system. For some herpesviruses and papillomaviruses, a 'hit and run' mechanism has been proposed, which implies virally induced irreversible damage at some point early in tumorigenesis, but no role later on in the maintenance of the malignant phenotype; the virus may therefore completely disappear from the cancer. Viruses may act as mutagens or, even more indirectly, by increasing the risk of mutations via induction of cell proliferation in the course of chronic inflammatory reactions. The etiologic significance of a virus is very difficult to prove in this case. Large-scale and long-term prospective epidemiological studies are the only way to define the risk of a given infection. As the virus may be present for short periods only, the infection is best monitored by the detection of specific antibodies. Previous studies focused on anticapsid antibodies, but with many DNA viruses the production of particles and oncogenic transformation appear to be mutually exclusive,

so it might be more promising to screen for antibodies against early nonstructural viral proteins.

Following the classical concepts of virus-induced cell transformation, a tumor virus induces new genetic material into host cells by establishing a persistent infection. The expression of one or more viral oncogenes may then affect cell proliferation and/or differentiation. This can be tested *in vitro* by transformation assays which may result in cells that are tumorigenic in nude mice. If a virus acts in this way, one has to find viral genomes as well as virus-specific transcripts and oncogenic proteins in transformed cells and also in cancer cells if the viral functions are essential for maintaining the malignant phenotype. Tumor viruses may alternatively contribute to carcinogenesis by inserting viral transcription control elements in the vicinity of cellular proto-oncogenes. Viral DNA has to be detectable in these cases adjacent to relevant proto-oncogenes of cancer cells. A harmful integration is typically a very rare event among many random insertions, which is selected for by tumor growth *in vivo*. This mechanism cannot be mimicked by *in vitro* transformation assays.

The physical association of viral genes with tumor cells, in combination with data on oncogenic activity from cell culture or animal experiments, is usually regarded as a most convincing evidence for a role for a virus in human carcinogenesis; however, even better proof of the effective contribution of the virus is obtained from longitudinal epidemiological studies. Finally, the most convincing argument in favor of a necessary role for a tumor virus will be cancer prevention by intervention directed at the virus, such as specific vaccination.

Burkitt's Lymphoma

The malignant lymphoma that was recognized by Denis Burkitt in 1958 as a distinct disease entity in Africa was the first human neoplasm to be linked with a viral etiology. Burkitt's lymphoma (BL) is the most frequent type of childhood tumor found in some hot and humid lowland areas of Central and Eastern Africa, but sporadic cases of the disease, defined by the peculiar histopathology and chromosome translocations, occur throughout the world. Although, in the majority of cases, BL presents in younger children around 8 years of age as a unilateral swelling of the jaw, the disease is usually multifocal at diagnosis due to early metastasis; it characteristically involves liver, kidneys, ovaries or testes, lymphoid tissues in the gut, and endocrine or exocrine glands. In older children and young adults, mostly in the sporadic cases outside Africa, abdominal masses are the first sign of disease.

Lymphoid tumor cell lines derived from BL biopsies led to the discovery in 1964 by Epstein and colleagues of a new herpesvirus, termed Epstein–Barr virus (EBV). The cell lines usually contain EBV genomes as nonintegrated covalently closed circular double-stranded DNA (dsDNA) genomes of about 172 kb in high multiplicity; some lymphoblastoid cell lines carry the EBV genomes integrated into the cellular genomes. EBV is capable of immortalizing human B lymphocytes. The transformed cells express a viral nuclear antigen complex, termed EBNA, which consists of at least six proteins, and in addition latent membrane proteins (LMP). Genetic experiments identified EBNA-2 and LMP as the growth-transforming factors. BL-derived lymphoblastoid cell lines typically express EBNA-1, while, remarkably, the other EBNA proteins and LMP are not detectable. Some lymphoblastoid lines are in a semipermissive state; they produce mature herpesvirus particles spontaneously or can be induced to do so by substances such as the tumor-promoting phorbol ester, phorbol 12-myristate 13-acetate (PMA). BL lymphoma cells and lymphoblastoid tumor cell lines have the surface antigen markers of B lymphocytes; this correlates with a narrow host range of the virus; *in vitro* the virus selectively adsorbs and penetrates by binding of the virion envelope glycoprotein gp350/220 to the complement receptor 2 (CR2; CD21) of B lymphocytes or their precursors. In epithelial cell lines, transcripts encoding CD21 have been detected; however, expression of this receptor on primary epithelial cells remains to be demonstrated.

Indirect immunofluorescence testing with EBV-producing lymphoblastoid cell lines provided the basis for seroepidemiology. It showed that EBV is a ubiquitous virus that occurs in the majority of populations on a worldwide scale. Primary infection can lead to infectious mononucleosis or may remain inapparent without overt disease. BL typically follows early seroconversion during infancy; tumor-bearing children have high antibody titers against structural and early EBV proteins.

In spite of a general association between development of BL and the viral markers, the exact role of EBV in tumor pathogenesis has remained controversial. More than 90% of the tumors in areas of endemic BL contain EBV DNA and express EBNA, and the children have generally high titers of antibodies; however, in sporadic forms of BL that occur in other parts of the world the viral DNA and protein markers are present in only about 15–20%, and elevated antibody titers are not observed in the case of tumors devoid of viral markers. Thus, the presence of EBV persisting in certain compartments of B cells or their precursors may be a risk factor for the develop-

ment of BL, but it cannot be the sole driving force for oncogenesis. So far, it is also difficult strictly to exclude the possibility that EBV is only a passenger of the tumor without contributing to its generation. Common denominators of all BL forms, endemic and sporadic, are certain chromosome translocations. Typically, the gene locus for the proto-oncogene c-*myc* (chromosome 8q29) is juxtaposed to the immunoglobulin heavy chain locus on the long arm of chromosome 14. The break points on the c-*myc* gene are located upstream of the promoter, in the first exon, or between the first and second exon of the proto-oncogene. Less frequently, c-*myc* is translocated to the immunoglobulin (chromosome 2p11) or (chromosome 22q11) light chain genes. It is generally believed that the translocations result in some form of c-*myc* deregulation, perhaps at the level of transcriptional control, mRNA processing or protein synthesis. A unifying concept to explain the role of EBV in BL proposes that the virus initiates the process of tumorigenesis through growth stimulation of a certain B-cell population. Polyclonal B-cell expansion, possibly favoured by malaria-induced impairment of T-cell functions, may enlarge the number of target cells for the necessary key event, chromosome translocation and deregulation of the proto-oncogene c-*myc*. An alternative may be the immortalization by EBV of a malaria-amplified pool of pre-existing c-*myc*-translocated B-cells. In both cases, early EBV infection and viral persistence would be an important risk factor for BL, primarily in its endemic forms.

EBV-Associated Lymphoproliferative Syndromes Other than BL

While primary infection with EBV usually remains without overt disease or manifests as benign infectious mononucleosis, rare cases develop into fatal lymphoproliferations with features of malignant B-cell lymphoma. About half of these cases occur in boys or male adolescents with an inherited defect in the immune system, termed X-linked lymphoproliferative syndrome (XLPS) or Duncan syndrome. The other cases are sporadic without a sex preponderance. Proliferating cells were shown to contain persisting EBV DNA and to express the viral latency genes EBNA and LMP. The infiltrating B-cell derivatives have been shown to be polyclonal in some cases; other processes appeared oligoclonal. The defects of the immune system leading to XLPS or fatal mononucleosis are not precisely known, as the children are not generally immunodeficient. Possibly it relates to functional impairment of natural killer (NK) cells, cytotoxic T cells or changes in the B cells that are the targets of EBV-induced growth stimulation. Notably, some of the patients do not have the normal antibody response to EBV antigens, particularly EBNA-1.

EBV sometimes induces fatal lymphoproliferative disease or lymphoma in globally immunodeficient individuals, either in cases of severe congenital immunodeficiencies or acquired immunosuppression in transplant recipients. Diffuse B-cell lymphomas, often localized in the central nervous system or other extranodular sites, are a frequent cause of morbidity and mortality in acquired immunodeficiency disease (AIDS). The tumors are often polyclonal initially, as they arise from several independent transformation events; they gradually evolve into an oligoclonal or even monoclonal proliferation. The tissues usually have the markers of persisting and actively transforming EBV, including EBNA and LMP expression. Similarly, the lymphomas of owl monkeys (*Aotus trivirgatus*) and cotton top marmosets (*Saguinus oedipus*) that are experimentally inoculated with EBV are considered to be immediate outgrowths of EBV-transformed B lymphoblasts. Presumably, deficiency in certain immune effector mechanisms, genetically determined or acquired, which are necessary to eliminate EBV-transformed B lymphoblasts from the body, leads to progressive lymphoproliferative diseases. On occasion it has been reported that human lymphomas with T-cell markers, such as nasal T-cell lymphomas, contain persisting EBV. A viral etiology of those tumors remains difficult to establish; the cell surface receptor for EBV on T cells is not yet known.

EBV genomes have been detected more recently in a large proportion (up to 60%) of cases of Hodgkin's disease. Distinct LMP-specific membrane and cytoplasmic staining has been found exclusively in Hodgkin and Reed–Sternberg cells of virus DNA-positive specimens, while EBNA-2 is not detected. This suggests a pattern of EBV gene expression different from that of B lymphoblastoid cells and BL. In view of the transforming potential of the LMP gene, it suggests a causal role of EBV in the case of Hodgkin's disease. Viral persistence and preferential expression of LMP has also been seen in some cases of CD30+ anaplastic large cell lymphoma, a heterogeneous group of high-grade malignant lymphomas at the borderline between Hodgkin's and non-Hodgkin's lymphomas. The role of EBV in angioimmunoblastic lymphoadenopathy remains to be determined.

Nasopharyngeal Carcinoma

Nasopharyngeal carcinomas (NPCs) are highly malignant neoplasms which mostly occur in adults between the ages of 20 and 50 years. Though the incidence is generally low in Europe and North

America, NPC is a frequent tumor in southern parts of China, in Tunisia, Central Africa and in the native population of Alaska. The prognosis is poor; most frequently, NPCs present with early metastasis into cervical lymph nodes and the skull. The association of EBV with NPC has been clearly established. Viral DNA is regularly found in all cases of poorly differentiated or lymphoepithelial NPC. Studies on the structure of the persisting episomal EBV genomes have indicated that the neoplastic epithelial cells contain a single clone of EBV in each case. Carcinoma cells consistently express EBNA-1 and, frequently, latent membrane proteins, while the EBNA-2 gene is not transcribed. There is a strong serological association between EBV and NPC. The sera of patients with NPC typically have high titers of immunoglobulin A (IgA) antibodies to structural viral components, latency proteins and replicative antigens. A similar association was found between EBV and several rare tumor forms that are also assumed to be derived from embryonic branchial cleft remnants. High IgA titers against EBV proteins and persisting viral DNA in the tumors have also been found in some forms of neoplasms from the parotid, thymus and lymphatic tissue of the oropharynx.

T-Cell Leukemia

Adult T-cell leukemia (ATL) was the first human disease to be causally linked to a retrovirus. ATL, first described as a frequent tumor form in southern regions of Japan, is a distinct disease entity that occurs in many other parts of the world, including Central Africa and the Caribbean basin. Epidemiology clearly demonstrated the association with human T-cell leukemia virus type 1 (HTLV-1). Serological surveys showed that antibodies against HTLV-1 were regularly found in leukemia patients, and frequently in their close contacts. Tumor cells mostly contain integrated proviral DNA; clonality of the tumor correlates with the monoclonal or oligoclonal integration pattern of the viral genomes. HTLV-1 can be detected by polymerase chain reaction (PCR) in peripheral lymphocytes of latently infected persons and ATL patients. If peripheral mononuclear blood cells are infected with HTLV-1 in cell culture, T cells are immortalized, resulting in continuous growth in suspension culture. The cell surface marker phenotype largely resembles the tumor cells which express high levels of IL-2Rα (α chain of the interleukin-2 receptor) and CD4. HTLV-1 has also been shown to be oncogenic in animal model systems.

ATL usually develops 30–50 years after perinatal infection with HTLV-1. The virus is most frequently transmitted by breast feeding and sexual contacts. The malignancy, arising after the long carrier phase, may be manifested as a lymphoma or acute leukemia, with high white blood cell count and a poor prognosis, usually being fatal within 6–8 months. Smoldering and chronic forms of ATL can convert into an acute course. Approximately 3% of the perinatally infected will develop ATL during their lifetime. Unlike retroviruses of other subgroups, HTLV-1 contains a 1.6 kb proviral genomic region coding for at least two regulatory polypeptides, the 40 kDa transcriptional transactivator Tax and the 27 kDa phosphoprotein Rex, which is required for the cytoplasmic targeting for structural protein mRNAs. Tax, a transactivator of the viral promoter and modulator of numerous cellular genes, appears to mediate the oncogenic effects of the virus; it induces polyclonal expansion of CD4+ T lymphocytes. The early virus-induced helper T-cell proliferation may precede a long period of tumor progression as growth becomes autonomous, turning independent of Tax expression.

HTLV-2, which frequently occurs in some tribes of native Americans, has been isolated repeatedly from the T-cell variant of hairy cell leukemia, but the etiology of T-cell malignancies by HTLV-2 has not been substantiated further until now. Like type 1, HTLV-2 is increasingly spread among intravenous drug abusers in European and American cities.

Genital Cancer

Clinical studies, and especially data from molecular biology, suggest that certain types of human papillomavirus (HPV) are of etiological importance for genital cancer. In developing countries, carcinoma of the cervix uteri is the most frequent type of female cancer. HPV DNA can be found in 80–90% of the tumors, and the early genes E6 and E7 are usually expressed. The most prevalent type in epidermoid carcinomas is HPV 16. HPV 18 may be preferentially associated with adenocarcinomas. Other types like HPV 31, 33, 35, 39, 45, 51, 52 or 56 have been detected in a few cases of squamous cell carcinoma each. HPV DNA was also demonstrated in the less prevalent carcinomas of the vulva, the vagina, the penis and the anus; HPV 16 is again the most frequent type, followed by HPV 18, HPV 6 and HPV 11. The significantly elevated prevalence of individual HPV types in cancers compared with the normal population led to the concept of HPVs with higher (HPV 16, 18, 45) and lower (HPV 6, 11) carcinogenic potential. This grouping is supported by first follow-up studies of the natural history of precancerous lesions associated with different HPVs and by differences in the *in vitro* transformation of keratinocytes.

HPV 16 and other members of the high-risk

papillomavirus group immortalize primary human keratinocytes and induce resistance to differentiation stimuli. Histological abnormalities can be observed in stratifying keratinocyte cultures that resemble those in precancerous, intraepithelial lesions *in vitro*. The cells are not tumorigenic in nude mice initially, but quickly change to an aneuploid karyotype, which is in keeping with frequently occurring abnormal mitoses in HPV 16-positive lesions. At higher passage level, malignant clones reproducibly arise, which indicates that HPV infection is sufficient to induce cancer cells in combination with additional spontaneous or virus-induced modifications. The viral genes E6 and E7 are required to trigger these effects. They encode proteins that are transactivators of transcription and are able to interact with the cellular proteins p53 and p105-RB (the retinoblastoma protein), respectively. The known cell-cycle regulating functions of these proteins are likely to be disturbed by this complex formation with the viral proteins. The E6 and E7 proteins of low-risk viruses display much lower affinities to the cellular proteins, in parallel with a lower or not detectable transforming potential *in vitro*.

Much attention has been paid to the possible role of viral DNA integration in tumor progression. HPV 18 DNA appears integrated into the cellular genome in almost all cervical cancers, and HPV 16 DNA in about two-thirds of the cases. This is in contrast with benign and premalignant lesions, where the viral DNA usually persists extrachromosomally. There is no evidence for a specific integration site, but HPV DNA has been repeatedly detected in the vicinity of the *myc* proto-oncogene in combination with an overexpression of the cellular gene.

The opening of the circular viral genome during integration frequently disrupts the regulator genes E1 and/or E2. Engineered mutants in these genes revealed increased transformation efficiency *in vitro*, so that naturally occurring inactivation may quantitatively enhance cell transformation.

In addition to the disruption of viral control mechanisms, there seems to be a failure of a cellular regulation of viral gene expression in malignant cells. The analysis of hybrids between HPV DNA-positive cervical cancer-derived cells and primary keratinocytes suggested that an inducible control system, possibly encoded by chromosome 11, can normally suppress viral transcription.

The persistence of viral DNA and the continual expression of transforming genes in advanced cancers suggest that HPV functions are also involved in the maintenance of the malignant phenotype. An experimental suppression of E6 and E7 expression inhibited the proliferation of HPV-positive cervical cancer cell lines and reduced the cloning efficiency in semisolid medium, thus indicating that the viral proteins are still modulating the growth of malignant cells.

The genital tract HPVs are also responsible for many HPV infections at extragenital mucosal sites such as the oral cavity and most notably the larynx. However, cancers arising in this field only rarely harbor HPV DNA. Case reports describe the presence of HPV 2, 6, 11, 16 or 30 in carcinomas of the tongue, the oral and nasal cavities, the larynx, the hypopharynx and the lung. The reason for the striking difference between the genital and aerodigestive tracts is not known. Either the etiology of oral and laryngeal cancers is unrelated to HPV, or the relevant HPV types are not yet characterized or the viral DNA is no longer necessary for cancer cells and is finally lost.

Skin Cancer

HPVs induce various proliferative skin lesions that are benign, like plantar, common and flat warts. An association between HPV and skin cancer becomes obvious in epidermodysplasia verruciformis (EV). EV patients are infected with a subgroup of HPVs, which induce characteristic persisting macular lesions disseminated over the body. Many EV patients develop squamous cell skin carcinomas, mainly at sun-exposed sites, which suggests a cocarcinogenic effect of ultraviolet light. The DNA of HPV 5 or 8 persists extrachromosomally in high copy number in more than 90% of the cancers. HPV14, 17, 20 or 47 were occasionally detected. The prevalence of specific HPVs is in striking contrast with the plurality of HPV in benign lesions and has been interpreted as reflecting a higher oncogenic potential of these types.

Oncogenes of EV-HPVs were mainly identified by their effects on rodent fibroblasts. The E6 gene induces altered morphology, reduced serum requirement, and anchorage-independent growth. In contrast with genital HPV, no complex formation could be detected between HPV8 E6 and the cellular p53 protein, which suggests different strategies of transformation. The HPV8 E2 gene, which encodes a transactivator of transcription, leads to reduced serum requirement and growth in soft agar. There is some indication of an increased transforming activity of E6 from HPV5, 8 and 47 when compared with E6 from related HPVs, which have not yet been detected in carcinomas.

A high prevalence of HPV DNA in squamous and basal cell carcinomas of the skin, particularly of immunosuppressed but also of immunocompetent patients, has most recently been demonstrated by highly sensitive PCRs. Evidence is accumulating for many novel HPV types related to EV HPVs and

cutaneous types. Genital mucosal HPV types have also been found. That there is a strong association between genital HPV16 and squamous cell neoplasms from the finger is remarkable. Individual skin tumors were frequently noted to be infected by several HPVs. No single HPV type predominates in skin cancers of non-EV patients, so far as is known. The need for highly sensitive detection methods suggests that HPV DNA persists at very low concentrations in many skin cancers, perhaps at less than one genome copy per cancer cell. The relevance of these findings to the pathogenesis of cutaneous cancer remains to be determined. The possibilities discussed above for carcinomas of the aerodigestive tract are also valid for skin carcinomas.

Hepatocellular Carcinoma

Primary liver cancer is among the most common fatal malignancies of humans worldwide. An association with hepatitis B virus (HBV) from the hepadnavirus family was suggested by the geographical coincidence of a high incidence of hepatocellular carcinoma in southeast Asia and equatorial Africa with high rates of chronic HBV infections, generally contracted congenitally. Prospective studies demonstrated about a hundredfold increased risk of hepatoma among carriers of the HBV surface antigen (HBsAg). Integrated HBV DNA can be detected in a large proportion of the tumors from high-risk areas and in hepatoma-derived cell lines.

Liver cancer usually develops only after several decades of chronic HBV-induced hepatitis and may thus be triggered by accumulating genetic damage due to inflammation and continuous cell regeneration. A specific contribution of HBV might be expected from *cis* effects following integration of viral DNA, but except for a few case reports no consistent evidence has been obtained for the activation of particular proto-oncogenes. A transactivation of transcription may be more relevant; this can be achieved by the viral X protein, the large surface protein and a truncated $preS_2/S$ protein. The viral $preS_2/S$ gene, which normally encodes a surface protein, appears frequently disrupted in cancers as a consequence of DNA integration and then gives rise to the transactivator. All HBV transactivators exert pleiotropic effects via the protein kinase C/raf-controlled signal pathway, finally activating transcription factors such as AP-1 and NF-κB and proliferation. The analysis of viral integration patterns and functional assays suggest that at least one transactivator may function in most hepatomas. Multifocal nodular hyperplastic liver disease developed in mice transgenic for the HBV surface protein genes, and liver cancer arose in mice transgenic for the X gene. Mutations in the p53 tumor suppressor gene occur in about 30% of human hepatomas. They are observed more often in countries with dietary contamination by mutagenic aflatoxin and seem to be a late event in liver carcinogenesis. The complex formation between p53 and X protein as well as truncated $preS_2/S$ may be relevant to p53 inactivation in patients with wild-type p53. The X protein was also shown to interact with elements of the DNA repair system, which may increase the mutation rate of p53.

HBV is the first human tumor virus against which vaccination programs have been initiated on a broad basis. First signs of a decrease in the incidence of hepatoma in populations vaccinated in the 1970s substantiate the viral role in cancer development.

More recently, seroepidemiological evidence was obtained for a correlation between hepatitis C virus (HCV) infections and hepatoma. Antibodies against HCV were detected in between 13% and over 80% of liver cancer patients around the world. Over 60% of acute hepatitis C becomes chronic and may progress to cirrhosis and hepatocellular carcinoma. Latency periods between primary infection and cancer are usually measured in decades but in some cases the intervals are rather short (5–10 years). The cumulative prevalence of hepatoma in cirrhotic HCV-infected patients is over 50%, indicating that HCV substantially increases the risk of hepatocellular carcinoma. HCV is related to flaviviruses and pestiviruses and is the first human tumor-related virus with an RNA genome and no DNA intermediate during replication. The role of the virus in carcinogenesis is not yet clear. Liver injury during chronic hepatitis may be responsible for malignant conversion but there is also some evidence that HCV is more directly involved. HCV appears to persist and replicate in hepatocytes during malignant transformation. The core protein has been shown to cooperate with the *ras* oncogene in transformation of primary rat embryo fibroblasts, to activate transcription of the c-*myc* proto-oncogene and to suppress the c-*fos* promoter. As regulation of cell death is now appreciated as an important factor in the pathogenesis of human malignancies, it is interesting to note that the HCV core protein can suppress cisplatin-mediated apoptosis in human cervical epithelial cells. Core gene variants that predominate in cancerous versus noncancerous lesions may point to a different oncogenic potential of various representatives of the HCV quasispecies. Experimental expression of the nonstructural protein NS3, a serine proteinase, leads to transformation of NIH3T3 cells and it has been speculated that NS3 may cleave and activate cellular proto-oncogene products.

Kaposi's Sarcoma

Kaposi's sarcoma (KS) was first described by Moriz Kaposi as 'multiple idiopathic pigmented sarcoma of the skin' in 1872. It is a multifocal, proliferative lesion of spindle-shaped cells with slit-like vascular spaces in skin and mucous membranes of the oral cavity, gastrointestinal tract and pleura. The tumor cells, termed KS spindle cells, are thought to be of endothelial origin. These tumor cells are likely not monoclonal in the early stages of KS. The 'classic' form of KS is a rare and often benign tumor of elderly males, mostly of Mediterranean or Jewish descent. Usually, the skin lesions of classic KS arise multifocally on the lower limbs and develop from patches to plaques and nodules over several years.

More aggressive, disseminated forms of KS have been described in Africa in the 1950s, and later in immunosuppressed organ transplant recipients. The etiology of this peculiar semimalignant neoplasia has always been an enigma, the favored hypothesis being a model where proliferation and transformation of endothelial cells are triggered in a paraendocrine manner by a host of proinflammatory cytokines and growth factors. Although this model is in agreement with some of the clinical features of this uncommon malignancy, it does not answer the question of what initiates the abnormal release of cytokines. An early hypothesis linked KS to infectious agents. The epidemiology of KS amongst patients with AIDS favored the latter hypothesis.

During the first decade of the AIDS epidemic, KS was at least 20 000 times more common in patients with AIDS than in the general population. Most notably, 20% of homosexual and bisexual patients developed KS, but only 1% of men with hemophilia. By employing the PCR-based representational difference analysis (RDA), Chang, Moore and colleagues searched for DNA sequences present in KS lesions but absent from uninvolved skin. This work resulted in the identification of DNA fragments of a novel human herpesvirus, now termed human herpesvirus 8 (HHV-8) or Kaposi's sarcoma-associated herpesvirus (KSHV). Numerous studies soon revealed that HHV-8 DNA is invariably found in all types of KS, where the majority of KS spindle cells are positive for HHV-8 DNA and transcripts. Searches in normal and diseased tissues other than KS showed that, at least in Northern Europe and the USA, HHV-8 DNA is infrequently detected in Caucasians. Only body cavity-based lymphomas (BCBL) and multifocal Castleman's disease, two rare B-lymphoproliferative disorders, were unequivocally HHV-8 DNA positive.

HHV-8 cell lines established from AIDS-associated cases of BCBL allowed for first studies of HHV-8 serology. In summary, the numerous studies available today clearly show that HHV-8 seropositivity closely reflects the risk of KS development. Antibodies against HHV-8 are usually detectable in 75–95% of KS patients but only in 1–5% of healthy blood donors in Northern Europe and the USA. Although the virus appears to be much more prevalent in Africa and Southern Europe, HHV-8 is closely linked to Kaposi's sarcoma. This is based both on seroepidemiology and DNA detection by PCR and *in situ* hybridization, suggesting a causal relationship between HHV-8 infection/reactivation and KS development.

Studies of HHV-8 genome structure and function also support the hypothesis of viral etiology. The complete genomic sequence revealed that HHV-8 belongs to the rhadinovirus subgroup of herpesviruses, whose best-characterized member is herpesvirus saimiri, an oncogenic virus of New World primates. The HHV-8 genome encodes an unusually high number of genes that have been captured from the host cell. Amongst them are several genes known to be involved in regulation of cell growth (D-cyclins) and apoptosis (bcl-2, v-Flip), signal transduction (IL-6, β-chemokines, IL8-receptor, interferon regulatory factor). Several HHV-8 genes have been found to have transforming potential in cell culture. Although further studies are required to substantiate the mechanism of oncogenesis by HHV-8, it becomes increasingly evident that HHV-8 is a causal factor for KS development.

Future Perspectives

Some two decades ago, only one human virus, EBV, was accepted as a candidate human tumor virus. Since then, the oncogenic potential of human papillomavirus, hepadnavirus and retrovirus has been clearly established. Now it has become evident that virus infections that may result in tumor formation are quite frequent. On a worldwide scale, more than 20% of all malignant tumor forms in females and about 8% of the tumors in males may be the late consequence of a previous virus infection. In most cases, long latency periods of many years or several decades elapse between primary infection by tumor viruses and first symptoms of cancer (**Table 1**). All human tumor viruses are widespread in the world population. They contribute to tumor disease, mainly by initiation of oncogenesis, but are not sufficient by themselves to cause a tumor. Thus, all human tumor viruses are important risk factors for cancer, but require additional events. This implies that mere proof of an infection with a tumor virus is of limited value for the management of patients and cancer prevention. Specific diagnostic tests have to be

Table 1 Latency periods for virus-associated tumors

Virus	*Tumor*	*Latency period (years)*
Hepatitis B virus	Hepatocellular carcinoma	30–50
Human T-cell leukemia virus type 1	T-cell leukemia	20–50
Human herpesvirus 8	Kaposi's sarcoma in immunosuppression	3–10
Human papillomavirus 5, 8	Squamous cell skin carcinoma of patients with epidermodysplasia verruciformis	5–15
Human papillomavirus 16, 18	Cervical cancer	5–25
Human papillomavirus 16, 18	Penile and vulval carcinoma	20–50
Epstein–Barr virus	Burkitt's lymphoma	3–12
Epstein–Barr virus	Nasopharyngeal carcinoma	30–40

Modified from zur Hausen H (1986) Intracellular surveillance of persisting viral infections. *Lancet* ii: 489.

designed which evaluate parameters of the viral infection more closely related to malignant conversion. Often the viruses cannot be traced unambigously in the tumor; conventional virus isolation procedures usually remain unsuccessful: tumor tissues are usually not infectious. The neoplastic phenotype of HPV-positive genital carcinoma cells seems to be affected by viral functions, which raises the prospect of virus-specific pharmacological interference for adjuvant cancer therapy. In many cases, however, continuous viral expression is not detectable in malignant tumors. Primary infection and initial growth transformation apparently lead, through tumor progression, to a constitutive form of proliferation where viral gene products are not necessary for growth and the receptor molecules may no longer be functional. Thus, even in the longer term, antiviral gene therapy may not be successful for the treatment of the tumors, as they have become autonomous. Also in the distant future, the most efficient method for prophylaxis of virus-induced human tumors will probably be vaccination trials to prevent primary infection with the viruses.

See also: **Epstein-Barr virus (*Herpesviridae*): General features, Molecular biology; Hepadnaviruses (*Hepadnaviridae*): Avian hepatitis B virus, General features, Molecular biology; Hepatitis C virus (*Flaviviridae*); Human immunodeficiency viruses (*Retroviridae*): Molecular biology, Antiretroviral agents, General features; Human T-cell leukemia viruses (*Retroviridae*): HTLV-1, HTLV-2; Retroviral Oncogenes; Papillomaviruses – human (*Papovaviridae*): General features, Molecular biology; Transformation: Animal viruses.**

Further Reading

Chang Y, Cesarman E, Pessin MS *et al* (1994) Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* 266: 1865.

Di Bisceglie AM (1995) Hepatitis C and hepatocellular carcinoma. *Semin. Liver Dis.* 15: 64.

IARC Working Group on the Evaluation of Carcinogenic Risks to Humans (1994) *Hepatitis viruses. IARC Monogr.* 59.

IARC Working Group on the Evaluation of Carcinogenic Risks to Humans (1995) *Human papillomaviruses. IARC Monogr.* 64.

Laimins LA (ed.) (1996) Viruses in human cancers. *Semin. Virol.* 7: 293.

Neipel F, Albrecht J and Fleckenstein B (1997) Cell-homologous genes in the Kaposi's sarcoma-associated rhadinovirus human herpesvirus 8: determinants of its pathogenicity? Minireview. *J. Virol.* 71: 4187

Pfister H (1996) The role of human papillomavirus in anogenital cancer. *Obstet. Gynecol. Clin. North Am.* 23: 579.

Pfister H and ter Schegget J (1997) Role of HPV in cutaneous premalignant and malignant tumors. *Clin. Dermatol.* 15: 335.

Rickinson AB (ed.) (1992) Viruses and human cancer. *Semin. Cancer Biol.* 3: 2049.

Smith MR and Greene WC (1991) Molecular biology of the type 1 human T-cell leukemia virus (HTLV-1) and adult T-cell leukemia. *J. Clin. Invest.* 87: 761.

Turkey Herpesvirus *see* **Marek's Disease Virus (Herpesviridae)**

TYMOVIRUSES

Adrian Gibbs, Research School of Biological Sciences, Australian National University, Canberra, Australia

History and Distinguishing Features

Yellow mosaic and vein clearing diseases of several species of brassica found in the UK in the 1940s, were found to be caused by a stable sap and beetle-transmitted virus that was named turnip yellow mosaic virus (TYMV). Other similar viruses have been isolated since and found to form a clearly definable virus genus, which has been named *Tymovirus*, after the first found and type species. They have not been assigned to a family.

Around twenty tymoviruses are known. They infect dicotyledonous angiosperms, cause yellow mosaic diseases, are transmitted by beetles, but rarely by seed. They are readily transmitted experimentally by sap inoculation. Their virions are isometric and 25–30 nm in diameter. Their genomes are single molecules of single-stranded messenger-sense RNA about 6300 nucleotides long; 34–42% cytidylic acid. Their 5′-termini are capped, and most have a tRNA-like 3′-terminus. Their genomes replicate in small vesicles which form as invaginations of the outer membrane of chloroplasts.

Geographic Distribution

Tymoviruses have only been isolated from dicotyledonous plants, most of them wild. Most tymovirus species have natural and experimental host ranges restricted to one or a very few plant families and their geographic ranges are also mostly restricted. The species (and their normal geographic ranges) are Andean potato latent (the Andes), belladonna mottle (Europe), cacao yellow mosaic (Sierra Leone), calopogonium yellow mosaic (Malaysia), chayote mosaic (Central America) clitoria yellow vein (East Africa), desmodium yellow mosaic (USA), desmodium yellow mottle (Singapore), dulcamara mottle (UK), eggplant mosaic (Antilles), erysimum latent (Europe), kennedya yellow mosaic (Australia), melon rugose mosaic (Yemen), okra mosaic (Ivory Coast), ononis yellow mosaic (UK), passiflora yellow mosaic (Brazil), physalis mosaic (USA) (this virus was first called belladonna mottle virus-Iowa strain), plantago mottle (USA), scrophularia mottle (Germany), turnip yellow mosaic (Australia and Europe), voandezia necrotic mosaic (West Africa) and wild cucumber mosaic (USA) viruses, and also poinsettia mosaic virus (worldwide), which is a tentative tymovirus species.

Poinsettia mosaic virus has been carried internationally by the horticultural trade, and closely related, but distinct populations of turnip yellow mosaic virus have been found in Europe and Australia. However, every other tymovirus species seems to be confined to a single continent, and reports of wider distributions result from the use of serological tests, which do not adequately distinguish between tymovirus species. For example belladonna mottle virus was initially reported from both Europe and the USA, and clitoria yellow mosaic virus from both East Africa and Malaysia. However, in both instances, gene sequencing showed that the second record was not the same species; these are now known as physalis mosaic and calopogonium yellow mosaic viruses, respectively.

Host Ranges

Tymoviruses have been isolated from dicotyledonous plants; there is only one unconfirmed report of a tymovirus experimentally infecting a monocotyledonous plant. Most infect plants that use the C3 photosynthetic pathway, and only a very few C4 plants are susceptible. Individual tymoviruses have restricted natural host ranges and usually infect a few species from one family. Most have been isolated from wild plants, whereas species of all other genera/groups of plant viruses have mostly been isolated from crop plants. Most viruses that naturally infect wild plants cause few or no symptoms and do not affect growth, however tymoviruses cause bright yellow mosaic symptoms, and depress the growth of their wild natural hosts, but at least one such species, kennedya yellow mosaic virus, compensates for such damage by protecting its wild plant host against herbivores.

Tymoviruses have broader, but still restricted, experimental host ranges. They infect more species from the family of their natural hosts than from other families, and also a greater proportion of plant species from the same major division of the dicotyledons as their natural hosts than species from other divisions.

Molecular Biology

Virions

Tymoviral virions are isometric c.28 nm in diameter. Their shells are regular icosahedra of 180 subunits of a single protein species that cluster in fives or sixes in the surface of the virion shell to form the 32

morphological subunits, which can be seen in negatively stained virions. Purified virions sediment as two components. Those which sediment at 110–120S are nucleoprotein and each contains a single copy of the viral genome, which constitutes 35% of their mass. Some also contain a variable number of tRNA or virion protein mRNA molecules, and a variable number of polyamine molecules. The other virion component sediments at 50–55S and consists of the protein shell of the virion, and those of the solanaceous tymoviruses and ononis yellow mosaic virus also contain variable amounts of nongenomic RNA molecules.

Genome and encoded proteins

Tymoviral genomes are single molecules of about 6300 nucleotides of single-stranded RNA, and, when separated chemically from the virions, they are infectious. The genomes have a characteristic nucleotide composition; G 15–20%, A 17–24%, C 31–42% and U 20–29%. There are untranslated regions of the genome at both termini and between some open reading frames (ORFs) but these constitute only about 3% of the genome. They have a 5′-terminal m7GpppGp cap structure. Most tymovirus genomes also have a 3′-terminal sequence that can form a tRNA-like structure and that can be specifically valylated, but three tymoviruses, belladonna mottle, dulcamara mottle and erysimum latent, have a 3′-terminal sequence that can only form part of a tRNA-like structure, and dulcamara mottle has a 3′-terminal poly(A) sequence. All tymovirus genomes have three ORFs that are of similar size and arrangement in the genome. The largest is also the most conserved and spans most of the genome. It encodes a large replicase protein (RP: 1747–1874 residues, M_r 194 000–210 000) that has sequence motifs (NH_2- to -COOH-) characteristic of a *N*-methyltransferase, a papain-like protease, a helicase/nucleotide-binding fold and a replicase. Overlapping the 5′-terminal third of the RP, and always starting seven nucleotides to the 5′ side of the RP start codon, is an ORF that encodes an overlapping protein (OP) of variable length (440–750 residues, M_r 49 000–82 000) that is the least conserved of the proteins encoded by the virus. The OPs are very basic (pI 10.9–11.9), and may aid systemic spread of the virus in the plant. The third ORF, at the 3′ end of the genome, is of the virion protein (VP: 188–202 residues, M_r 19 600–21 500). The VP ORF is in different reading frames, relative to the RP/OP gene doublet, in different tymoviruses, and, in some, overlaps by a few nucleotides the 3′-terminal region of the RP ORF.

A region of about 50 nucleotides to the 5′ side of the start of the VP ORF, and hence in the 3′-terminal part of the RP ORF, is similar in all tymoviruses and two blocks of it are particularly conserved. One, which has been named the 'tymobox', is probably the VP mRNA promoter. It is 28–44 nucleotides from the start of the VP ORF and is 16 nucleotides in length. Eleven tymoviruses have tymoboxes with the same sequence (-GAGUCUGAAUUGCUUC-), there is a single nucleotide difference in three, and there are four differences in the tymobox of wild cucumber mosaic virus. Part of the tymobox sequence encodes the sequence, -ELL-, found near the C-terminus of all tymoviral RPs. The second conserved region occurs between the start of the VP ORF and the tymobox, seven or eight nucleotides from the latter. This is the translation initiation box -CAAU- (-CAAG- in one), and includes the 5′-terminal -AAU- found in the VP subgenomic mRNA of three tymoviruses.

The tymobox is useful as a genus-specific target for oligonucleotide probes and primers to isolate and sequence the VP gene of most tymoviruses.

Virion protein structure

The structure of the genome-containing virions of TYMV has been determined to a resolution of 0.32 nm. Each virion has a shell built from 180 identical VP molecules surrounding the folded genome. Each VP molecule is of 189 amino acid residues arranged as an elongated eight-stranded antiparallel β-barrel (jellyroll). The VP molecules are arranged in the shell as pentameric or hexameric clusters at the vertices of an icosahedron with deep furrows at the quasi-threefold axes; the outer radius of the shell is 15.9 nm from its center with the furrows at 12.1 nm. The C-termini of the VP molecules are external to the virion, and the N-termini are internal, and those grouped around the sixfold axes interact to form an annulus. During infection, histidine residues inside the VP may become deprotonated to aid disassembly. The genomic RNA also has some defined structure, possibly partial icosahedral symmetry, and there is a solvent-filled space of radius 2.5 nm at the center of the virion.

Only 73 of the amino acid residues of the VP are exposed at the surface of the virion, however not all are equally immunogenic or antigenic, indeed, despite being internal, the N-terminal peptide is a dominant immunogen. Thus the serological behavior of tymovirus virions is complex, involves only a small number of the amino acid residues of the VP, and may be an unsubtle indicator of tymovirus relationships.

Replication Strategy

Tymovirus genomes probably replicate in the vesicles

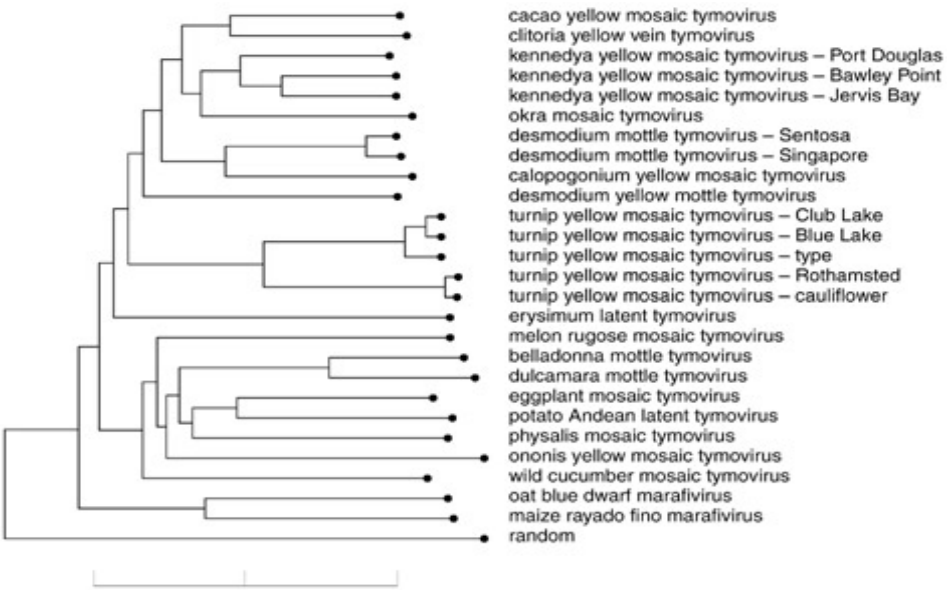


Figure 1 A neighbor-joining tree calculated from the percentage nucleotide differences (excluding gaps) between the virion protein genes of 24 tymovirus isolates and two marafiviruses aligned by the program CLUSTAL V (using default parameters). A random sequence was generated from the alignment by choosing randomly among the different nucleotides that occurred at each position in the alignment, and this was used as an outgroup. The bar represents a 20% nucleotide difference uncorrected for multiple changes.

that form in the margins of the chloroplasts of tymovirus-infected plants; these vesicles have been shown to contain the viral replicase complex and double-stranded RNA 'replicative intermediate' of the virus. It is probable that the OP and RP ORFs are translated directly from genomic RNA, but that of the VP from a subgenomic mRNA is transcribed from the negative genomic strand using the tymobox sequence as a promoter. Tymovirus proteins are produced by cytoplasmic ribosomes. The RP polyprotein is probably *cis*-hydrolyzed into its component parts by a viral protease in the central region of the RP; the first cut is between the helicase and replicase regions, and then the helicase and protease are separated.

Virions of the virus assemble at or near where the chloroplast vesicles connect with the cytoplasm. Virions and genome-free virion shells are found particularly around patches of vesicles, but also throughout the cytoplasm and all parts of infected plants. Virion shells may also accumulate in large numbers in nuclei.

Taxonomy and Evolution

Figure 1 shows a neighbor-joining tree calculated from the nucleotide sequence differences of the virion proteins of 24 tymoviruses. It can be seen that different isolates of each tymovirus species always cluster together, and that species fall into larger groupings that mostly correlate with the family of the plant from which they were isolated. The largest of the groups includes most of the tymoviruses isolated from legumes (**Fig. 1**), these form a sister group to the brassica-infecting tymoviruses, and the remaining major group includes all those isolated from solanaceous plants and cucurbits. The tymoviruses form a sister group to the marafiviruses. The replicase genes are known for seven tymoviruses and one marafivirus and their sequence relationships are totally congruent with those shown by the virion protein genes.

Tymoviruses are variable. Tymovirus isolates that infect the same range of plant species and cause closely similar symptoms are considered to be isolates of a single species. However, they may have virions that differ serologically and in their surface charge, and have genomes with sequence differences of up to 27% (turnip yellow mosaic virus). Some species have distinct subspecies, for example there are two subspecies of turnip yellow mosaic (the type and cauliflower subspecies), and three of kennedya yellow mosaic (the Port Douglas, Jervis Bay and Bawley Point subspecies).

The marafiviruses, which include oat blue dwarf (OBDV), maize rayado fino and Bermuda grass etched line viruses, form a 'sister' genus of the tymoviruses, and are closely related to them. All have particles of

similar size and morphology, and the genome of OBDV has been found to be similar in size (6.509 kb), composition (42.8% C) and sequence to those of the tymoviruses; the complete replicase polyproteins of five tymoviruses differ from one another by up to 65% in sequence, whereas that of OBDV differs from the tymoviruses by 70%. The OBDV genome has a 'tymobox' sequence with 13/16 nucleotide identity to those of most tymoviruses and it encodes the same -ELL- motif. Marafiviruses differ from tymoviruses in several biological features. They mostly infect monocotyledonous plants (only OBDV is known to infect dicotyledons), they seem only to replicate in the phloem or in enations formed by hyperplasia of the procambium of the phloem, and their replication is not associated with chloroplasts or other cell organelles. They have leafhopper vectors and replicate in those vectors. Marafiviruses also differ significantly from tymoviruses in their molecular biology. Their genome does not encode an overlapping protein like that of the tymoviruses, and their virions have proteins of two sizes, that are mostly identical in sequence; the larger is produced by hydrolysis of the genomic transcript and the smaller is transcribed from a subgenomic mRNA, and lacks the N-terminal sequence of the larger protein.

The sequence motifs of the replicase protein (RP) of tymoviruses and marafiviruses also show clear similarities to those of the RPs of the capilloviruses, carlaviruses, closteroviruses and potexviruses, indicating that the RPs of all these viruses have a common ancestor; the RPs of the tymoviruses and OBDV differ from those of potato X and other potexviruses by 90–95% in sequence. The sequence motifs of the RPs also indicate that they may be very distantly related to the 'alpha-like viruses', which include alpha-, furo-, hordei-, rubi-, tobamo-, tobra- and tricornaviruses. Some of these viruses have isometric or bacilliform virions, which may have virion proteins that have a similar β-barrel (jellyroll) structure to those of the tymoviruses, and hence their VP genes may have a shared ancestor. However, all the viruses with filamentous or rod-shaped virions probably have four-stranded α-helix virion proteins encoded by genes unrelated to those encoding the jellyroll VPs. None of the viruses with replicase genes related to those of the tymoviruses has an overlapping (OP) gene, and hence the OP gene probably arose *de novo* in the 'proto-tymovirus'.

Epidemiology and Transmission

The known natural vectors of tymoviruses are all chrysomelid beetles (Halticidae and Galerucidae) except that of TYMV in Australia, which is a pill beetle (Byrrhidae). The vector beetles prefer feeding on plants that are tymovirus-infected rather than virus-free. A few tymoviruses are transmitted by the seed of a limited number of plant species, but not by their pollen. All may be transmitted by sap inoculation, but none by plant contact.

Pathogenicity and Symptoms

Tymoviruses cause yellow mosaics, vein-clearing and mottles. All plants infected with tymoviruses develop small characteristic vesicles within their chloroplasts attached to their outer membranes. These vesicles form as invaginations of the outer chloroplast membranes, and the bilayer membranes of those membranes and the vesicles are confluent.

Prevention and Control

Tymoviruses have mostly been found in uncultivated plants, and none is known to cause economically important diseases of crops.

See also: **Marafiviruses (Marafivirus); Potexviruses.**

Further Reading

Brunt A, Crabtree K, Dallwitz M, Gibbs A and Watson L (eds) (1996) *Viruses of Plants*. Oxon: CAB International.

Canady MA, Larson SB, Day J and McPherson A (1996) Crystal structure of turnip yellow mosaic virus. *Nature Struct. Biol.* 3: 771.

Gibbs A and Mackenzie AM (1998) Tymovirus isolation and genomic RNA extraction. In: Foster GD and Taylor SC (eds) *Plant Virology Protocols. Methods Mol. Biol.* 81: 219.

Hellendorn K, Michiels PJ, Buitenhuis R and Pleij CW (1996) Protonatable hairpins are conserved in the 5′-untranslated region of tymovirus RNAs. *Nucleic Acids Res.* 24: 4910–4917.

Hirth L and Givord L (1988) Tymoviruses. In: Koenig R (ed.) *The Viruses: the Plant Viruses 3*, p. 163. Plenum Press: New York and London.

Ty Elements *see* **Retrotransposons of Fungi**

U

UMBRAVIRUSES

DJ Robinson and **AF Murant**, Scottish Crop Research Institute, Invergowrie, Dundee, UK

History

Several of the viruses now placed in the genus *Umbravirus* have been known since the early days of plant virology but, because of their peculiar properties and the attendant difficulties in working with them, most have been little studied. The first to be described was tobacco mottle virus (TMoV), reported from Zimbabwe and Malawi by K. M. Smith in 1946. TMoV was shown to be mechanically transmissible but to depend on a helper virus that is not mechanically transmissible, tobacco vein distorting virus, for transmission by aphids in a persistent (circulative) manner. The two viruses together caused a disease in tobacco called 'rosette'. Several virus complexes with similar properties are now known and the dependent viruses in these complexes are placed together in the genus *Umbravirus*. The name was coined from Latin, *umbra,* meaning 'shade', 'shadow' and from English, *umbra,* 'a shadow', 'an uninvited guest accompanying an invited one'. All the helper viruses that have been identified are definitive or tentative members of the family *Luteoviridae*.

Most attention has been paid to umbraviruses associated with two diseases of major economic importance, carrot motley dwarf, which is damaging worldwide, and groundnut rosette, which is very serious and epidemic in Africa. The umbravirus carrot mottle virus (CMoV) was first described in 1964 from motley dwarf-diseased carrots in the UK. A similar but distinct umbravirus, called carrot mottle mimic virus (CMoMV), has recently been found in carrots in Australia. In both instances the helper virus is carrot red leaf luteovirus (CaRLV). In 1969, groundnut rosette virus (GRV) and its helper luteovirus, groundnut rosette assistor virus (GRAV), were recognized as causal agents of groundnut rosette disease, but it was not until 1988 that a satellite RNA of GRV was shown to be the main cause of the symptoms of rosette disease. Recently, pea enation mosaic virus (PEMV), a virus hitherto considered to have a bipartite genome and classified as the sole member of the genus *Enamovirus,* has been shown to be a complex of two viruses. PEMV-1, formerly PEMV RNA-1, is classified in the genus *Enamovirus,* family *Luteoviridae,* and PEMV-2, formerly PEMV RNA-2, is considered to be an umbravirus. The above-mentioned umbraviruses and others which are of lesser or unknown importance, are listed in **Table 1**, together with their helper viruses, where known, and their aphid vectors. The tentative members have either not been adequately characterized, or their putative helper virus has not been detected or identified.

Taxonomy and Classification

Umbraviruses have monopartite single-stranded RNA (ssRNA) genomes with a characteristic arrangement of open reading frames (ORFs) (**Fig. 1**). Amino acid sequence comparisons show that the putative RNA-dependent RNA polymerases encoded by the genomic RNA molecules of CMoMV, GRV and PEMV-2 belong to the so-called supergroup 2 of RNA polymerases, as do those of viruses in the genera *Carmovirus, Dianthovirus, Luteovirus, Machlomovirus, Necrovirus* and *Tombusvirus*. These enzymes are the only universally conserved proteins of positive-strand RNA viruses, and their affinities suggest that the genus *Umbravirus* might be considered to be in or close to the family *Tombusviridae*. Presently *Umbravirus* remains a genus unassigned to a family.

Virus Properties

In plants infected by umbraviruses unaccompanied by the helper virus, no particles of the kind associated with most plant viruses can be found by electron microscopy in leaf extracts, but infected leaves yield abundant infective ssRNA. However, the infectivity of CMoV and GRV in buffer extracts of leaves is surprisingly stable, surviving for about a day at room

Table 1 Umbraviruses, their helper luteoviruses and their main aphid vectors

Umbravirus	*Helper luteovirus*	*Main aphid vector*
Definitive Umbravirus species		
Bean yellow veinbanding virus (BYVBV)	Bean leaf roll virus (BLRV) Pea enation mosaic virus-1 (PEMV-1)	*Acyrthosiphon pisum*
Carrot mottle virus (CMoV)	Carrot red leaf virus (CaRLV)	*Cavariella aegopodii*
Carrot mottle mimic virus (CMoMV)	Carrot red leaf virus (CaRLV)	*C. aegopodii*
Groundnut rosette virus (GRV)	Groundnut rosette assistor virus (GRAV)	*Aphis craccivora*
Lettuce speckles mottle virus (LSMV)	Beet western yellows virus (BWYV)	*Myzus persicae*
Pea enation mosaic virus-2 (PEMV-2)	PEMV-1	*Acyrthosiphon pisum*
Tobacco mottle virus (TMoV)	Tobacco vein distorting virus (TVDV)	*M. persicae*
Tentative Umbravirus species		
Sunflower crinkle virus (SCV) (Sunflower rugose mosaic virus)	Unknown	*Aphis gossypii*
Sunflower yellow blotch virus (SYBV) (Sunflower yellow ringspot virus)	Unknown	*Aphis gossypii*
Tobacco bushy-top virus (TBTV)	Unknown	*M. persicae*
Tobacco yellow vein virus (TYVV)	Tobacco yellow vein assistor virus (TYVAV)	*M. persicae*

temperature and for up to 15 days at 4–5°C. It is, however, very sensitive to treatment with organic solvents, and this suggests that the infective RNA is protected in lipid-containing structures. Vesicular structures about 50 nm in diameter occur in the cell vacuoles of CMoV-infected *Nicotiana clevelandii*, often associated with the tonoplast, and have also been found in partially purified preparations. Similar structures have been seen in plant cells infected with bean yellow veinbanding virus (BYVBV), lettuce speckles mottle virus (LSMV) and GRV. At first it was thought that these structures might be virus particles of a kind unusual among plant viruses but it now seems likely that they may be involved in virus replication and/or serve to protect the RNA. The present view is that umbraviruses do not form conventional virus particles but depend on the coat protein of the associated luteovirus for encapsidation and for transmission by the aphid vector of the helper.

Properties of the Genome

The genomes of umbraviruses consist of one linear segment of ssRNA. They are probably not polyadenylated at their 3′ends, but there is no information about structures at their 5′ends. The genomic RNA sequences of CMoMV, GRV and PEMV-2 comprise 4201, 4019 and 4253 nucleotides, respectively. Gel electrophoresis of double-stranded RNA (dsRNA) forms indicates that the genomes of other umbraviruses are of similar size.

The genomes of CMoMV, GRV and PEMV-2 each contain four ORFs (**Fig. 1**). A very short 5′noncoding region is followed by ORF 1, which codes for a potential product of M_r 31 000–37 000. ORF 2 overlaps ORF 1 in a different reading frame and could encode a product of M_r 64 000–65 000. The function of the ORF 1 product is unknown, but the potential ORF 2 product contains sequence motifs characteristic of viral RNA-dependent RNA polymerases. An untranslated region separates ORF 2 from the remaining two ORFs (3 and 4), which overlap one another almost completely in different reading frames. The putative products of these two ORFs are of M_r 26 000–29 000, and the ORF 4 product contains sequences typical of plant virus movement proteins. The ORF 3 products of CMoMV, GRV and PEMV-2 have 42–50% similarity to each other but no significant similarity to any other viral or nonviral proteins. The sequence of the predicted ORF 3 product therefore gives no clue to its possible function.

All three genomes lack genes for plausible coat proteins.

Replication

Strategy of replication of nucleic acid

Leaf tissue of plants infected with umbraviruses contains abundant dsRNA, including a prominent species of about 4.2–4.8 kbp corresponding in size to that expected for a double-stranded form of the genomic RNA. Replication of umbravirus RNA therefore presumably involves the ORF 2-encoded RNA-dependent RNA polymerase and a dsRNA

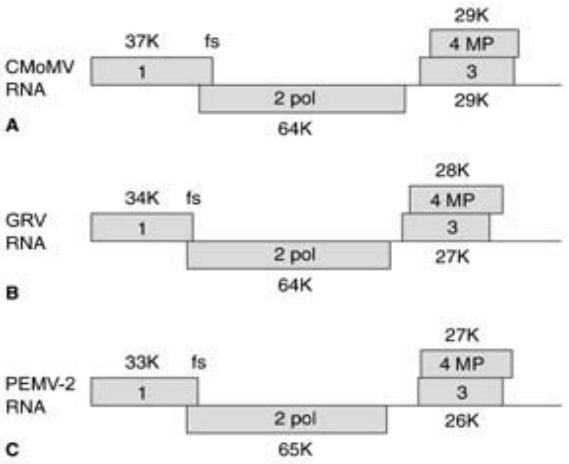


Figure 1 Diagram showing the arrangement of the ORFs in (**A**) CMoMV RNA, (**B**) GRV RNA and (**C**) PEMV-2 RNA. In each case the continuous horizontal line represents the RNA and the numbered blocks the correspondingly numbered ORFs. The M_r of the predicted translation product is shown adjacent to each ORF. ORFs that encode probable RNA-dependent RNA polymerases are marked 'pol', and those that encode probable cell-to-cell movement proteins are marked 'MP'. The positions of probable frameshift events are marked 'fs'. (Adapted from Taliansky M, Robinson DJ and Murant AF (1997) Complete nucleotide sequence and organization of the RNA genome of groundnut rosette umbravirus. *Journal of General Virology* **77:** 2335.)

intermediate, but no further details of the replication mechanism have been elucidated. A second prominent species of dsRNA, present in all plants infected with umbraviruses that have been examined, is of 1.1–1.5 kbp, and may be an intermediate in the synthesis of subgenomic RNA.

Characterization of translation

ORFs 1 and 2 are probably translated from genomic RNA as a single polypeptide of M_r 94 000–98 000 by a mechanism involving a −1 frameshift. ORF 2 does not have an appropriately positioned AUG initiation codon. Moreover, a 'shifty' heptanucleotide sequence is present just upstream of the ORF 1 termination codon and is flanked by sequences that could form stable stem–loop structures. These features are similar to ones associated with frameshifting sites in the genomes of several other plant and animal viruses.

The 1.1–1.5 kbp dsRNA found in umbravirus-infected plants is of the size expected for a subgenomic RNA for ORFs 3 and 4. For CMoMV, this dsRNA has been shown to include the sequences of ORFs 3 and 4, and the 3′ untranslated region. For GRV, corresponding ssRNA has been identified, which runs as two closely spaced bands in gel electrophoresis. Thus it is uncertain whether ORFs 3 and 4 are translated from the same subgenomic RNA species or two different ones.

Satellite RNA

Additional dsRNA species are associated with infection by some umbraviruses and, in the case of GRV, this dsRNA is known to represent a satellite RNA. GRV satellite RNA is found in all naturally occurring isolates of GRV, and is largely responsible for the symptoms of rosette disease in groundnut plants. Different variants of the satellite are responsible for different forms of the disease. Thus the agents of groundnut rosette disease comprise three components: the satellite RNA, GRV acting as a helper for replication of the satellite RNA, and GRAV acting as a helper for the encapsidation and aphid-transmission of GRV and its satellite RNA. Furthermore, for some reason that is not understood, the satellite RNA is also required for the GRAV-dependent aphid transmission of GRV, i.e. unlike most satellites, it is essential for the biological survival (though not the replication) of its helper virus. Most satellite RNA variants do not affect replication of the helper virus but at least one variant strongly downregulates GRV RNA synthesis.

Evolution

As mentioned above, umbraviruses have some affinities with viruses in the family *Tombusviridae* but whether they have lost or never had coat protein genes is idle speculation. Dependence of one virus on another for one or more gene functions is a fairly common feature among plant viruses. In most instances, the dependence of umbravirus on luteovirus is not reciprocated, but the PEMV complex appears to be a notable exception. Like other members of the family *Luteoviridae*, PEMV-1 lacks the ability to move outside the vascular system of infected plants but can 'borrow' the cell-to-cell movement function encoded by the umbravirus PEMV-2. It is thereby released from the confinement to the phloem that is typical of its family and as a result becomes transmissible by manual inoculation. Another member of the *Luteoviridae*, beet western yellows virus, has been reported to acquire limited manual transmissibility when in the presence of LSMV, and this may represent another example of the same phenomenon. The PEMV complex may perhaps illustrate the development of mutual interdependence that may have led to the evolution of multipartite genomes in other virus genera.

Serology

Because umbraviruses do not encode their own coat proteins but depend on their helper viruses for encapsidation, there is no information about serological relationships among umbraviruses.

Geographic Distribution

Carrot motley dwarf and pea enation mosaic diseases occur worldwide wherever their crop hosts are grown. However, it is unclear which of the viruses CMoV or CMoMV is associated with carrot motley dwarf disease in different parts of the world. Groundnut rosette disease occurs only in Africa, south of the Sahara. BYVBV is reported only from Europe, and LSMV only from California, USA. All other umbraviruses are reported only from Africa.

Host Range and Virus Propagation

Individual umbraviruses are confined in nature to one or a few host plant species. Their experimental host ranges are broader but still restricted. The host ranges of umbraviruses overlap, but do not necessarily coincide, with those of their helper viruses. The symptoms induced in infected plants are usually mottles or mosaics.

Experimentally, umbraviruses can be transmitted by manual inoculation, although sometimes with difficulty. They can be propagated in their natural hosts, but often an experimental host is more convenient. *Nicotiana benthamiana* and *N. clevelandii* have proved useful for propagation of CMoV, CMoMV, GRV and several other umbraviruses.

Transmission

In nature, umbraviruses are transmitted by aphids (**Table 1**), but only from plants that also contain a helper virus (**Table 1**). All helper viruses that have been characterized are definitive or tentative members of the family *Luteoviridae*. The mechanism of the dependent transmission is the encapsidation of the umbravirus RNA by the coat protein of the luteovirus. Transmission of the dependent umbravirus therefore occurs in the same persistent (circulative, nonpropagative) manner as that of the helper virus.

Epidemiology

Between crop growing seasons, umbraviruses must survive in plants that are also both hosts of the helper virus and feeding hosts of the vector. Sometimes the aphid vector too will survive on the same plants, but in cold winters in temperate climates, it may survive only in the form of eggs, and these may be laid on an alternate host that is not a host of the viruses. For example, the aphid *Cavariella aegopodii*, the vector of the CMoV/CaRLV complex, lays its eggs on willow (*Salix* spp.), which is not a host of either virus. The aphids produced on willow in the spring are therefore nonviruliferous and must re-acquire the virus complex from a perennial umbelliferous host such as *Anthriscus cerefolium* (cow parsley, Queen Anne's lace), or from overwintering carrot plants.

The components of the pea enation mosaic virus complex infect a wide range of leguminous plant species, of which the most important as overwintering hosts are probably *Medicago sativa* (alfalfa, lucerne) and *Vicia sativa* (common vetch). No nonleguminous natural hosts are known. The most important vector in nature is the pea aphid (*Acyrthosiphon pisum*).

Groundnuts, which originated in South America, are affected by rosette disease only in Africa south of the Sahara, and not in any other parts of the world to which they have been introduced. The causal viruses, GRV and GRAV, must therefore have come from wild plants indigenous to Africa. However, attempts to find an African plant species that is a host of both viruses have so far been unsuccessful. The viruses may now survive in overseasoning groundnut plants. Groundnut rosette is present in most seasons, but periodically becomes epidemic, causing devastating losses of great economic and social importance. The factors that determine the onset of epidemics are not understood, but are undoubtedly linked with the biology of the vector, *Aphis craccivora*.

Little is known about the ecology and epidemiology of other umbraviruses.

Pathology and Histopathology

Although all umbraviruses depend on a helper virus for transmission by vector insects, several of them are equally or more important than their helpers in the causation of disease symptoms. The umbravirus of greatest economic importance is GRV, which causes the most devastating virus disease of groundnut in Africa. However, in this case, as already mentioned, it is a GRV-dependent satellite RNA that is the actual cause of the symptoms.

Umbraviruses, even in the absence of their helper viruses, exhibit rapid systemic spread in plants. They infect cells throughout the leaf, though presumably the aphid-transmissible particles are restricted to the same tissues (in most instances the phloem) as the luteoviruses that provide their coat protein. In mesophyll cells infected with CMoV there is extensive development of cell wall outgrowths sheathing elongated plasmodesmatal tubules.

Prevention and Control

Vector Control

Preventing the spread of umbraviruses by controlling the aphid vector is rarely practical commercially, because severe disease outbreaks are erratic and difficult to predict. Also, especially in the case of GRV, which occurs mostly in the poorer African countries, use of insecticides may be too expensive. However, in many countries, carrot crops are sprayed intensively with insecticides, primarily for the prevention of carrot fly (*Psila rosae*) infestation, and this incidentally provides effective control of carrot motley dwarf disease.

Avoidance of Infection

For the avoidance of pea enation mosaic disease, it is sometimes recommended that pea or faba bean crops should be sited away from alfalfa or clover fields. In Africa, early planting and close spacing of groundnut are effective ways of avoiding rosette disease, because the flying aphid vector has difficulty in recognizing the crop once the plant cover is complete. This approach is also effective against tobacco rosette disease.

Host Plant Resistance

If resistant plant cultivars are available, this is undoubtedly the best approach. Resistance to GRV, inherited as a double recessive character, is found in some groundnut germplasm, and groundnut lines possessing this resistance have been developed, though they are often not preferred for commercial or cultural reasons.

See also: **Vector transmission of plant viruses; Luteovirus; Pea enation mosaic virus (*Luteoviridae*).**

Further Reading

Demler SA, de Zoeten GA, Adam G and Harris KF (1996) Pea enation mosaic enamovirus: properties and aphid transmission. In: Harrison BD and Murant AF (eds) *The Plant Viruses, Polyhedral Virions and Bipartite RNA Genomes*, vol. 5, p. 303. New York: Plenum Press.

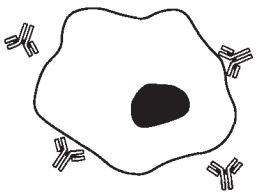
Murant AF (1990) Recent research on the aetiology of groundnut rosette disease. *Ann. Rept. Scott. Crop Res. Inst. 1989* p. 138.

Murant AF (1993) Complexes of transmission-dependent and helper viruses. In: Matthews REF (ed.) *Diagnosis of Plant Virus Diseases*, p. 333. Boca Raton: CRC Press.

***Ustilago Maydis* Viruses** *see* **Totiviruses**

V

VACCINES AND IMMUNE RESPONSE



Gordon L Ada, Australian National University, Canberra, Australia

History

Any history of vaccination to control viral infections, however brief, begins with the story of smallpox and its final eradication. There were bicentenary celebrations in 1996 to celebrate the famous experiment of Edward Jenner in which he inoculated a boy, James Phipps, with cowpox material and demonstrated that he subsequently resisted a challenge with smallpox. Almost 100 years later, Louis Pasteur developed the rabies virus vaccine, and in 1935, an attenuated viral preparation to combat yellow fever was made and remains to this day a safe and effective vaccine. The first inactivated influenza virus vaccine became available in 1936. The production of these two vaccines was possible because of the newly developed technique of growing virus in the embryonated eggs of chickens.

However, the major event that accelerated viral vaccine development was the ability to grow many viruses in tissue culture. This began in the 1920s, but it was the studies by John Enders and colleagues in successfully growing polioviruses in cell culture which initiated an explosion of activity in this field. Jonas Salk produced the trivalent formalin-inactivated poliovirus vaccine and this was followed shortly afterwards by the oral live attenuated polio vaccines of Albert Sabin. Most viral vaccines in use today and those in clinical trials and probably close to registration (**Table 1**) are attenuated preparations.

A subunit preparation consisting of the surface glycoproteins of influenza virus, was subsequently made and is widely used because of its lower reactogenicity compared to whole virus. A new era of viral vaccine development was initiated when the hepatitis B surface antigen, originally isolated from infected blood, was prepared from DNA-transfected yeast cells for use as a vaccine.

Smallpox was declared eradicated in 1980, three years after the last case of endemic smallpox was detected, and nearly 14 years after the World Health Organization (WHO) had begun an intensified campaign to eradicate this disease. In 1985, the Pan American Health Organization (PAHO) initiated a program to eradicate indigenous wild-type polio from the Americas, and eradication was declared in 1994, three years after the last case was detected. WHO has declared the year 2000 as the target date for global eradication and there is a reasonable chance that this will be achieved. Following elimination of indigenous measles from some countries (e.g. Finland, the Caribbean Islands), PAHO has nominated the year 2000 as the date for eradication of this disease from the Americas.

Two Main Requirements of a Vaccine

The safety of a candidate vaccine has become of prime importance. Most of the vaccines listed in **Table 1** are very safe, but two in particular are less so. During the eradication campaign, most smallpox vaccines (vaccinia virus) had a level of side effects which made them unacceptable for current use. Safer preparations have since been made by the deletion of specific DNA sequences. Type 3 poliovirus in OPV can revert to virulence, and poliomyelitis occurs at the rate of about 3×10^6 doses of vaccine in recipients or their close contacts. Attenuated live vaccines may pose a risk to immunodeficient or -compromised people, but at least in the case of measles virus, even this is a very low risk.

The second requirement is efficacy, which is assessed as the ability to protect recipients from disease after a subsequent exposure to the wild-type infectious agent. Vaccine efficacy depends almost entirely on the nature and persistence of the induced immune response. Vaccines are usually given prophylactically and, as the time interval between vaccination and the subsequent exposure to challenge may be short (weeks) or long (years), efficacy depends on stimulation of the adaptive immune response, the

Table 1 Viral vaccines

	Infectious agents	
Type	*Current vaccines*	*Products close to registration*
Live, attenuated	Vaccinia	Cytomegalo
	Measles	Hepatitis A
	Yellow fever	Influenza
	Mumps	Dengue
	Polio (OPV)[a]	Parainfluenza
	Rota[a]	Japanese encephalitis
	Adeno[a]	
	Rubella	
	Varicella-Zoster	
Inactivated	Polio (IPV)	Hepatitis A
	Infuenza	
	Rabies	
	Japanese encephalitis	
Subunit	Hepatitis B	
	Influenza	
Combination	Measles, mumps, rubella (MMR)	

[a] Administered orally

OPV, Live polio virus; IPV, Inactivated polio virus.

lymphocytes, which have immunological memory. If a vaccine is used therapeutically, which may be deliberate or occur naturally if the vaccine is used in an endemic area, activated innate components may contribute to a protective effect. The efficacy of many vaccines may be predicted by the level of protective antibodies induced by vaccination, but where antigenic variation occurs, a high level of cell-mediated immune (CMI) responses may indicate a degree of protection.

Roles of antibodies

Specific antibody has three main roles: (1) to neutralize the infectivity of a virus; (2) to lyse infected cells which express viral antigens at the cell surface; and (3) to complex with viral debris from infected cells and help in its removal. Following vaccination, the continuing presence of specific antibody of increasing affinity which will bind to virus and prevent infection is regarded by vaccinologists as the most critical requirement of a vaccine. Many viruses naturally infect via a mucosal surface, and here, secretory immunoglobulin A (s.IgA) fulfills this role. In contrast, many vaccines are given parenterally, in which case, the IgM and IgG formed does not prevent the initial infection, but should prevent systemic spread, eg. polio and measles infection.

The mechanism of infectivity prevention by antibody varies between viruses, and even different immunoglobulin isotypes may have different effects. Viral replication may be prevented whether the antibody prevents virus adsorption to the cell, entry into the cell or expression of the viral genome.

Antiviral antibody may bind to antigens expressed on the infected cell plasma membrane and cause lysis – antibody-dependent cellular cytotoxicity (ADCC). Alternatively, lysis may be caused by certain antibody isotypes binding complement and the complex then binds to complement receptors on the infected cell surface. Antibody has been shown to clear some viral infections in T cell-deficient mice, eg. influenza and vaccinia virus infections. In contrast, antibody is inefficient at clearing ectromelia, lymphocytic choriomeningitis virus (LCMV) or Theiler's virus, which are natural pathogens of mice. However, in persistent infections such as LCMV in mice, antibody can prevent virus reaching sites such as the epidermis where CTLs are less effective.

Viral epitopes recognized by protective antibodies Generally, only one or a few antigens of a virus, those exposed to the medium, contain epitopes recognized by neutralizing antibodies. For the virus to survive extracellularly, it must be resistant to enzymes, particularly proteases, and this is usually achieved by having polymers of viral antigens – trimers (influenza hemagglutinin and human immunodeficiency virus envelope) or multimers (VP1 of polio) – so that the important epitopes are usually discontinuous sequences.

Roles of effector T cells

There are two classes of T cells – CD4+ (class II MHC-restricted) and CD8+ (class I MHC-restricted) – and two subclasses of each – CD4+Th1 and Th2, and CD8 + Tc1 and Tc2. They are distinguished by their patterns of cytokine secretion. Because of similarities in these patterns, Th2 and Tc2 are called type 2 T cells, and Th1 and Tc1, type 1 T cells (*see also* **Immune Response, general features**). The main role of Th2 cells is 'helping' B cells make antibody. Th1 cells do this to a lesser extent, but in contrast to Th2 cells, they mediate delayed-type hypersensitivity reactions. From a vaccine development point of view, a major role of Tc1 is their ability to lyse virus-infected cells a long time before viral progeny is formed, so they are frequently called cytotoxic T cells (CTLs). **Table 2** lists some examples of viral infections in mice where there has been clearance or control of virus replication following transfer of specific CTLs.

Table 2 Examples of clearance or control of virus replication in infected mice following transfer of specific CD8+ cytotoxic T cells (CTLs, Tc1)

Orthomyxovirus (influenza virus)
Paramyxovirus (Sendai virus)
Poxvirus (vaccinia virus)
Pneumovirus (respiratory syncytial virus)
Arenavirus (lymphocytic choriomeningitis virus)
Alphaherpesvirus (herpes virus)
Betaherpesvirus (cytomegalovirus)

There are 'experiments of nature' which suggest a protective role for CTLs in infected people. In four situations where people were known to have been exposed to HIV but who were seronegative and virus could not be isolated, CTLs specific for known HIV CTL epitopes could be isolated from their blood.

Though cultured or cloned Th1 cells may also be cytotoxic, primary cells (without *in vitro* culture) are rarely so. Th (presumably Th1) cells have been shown to be protective in a herpes 2 virus infection – the zosteriform spread model. *In vitro,* CTL production is usually enhanced by the presence of TH1 cells, but *in vivo,* there are several examples where 'normal' levels of CTL activity were found following a viral infection in doctored mice lacking CD4+ T cells. However, it has also been shown that if the virus infection persists, CTL activity decreases over time compared to the situation in normal mice, which is similar to what occurs in many HIV-infected individuals when levels of CD4+ T cells greatly decrease and CTL activity disappears.

Sources and properties of T cell epitopes In contrast to the restricted number of viral antigens possessing epitopes recognized by neutralizing antibodies, potentially many viral antigens may contain T cell epitopes. They may be detected as follows.

1. Individual antigens are screened, sometimes using cells infected by a chimeric live vector.
2. A series of overlapping peptides is synthesized and each is tested for its ability to sensitize a target cell for lysis by CTLs.
3. Whereas peptides binding to class II MHC antigens vary considerably in length, averaging about 15 amino acids, those binding to class I MHC antigens are more homogeneous, the great majority being nonamers. The nonamers have a motif specific for MHC molecules of different allelic specificities, possessing two anchor positions that are critical for ligand binding. This has allowed protein sequences to be screened to find ligands potentially specific for different MHC antigens.
4. Peptide ligands can be 'stripped' from an infected cell and sequenced.

There were some general findings.

1. Where comprehensive studies have been made, e.g. influenza virus and HIV, the great majority of antigens possess potential CTL epitopes. With flaviviruses, the nonstructural proteins are the major source of CTL epitopes.
2. With both influenza and HIV, the internal antigens contain conserved sequence epitopes.
3. Not infrequently, one or a few epitopes may be dominant and these may be in a variable region or subject to mutation, both being potential mechanisms for the virus to escape immune control. A vaccine might need to include less dominant epitopes from conserved regions where mutations do not occur.
4. Even though a peptide will bind firmly to a MHC antigen of a given specificity, not all people with that allelic specificity but differing in the rest of the haplotype will respond to that peptide, because of crosstolerance with different self peptides.

Secondary Responses

Immunization/vaccination should achieve two major goals. The first is the continuing presence of high affinity specific antibody which will generally neutralize most (>95%) of the challenge virus. Probably, vaccines rarely if ever give 'sterilizing' immunity (100% prevention of infection). If sufficient virus escapes neutralization by pre-existing antibody to infect enough antigen presenting cells (APCs) the second effect is an enhanced CMI response, particularly CTLs. *In vitro,* limit dilution analyses show that following priming, there is an increase in the frequency of T cells of the particular specificity. However, these memory cells are also more easily activated than naive cells, and this may be a more important reason why a more rapid and greater CTL response is seen.

If the antigenic specificity (for neutralizing antibody) of the challenge virus differs grossly from that of the virus used for vaccination, as happens when antigenic shift occurs with influenza, or between different clades of HIV, the control and recovery from the challenge infection will depend very largely on an enhanced CMI response.

Table 3 Some factors favoring or obstructing the development of an effective viral vaccine

Favoring
1. Infection by wild-type agent induces immunity which clears the infection
2. Few important serotypes (DNA viruses); little or no antigenic drift (RNA viruses)
3. Antigens that are a source of protective B cell epitopes or important T cell epitopes are readily identified
4. The virus is not highly infectious
5. Infection is confined to one or few organs, e.g. the lung with influenza virus
6. There is readily available a relatively inexpensive animal model that mimics the human infectious process with onset of characteristic disease
Obstructing
7. Marked antigenic shift or drift
8. Viral DNA/cDNA integrates into the host cell genome; latency occurs
9. Virus infects a variety of cells distributed widely throughout the body
10. A latent infection may be transmitted by infected cells
11. Protective immunity does not occur after natural infection
(a) infection persists due to suppression/evasion of CMI responses;
(b) escape mutants (T and B cells) occur over time
12. Crucial cells of the immune system are infected or affected
13. Antibodies may enhance infection of susceptible cells
14. Lack of a suitable animal model

Protection Following Immunization by Different Routes

The area of mucosal surface in mammals is far greater than the area of skin, and many viruses infect via this route. The main sites of entry are oral, rectal, respiratory, urogenital and ocular. Following vaccination via a mucosal route such as the gut, the first line of defense is s.IgA, and the CMI responses originate from local lymphoid tissues such as the Peyer's patches. For example, OPV induces both a mucosal and systemic response whereas IPV induces only the latter which, however, is sufficient to prevent infection of the central nervous system and poliomyelitis. Measles vaccine is given parenterally; viremia occurs so that some virus reaches the respiratory tract and a mucosal, as well as sytemic response, occurs. There is still interest in administering this vaccine intranasally to achieve a stronger mucosal response which might also be less affected by maternally derived antibody.

There is a 'common mucosal system' so that an immune response induced say in the gut will cause traffic of immune cells to other mucosal sites. An example is the adenovirus vaccine. This is given orally but induces significant protection in the respiratory tract where the natural infection occurs. In contrast, using this approach, it seems to be more effective to administer antigen intranasally rather than orally to achieve immunity in the urogenital tract. For reasons that are not yet well understood, mucosal immunity tends to be shortlived.

Immunological Requirements of a Vaccine

1. Activation of APCs to facilitate antigen processing and enhancement of costimulator and cytokine production;
2. Stimulation of both T and B cells so that there is a high yield of memory cells;
3. Generation of type 1 and 2 T cells to several epitopes, preferably in conserved regions, to overcome the variation in the immune response in the population due to MHC polymorphism;
4. Persistence of antigen in its natural conformation on follicular dendritic cells (FDCs) in lymphoid tissues, where B memory cells are formed and recruited to form antibody-secreting cells (ASCs) so that antibody of high affinity is continually present.

Factors Affecting the Feasibility of Vaccine Development

Table 3 lists some of the factors which make it more straightforward to develop a vaccine against a virus, and these can be contrasted with those which tend to increase the difficulty. It can be readily seen that most of the latter points apply to HIV.

See also: **Immune response: General features, Cell mediated immune response; Persistent viral infection; Virus structure: Principles of virus structure.**

Further Reading

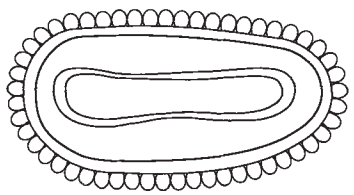
Ada GL and McElrath MJ (1996) Perspective. HIV-1 vaccine-induced cytotoxic T cell responses: potential role in vaccine efficacy. *AIDS Res. Hum. Retrov.* 13: 243.

Ada G and Ramsay A (1997) *Vaccines, Vaccination and the Immune Response*. Philadelphia: Lippincott–Raven.

Plotkin SA, Mortimer EA and Orenstein W (eds) (1998) *Vaccines*, 3rd edn. Philadelphia: WB Saunders.

Stratton KR, Howe CJ and Johnston RB (1994) *Adverse Events Associated with Childhood Vaccines: Evidence Bearing on Causality*. Washington, DC: Institute of Medicine, National Academy Press.

VACCINIA VIRUS (*POXVIRIDAE*)



Riccardo Wittek, Université de Lausanne, Lausanne, Switzerland

History

In 1980, the World Health Assembly announced that smallpox, once the most serious infectious disease of humankind, had been eradicated from all the countries of the world. Several factors were important in making the eradication campaign a unique success in the history of medicine. Among these was the active role played by WHO which organized the campaign and provided expertise in all relevant areas. Furthermore, variola virus, the agent of smallpox, had no animal reservoir and consequently, interrupting the chain of human-to-human transmission eliminated the virus. Finally, with vaccinia virus, an excellent vaccine for smallpox was available. The vaccine had a low production cost, could be freeze-dried and in this form was very heat stable obviating the need of a cold chain. In addition, the vaccine was easy to administer even by relatively untrained field workers and left a characteristic scar providing permanent evidence of vaccination which was important for WHO in assessing vaccination coverage.

It is not known when vaccinia virus was introduced as a smallpox vaccine. Edward Jenner, who was the first to demonstrate, at the end of the 18th century, that inoculation of a related poxvirus provided protection against challenge with variola virus, isolated his vaccine virus from a milkmaid infected with 'cowpox'. It is unclear what this virus was. Although, in modern times, vaccinia virus was occasionally isolated from skin lesions of infected cows, these cases were usually associated with contact with vaccinated humans and therefore, the cow does not seem to be the natural host of the virus. The origin of vaccinia virus thus remains a matter for speculation (see also: 'Evolution').

Taxonomy and Classification

Vaccinia virus is a member of the family *Poxviridae* which comprises a group of complex animal DNA viruses. The family is further subdivided into the two subfamilies, *Chordopoxvirinae* and *Entomopoxvirinae* whose representatives infect vertebrate and insect hosts, respectively. Within the subfamily, *Chordopoxvirinae* vaccinia virus belongs to the genus *Orthopoxvirus*.

Properties of the Virion

Poxviruses are among the largest and most complex viruses known. The particles have a typical brick-shape and measure about 300 × 250 nm. Two types of particles can be distinguished. Virions that are naturally released from the infected cells are surrounded by a Golgi-derived envelope. Virions that are released by experimental cell lysis lack this envelope, but are also infectious. Electron microscopy of negatively stained specimens reveals that the surface of nonenveloped particles is composed of tubular structures ('surface tubules'), which give the particles a characteristic appearance (**Fig. 1A**). However, more recent cryoelectron microscopy (**Fig. 1B**), which allows examination of hydrated virus particles, provided no evidence of surface tubules and it is conceivable that these structures represent a shrinkage artifact resulting from dehydration of particles prepared for electron microscopy by conventional procedures.

In thin sections of virions examined by conventional electron microscopy, the internal core structure can be visualized, which consists of an oval biconcave disk. The concavities of the core accommodate the two lateral bodies. Again, these structures are not

Further Reading

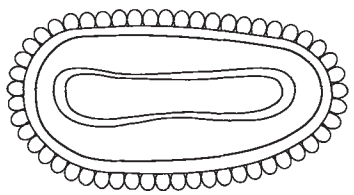
Ada GL and McElrath MJ (1996) Perspective. HIV-1 vaccine-induced cytotoxic T cell responses: potential role in vaccine efficacy. *AIDS Res. Hum. Retrov.* 13: 243.

Ada G and Ramsay A (1997) *Vaccines, Vaccination and the Immune Response*. Philadelphia: Lippincott–Raven.

Plotkin SA, Mortimer EA and Orenstein W (eds) (1998) *Vaccines*, 3rd edn. Philadelphia: WB Saunders.

Stratton KR, Howe CJ and Johnston RB (1994) *Adverse Events Associated with Childhood Vaccines: Evidence Bearing on Causality*. Washington, DC: Institute of Medicine, National Academy Press.

VACCINIA VIRUS (*POXVIRIDAE*)



Riccardo Wittek, Université de Lausanne, Lausanne, Switzerland

History

In 1980, the World Health Assembly announced that smallpox, once the most serious infectious disease of humankind, had been eradicated from all the countries of the world. Several factors were important in making the eradication campaign a unique success in the history of medicine. Among these was the active role played by WHO which organized the campaign and provided expertise in all relevant areas. Furthermore, variola virus, the agent of smallpox, had no animal reservoir and consequently, interrupting the chain of human-to-human transmission eliminated the virus. Finally, with vaccinia virus, an excellent vaccine for smallpox was available. The vaccine had a low production cost, could be freeze-dried and in this form was very heat stable obviating the need of a cold chain. In addition, the vaccine was easy to administer even by relatively untrained field workers and left a characteristic scar providing permanent evidence of vaccination which was important for WHO in assessing vaccination coverage.

It is not known when vaccinia virus was introduced as a smallpox vaccine. Edward Jenner, who was the first to demonstrate, at the end of the 18th century, that inoculation of a related poxvirus provided protection against challenge with variola virus, isolated his vaccine virus from a milkmaid infected with 'cowpox'. It is unclear what this virus was. Although, in modern times, vaccinia virus was occasionally isolated from skin lesions of infected cows, these cases were usually associated with contact with vaccinated humans and therefore, the cow does not seem to be the natural host of the virus. The origin of vaccinia virus thus remains a matter for speculation (see also: 'Evolution').

Taxonomy and Classification

Vaccinia virus is a member of the family *Poxviridae* which comprises a group of complex animal DNA viruses. The family is further subdivided into the two subfamilies, *Chordopoxvirinae* and *Entomopoxvirinae* whose representatives infect vertebrate and insect hosts, respectively. Within the subfamily, *Chordopoxvirinae* vaccinia virus belongs to the genus *Orthopoxvirus*.

Properties of the Virion

Poxviruses are among the largest and most complex viruses known. The particles have a typical brick-shape and measure about 300 × 250 nm. Two types of particles can be distinguished. Virions that are naturally released from the infected cells are surrounded by a Golgi-derived envelope. Virions that are released by experimental cell lysis lack this envelope, but are also infectious. Electron microscopy of negatively stained specimens reveals that the surface of nonenveloped particles is composed of tubular structures ('surface tubules'), which give the particles a characteristic appearance (**Fig. 1A**). However, more recent cryoelectron microscopy (**Fig. 1B**), which allows examination of hydrated virus particles, provided no evidence of surface tubules and it is conceivable that these structures represent a shrinkage artifact resulting from dehydration of particles prepared for electron microscopy by conventional procedures.

In thin sections of virions examined by conventional electron microscopy, the internal core structure can be visualized, which consists of an oval biconcave disk. The concavities of the core accommodate the two lateral bodies. Again, these structures are not

seen by cryoelectron microscopy and their significance remains to be established. Cores prepared by treatment of virions with 2-mercaptoethanol and a nonionic detergent have a similar appearance both by conventional and cryoelectron microscopy (**Fig. 1C**).

Properties of the Genome

Vaccinia virions contain a large double-stranded DNA genome which has been entirely sequenced in the Copenhagen strain and shown to consist of 191 636 base pairs. Characteristic features of the molecules are the crosslinks that join the two DNA strands at both ends. The terminal 100 nucleotides or so consist of single-stranded loops. The genome is further characterized by the presence of long inverted terminal repeats of about 10 kbp. The terminal 3.5 kbp of these are mainly composed of a tandemly repeated 70 bp sequence.

Properties of the Proteins

The DNA sequence of the viral genome provides evidence for the presence of about 200 potential protein-coding sequences, but only relatively few proteins have been assigned a function. The majority of polypeptides with known or suspected functions are enzymes involved in nucleic acid metabolism. Examples are the subunits of RNA polymerase, enzymes involved in capping and polyadenylation of mRNA, DNA polymerase, thymidine and thymidylate kinase, DNA ligase and several more. Other proteins are structural components of the virion. Together with some enzymes, which are also packaged in the virus particle, the total number of virion polypeptides may be as high as 100. The envelope of extracellular virions contains at least six proteins, five of which are glycosylated. The 37 kDa major envelope antigen is acylated.

Physical Properties

The development of a stable smallpox vaccine was greatly facilitated by the fact that the infectivity of vaccinia virus is relatively unaffected by environmental conditions which inactivate most other viruses. This property, together with the large particle size, was also the reason why vaccinia virus was the first

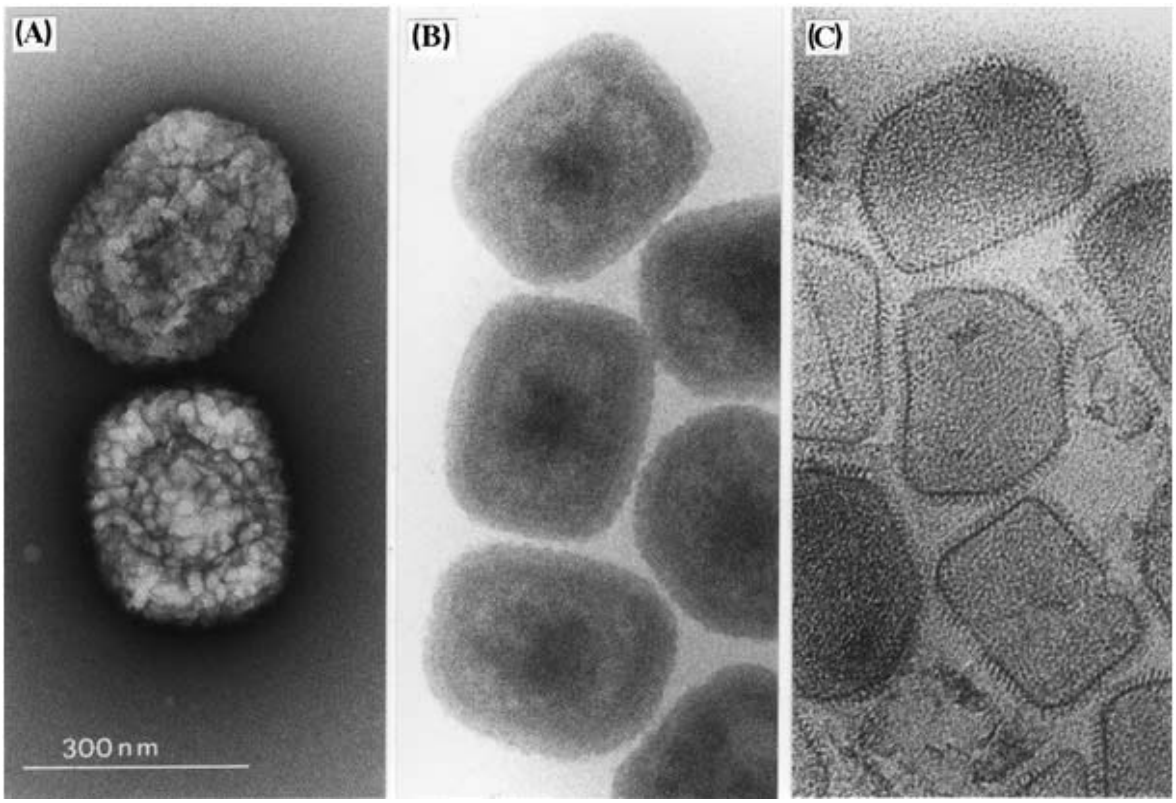


Figure 1 Structure of vaccinia virus including the viral core. (**A**) Conventional preparation of the virus by negative staining. (**B**) Vitrified sample observed by cryoelectron microscopy. The virus particles are in the native state and are floating in a thin layer of vitrified solution kept for observation at c. −160°C and are neither stained nor chemically fixed. (**C**) Core particles prepared as in (**B**). (Courtesy of M. Adrian and J. Dubochet.)

animal virus to be purified extensively. Heating at 55°C for 60 min, or at 50°C for 90 min completely destroys infectivity. Other methods for destroying infectivity were tested in attempts to produce an inactivated smallpox vaccine. Ultraviolet irradiation, formaldehyde treatment, photodynamic inactivation with methylene blue and gamma irradiation were all found to inactivate vaccinia virus, but some of these procedures also resulted in a loss of antigenicity.

Complete solubilization of vaccinia virus is achieved by heating at 100°C in the presence of sodium dodecylsulfate and reducing agents.

Strategy of Replication of Nucleic Acid

Poxviruses are unusual among DNA viruses in that their replication cycle takes place in the cytoplasm of the infected host cell. Upon penetration, the virus sheds its outer protein layers resulting in the release of the viral core containing the DNA and enzymes involved in the synthesis and modification of mRNA. A first burst of RNA synthesis occurs. Following expression of the early genes, the DNA is released from the core and replicated. This allows expression of the intermediate genes, the transcription of which requires a naked DNA template. After transcription of intermediate genes, late genes, many of which encode structural proteins, are expressed.

Replication of the genome occurs through synthesis of long concatemeric intermediates which are subsequently resolved into unit-length genomes. Concatemer resolution is a highly specific process and depends on a 20 bp element located adjacent to the hairpin loop in the mature DNA molecule. For a long time DNA replication itself was not believed to require specific origins of replication since any DNA transfected into vaccinia virus-infected cells is replicated to some extent. More recently, however, it was shown that optimal replication efficiency depends on 200 bp of sequences from the viral telomere.

Characterization of Transcription

Transcription of the different temporal classes of vaccinia virus genes requires specific promoters, which are short, a multisubunit RNA polymerase and stage-specific transcription factors.

Transcription of early genes depends on early promoter elements, which are about 30 bp long. An early transcription factor binds to these regulatory sequences. Termination of early transcription requires the virus-encoded capping enzyme and occurs about 50 bp downstream of a UUUUUNU signal in the nascent RNA chain. Early mRNAs are capped and polyadenylated and typically contain short 5′ untranslated leader sequences.

Intermediate gene transcription requires the presence of the viral capping enzyme and two intermediate gene transcription factors which are able to activate intermediate gene transcription only after the DNA has been replicated. Interestingly, one of the intermediate gene transcription factors has also been purified from uninfected HeLa cells but not from nonpermissive cells. This finding challenges the dogma that exclusively virus-encoded proteins are used for transcription in vaccinia virus.

Late gene promoters also consist of about 30 bp and contain the highly conserved TAAAT motif in which transcription initiation occurs. Late mRNAs are heterogeneous in length, are polyadenylated and have a capped poly(A) leader sequence of about 35 Å residues. The leader is not encoded in the genome but is produced by stuttering of RNA polymerase in the TAAAT motif. In addition to RNA polymerase, four transcription factors are required for late gene transcription. Three of these are products of intermediate genes, the fourth is synthesized both before and after DNA replicaton.

Transcription in vaccinia virus is characterized by a cascade in which transcription of each temporal class of genes requires the presence of specific transcription factors which are made by the preceding temporal class of genes. Thus, early gene transcription factors are made late in infection, packaged in virions and used in the subsequent round of infection. Intermediate gene transcription factors are encoded by early genes, and late transcription factors by intermediate genes. The recent discovery of a cellular protein involved in intermediate gene transcription and the fact that one late gene transcription factor is made before and after DNA synthesis, requires some revision of the cascade model.

Characterization of Translation

Infection of cells by vaccinia virus results in rapid shut-off of host cell DNA, RNA and protein synthesis. Phosphorylation of ribosomal proteins and short polyadenylated viral RNAs has been implicated in the inhibition of cellular protein synthesis.

The preferential translation of temporal classes of mRNAs is probably a direct consequence of the half-life and abundance of mRNAs at a given time of infection.

Post-translational Processing

Several types of post-translational processing events occur in the maturation of viral proteins. The mature

brought in by a co-infecting virus. The gene to be expressed, under the control of the T3 or T7 promoter elements, is introduced into the cells either as an expression plasmid or as part of a vaccinia virus vector. These approaches allow for controlled expression, which is especially important when the protein product is cytotoxic.

Poxvirus expression systems have been used to express hundreds of proteins of biological and medical importance. The practical application of vaccinia virus vaccine candidates has been demonstrated in both the veterinary and human fields. Vaccinia was used successfully as a vaccine for the eradication of smallpox. Other examples of its use include vaccinia virus recombinants to protect raccoons against rabies, a capripoxvirus vector to protect cattle against rinderpest, a swinepox vector to protect pigs against pseudorabies, and fowlpox vectors to protect chickens against influenza virus, Newcastle disease virus and infectious bursal virus. Clinical trials with a vaccinia-based HIV-1(IIIB) *env* recombinant have been initiated in the USA. Results from these studies clearly demonstrated the ability of this recombinant to prime an immunological response in the recipients, although the response was suboptimal in some individuals. A vaccinia virus-based vector expressing the Epstein–Barr virus membrane glycoprotein has been used in China with some success in preventing natural infection.

New vectors have recently been developed that address some of the safety concerns of the original vectors. A strain of vaccinia virus with 18 genes implicated in the virulence and host range of the virus deleted has been developed. Another vector, based on a canarypoxvirus, replicates only in avian species. When this vector is introduced into nonavian cells, the transgene is expressed. Preliminary studies demonstrate that both are effective in generating high levels of transgene expression, and that in a vaccine setting they can both induce protective immunity. A canarypoxvirus-based vector expressing the rabies G glycoprotein has been assessed in phase I clinical trials in Europe and the USA, with encouraging results. In addition, vectors expressing the human cell surface protein CD80 are in clinical trials. This protein is involved in T cell recognition of target cells, so its expression in tumor cells potentially enhances the ability of the immune system to recognize cancer cells. This tumor vaccine approach is expanding, as poxvirus vectors encoding a variety of tumor antigens are currently undergoing preclinical testing. Given the large capacity of the vectors, genes encoding both tumor antigens and immune system recognition enhancing molecules could be inserted into a single virus.

Poxvirus vector-based systems have demonstrated their utility for expression of foreign genes for study in tissue culture, for use as a vaccine vehicle in both animals and humans, and they are beginning to be used for treating cancer. Because of the wide variety of available vectors, and the safety features that have been built into them, poxvirus vectors will increasingly become the vector of choice for a variety of applications.

Baculovirus Expression Vectors

Baculoviruses are pathogenic insect viruses that have been investigated for possible use as biological control agents for insect pests. Recently, these viruses have proven useful in the development of helper-independent vectors for overexpression of foreign genes in eucaryotic cells. Baculoviruses contain a double-stranded DNA genome of around 130 kb, and the DNA is infectious. The main attraction of this system is the ability to produce very large quantities of transgene protein, but until recently the system was limited to insect cells. Recent developments have considerably expanded the potential of the baculovirus vector system for use in mammalian cells.

Baculovirus vectors are generally produced by homologous recombination between a shuttle vector and a wild-type genome, using the viral polyhedrin gene as the recombination target. Shuttle vectors often contain the transgene of interest plus a selectable marker gene, although inactivation of the polyhedrin gene through transgene insertion gives rise to plaques that can be differentiated from those of wild-type virus. The polyhedrin gene of baculoviruses such as *Autographa californica* nuclear polyhedrosis virus (AcMNPV) is expressed at very late times of infection and produces a protein that surrounds the mature virus particle to form an occlusion body in insect larvae. Polyhedrin protein expression in infected tissue culture cells can continue for days following initial infection and often amounts to 50% of the total cell protein. The polyhedrin protein is not essential for replication of the virus in tissue culture, and transgenes placed under the control of its promoter can be expressed to levels approaching that of the polyhedrin itself. The virus has a rod-shaped capsid and has a large capacity for packaging foreign DNA. Baculoviruses do not productively infect vertebrate cells, but modifications to the system have allowed transgene expression in a variety of mammalian cell lines, further expanding the potential of the vector system.

Baculovirus vectors have found their greatest use in the production of large quantities of protein for purification. While insect cells can post-translation-

ally modify proteins correctly, there were concerns that they could not process high-mannose glycans like mammalian cells. However, recent reports on human plasminogen expressed in insect cells show that the enzymes necessary for processing high-mannose glycans can be activated with glycan processing identical to that found in the human-expressed protein. These are important considerations if baculovirus-expressed products are to have the same biological and immunological properties as that of the native protein. Baculovirus-expressed protein was used in the first human clinical trials for an acquired immune deficiency syndrome (AIDS) vaccine, using an HIV-1 (IIIB) *env* gene product purified from insect cells. Its general utility for production of purified proteins for clinical use remains to be demonstrated.

Herpesvirus Vectors

Two herpesviruses are emerging as alternatives for the development of gene transfer vectors: herpes simplex virus (HSV) and Epstein–Barr virus (EBV). HSV has a genome of approximately 155 kb in length, which is maintained as a concatemerized circular or linear episome in infected cells. It efficiently infects cells, including nondividing cells, from a wide variety of organisms, and is able to establish a persistent infection. Its genome can accommodate large amounts of foreign DNA, and a number of vector systems have been developed. The EBV-based systems have not been developed as extensively and currently employ replication-defective EBV strains. Unlike HSV, EBV vectors have a very limited tropism and their possible usefulness remains to be explored. The main difficulty using these two systems is the need for almost complete virus genomes that are very complex and large in size, making manipulations exceedingly difficult. The eventual usefulness of these systems will be dictated by further simplifications that render them more manageable in the laboratory and that facilitate vector production.

Helper-dependent and -independent systems have been developed for preclinical evaluation. The helper-independent systems have basically the complete viral genome with one or more transgenes substituted for nonessential viral genes. These vectors are rendered defective for growth by deletion of essential genes, permitting their growth only on complementing cell lines. So far these vectors have met with limited success in model systems because of leakiness in the system, which leads to cytotoxicity and cell death. The requirements for maintenance of gene expression during the latent state in neuronal cell types are also currently unknown, so prolonged transgene expression has been difficult to obtain. The second type of HSV vector is the helper-dependent system. The vectors contain only the sequences required for replication and packaging, but all the other functions must be supplied in *trans* from a helper virus. While such systems are capable of producing high-titer amplicon-containing virus, they are almost invariably contaminated with large quantities of the helper virus. Recent improvements in the amplicon system have allowed the generation of relatively high-titer amplicon preparations with much lower levels of contamination with helper virus. These systems hold promise for use in clinical situations, especially in gene transfer to neuronal cells, but further development of the system is required.

Retrovirus Vectors

Retroviruses are ubiquitous in nature and have a relatively simple RNA genome of around 9 kb in length. By reverse transcription, the RNA genome becomes a double-stranded DNA that integrates into the host chromosome and is stably transmitted to progeny cells. Specific aspects of the life cycle of retroviruses and their relatively simple genome have facilitated their development into gene transfer vectors. Most of the vectors currently used for basic science and for clinical applications are based on murine leukemia viruses. Because of the high efficiency with which retroviral vectors can transduce a variety of cell types from many different species, they have become a method of choice for gene transfer experiments.

Retrovirus vectors can be produced by either transient transfection of cells, or by the generation of stable producer cell lines. In both cases, the transcription units for the packaging functions for the virus particle and that for the transgene are on separate genetic elements. The packaging proteins are provided in *trans*, either by a co-transfected second plasmid that encodes the proteins needed for particle formation, reverse transcription and integration of the vector (transient production), or by a packaging cell line that contains these genes integrated into the chromosome (**Fig. 2**). Separation of the *trans* functions into several plasmids is important to minimize the chance that a wild-type replication competent virus may be generated by a recombination event. Packaging cell lines are able to generate large numbers of virus particles without a genome. These particles are empty because the packaging construct does not contain the information needed for specific incorporation of RNA into the virus particle. This is an important aspect of the system because it prevents the transmission of genomic information encoding virus proteins. The vector plasmid contains all of the *cis*

replication in cultured cells but that their presence confers a selective advantage for virus propagation in the organism.

Evolution

Several hypotheses for the origin of vaccinia virus have been advanced, but none is entirely convincing. According to some of these, vaccinia virus was either derived from variola or cowpox virus, or is a hybrid between the two. Other authors propose that vaccinia virus is a 'fossil' and represents the maintenance in the laboratory of a virus of a domestic or wild animal that has otherwise become extinct. This view is supported by the fact that with the exception of buffalopox virus all vaccinia virus strains form a homogeneous group of viruses both with respect to biological properties and genome structure and are not more closely related to cowpox virus or variola virus than to other orthopoxvirus species. Therefore, the virus that was introduced by Jenner for smallpox vaccination and subsequently distributed worldwide, may indeed have been vaccinia virus.

At the genome level, differences between vaccinia virus strains are mainly due to variability in the terminal regions of the molecule, whereas the central part is highly conserved. Comparison of protein-coding regions between different strains typically show greater than 99% identity in amino acid sequences.

Buffalopox virus is also considered as a vaccinia virus strain and was probably derived from smallpox vaccine. The virus appears to have evolved more rapidly and has become a separate subspecies which is maintained in buffalo herds in certain parts of India.

Serologic Relationships and Variability

All orthopoxviruses show extensive serological cross-reactivity which is even more marked between individual vaccinia virus strains. Strains cannot, therefore, be distinguished by serological means. Restriction enzyme analysis of the viral genome has become the most reliable method for differentiating between vaccinia virus strains, and has largely replaced methods based on biological criteria such as pock morphology on the chorioallantoic membrane, or ceiling temperature.

Epidemiology

Vaccinia virus was occasionally spread from newly vaccinated humans to nonvaccinated individuals. This could lead to serious complications in cases where contraindications for vaccination of the unvaccinated contact existed. Transmission also occurred to domestic animals, such as cows and pigs and these animals in turn, represented a source of infection for humans. Buffalopox virus which probably also originated from vaccinia virus, was spread between animals by farmers. According to recent reports from India, buffalopox virus has acquired considerable pathogenicity for humans. Most outbreaks of rabbitpox in colonies of laboratory rabbits were caused by a vaccinia virus strain which was accidentally transmitted to the animals by laboratory workers. In some outbreaks, the source of infection was not traced.

Transmission and Tissue Tropism

The smallpox vaccine was usually introduced into the epidermis over the deltoid muscle. Jenner inoculated human subjects by a light scratch of the skin through a drop of vaccine and variations of this technique were used for a very long time. During the smallpox eradication campaign, other techniques for delivering vaccine were developed. Jet injectors were widely used in West Africa and Brazil. The major advance was the development of the bifurcated needle which could easily be used even by inexperienced vaccinators and which required no special maintenance.

Accidental transmission of vaccinia virus between humans or between humans and animals occurred by direct contact through minute skin lesions. In most outbreaks of rabbitpox, spread appeared to occur by the respiratory route; direct contact between animals was not necessary.

After smallpox vaccination of humans, virus replicated in the epidermis and remained confined to the site of inoculation. Spread to other parts of the body was only observed in the rare cases of complications. After infection of rabbits with rabbitpox virus by the respiratory route, virus spread through the body in a stepwise manner and replication occurred in various tissues.

Pathogenicity

The frequency of the rare, but serious complications of smallpox vaccination was related to the use of particular vaccinia virus strains. For instance, after the Bern strain, once in Austria, Switzerland and West Germany, was replaced by the Lister strain in 1971, the occurrence of complications of the central nervous system declined. Other strains with a good safety record are the New York City Board of Health strain and the Japanese LC16m8 strain. Since the residual virulence of vaccinia virus is the major obstacle for the introduction of recombinant virus as live vaccines against other pathogens, considerable effort is being

made to make the virus a safer vector. Several nonessential genes have been deleted from the viral genome and in each case, the resulting mutant virus showed an attenuated phenotype in experimental animal models.

Rabbitpox virus is a highly virulent vaccinia virus strain for rabbits causing a rapidly lethal disease. Frequently death occurs before skin lesions have time to develop.

Clinical Features of Infection

After primary smallpox vaccination a papule developed in 3–5 days, rapidly became a vesicle and later became pustular, reaching its maximum size after 8–10 days. A scab then formed, which separated at 14–21 days leaving a typical vaccination scar. Vaccination produced a generalized infection with swelling and tenderness of the draining lymph nodes and mild fever. The vaccinees frequently felt miserable for a few days.

Apart from the 'normal' vaccination reactions, which were more intense than those seen with other human vaccines, a number of rare but serious complications occurred. These were of two kinds: those affecting the skin and those affecting the central nervous system. In the first group, progressive vaccinia was the least frequent, but most serious complication and was observed only in vaccinees with a deficient cell-mediated immune system. The clinical features of progressive vaccinia were a failure of the primary vaccination lesion to heal, appearance of lesions elsewhere on the body and progression of all lesions until the patient died. The fatality-rate was extremely high. Eczema vaccination was the second most serious skin affection and occurred among persons with eczema. This complication was characterized by the appearance of lesions on areas of the skin that were eczematous at the time or had previously been so. The fatality-rate was about 30%. Generalized vaccinia followed virus spread via the bloodstream. Lesions similar to the vaccination lesions appeared on many parts of the body. Generalized vaccinia had a good prognosis. Accidental infection occurred among laboratory workers in research facilities or vaccine production centers or in contacts of newly vaccinated persons and were most serious when they affected the eye. Complications affecting the central nervous system were encephalopathy, usually in children less than 2 years of age, and encephalomyelitis in older children and adults. These complications were of particular concern since they occurred in persons with no obvious contraindications for vaccination and had a high case-fatality rate.

Pathology and Histopathology

The pathology and histopathology of 'normal' skin lesions at various stages after smallpox vaccination were studied in biopsies. For more information, the reader is referred to the specialized literature which also deals with the pathology of the rare complications of vaccination.

Immune Response

Smallpox vaccination with vaccinia virus provided complete protection against smallpox for at least 5 years and some protection for over 30 years. Both humoral and cell-mediated immune responses were observed. Studies in mice indicated that cytotoxic T cells are more important for protection against mousepox than circulating antibodies. In humans, progressive vaccinia developed in patients with an impaired cell-mediated immune system, but normal humoral antibody responses, underlining the importance of cytotoxic T cells for limiting vaccinia virus spread and presumably also for providing protection against smallpox.

Evasion of the Host Immune Response

The recent discovery that poxviruses encode a whole arsenal of proteins whose functions are to counteract the host response to infection, has opened up a new field of exciting research. The list of such proteins includes a viral protein that blocks complement activation, thereby protecting intracellular virus from complement-mediated neutralization of infectivity. Other proteins target the host interferon response either by acting as soluble interferon receptors or by neutralizing the action of interferon-induced antiviral host proteins. Still other proteins interfere with the production of interleukin or compete for the binding of this cytokine to its cell surface receptor, or delay apoptosis. Some of these viral proteins exhibit unusual properties. For instance, in contrast to its cellular counterpart the virus-encoded secreted receptor for interleukin-1 binds only interleukin-1b but not interleukin-1a. The soluble viral interferon receptors on the other hand bind interferons from several different species. The study of these interesting viral proteins is expected to provide insight into the functions of host defense mechanisms which are particularly relevant for the control of infection by certain viruses.

Prevention and Control

After the eradication of smallpox, all countries have abandoned general, compulsory smallpox vaccina-

tion. Whether or not the staff in research institutes working with vaccinia virus should be vaccinated is a matter of controversy. Apart from the risk associated with smallpox vaccination, it will become increasingly more difficult in future to obtain vaccine and to find medical doctors proficient in the vaccination procedure.

Future Perspectives

Clearly, in future vaccinia virus vectors will continue to play an important role in basic and applied research. The future of vaccinia virus as a vaccine will depend on whether recombinant virus-expressing antigens of other pathogens will be accepted as live virus vaccines. The main argument against the introduction of such recombinants are the rare complications associated with the use of vaccinia virus as the smallpox vaccine. Considering the great potential of recombinant virus, the decision of whether or not to introduce such vaccines should only be made after a thorough risk–benefit evaluation and the development of safer vectors might influence the decision favorably.

See also: **Smallpox and monkeypox viruses (*Poxviridae*); Vaccines and immune response; Vectors: Animal viruses.**

Further Reading

Fenner F, Wittek R and Dumbell KR (1989) *The Orthopoxviruses*. New York: Academic Press.

Moss B (1996) Poxviridae: the viruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 2637. Philadelphia: Lippincott-Raven.

Smith GL (1994) Virus strategies for evasion of the host response to infection. *Trends Microbiol.* 2: 81.

VARICELLA-ZOSTER VIRUS (*HERPESVIRIDAE*)

Contents

General Features

Jeffrey I Cohen and **Stephen E Straus**, Medical Virology Section, Laboratory of Clinical Investigation, National Institutes of Health, Bethesda, Maryland, USA

History

Descriptions of vesicular rashes characteristic of chickenpox (varicella) date back to the ninth century. In 1875 Steiner showed that chickenpox was an infectious agent by transmitting the disease from chickenpox vesicle fluid to previously uninfected people. Shingles (zoster) has been recognized since ancient times. In 1909 Von Bokay suggested that chickenpox and shingles were related infections, an idea that was experimentally confirmed in the 1920s and 1930s when children inoculated with fluid from zoster vesicles were shown to contract chickenpox. By virtue of the remarkable dermatomal confinement of most zoster lesions, Garland suggested in 1943 that zoster was due to reactivation of varicella virus that had remained dormant or latent in sensory nerve ganglia.

The viral agents of varicella and zoster were first cultivated by Weller in 1952 and shown on morphologic, cytopathic and serologic criteria to be identical. In 1984 Straus and colleagues showed that viruses isolated during sequential episodes of chickenpox and zoster from the same patient had identical restriction endonuclease patterns, proving the concept of prolonged latent carriage of the virus. However, demonstration of latent virus within dorsal root ganglia awaited subsequent molecular studies using *in situ* hybridization and polymerase chain reaction (PCR) techniques.

Taxonomy and Classification

Varicella-zoster virus (VZV) is a member of the *Alphaherpesvirinae* subfamily, genus *Varicellovirus* of the family *Herpesviridae*. Other alphaherpesviruses that infect humans include herpes simplex viruses 1 and 2, and rarely cercopithicine herpesvirus (B virus). All of these agents exhibit relatively short

tion. Whether or not the staff in research institutes working with vaccinia virus should be vaccinated is a matter of controversy. Apart from the risk associated with smallpox vaccination, it will become increasingly more difficult in future to obtain vaccine and to find medical doctors proficient in the vaccination procedure.

Future Perspectives

Clearly, in future vaccinia virus vectors will continue to play an important role in basic and applied research. The future of vaccinia virus as a vaccine will depend on whether recombinant virus-expressing antigens of other pathogens will be accepted as live virus vaccines. The main argument against the introduction of such recombinants are the rare complications associated with the use of vaccinia virus as the smallpox vaccine. Considering the great potential of recombinant virus, the decision of whether or not to introduce such vaccines should only be made after a thorough risk–benefit evaluation and the development of safer vectors might influence the decision favorably.

See also: **Smallpox and monkeypox viruses (*Poxviridae*); Vaccines and immune response; Vectors: Animal viruses.**

Further Reading

Fenner F, Wittek R and Dumbell KR (1989) *The Orthopoxviruses*. New York: Academic Press.

Moss B (1996) Poxviridae: the viruses and their replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*, 3rd edn, p. 2637. Philadelphia: Lippincott-Raven.

Smith GL (1994) Virus strategies for evasion of the host response to infection. *Trends Microbiol.* 2: 81.

VARICELLA-ZOSTER VIRUS (*HERPESVIRIDAE*)

Contents

General Features

Jeffrey I Cohen and **Stephen E Straus**, Medical Virology Section, Laboratory of Clinical Investigation, National Institutes of Health, Bethesda, Maryland, USA

History

Descriptions of vesicular rashes characteristic of chickenpox (varicella) date back to the ninth century. In 1875 Steiner showed that chickenpox was an infectious agent by transmitting the disease from chickenpox vesicle fluid to previously uninfected people. Shingles (zoster) has been recognized since ancient times. In 1909 Von Bokay suggested that chickenpox and shingles were related infections, an idea that was experimentally confirmed in the 1920s and 1930s when children inoculated with fluid from zoster vesicles were shown to contract chickenpox. By virtue of the remarkable dermatomal confinement of most zoster lesions, Garland suggested in 1943 that zoster was due to reactivation of varicella virus that had remained dormant or latent in sensory nerve ganglia.

The viral agents of varicella and zoster were first cultivated by Weller in 1952 and shown on morphologic, cytopathic and serologic criteria to be identical. In 1984 Straus and colleagues showed that viruses isolated during sequential episodes of chickenpox and zoster from the same patient had identical restriction endonuclease patterns, proving the concept of prolonged latent carriage of the virus. However, demonstration of latent virus within dorsal root ganglia awaited subsequent molecular studies using *in situ* hybridization and polymerase chain reaction (PCR) techniques.

Taxonomy and Classification

Varicella-zoster virus (VZV) is a member of the *Alphaherpesvirinae* subfamily, genus *Varicellovirus* of the family *Herpesviridae*. Other alphaherpesviruses that infect humans include herpes simplex viruses 1 and 2, and rarely cercopithicine herpesvirus (B virus). All of these agents exhibit relatively short

replicative cycles, destroy the infected cell and establish latent infection in sensory ganglia.

Three subgroups of simian viruses cause severe varicella-like illnesses in nonhuman primates. These include the delta herpesvirus, Medical Lake macaque virus and chimpanzee herpesvirus. While these simian viruses are antigenically related to VZV, they are more homologous to each other than to VZV and are not known to infect humans.

Geographic and Seasonal Distribution

Varicella and zoster infections occur worldwide. Over 90% of varicella occurs during childhood in industrialized countries located in the temperate zone, but infection is commonly delayed until adulthood in tropical regions. Zoster may occur less frequently in tropical areas because of later acquisition of primary infection.

Varicella infection is epidemic each winter and spring, while zoster occurs throughout the year, without a seasonal preference.

Host Range and Virus Propagation

The reservoir for VZV is limited to humans. The virus inherently grows poorly in nonhuman animals or cell lines. Myers and colleagues, however, developed a guinea pig animal model of VZV infection by adapting the virus for growth in guinea pig embryo cells *in vitro*. Inoculation of animals results in a self-limited viremic infection and the emergence of both humoral and cellular immunity. Virus replicates initially in the nasopharynx and can be transmitted to other guinea pigs. Latent VZV DNA has been demonstrated in dorsal root and trigeminal ganglia. The same investigators extended their animal model to congenitally hairless Hartley guinea pigs with the advantage that some animals develop a papular erythematous rash. Rats and mice inoculated with VZV develop latent infection of dorsal root ganglia. Inoculation of VZV into fetal thymus and liver implants in SCID mice results in virus replication in T cells; inoculation of virus into subcutaneous fetal skin implants reproduces many of the histopathologic features of varicella.

An alternative, but less ideal animal model involves the common marmoset (*Callithrix jacchus*). VZV replicates in the lungs with a mild pneumonia and a subsequent humoral immune response. Inoculation of chimpanzees with VZV results in a transient rash containing viral DNA and evokes a modest humoral immune response. None of these animal models has, as yet, reproduced the disease pattern seen in humans, namely a vesicular rash and spontaneous reactivation from latency.

VZV is usually cultured in human fetal diploid lung cells in clinical laboratories. The virus has been cultivated in numerous other human cells including melanoma cells, primary human thyroid cells, astrocytes, Schwann cells and neurons, and can be grown in some simian cells including African green monkey kidney cells and Vero cells, and in guinea pig embryo fibroblasts.

VZV is extremely cell-associated. The titer of virus released into the cell culture supernatant is very low, and preparation of cell-free virus, by sonication or freeze-thawing cells, usually results in a marked drop in the viral titer. Therefore, virus propagation is usually performed by passage of infected cells on to uninfected cell monolayers. VZV is detected by its cytopathic effect, with refractile rounded cells that gradually detach from the monolayer, or by staining with fluorescein-labelled antibody.

Genetics

Several markers can be used to distinguish different strains of the virus. These include temperature sensitivity, plaque size, different effects on host cell lipid metabolism, antiviral sensitivity and restriction endonuclease cleavage patterns. The molecular basis for most of these strain differences are unknown, but viruses that are resistant to acyclovir are usually found to have mutations in their thymidine kinase gene. Other resistant strains have mutations in the DNA polymerase gene.

The entire genome of one prototypical laboratory strain of VZV consists of 124 884 bp. The identification of some viral genes was made by analogy to herpes simplex virus type 1 genes with similar sequences, and by genetic complementation studies in which cell lines expressing selected VZV proteins were used to support the growth of temperature-sensitive mutants of herpes simplex type 1 viruses. Recombinant VZV containing Epstein–Barr virus, hepatitis B virus or herpes simplex virus antigens have been constructed. Recently, a versatile cosmid transfection system has been developed that has allowed targeted deletion, insertion or mutagenesis of a number of individual viral genes products. For example, deletion of the VZV ORF10 gene, the homologue of the essential herpes simplex virus gene VP16, resulted in a virus that was unimpaired for growth *in vitro*.

Evolution

Comparison of the nucleotide and predicted amino acid sequences of VZV with herpes simplex virus types 1 and 2 indicates that these viruses originated

from a common ancestor. They share similar gene arrangements and only five genes of VZV do not appear to have herpes simplex virus counterparts.

VZV is more distantly related to all other human herpesviruses, but many of the nonstructural proteins involved in viral replication have conserved elements and activities. Comparison of VZV, for example, with Epstein–Barr virus shows that the majority of VZV genes are homologous with Epstein–Barr virus. Three large blocks of genes are conserved, although rearranged within the two genomes.

Serologic Relationships and Variability

There is only one serotype of VZV. Antibodies detected by the complement fixation test and virus-specific IgM antibodies decline rapidly after convalescence from varicella. Other, more sensitive serologic tests recognize antibodies that persist for life, including immune adherence hemagglutination (IAHA), fluorescence antibody to membrane antigen (FAMA) and enzyme linked immunosorbent assay (ELISA). VZV-specific antibodies are boosted both by recrudescent infection (zoster) and by exposure to others with varicella.

Variability of VZV strains has been shown primarily by differences in restriction endonuclease patterns. Passage of individual strains *in vitro* eventually results in minor changes in restriction endonuclease patterns, predominantly through deletion or reiteration of small repeated elements scattered throughout the genome. Other than these sites, the genome sequence is remarkably stable. For example, the sequence of the thymidine kinase gene has been determined for several epidemiologically unrelated wild-type and acyclovir-resistant strains and found to possess >99% nucleotide and amino acid identity.

Epidemiology

Varicella may occur after exposure of susceptible persons to chickenpox or to herpes zoster. Over 95% of primary infections result in symptomatic chickenpox. Over 90% of individuals in temperate countries are infected with VZV before age 15.

Zoster is due to reactivation of VZV in patients who have previously had chickenpox; some of these patients may not recall the primary infection. Zoster is not clearly related to exposure to chickenpox or to other cases of zoster. About 10–20% of individuals ultimately develop herpes zoster; the risk of which rises steadily with age (**Fig. 1**). Severely immunocompromised patients, such as those with the acquired immune deficiency syndrome (AIDS), have a particularly high incidence of zoster. Recurrent zoster is uncommon; less than 4% of patients experience a second episode. Asymptomatic viremia has been detected in bone marrow transplant recipients and has been followed by recovery of cell-mediated immunity.

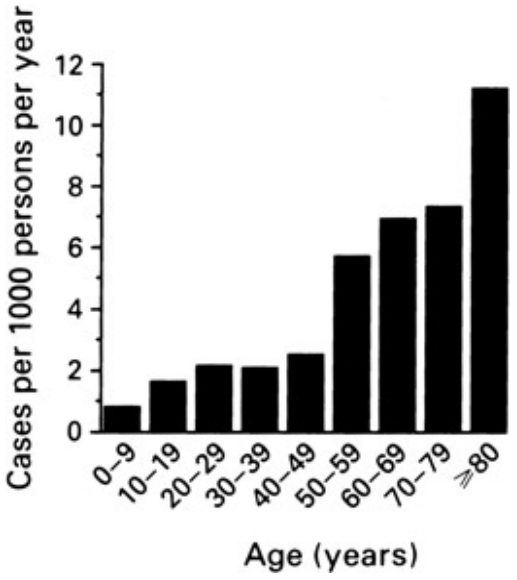


Figure 1 Incidence of herpes zoster per 1000 persons. (Reproduced with permission from Kost and Straus (1996).)

Transmission and Tissue Tropism

VZV is transmitted by the respiratory route. VZV has been detected by PCR in room air from patients with varicella or zoster. Intimate, rather than casual, contact is important for transmission. Chickenpox is highly contagious; about 60–90% of susceptible household contacts become infected. Herpes zoster is less contagious than chickenpox. Only 20–30% of susceptible contacts develop varicella. Patients with varicella are infectious from 2 days before the onset of the rash until all the lesions have crusted.

Primary infection with VZV results in viral replication in the upper respiratory tract and oropharynx, with lesions present on the respiratory mucosa. The infection subsequently spreads to the lymphatics and a mild viremia develops. Mononuclear cells are thought to support viral replication and convey the virus throughout the body. VZV DNA has been detected by PCR in the oropharynx, and virus has been cultured from the blood early during varicella. Further viral replication occurs in the reticuloendothelial system. A brisk secondary viremia results in spread to the periphery. Skin lesions begin with infection of the vascular endothelial cells and virus spreads to epithelial cells. At some time during the course of the infection the virus spreads to both

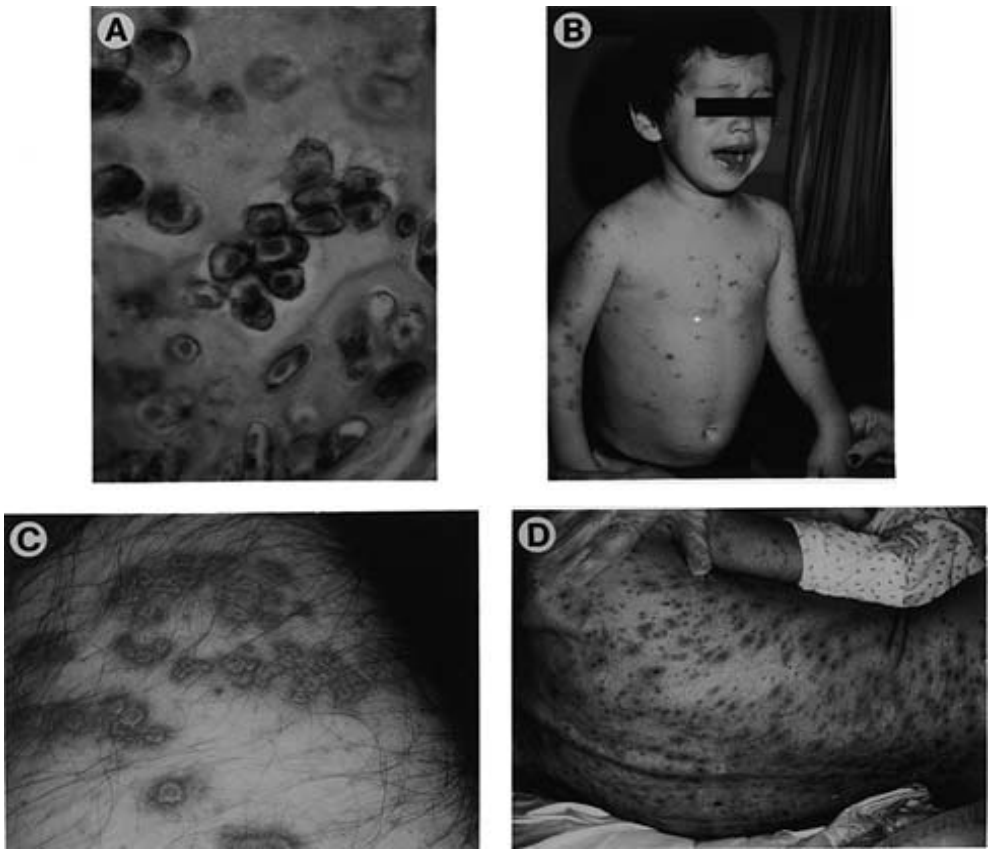


Figure 2 Histopathology and clinical findings of varicella-zoster virus infections. (**A**) Eosinophilic intranuclear inclusions from a skin biopsy in a patient with herpes zoster original magnification ×400. (**B**) Chickenpox in a child. (**C**) Localized zoster in an adult. (**D**) Disseminated zoster in a patient with chronic lymphocytic leukemia. (Reproduced with permission from Straus SE, Ostrove JM, Inchauspe G *et al* (1988) Varicella-zoster virus infections: biology, natural history, treatment, and prevention. *Ann. Intern. Med.* 108: 221–237.)

neurons and their surrounding satellite cells. During latency, VZV DNA can be detected in thoracic and trigeminal ganglia by PCR, but there are conflicting data regarding the cell types harboring the latent virus. Using *in situ* hybridization, VZV has been detected in neurons, in satellite cells, or in both cell types. During latency only about four of the 70 known viral genes remain expressed.

Zoster is due to reactivation of virus in the sensory ganglia. The factors leading to its reactivation are not known, but are associated with neural injury and cellular immune impairment. Reactivated virus spreads down the sensory nerve to the skin where the resulting vesicles are typically confined to a single dermatome. Viremia and subsequent cutaneous or visceral dissemination of lesions may occur in zoster, especially in immunocompromised patients.

Pathogenicity

Passage of wild-type VZV in cell culture by Takahashi in 1974 led to attenuation of the virus and changes in its temperature sensitivity and infectivity for certain cell lines. The resulting Oka vaccine strain has several differences in restriction endonuclease patterns from wild-type strains, but it is not known which of these differences contribute to attenuation.

Clinical Features of Infection

The incubation period for chickenpox is 2 weeks, with a range of 10–21 days. The disease begins with fever and malaise, followed by a generalized vesicular rash (**Fig. 2B**). Lesions tend to appear first on the head and trunk and then spread to the extremities. New lesions usually follow viremic waves for 3–5 days and, in the normal host, most lesions are crusted and healed by 2 weeks. Lesions in different stages coexist in an individual. The disease is usually self-limited in the normal host.

Complications of varicella are more common in neonates, in children with malnutrition, in immunocompromised patients (e.g. malignancy or immunosuppressive therapy), in pregnant women, and in

older adults. These complications include bacterial superinfection of the skin, pneumonia, hepatitis, encephalitis, thrombocytopenia and purpura fulminans. Reye syndrome occurs in rare children who take aspirin to treat varicella fevers. There are about 3–4 million cases of varicella each year in the USA, with about 100 deaths.

Zoster usually presents with pain and dysesthesias 1–4 days before the onset of the vesicular rash. The rash is usually painful and confined to a single dermatome (**Fig. 2C**), but may involve several adjacent dermatomes. Fever and malaise often accompany the rash. Vesicles are often pustular by day 4 and become crusted by day 10 in the normal host.

Postherpetic neuralgia, manifested by pain lasting for weeks to several years in the area of the initial rash, is the most common and disconcerting complication of zoster in the normal host. Less common complications include encephalitis, myelitis, the Ramsay Hunt syndrome (lesions in the ear canal, with auditory and facial nerve involvement), ophthalmoplegia, facial weakness and pneumonitis. Immunocompromised patients with zoster are more likely to develop disseminated disease with neurologic, ocular or visceral involvement (**Fig. 2D**). Patients with AIDS have a high frequency of zoster and may develop recurrent or chronic disease with verrucous, hyperkeratotic skin lesions.

Pathology and Histology

Varicella lesions are readily recognized in the skin and mucous membranes. However, similar lesions also occur in the mucosa of the respiratory and gastrointestinal tracts, liver, spleen and any tissue, and remain unrecognized except in severe cases. With severe disease there is inflammatory infiltration of the small vessels of most organs. Zoster causes inflammation and necrosis of the sensory ganglia and its nerves, and skin lesions which are histopathologically identical to those seen with varicella.

Cutaneous lesions due to VZV begin with infection of capillary endothelial cells, followed by direct spread to epidermal epithelial cells. The epidermis becomes edematous, with acantholysis and vesicle formation. Mononuclear cells infiltrate the small vessels of the dermis. Initially, vesicles contain clear fluid with cell-free virus, but later the vesicles become cloudy and contain neutrophils, macrophages, interferon and other cellular and humoral components of the inflammatory response pathways. Subsequently, the vesicles dry, leaving a crust that usually heals without scarring. Cells infected with VZV show eosinophilic intranuclear inclusions with multinucleate giant cell formation (**Fig. 2A**). These changes are not specific for VZV, as they are seen with herpes simplex and cytomegalovirus infections.

Immune Response

Infection with VZV elicits both a humoral and cellular immune response. The ability of VZV immune globulin (VZIG) to attenuate or prevent infection in exposed children (see Prevention and Control, below) indicates that virus-specific antibody is important in protection from primary infection. The presence of VZV-specific IgG does not correlate, however, with protection from zoster. Antibody to VZV is often present by the time the rash of varicella first appears. Virus-specific IgM, IgG and IgA are present within 5 days of symptomatic disease; however, only IgG persists for life. Antibodies to viral glycoproteins gE (gpI), gB (gpII), gH (gpIII) and the immediate early gene 62 product have been detected during acute infection, and the titers of antibodies to these proteins are boosted during recurrent infection. The mere presence of antibody to VZV glycoproteins in children with leukemia who had received live varicella vaccine was not adequate to prevent breakthrough varicella or zoster.

The cellular immune responses are thought to be more important in recovery from acute varicella infection and for prevention of, and recovery from, zoster. The level of cellular immunity correlates with disease severity during acute varicella. Cytotoxic T cells that lyse virus-infected cells are present by 2–3 days after the onset of the rash of varicella. Cell-mediated immunity, as measured by lymphocyte proliferative response, is directed against cells expressing glycoproteins gE, gB, gH, gI and gC (gpI–gpV, respectively), the immediate early genes 62 and 63, and presumably other gene products as well. Interferon is present in VZV vesicles.

Most varicella infections result in lifelong immunity to reinfection. Second episodes of varicella are rare; these individuals tend to have reduced humoral and cellular immunity to VZV at the time of the second infection. Zoster is associated with a reduction in cellular immunity to VZV that, in the normal host, is partially restored in response to this recurrent infection. Recurrent zoster is uncommon, except in severely immune deficient patients, such as those with AIDS.

Prevention and Control

Prevention of varicella can be achieved by restricting exposure or by resorting to either immunoglobulin prophylaxis or vaccination with live, attenuated virus. If given within 4 days of exposure to the virus,

VZIG prevents or attenuates varicella in seronegative persons. The preparation has no effect in modifying zoster. VZIG is recommended for individuals (1) with recent, close contact to patients with varicella or zoster, (2) who are susceptible to varicella, *and* (3) who fall in a high-risk category. The last group includes premature or certain newborn infants, pregnant women, and patients with congenital or acquired cellular immune deficiencies.

The live, attenuated varicella vaccine (Oka strain) was licensed in the USA in 1995 and is recommended for vaccination of all children and some susceptible adults. The vaccine protects normal children and adults, as well as children with malignancies, from clinical varicella. Most children develop adequate humoral and cellular immunity to varicella after a single dose of vaccine; additional doses enhance the degree of immunity and are recommended for adults. A rash may follow vaccination. It is usually mild, but can be severe if the vaccine is given to patients experiencing periods of profound cellular immune impairment. The live vaccine virus establishes neural latency and can reactivate. Thus, zoster has been reported in vaccinees, especially those who are immunocompromised, but the rate appears to be no higher than that following natural infection. Vaccination may be combined with VZIG for postexposure prophylaxis.

Patients with varicella or zoster should be isolated from susceptible persons until all lesions have crusted. This is particularly important for hospital workers and immune-deficient patients.

Acyclovir, vidarabine and leukocyte interferon have been used in the treatment of varicella and zoster in immunocompromised patients. Interferon proved to be an inadequate and impractical therapy. Vidarabine must be administered intravenously and is also less effective and more toxic than acyclovir. Acyclovir is the current treatment of choice for selected infections. It results in a shorter duration of symptoms and decreased visceral dissemination of varicella or zoster in the immunocompromised host. Acyclovir also prevents spread of trigeminal zoster to the eye and modestly shortens the duration of varicella and zoster symptoms in the normal host. Analogues of acyclovir, such as famciclovir and valaciclovir, result in higher levels of antiviral activity and have been licensed for oral therapy of zoster in the USA.

Acyclovir-resistant strains of VZV have been reported in patients with AIDS; these infections are best treated with foscarnet. Corticosteroids, when used early during zoster, reduce acute pain. Herpes zoster, particularly in elderly patients, may lead to prolonged and severe postherpetic neuralgia. Treatment of postherpetic neuralgia is difficult and often unsatisfactory, but many patients experience improvement with tricyclic antidepressant drugs like amitriptyline.

Future Perspectives

Widespread vaccination of children (both normal and immunocompromised) with the attenuated, live varicella vaccine should reduce the incidence and severity of varicella. The increase in humoral and cellular immunity after vaccination of elderly patients suggests that the vaccine might also reduce the frequency and severity of zoster and a large clinical trial is being planned to test this hypothesis. Because of the potential for the live vaccine to cause zoster, subunit vaccines (containing viral glycoproteins) may prove preferable.

See also: **Cytomegaloviruses (*Herpesviridae*): Animal cytomegaloviruses, General features (human), Molecular biology (human), Murine cytomegaloviruses; Herpes simplex viruses (*Herpesviridae*): General features, Molecular biology; Herpesviruses – baboon and chimpanzee (*Herpesviridae*); Immune response: Cell mediated immune response, General features.**

Further Reading

Arvin A, Moffat JF and Redman R (1996) Varicella-zoster virus: aspects of pathogenesis and host response to natural infection and varicella vaccine. *Adv. Virus Res.* 46: 263.

Cohen JI and Straus SE (1996) Varicella-zoster virus and its replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology* 3rd edn, p. 2525. Philadelphia: Lippincott-Raven.

Ellis RW and White CJ (eds) (1996) The varicella-vaccine. *Infect. Dis. Clin. North Am.* 10: 457.

Kost RG and Straus SE (1996) Postherpetic neuralgia – pathogenesis, treatment, and prevention. *N Engl J Med* 335: 32.

Rentier B (ed.) (1995) Updated proceedings of the second international conference on the varicella-zoster virus. *Neurology* 45 (suppl. 8): S1.

Molecular Biology

William T Ruyechan and **John Hay**, Department of Microbiology, School of Medicine, State University of New York at Buffalo, Buffalo, New York, USA

Properties of the Virion

The morphology of the varicella-zoster virus (VZV) virion is similar to that of other herpesviruses. The virion is 180–200 nm in diameter and is made up of four structurally distinct elements. The linear duplex viral DNA genome is packaged in a central 75 nm core, contained within an icosahedral nucleocapsid 100 nm in diameter, with 5:3:2 axial symmetry. The nucleocapsid is composed of 162 hexameric and pentameric capsomeres with central hollows wider on the outside than the inside. A proteinaceous tegument surrounds the nucleocapsid and is, in turn, surrounded by a lipid bilayer potentially derived from a number of cellular membranes, including the nuclear membrane and Golgi membranes. This lipid coat contains at least six virus-encoded glycoproteins which are visualized by electron microscopy as spikes or studs approximately 8 nm in length.

A minimum of 30 proteins ranging in size from ~250 to 17 kDa have been identified as components of the VZV virion. The capsid is composed primarily of the 155 kDa VZV major capsid protein which makes up the capsomeres. In addition, some 8–10 other polypeptides are also present in purified nucleocapsids based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) analysis. Thus the capsid proteins and the glycoproteins account for somewhat less than half the virion proteins. The remainder are, by a process of elimination, contained in the tegument of the virus. Our knowledge of the VZV tegument proteins is incomplete. Recent work, however, has shown that two of the tegument components (the ORF10 protein and the IE62 protein) are the VZV homologues of the herpes simplex virus (HSV) alpha-*trans*-inducing factor (alpha-TIF) and the major immediate early transcriptional regulatory protein ICP4 (IE175). In addition, the products of VZV gene 47 (a protein kinase), gene 9 and the IE4 and IE63 proteins (both gene regulatory proteins) also appear to be present in the virion. The significance of the presence of these proteins is discussed in subsequent sections.

Properties of the Genome

The genome of VZV is a linear duplex DNA molecule with a bouyant density of 1.706 g cm^{-3} and an overall G + C content of 46%. The genome of VZV strain Dumas has been sequenced in its entirety and contains a total of 124 884 base pairs. The size and organization of VZV DNA indicated by this sequence are in good agreement with experimental data derived from a number of different strains over the last decade and it has been taken as the paradigm by workers in the field. The VZV genome is composed of two covalently joined segments designated L (long segment) and S (short segment) and is subdivided into four distinct domains based on sequence redundancy. These include a long unique region, U_L, ~105 kilobase pairs (kbp) in length and a short unique region, U_S, ~5.2 kbp in length, both of which are composed primarily, although not exclusively, of single-copy sequences. The U_L region is bounded by a short, 88.5 bp element (R_L) which is present in inverted orientation at the external (TR_L) and internal (IR_L) termini of the L segment. The S segment is also bounded by a set of inverted repeats termed TR_S and IR_S. These repeat elements, however, are much larger, being ~7.3 kbp in size. Both repeat elements are considerably higher in G+C content than the U_L and U_S segments with average values of 59% and 68% for R_S and R_L, respectively, as compared to ~43% for the single copy sequences.

The VZV genome exists as two predominant isomeric forms which result from inversion of the S segment. These two isomers, which have been designated P (prototype) and I_S (inverted S), are present in equimolar amounts and represent 90–95% of packaged VZV DNA. The remaining two isomeric possibilities, involving inversion of the L segment (I_L) and of both L and S (I_{LS}) with respect to the prototype, are also present but represent only 5–10% of the viral DNA. The four isomeric forms of VZV DNA are believed to result from general recombination during viral DNA synthesis. Why only two isomers predominate, as opposed to the four equally present in HSV is not clear, and this finding could indicate a preferential recognition of specific L-S joints by the VZV cleavage apparatus. Finally, a small and variable percentage of molecules (0.1–5.0%) can be isolated from virions as full-sized circular genomes. The origin of these molecules, the modes of their packaging and circularization, and the role they may play in the infectious cycle of the virus are unknown.

Properties of Viral Proteins

The VZV genome has been shown to contain at least 69 genes, three of which are diploid, encoding proteins that range in size from 8079 to 306 325 Da. Currently, approximately one-third of these proteins and/or their associated activities have been identified

in VZV-infected cells. The functions of roughly another one-third have been inferred based on comparison of their genome location and predicted primary amino acid sequences with those of known HSV functions. Five of the open reading frames (ORFs) appear to be unique to VZV and one of these encodes a thymidylate synthetase activity. Not unexpectedly, several VZV proteins are not absolutely required for growth in cell culture; these include the products of ORFs 1, 8, 9A, 10, 13, 19, 36, 47 and 66. The VZV genes and their characteristics are given in **Table 1.**

Among the most extensively studied VZV protein species are the viral glycoproteins designated gB, gC, gE, gH, gI and gL (originally named gpII, gpV, gpI, gpIII, gpIV and gpVI). These glycoproteins are homologous to the HSV proteins of the same names, are incorporated into the lipid coat of the virion during maturation, and may appear on several cellular membranes in infected cells. Based on sequence homologies, there are likely to be several additional glycoprotein/membrane proteins that remain to be described. All of the known VZV glycoproteins appear to be heavily modified post-translationally.

Glycoprotein E (the most abundant VZV glycoprotein) and gI are believed to form an IgG Fc receptor and thus may have roles similar to that of their HSV counterparts. Glycoprotein B is assumed to be involved in viral attachment and fusion, whereas gH and gL form complexes that are transported to the cell surface, playing roles in cell fusion and cell-to-cell spread of VZV. Glycoprotein C varies considerably in size from strain to strain, has homology to the MHCII complex and may have a role in the pathogenesis of VZV in human tissue.

Homologues of all of the seven genes required for origin-dependent DNA synthesis in the HSV-1 system are present in the VZV genome. Of these seven predicted VZV gene products, only three have been characterized to any significant extent. These are the viral DNA polymerase, the major single-strand-binding protein and the origin-binding protein, that specifically recognizes DNA sequences at the VZV replication origin. The VZV DNA polymerase has been partially purified and is, like other herpesvirus enzymes, activated by monovalent cations with a peak activity at approximately 80 mM KCl. Finally, the VZV DNA polymerase is sensitive to phosphonoacetate and acyclovir, although to a lesser extent than the HSV-1 DNA polymerase. The VZV gene 29 single-stranded (ss) DNA binding protein is a major species in VZV-infected cells, contains a potential divalent metal binding site (zinc finger) and is present primarily in the nuclei of infected cells. The pattern observed is similar to the punctate and localized patchy staining seen with the HSV-1 major DNA-binding protein, suggesting a role in organization of proteins involved in DNA replication complexes, analogous to that of HSV ICP8. The remaining putative VZV origin-dependent replication genes (6, 16, 52 and 55) have not been examined in any detail.

VZV encodes proteins in ORFs 4, 61, 62 and 63 that are homologues of four of the five HSV immediate early genes. It is not yet clear that these are all 'immediate early' products in VZV, but three of them seem to be (IE4, IE62 and IE63); all are known to be involved in transactivation and transrepression of VZV transcription. VZV IE4 is homologous in genome location and, to a limited extent, in predicted amino acid sequence with the HSV U_L54 gene which encodes ICP27 (IE63). Based on transient expression assays, it is a potent transactivator of both homologous and heterologous promoters and can act synergistically with IE62 to enhance transcription. No evidence of a trans repressor activity has been identified, distinguishing IE4 from its HSV analogue, which acts both as a trans activator and a trans repressor. The ORF 61 product is phosphorylated and contains a RING finger domain, involved in metal binding and important for its functions. A similar region is found in the HSV-1 homologue (ICP0; IE110), a promiscuous trans activator of transcription. Used singly, the ORF 61 product can trans activate several VZV promoters and can further trans activate or repress the activating ability of both VZV IE4 and IE62; its activity appears to be cell type-specific. VZV IE62 is a phosphoprotein that both occurs in the virion tegument and will substantially enhance viral DNA infectivity. It is homologous to the HSV ICP4 (IE175) and is likely to be the major gene regulatory factor for VZV. It strongly trans activates VZV promoters of all kinetic classes through an N-terminal activation domain, reminiscent of a similar domain in HSV VP16. Thus, VZV IE62 may play some roles in VZV assigned to two proteins in HSV. IE62 is a sequence-specific DNA-binding protein, capable of regulating the activity of its own promoter. This promoter contains octamer-binding sites, as well as sites for CE/B and ATF binding. VZV IE63 is homologous to HSV ICP22 (IE68), and some evidence exists that it may, either singly, or in combination with IE62, play a role in VZV gene expression regulation. Also in the context of gene regulation, the ORF 10 tegument product (homologous to HSV VP16) is able to trans activate VZV promoters, but is much weaker than VP16 (or IE62).

Other VZV proteins that have been characterized include two protein kinases (from ORFs 47 and 66).

Table 1 Functions of VZV open reading frames

Gene	*HSV*	*Mol. wt*	*Properties*	*Function*
1		12103	phobic/C	Membrane[a]
2		25983		
3	UL55	19149		
4	UL54	51540	philic/N	(IE) Transcriptional regulator
5	UL53	38575	phobic	Glycoprotein K[a]
6	UL52	122541		DNA helicase/primase complex[a]
7	UL51	28245		
8	UL50	44816		dUTPase
9	UL49	32845	philic	Trafficking protein (cf. HSV VP22)
9A	UL49.5	9800		Membrane
10	UL48	46573		Tegument; transactivator
11	UL47	91825	philic/acidN	Tegument[a]
12	UL46	74269		
13		34531		Thymidylate synthetase
14	UL44	61350		Glycoprotein C
15	UL43	44522	phobic	Membrane?
16	UL42	46087		DNA polymerase accessory[a]
17	UL41	51365		Virion shut-off[a]
18	UL40	35395	acid	Ribonucleotide reductase (small)
19	UL39	86823		Ribonucleotide reductase (large)
20	UL38	53969		Capsid[a]
21	UL37	115774		Late/transcription regulation?[a]
22	UL36	306325		Tegument[a]
23	UL35	24416	phil/STQrich	Capsid[a]
24	UL34	30451	phobic/C	Phosphoprotein[a]
25	UL33	17460	philic/acidN	Virion[a]
26	UL32	65692		Virion?[a]
27	UL31	38234	philic/baseN	
28	UL30	134041		DNA polymerase
29	UL29	132133		Major ssDNA-binding protein
30	UL28	86968		Virion?[a]
31	UL27	98026		Glycoprotein B
32		15980	philic/acid	
33	UL26	66043		Protease/Capsid[a]
33.5	UL26.5	~34000		Assembly protein[a]
34	UL25	65182		Virion[a]
35	UL24	28973	basic	
36	UL23	37815		Pyrimidine deoxynucleoside kinase
37	UL22	93646		Glycoprotein H
38	UL21	60395		
39	UL20	27078	phobic	Membrane?[a]
40	UL19	154971		Major capsid
41	UL18	34387		Capsid[a]
42	UL15	82752		
45	ex1/2	(spliced)		
43	UL17	73905		
44	UL16	40243		
46	UL14	22544		
47	UL13	54347		Protein kinase
48	UL12	61268		Deoxyribonuclease
49	UL11	8907	philic	Virion[a]
50	UL10	48669	phobic	Glycoprotein M[a]

Table 1 Continued

Gene	*HSV*	*Mol. wt*	*Properties*	*Function*
51	UL9	94370		*ori*-binding protein
52	UL8	86343		DNA helicase/primase complex[a]
53	UL7	37417		
54	UL6	86776		Virion[a]
55	UL5	98844		DNA helicase/primase complex[a]
56	UL4	27166	S, T rich	
57		8079	philic/basic	
58	UL3	25093	philic/basic	
59	UL2	34375		Uracil-DNA glycosylase[a]
60	UL1	17616	acidic	Glycoprotein L
61	IE110	50913	philic	Transcriptional regulator
62	IE175	139989		(IE) Transcriptional regulator
63	US1	30494	philic/acid	(IE) Transcriptional regulator
64	US10	19868		Virion[a]
65	US9	11436	phobic	Tegument[a]
66	US3	43677		Protein kinase[a]
67	US7	39362		Glycoprotein I
68	US8	69953		Glycoprotein E

[a]Inferred from data with HSV.

The ORF 47 enzyme is found in the virion tegument, and can phosphorylate IE62, but not other VZV IE proteins. In addition, several enzymes of DNA metabolism are coded by VZV genes, including ribonucleotide reductase and thymidylate synthetase.

Physical Properties and Sensitivity to Environment

Due to the highly cell-associated nature of VZV and the relatively low yields of virus obtained from infected cells, very little work has been done on the physical properties and chemical composition of the virion. It is assumed that, based on its overall structural similiarity to other herpes virions, that the relative proportions of protein, nucleic acid and lipid are typical, as are the bouyant density and overall virion mass.

The VZV virion is highly sensitive to physical and chemical agents. The infectious particle is very labile, and cell-free virus preparations have very high particle:infectivity ratios. Virus from vesicle fluid appears to be more stable. VZV is temperature-sensitive and is inactivated rapidly and completely at 60°C and somewhat more slowly at lower temperatures. VZV is also sensitive to freezing, a property which contributes to difficulties in preparation and storage of high titer stocks. Quick freezing of the virus or infected cells and subsequent storage at −70°C, however, can result in less than 10% loss in titer. In contrast, the titer is rapidly lost on storage at −10°C. Recent reports indicate that lyophilization of both infected cells and cell-free virus in the presence of sugar followed by storage at temperatures as high as −20°C results in complete titer preservation.

VZV is sensitive to pH, with loss of infectivity occurring below pH 6.2 and above pH 7.8. It is also sensitive to UV irradiation, to an extent similar to HSV-1 and HSV-2. VZV is susceptible to mechanical disruption, with 80–99% of infectivity lost after 2 min of sonication. This property may be responsible for some of the loss of infectivity seen on purification of the virus by ultracentrifugation, when a large number of damaged cell-free virions are observed in the electron microscope. HSV virions treated in the same manner are largely intact. A likely explanation for at least part of this VZV property is that virus particles may mature through lysosomal vesicles, allowing digestion of virion components by glycosidases and peptidases.

Replication of Nucleic Acid

The replication of VZV DNA has not been subject to extensive analysis, primarily due to the difficulty in obtaining sufficient numbers of synchronously infected cells. Consideration of the relative proportions of isomeric forms of the viral DNA and the frequency of novel junctions has led to the following model. On entry into the infected cell, the viral genome circularizes and undergoes a limited number of rounds of bidirectional replication. This initiates at the viral

origin (ori) sequences (in the RS regions) consisting of an AT-rich palindrome and a CGTTCGCACTT sequence. During this phase, segment inversion may take place by intramolecular recombination between the inverted repeats. Replication, generating head-to-tail concatemers via a rolling circle mechanism, then accounts for the bulk of viral DNA synthesis. Finally, the newly synthesized DNA is cleaved into unit length molecules and packaged into preassembled capsids in the nucleus. The non-random proportions of isomeric DNA forms are postulated to result from a differential recognition of the normal and novel L-S joints by the viral cleavage system.

The full complement of viral and cellular proteins required for the complete replication of VZV DNA has not been identified. However, as indicated above and in **Table 1**, analogues of the seven genes required for origin-dependent replication of HSV have been identified in the VZV system and three of these have been partially characterized.

Characterization of Transcription

The enzyme responsible for transcription of VZV mRNAs is, presumably, the RNA polymerase II encoded by the host cell. As with other herpesviruses, no viral encoded RNA polymerase activity has been identified. Although 78 relatively abundant transcripts (reading from both strands) have actually been mapped to the VZV genome, as well as 33 less abundant and 29 large transcripts (6–11 kb), these numbers are underestimates of the true transcriptional capability of the genome.

Based on the extensive colinearity of the VZV and HSV genomes and the relatively high degree of functional homology predicted between VZV and HSV gene products, it is reasonable to assume that VZV transcription is regulated in the coordinated cascade scheme seen with other alphaherpesviruses. Direct experimental evidence for this scheme has been difficult to obtain due to the low titers of VZV obtained in cell culture. However, time course studies of protein synthesis have been carried out, which indicate that under conditions of near-synchronous infection, three major classes of VZV-specific proteins are apparent. They have been named immediate early (IE), early (E) and late (L) by analogy with HSV, and each temporal class contains proteins which are representative of such classes in other herpesvirus systems. For example, IE62 is synthesized in the IE phase, the gene 29 major DNA-binding protein is synthesized during the E phase, and the major capsid protein is synthesized at peak levels during the L phase of protein synthesis. Despite these similarities, though, VZV transcription is often different from that of HSV-1. For example, the VZV ORF 10 protein, corresponding to the HSV VP16 (virion trans activator) lacks the acidic carboxy-terminal region which is responsible for the trans inducing activity of that protein. VZV has apparently evolved a separate strategy for the efficient initial transcription of its immediate early genes, probably involving IE62.

Other general features of VZV transcription are apparent from studies of transcription from specific genes. As of this writing, detailed transcript mapping has been carried out on relatively few VZV genes. One finding from these studies is that VZV may use atypical *cis*-acting transcriptional control elements. For example, several consensus TATA box elements may be present upstream of transcriptional start sites but are not used, whereas nonconventional AT-rich regions function to initiate transcription. Similarly, transactivation of VZV promoters often occurs using unusual upstream sequences. Analysis of the 3′ends of VZV genes also reveals differences in the utilization of *cis*-acting polyadenylation sites. Canonical polyadenylation signals for transcripts are often ignored and atypical sequences are utilized preferentially, although GU-rich elements (as in HSV) are usually present. Generally, multiple transcripts are often derived from one gene sequence, but there is no documented evidence for splicing. Indeed, in some VZV genes with spliced mRNAs in their HSV counterparts, there is no spliced VZV mRNA; it is predicted from the genome sequence that ORFs 42/45 will be spliced, however.

Characterization of Translation

Little is known about the details of translation in VZV-infected cells. It is assumed that mRNAs are translated in a fashion similar to that seen in HSV-infected cells and that translation occurs both on free and membrane-bound polyribosomes. Following translation, the majority of viral proteins are efficiently transported to the nucleus where they carry out their roles in viral replication.

Post-translational Processing

Thus far, the most extensively studied post-translationally processed VZV proteins are the glycoproteins. All are N-linked glycosylated and the majority show additional modifications including O-linked glycosylation, sulfation and phosphorylation. For example, VZV gE contains both O-linked and N-linked glycans, and is heavily sulfated and phosphorylated, with phosphorylation occurring at the level of both the polypeptide chain and glycosyl residues. The phosphorylated amino acids are serine

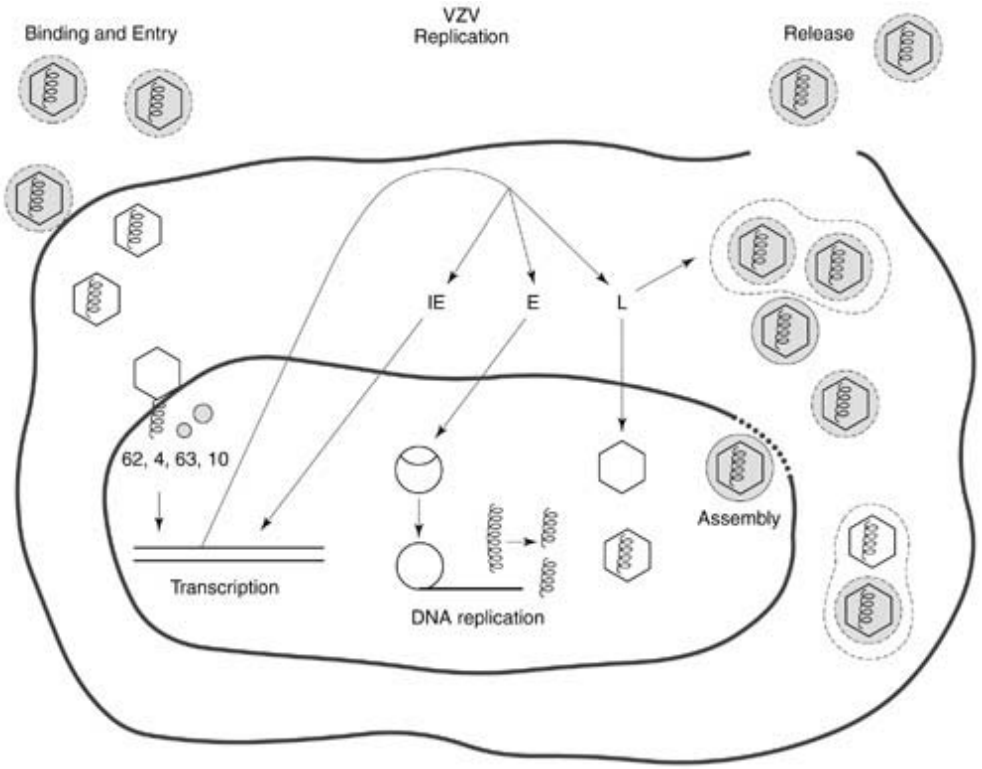


Figure 1 Schematic diagram of the proposed infectious cycle of VZV during lytic infection. Infection is initiated by binding of the virus to the plasma membrane of susceptible cells followed by fusion of the viral envelope and entry of the virus. Following uncoating, the viral DNA, along with tegument components, enters the nucleus where three temporal classes of proteins (IE, E and L) are produced. Transcription of IE mRNA may be enhanced by the IE62 protein which is carried into the cell as a major tegument component. DNA synthesis takes place during the E phase possibly utilizing both bidirectional and rolling circle mechanisms. During the L phase, progeny DNA are packaged into preformed capsids, in the nucleus. These may be enveloped by budding through the nuclear membrane. Enveloped particles acquire a second envelope by budding into cytoplasmic vesicles, which may fuse with lysosomal vesicle. Nucleocapsids may also cross the nuclear membrane and become enveloped in large cytoplasmic vesicles. The membranes surrounding all of these vesicles may ultimately fuse with the plasma membrane, resulting in release of the virus. In cell culture, much of this released virus may have damaged envelope structures.

and threonine but not tyrosine, and the enzymes involved are probably cellular casein kinases I and II. The enzyme responsible for the phosphorylation of the complex oligosaccharides has not been identified, nor has a specific role for the phosphorylated or sulfated residues.

Other viral proteins are modified, particularly by phosphorylation, including IE62 and the ORF 61 protein. IE62 has a predicted molecular weight of 140 kDa, but migrates at approximately 175 kDa on SDS–PAGE, showing evidence of differently phosphorylated forms. The functional significance of the phosphorylation of VZV proteins is unknown.

Assembly Site Uptake, Release, Cytopathology

Models for assembly and release of VZV virions are derived both from extensive, well-established electron microscopic studies and from analyses of VZV glycoprotein biosynthesis and trafficking in infected cells, but no consensus exists (**Fig. 1**). Fully assembled capsids containing unit length genomes comprising all four possible isomeric forms appear to be released from the nucleus, either naked, or having acquired a lipid envelope from the nuclear lamella. In the former case, nucleocapsids acquire their envelope by budding into large cytoplasmic vesicles into which viral glycoproteins have been inserted (via the Golgi), and are released from the cell through reverse endocytosis. In the latter case, vesicles with enveloped particles fuse with lysosomes, in which degradation of virions may take place. The involvement of lysosomes in this case is proposed to rest on their mannose-6-phosphate receptors binding this sugar on the surface of VZV particles. This apparently will not occur in the human host, since the cells in the superficial layers of the skin lack lysosomal vesicles. Perhaps this is also why vesicle fluid virus tends to have a higher and more stable titer.

In tissue culture, cells infected with VZV initially show increased refractility followed by rounding and swelling. Multinucleated cells are common, although the extent of syncytium formation is less than that seen with HSV strains of syncytial plaque morphology. Eventually, the infected cells detach from the support surface, producing readily discernible plaques. The nuclei of infected cells are larger than those of uninfected cells with marginally located chromatin and peripherally located nucleoli. Eosinophilic proteinaceous intranuclear inclusion bodies are present which occupy the sites of viral DNA replication. The nature and function of these inclusion bodies are unknown. Chromosomal aberrations and chromosome and chromatid breakage have also been observed.

Subjects for Further Investigation

A variety of interesting and fundamental questions remain to be answered concerning this important human herpesvirus. One of these is the cellular location and nature of latent VZV infection. Several early reports indicated that the sites of latency were neuronal cells within the dorsal root ganglia but later studies pointed to satellite cells surrounding the neuron. This has led to a model for reactivation of latency in which virus reactivating in satellite cells or neurons lytically infects the neuron resulting in cell destruction. This model is consistent both with the normally singular recrudescence of the virus, and with neuronal damage and postzoster neuralgia associated with reactivation of VZV. Another important feature of VZV latency is the state of expression from the viral genome. Unlike HSV, which appears to produce only a single transcript in the latent state known as the latency associated transcript (LAT), several messages are expressed in cells latently infected with VZV, corresponding to IE4, IE62, IE63, ORF 21 and ORF 29. Assuming translation of these mRNAs in latently infected cells, four of these 'latency proteins' have gene regulatory capacity, and may serve to control the latent state.

A second question involves the factors delineating VZV pathogenesis. Currently there are two obvious areas for investigation. The first involves the identification and understanding of both the *cis*- and *trans*-acting factors involved in the regulation of expression of VZV genes. Why, for example, has the virus evolved to use noncanonical transcription start and stop sites? Which specific viral polypeptides are involved in recognition of such sequences and how many cellular factors are involved? The roles of the known transcriptional regulatory proteins (IE4, IE62, IE63, ORF 61 and ORF 10) will have to be evaluated *in vivo*, in order to define their true roles during productive infection in the human host. Such an approach could also lead to the identification of host cell factors which influence the control of VZV expression. A second set of proteins for which we have only vague functional ideas are the glycoproteins. Some obvious questions are: why does VZV have fewer glycoproteins than HSV and how does this affect its pathogenesis? And do the VZV glycoproteins modulate tissue tropism of VZV?

These questions are all germane to the fact that an effective live attenuated vaccine strain (Oka) has been developed and is in widespread use in children in the industrialized world. However, the specific molecular nature of its attenuation is unknown. In recent studies in a SCID-hu model for VZV infection, it has been demonstrated that the Oka vaccine virus grows well in T-lymphocytes but not in skin cells, consistent with its antigenicity, as well as its relative inability to cause the typical varicella rash. In analogous experiments, VZV lacking gC has been shown to replicate poorly in skin cells, suggesting that this glycoprotein is a virulence factor for the virus. Now that we have a good animal model, as well as a reliable method for construction of viral mutants, we can anticipate substantial new data on VZV replication, both *in vivo* and *in vitro*.

See also: **Herpes simplex viruses (*Herpesviridae*): General features, Molecular biology; Latency; Persistent viral infection.**

Further Reading

Arvin AM (1996) Varicella zoster virus. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields' Virology*. Philadelphia: Lippincott-Raven.

Arvin AM and Gershon AA (1996) Live attenuated varicella vaccine. *Annu. Rev. Microbiol.* 50: 59.

Cohen JI (1996) Varicella zoster virus. The virus. *Inf. Dis. Clin. North Am.* 10: 457.

Cohen JI and Straus SE (1996) Varicella zoster virus and its replication. In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology*. Philadelphia: Lippincott-Raven.

Hay J and Ruyechan WT (1994) Varicella zoster virus – a different kind of herpesvirus latency? *Semin. Virol.* 5: 241.

Variola Virus *see* **Smallpox and Monkeypox Viruses**

sequences seem only to be beneficial with respect to biological containment, since viral vectors will not survive for long in nature. On the other hand, new functional viral genomes may arise from recombinational events involving (disarmed) RNA vectors, co-infecting (helper-) viruses and transgenes, which could lead to undesired spread of pathogens. Although so far no evidence has been obtained for such events, it is clear that critical risk-assessment analyses should be performed prior to possible release of viral vectors in agricultural practice.

See also: **Bromoviruses (*Bromoviridae*); Geminiviruses (*Geminiviridae*).**

Further Reading

Ahlquist P and Pacha RF (1991) Gene amplification and expression by RNA viruses and potential for further application to plant gene transfer. *Physiol. Plant.* 79: 163.

Bakkeren G *et al.* (1989) Recovery of *Agrobacterium tumefaciens* T-DNA molecules in whole plants early after transfection. *Cell* 57: 847.

Beachy RN, Fitcher JH, Hein MB (1996) Use of plant viruses for delivery of vaccine epitopes. *Annals of The New York Academy of Sciences* 792: 43–9.

Brisson N and Hohn T (1988) Plant virus vectors: CaMV. In: Weissbach A and Weissbach H (eds) *Methods for Plant Molecular Biology*, p. 437. San Diego: Academic Press.

Donson J *et al.* (1991) Systemic expression of a bacterial gene by a tobacco mosaic virus-based vector. *Proc. Natl Acad. Sci. U.S.A.* 88: 7204.

Gray SM (1996) Plant virus proteins involved in natural vector transmission. *Trends in Microbiology* 4: 259–64.

Hohn B *et al.* (1987) Plant DNA viruses as gene vectors. *CIBA Found.* 133: 185.

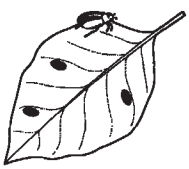
Joshi L, Joshi V and Ow DW (1991) BSMV genome mediated expression of a foreign gene in dicot and monocot plant cells. *EMBO J.* 9: 2663.

Matzeit V *et al.* (1991) Wheat dwarf virus vectors replicate and express foreign genes in cells of monocotyledonous plants. *Plant Cell* 3: 247.

Mushegian AR, Shepherd RJ (1995) Genetic elements of plant viruses as tools for genetic engineering. *Microbiological Reviews* 59: 548–78.

[This article is reproduced from the 1st edn (1994).]

VECTOR TRANSMISSION OF PLANT VIRUSES



Stewart M Gray, USDA, ARS and Department of Plant Pathology, Cornell University, Ithaca, New York, USA

D'Ann Rochon, Agriculture and Agri-Food Canada, Pacific Agri-Food Research Centre, Summerland, British Columbia, Canada

Introduction

It has been nearly 100 years since a leafhopper was confirmed as the vector of rice dwarf virus. Several hundred plant viruses have since been identified, a majority of which are dependent upon a vector for transmission between, and inoculation into, plant hosts. The plant viruses have evolved many interesting and biologically complex associations with their vectors, which include arthropods, nematodes and fungi. Although there is a great deal known about the general biology of most virus–vector interactions, we are only beginning to understand the molecular and cellular mechanisms that regulate the transmission processes and determine the efficiency of transmission.

General Mechanisms of Virus Transmission by Arthropods and Nematodes

In the early years viruses were said to be either mechanically transmitted or biologically transmitted by their vectors. Biological transmission was the specific association of a virus with a particular arthropod species or genus and the virus replicated in the vector. Mechanical transmission referred to the nonspecific transmission of viruses, usually by multiple vector species. The viruses did not replicate in the vector and transmission was thought to occur by the simple contamination of vector mouthparts. Most plant viruses do not replicate in their vectors, but it

was quickly realized the transmission process involved more than just contaminated mouthparts.

The early work on plant virus–vector associations was related to timing events, e.g. acquisition and inoculation periods, retention periods and latent periods (the time between ingestion of the virus and the ability of the vector to inoculate a healthy host). Therefore, the terminology evolved to describe time events. Nonpersistent viruses are not retained by the vector for more than a few hours. Semipersistent viruses are retained for days or possibly weeks. Nonpersistent and semipersistent viruses are acquired and inoculated within seconds or minutes, and do not require a latent period, nor do they replicate in the vector. Persistent viruses are retained for the life of the vector. These viruses require longer acquisition and inoculation times (hours to days), and latent periods of one day to several weeks.

As additional data were generated on the mechanisms of transmission, other variations of the terminology evolved. The nonpersistent and semipersistent viruses were found to associate specifically with the epicuticle that lines the stylets (mouthparts) or foregut, respectively, of their arthropod and nematode vectors and were often referred to as stylet-borne or foregut-borne viruses.

All of the above terms were developed for use with aphid and leafhopper vectors and are applicable to many plant viruses. However, as additional arthropod, nematode and fungal vectors were discovered and their associations with viruses were studied, terminology problems arose, especially with regard to fungal vectors.

In recent times the terms circulative and noncirculative have been widely used. Circulative refers to the requirements that the virus be actively transported across cell membranes and internalized by the vector. These viruses, which would include the persistent viruses, can be further divided into propagative viruses, which replicate in their arthropod vector in addition to their plant hosts, and nonpropagative viruses which only replicate in their plant hosts. Noncirculative viruses do not cross vector cell membranes and are carried externally either on the vector surface or on the cuticle lining of the vector's mouthparts or foregut. These would include both the nonpersistent and semipersistent viruses.

The noncirculative and circulative classification is simple and can be used for all plant viruses that require a vector for optimal existence in nature. There is of course some loss of definition and categorization, but subgrouping, such as nonpersistent and semipersistent, can be added if they pertain to a particular vector taxa. There would, of course, be the paradoxical virus–vector associations that may not easily fit into the proposed scheme. For example, beetle-transmitted viruses and the myirid bug-transmitted velvet tobacco mottle virus may utilize both circulative and noncirculative transmission mechanisms. Additionally, these terms have not been adapted for use with viruses transmitted by fungal vectors (see below).

Vector Feeding Mechanics and Behavior

Plant viruses are incapable of penetrating the plant cell wall and require a wound to gain entrance. However, the cell they enter must be viable so the virus can replicate and translate movement proteins that enable it to move to neighboring unwounded cells. A majority of arthropod and nematode vectors of plant viruses have piercing–sucking mouthparts which are ideal for inoculating plant viruses into plant cells. The hollow needle-like mouthparts can penetrate the plant cell wall by mechanical force and with the help of salivary and gut enzymes. The cell membrane is easily breached by mechanical force, making the cell contents available as food. The most significant feature of this type of feeding is that it does not irreparably damage the plant cell.

The general feeding behavior of many arthropod and nematode vectors also aids in the inoculation process. The acceptance or rejection of a plant host is accomplished by a series of brief probes into multiple epidermal cells. These 'taste-tests' are sufficient to inoculate the noncirculative, nonpersistent viruses. This type of transmission mechanism does not require the plant to be a host of the vector for the virus to establish an infection. Accordingly, most of the noncirculative, nonpersistent plant viruses are transmitted by numerous species within one vector taxon. For example, individual potyviruses are transmitted by numerous aphid species, but are not transmitted by whiteflies or leafhoppers.

If the brief feeding probes indicate the plant is an acceptable host or food source then the vector is likely to initiate prolonged feeding. This may occur in numerous epidermal or mesophyll cells, or more often the insect will seek out its preferred feeding site, the carbohydrate-rich phloem sap. Prolonged feeding allows for the inoculation of the semipersistent, noncirculative viruses as well as the circulative viruses. The transmission strategy of these viruses is to associate with one, or at most a few, vector species for the life of the vector. The virus and the vector share overlapping host ranges and this ensures the virus will be delivered to a new host when the vector moves to a new host.

There are insect vectors (mainly beetles) of plant viruses that have chewing mouthparts and a more

indiscriminate feeding behavior than the piercing–sucking insects and nematodes. Inoculation by beetles was, until recently, considered to be a mechanical process; virus contaminating the mouthparts was deposited into the wound, or virus in the gut was regurgitated as the beetle fed. The process is now known to be extremely specific and biologically complex. Viruses are inoculated into a gross wound resulting from the chewing action of the beetle because the virus can rapidly translocate in xylem elements away from the site of inoculation and infect cells away from the feeding site. Several viruses that are not transmitted by beetles can be acquired and are present in the hemolymph and gut regurgitant that is deposited into and around the feeding sites. The nontransmissible virus is apparently inactivated at the wound site or unable to gain entrance to a functional plant cell capable of sustaining a virus infection.

The transmission of plant viruses has been found to be extremely complex even in situations where initially it may have appeared to be a simple, nonspecific mechanical inoculation. The details of many of these molecular and cellular mechanisms regulating the transmission of plant viruses are described in subsequent sections.

Noncirculative Transmission

The noncirculative method of transmission is not widely associated with animal virus transmission, but it is the method of choice for a majority of plant viruses (**Table 1**). The noncirculative viruses are transmitted by arthropod and nematode vectors and can be further subdivided into nonpersistent and semipersistent categories. Semipersistent viruses tend to be associated with the cuticle lining the foregut of the vector (**Fig. 1**) and are retained for several days or weeks (months or years in some). Transmission efficiency increases as the acquisition feeding time increases, which suggests that virus is stably bound and accumulates until binding sites are saturated. In contrast, the nonpersistent viruses are retained only for a few hours and are easily lost during feeding probes. Furthermore, transmission efficiency rapidly decreases as acquisition feeding time increases. This suggests that bound virus is easily dislodged during prolonged feeding and subsequently ingested virus cannot associate with sites along the stylets.

There are two current theories for the mechanics of noncirculative, nonpersistent transmission. The ingestion–egestion model suggests that transmissible virus adheres to the cuticle at the stylet tips or further inside the mouthparts or foregut during ingestion of plant material. Bound virus is subsequently released during periods of regurgitation and salivation. The ingestion–salivation model acknowledges that virus can bind at multiple sites along the anterior alimentary canal, but the transmitted virus is limited to that which is bound to the proximal tip of the maxillary stylet where the food and salivary canals are fused. Virus is released by the act of salivation rather than by regurgitation (**Fig. 1**).

The most complete understanding of the mechanisms of noncirculative virus transmission comes from work on the aphid-transmitted potyviruses and caulimoviruses, both of which are nonpersistent and require a nonstructural, virus-coded protein referred to as a helper component, or aphid transmission factor to be transmitted (**Table 1**). Although there are several hypotheses for the role of helper in virus–vector interactions, one is emerging as the most plausible. The 'bridge' hypothesis suggests that the helper acts to mediate the attachment of virus to the cuticle lining of the vector mouthparts. Purified virus is not transmissible, but if aphids are given access to a solution containing helper prior to or along with purified virus then transmission can occur. Ultrastructural and immunolabeling evidence indicates that potyvirus fed to aphids along with helper becomes embedded in a matrix material bound to the cuticle, and helper protein is a component of the matrix. If aphids are fed on virus without helper, the matrix material is absent and virus is not retained.

The potyvirus coat protein contains a DAG amino acid motif located near the N-terminus. Mutations within this domain or adjacent to this domain abolish transmission and also prevent accumulation of virus in the stylets. Additionally, all of the potyvirus helper factors studied to date contain two characteristic amino acid motifs, a KITC box and a PTK box. Mutations in or adjacent to these motifs render the virus nontransmissible by the natural vector. A specific mutation of the KITC sequence to EITC abolished transmission, but did not affect the *in vitro* binding of virus to the helper. Furthermore, the virus was not observed on the stylets when acquired with the EITC-mutant helper, but was observed when acquired along with wild-type helper. These data indicate that the KITC box functions in aphid–helper interactions rather than helper–virus interactions. In contrast, mutations in the PTK box abolished helper–virus interactions *in vitro*; therefore this domain may play a role in attachment of the virus to the helper. Alternatively, mutations in the PTK box may prevent dimerization of the helper, which is the active configuration of the helper protein.

Analysis of the cauliflower mosaic caulimovirus (CaMV) helper provides further evidence of the bridge hypothesis. The CaMV helper accumulates in paracrystals in the cytoplasm of infected plant cells

Table 1 Mechanism of transmission and the principal vectors of plant virus families

Virus taxa	*Number of members*[a]	*Principal vector taxa*[b]	*Transmission mechanism*[c]	*Helper required*[d]
Caulimovirus	17	Aphids	NC-NP	Yes
Fabavirus	2	Aphids	NC-NP	No
Potyvirus	186	Aphids	NC-NP	Yes
Carlavirus	55	Aphids	NC-NP	No
Cucumovirus	3	Aphids	NC-NP	No
Alfamovirus	1	Aphids	NC-NP	No
Machlomovirus	1	Thrips/beetle	NC-NP	No
Macluravirus	2	Aphids	NC-NP	No
Potexvirus	55	Aphids (7/10), mites (2/10),	NC-NP	No
Badnavirus	16	Mealybugs (3/6), leafhopper (1/6)	NC-SP	No
Closterovirus	25	Aphids (10/19), whiteflies (6/19), mealybugs (2/19)	NC-SP	No*
Nepovirus	39	Nematodes	NC-SP	No*
Sequivirus	2	Aphids	NC-SP	No
Tobravirus	4	Nematodes	NC-SP	No
Trichovirus	6	Aphids (1/3), mealybugs (1/3), mites (1/3)	NC-SP	No
Waikavirus	3	Aphids (1/3), leafhopper (2/3)	NC-SP	No
Necrovirus	3	Fungi	*in vitro*	No
Tombusvirus	12	Fungi (1/12)	*in vitro*	No
Varicosavirus	4	Fungi	*in vitro*	No
Enamovirus	1	Aphids	C-Npr	No
Geminivirus				
Bigeminivirus	41	Whiteflies	C-Npr	No*
Hybrigeminivirus	2	Treehoppers	C-Npr	No
Monogeminivirus	11	Leafhoppers	C-Npr	No
Luteovirus	27	Aphids	C-Npr	No
Nanavirus	5	Aphids	C-Npr	No
Umbravirus	10	Aphids	C-Npr	Yes
Bromovirus	6	Beetles	C-Npr*	No
Carmovirus	22	Beetles (3/10)	C-Npr*	No
Comovirus	14	Beetles	C-Npr*	No
Sobemovirus	17	Beetles (6/8)	C-Npr*	No
Tymovirus	21	Beetles	C-Npr*	No
Rymovirus	7	Mites	?	No
Bymovirus	6	Fungi	*in vivo*	No
Furovirus	12	Fungi	*in vivo*	No
Bunyaviridae				
Tospovirus	5	Thrips	C-Pr	No
Marafivirus	3	Leafhopper	C-Pr	No
Reoviridae				
Phytoreovirus	5	Leafhopper	C-Pr	No
Fijivirus	6	Planthopper	C-Pr	No
Oryzavirus	2	Planthopper	C-Pr	No
Rhabdoviridae				
Phytorhabdovirus	32	Aphid (1/3), leafhopper (1/3), planthopper (1/3)	C-Pr	No
Cytorhabdovirus	17	Aphid (3/7), planthopper (4/7)	C-Pr	No
Nucleorhabdovirus	38	Aphid (7/17), leafhopper (4/17), planthopper (6/17)	C-Pr	No
Tenuivirus	10	Planthopper	C-Pr	No

[a] The number of members of each group was obtained from Brunt AA *et al* (1996) *Viruses of Plants.* Cambridge: CAB International.

[b] Indicates the vector taxa that is commonly associated with the transmission of the members of the virus group. In cases where multiple vector taxa have been reported to vector members of the virus group, the number of viruses vectored by that insect

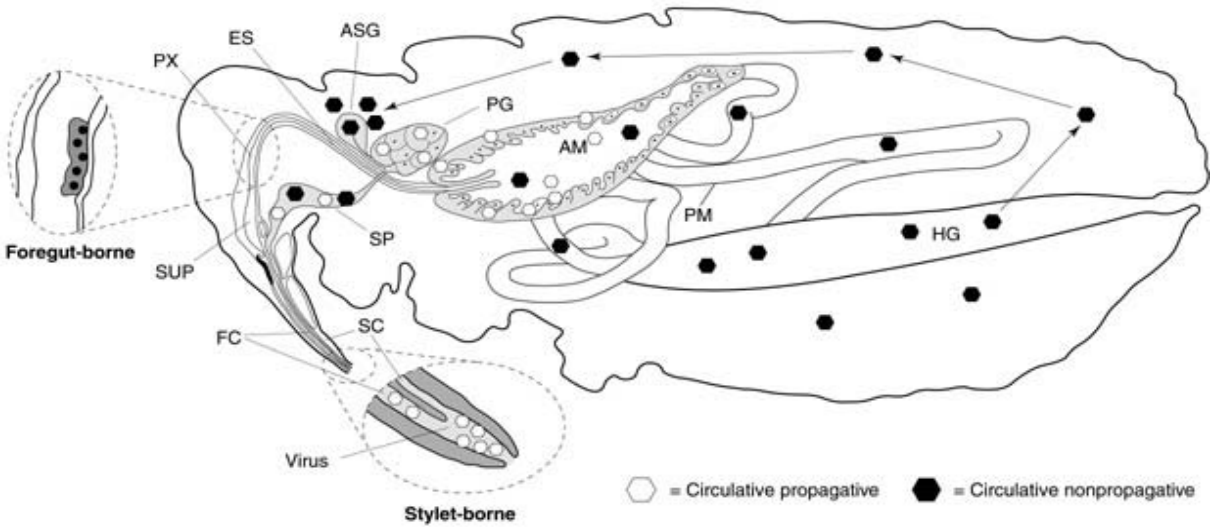


Figure 1 Mechanisms of transmission of plant viruses by arthropods with piercing–sucking mouthparts. The general anatomy of the alimentary system and the salivary system is shown; the areas relevant to virus transmission are labeled. One inset shows a detailed view of the distal end of the mouthparts where the food canal (FC) and salivary canal (SC) empty into a common space. The current model of transmission of stylet-borne (nonpersistent, noncirculative) viruses suggests that transmissible virus is retained at the distal tip of the stylets and then released by salivary secretions as the insect salivates during feeding. A second inset shows a detailed view of foregut-borne (semipersistent, noncirculative) viruses attached to the cuticle lining of the foregut, a region that would include the sucking pump (SUP), pharynx (PX) and esophagus (ES). Note the virus is embedded in a matrix material attached to the cuticle. The origin or composition of the matrix material is unknown. The circulative nonpropagative viruses will pass through the foregut into the anterior midgut (AM), posterior midgut (PM) and then into the hindgut (HG). They do not infect the gut cells but are transported through the posterior midgut or hindgut cells and released into the hemocele (body cavity). Current information indicates these viruses specifically associate with the accessory salivary glands (ASG) and are transported across the ASG cells and then released into the salivary canal (SC). The circulative propagative viruses will infect the midgut cells and subsequently infect other tissues. These viruses ultimately associate with the principal salivary glands (PG) and possibly ASG prior to their release into the SC. SP, salivary pump.

from which active helper can be solubilized. Mutations in the C-terminal domain of the CaMV helper abolish helper–virus binding *in vitro* and aphid transmission. Mutations in the N-terminus also abolish aphid transmission, but the helper retains its ability to bind to virions *in vitro*. These results suggest the C-terminus of the helper protein binds to virus particles, while the N-terminus of the helper protein is free to bind to sites in the aphid and bridge the indirect association of the virus particles to the insect cuticle.

Not all viruses transmitted in a noncirculative manner require a helper protein or helper virus (**Table 1**). Purified alfamoviruses, carlaviruses and cucumoviruses can be transmitted by aphids without helpers. Studies with cucumber mosaic cucumovirus have shown that transmission is regulated solely by the capsid protein. It is not known if these viruses are retained in similar locations in the vector.

The mechanisms of semipersistent transmission are not well studied. They may share some attributes with nonpersistent transmission but differences in sites of virus binding to the vector and times of retention indicate major differences in release of virus, if not in mechanisms of binding. There are few experimental data to explain how virus particles bound to the epicuticle substrate are released. The N-terminus of the potyvirus coat protein which binds the helper protein is often proteolytically cleaved *in vitro* without any deleterious effect on its infectivity.

taxa/the number of viruses tested for transmission is given. Information obtained from Brunt AA *et al* (1996) *Viruses of Plants.* Cambridge: CAB International.

[c] NC-NP, noncirculative, nonpersistent; NC-SP, noncirculative, nonpersistent; C-Npr, circulative, nonpropagative; C-Npr*, viruses transmitted by beetles do circulate in the insect hemolymph, but may also use a noncirculative mechanism of transmission as well; C-Pr, circulative, propagative. All mechanisms are defined in the text.

[d] Virus require a virus-encoded nonstructural helper protein or a helper virus for transmission; * indicates that there is some information that suggests a helper may be required for the transmission of some members of these virus families.

Similarly, the C-terminus of the nematode-transmitted tobacco rattle tobravirus can be cleaved from the particle without adversely affecting the virus. It is possible that proteases in the vector saliva or regurgitated gut secretions can act to cut the virus particle loose. Conformation differences in virus–helper–vector combinations and/or exposure to different enzymes and ionic conditions, depending on the site of retention, may account for differences between nonpersistent and semipersistent viruses and also for the differences in the specificity or transmission efficiency of vectors for the same virus.

Circulative Transmission

The mechanism of circulative transmission requires the virus to be internalized by the arthropod vector; there are no known circulative nematode transmitted plant viruses (**Table 1**). As previously mentioned, the circulative viruses are further divided into two subgroups: propagative viruses, those which replicate in their arthropod vectors (similar to the arboviruses); and the nonpropagative viruses.

Circulative, nonpropagative transmission

This type of transmission has been best studied with the luteoviruses and pea enation mosaic enamovirus (PEMV). The members of the luteovirus group and PEMV are each efficiently transmitted by one or, at most, a few aphid species. All the viruses share a common circulative pathway (**Fig. 1**) and biology within their aphid vectors. Ultrastructural studies have shown that ingested virus is not degraded or inactivated in the gut. Acquisition of virus into the aphid hemocele (body cavity) occurs either through the posterior midgut or hindgut epithelial cells by endocytosis. Virus is transported through the cell cytoplasm in vesicles that ultimately fuse with the basal plasmalemma, releasing particles into the space between the membrane and the basal lamina. Virus apparently moves rapidly across the basal lamina and into the hemocele. In most virus isolate–aphid species combinations, virus is acquired into the hemocele regardless if the aphid is a vector of that particular virus isolate. The gut does not appear to be a major barrier to luteovirus acquisition, although the process is specific for luteoviruses. Morphologically similar viruses can accumulate in the gut, but are not acquired into the hemocele.

Virus in the hemocele must pass through the salivary gland cells via an endocytosis pathway to be released by the vector into the plant. The salivary glands in aphids consist of two principal glands and two accessory salivary glands (ASG). Luteoviruses and PEMV associate exclusively with the ASG and more specifically with the anterior portion of these four celled glands. The ASG may function as an excretory organ in aphids and it is possible that luteoviruses have evolved to take advantage of specific excretory pathways to access the salivary ducts. An inability of luteovirus isolates to penetrate the ASG of nonvector aphids has long been known to contribute to the vector specificity. Recently it was shown that both the basal lamina and the basal plasmalemma function as independent barriers to transmission in different luteovirus isolate–aphid species combinations.

Studies of multiple luteovirus isolates in multiple nonvector aphid species has shown that the hindgut barrier or either of the two ASG associated barriers can function as the primary barrier of transmission for the same virus in different aphid species or in the same aphid for different virus isolates. This indicates that different membrane attachment sites (receptors) and/or different virus attachment protein domains are used at each transmission barrier by different virus isolate–aphid species combinations. Receptors on the aphid cell membranes have not been identified. However, the luteovirus and PEMV encoded proteins involved in aphid transmission have been studied and both virus groups share some structural features. The virus capsid contains a predominant coat protein (*c.* 22–24 kDa) and a minor amount of a larger protein translated via a readthrough of the coat protein stop codon. The full-length luteovirus coat protein readthrough is *c.* 72–74 kDa, but the C-terminal half of the 50 kDa readthrough domain is proteolytically processed to yield a 55–58 kDa coat protein readthrough commonly associated with purified virus preparations. It is not known if this type of processing actually occurs *in vivo*, but the C-terminal readthrough domain is not required for aphid transmission. The PEMV capsid also contains a coat protein readthrough, but the protein is inherently shorter than the luteovirus counterpart and does not undergo further processing.

The readthrough protein is not required for particle assembly or plant infection, and although particles containing only the 22–24 kDa coat protein are not aphid transmissible, ingested particles are found in the hemolymph. These results indicate that the coat protein regulates the uptake of the virus through the hindgut, and suggest the N-terminal portion of the readthrough domain regulates the virus–ASG associations. When luteovirus coat protein genes were expressed in insect cells using a baculovirus expression vector, virus-like particles (VLPs) were assembled. The readthrough-minus VLPs were purified and either fed to aphids through a Parafilm membrane or injected directly into the hemocele.

Ultrastructural examination of the aphids revealed the ingested particles were acquired through the gut into the hemocele, but, surprisingly, VLPs were observed in the accessory salivary gland cells and in the salivary ducts. These results are consistent with earlier studies showing that readthrough was not required for acquisition through the gut, but contrasted with the hypothesis that readthrough regulated the transport of virus through the accessory salivary gland. What then is the function of the readthrough domain, if any, in the aphid transmission process?

Aphids harbor endosymbiotic bacteria of the genus *Buchnera* in specialized cells located in the abdomen, called mycetocytes. Neither the aphid nor the bacteria are able to survive and reproduce without the other. All of the benefits that the bacteria provide for the aphid are unknown, but they are likely to provide essential amino acids the aphid is unable to synthesize. In addition, the bacteria produce copious amounts of a chaperonin protein named symbionin, a homologue of the *Escherichia coli* GroEL chaperonin protein.

Six luteoviruses and the related PEMV were all shown to bind specifically, but differentially, to *E. coli* GroEL and symbionin homologues from vector and nonvector aphids *in vitro*. The binding capacity was not correlated with transmission efficiency of the aphid, suggesting that if symbionin plays a role in transmission it does not play a role in vector specificity. A mutational analysis indicated the N-terminal portion of the readthrough domain contained the determinants for symbionin binding. Finally, virions that did not contain readthrough protein and did not bind symbionin *in vitro* were less persistent in the aphid hemolymph than wild-type virus. These studies provide convincing data that symbionin can interact specifically with luteoviruses and PEMV *in vitro* and may slow the degradation of virus in hemolymph. The mechanisms of degradation of virus in the hemolymph are unknown, nor is it known whether the attachment of symbionin to the virus protects the virus from targeting by the aphid immune system; alternatively, it may facilitate virus movement into the accessory salivary gland.

The coat protein readthrough appears to be a requirement for vector transmission of luteovirus and PEMV, but not for all circulative, nonpropagative viruses. Geminiviruses are single-stranded circular DNA viruses that have been divided into three taxonomic groups or genera (**Table 1**). The viruses within the monogeminivirus and hybrigeminivirus genera are each transmitted by a different species of leafhopper or treehopper. Viruses within the bigeminivirus genera are all transmitted by whiteflies. Geminiviruses have been observed in the gut epithelial cells and associated with salivary glands of whitefly vectors. They are assumed to follow a similar circulative route through the whitefly as the aphid transmitted luteoviruses, although no detailed ultrastructural studies have been published. Several lines of evidence suggest that the transmission mechanisms used by the geminiviruses differ from that of the luteoviruses.

The geminivirus coat protein has been shown to be the sole determinant of transmission of some whitefly-borne viruses, a property that was recently mapped to the N-terminus of the coat protein of abutilon mosaic bigeminivirus. The coat protein was also shown to be the sole determinant of whether a geminivirus was transmitted by a whitefly or a leafhopper. However, the coat protein does not solely determine the transmission phenotype of all geminiviruses. A genomic analysis of tomato golden mosaic bigeminivirus indicated that, although the coat protein was required for acquisition of the virus, both genomic components were required for transmission. DNA B was essential for the accumulation of virus in the whitefly, while DNA A was required for the successful inoculation of plants by viruliferous insects. It is not understood if either or both genomic sequences are directly influencing virus–insect interactions or plant–virus interactions that may indirectly influence transmission efficiency.

Studies of geminivirus titer over time in whiteflies have not been able to show conclusively an increase that would suggest virus replication, but the viral DNA does persist in the insect longer than its infectivity would suggest. No replicative forms of the viral DNA have been detected within the insect, which also argues against the replication of virus in the insect. However, squash leaf curl virus was observed in several whitefly tissues and the presence of virus was associated with cytopathological abnormalities in some tissues. Furthermore, the presence of the virus in the insect can have detrimental effects on the biology and reproduction of the vector. Both of these observations would suggest virus replication. Additionally, tomato yellow leaf curl virus was recently reported to be transmitted transovarily in its whitefly vector. This type of vertical transmission usually indicates the virus is replicating in the vector, but geminiviruses may have evolved a mechanism to cross the transovarial transmission barriers without replicating in that tissue, or perhaps there is some low level amount of infection of reproductive tissues. No cytopathological effects, deleterious reproductive effects or transovarial transmission have been documented for aphids fed on luteovirus-infected plants.

Circulative, propagative viruses

The plant-infecting viruses within this classification (**Table 1**) are those most closely related to the animal-infecting arboviruses. Indeed three of the five taxonomic groups considered here have animal infecting members: rhabdoviruses, reoviruses and bunyaviruses. The plant viruses within these groups could be considered as plant-infecting arboviruses or phytoarboviruses.

The phytoarboviruses, with few notable exceptions such as the tomato spotted wilt tospovirus, are not economically important and have not been intensively studied. Their genomes tend to be relatively large and complex and they have remained recalcitrant to many of the modern molecular biology techniques. In addition, it has been difficult to generate sufficient numbers of stable mutants with phenotypes related to vector transmission. These are problems that have also plagued arbovirus research but, on the other hand, arbovirus research has benefited tremendously from the establishment of cultured vector cell lines and the ability to conduct detailed genetic studies on vector populations. Both of these research strategies have been difficult to develop and apply to the insect vectors of the phytoarboviruses.

Similar to the circulative, nonpropagative viruses, the individual phytoarboviruses tend to be transmitted by only one or a limited number of closely related vector species. Furthermore, many of the individuals within a population of any given vector species are not able to transmit the virus, although many can support virus replication. Consequently much of the research on transmission has investigated the movement of virus in vectors and the mechanisms of vector specificity. The general pathway through the arthropod is similar for all these viruses (**Fig. 1**). Virus is imbibed along with the plant sap and attaches to and infects midgut cells, usually reaching high titers in these tissues. Virus is released into the hemocele and secondarily infects other tissues, including reproductive tissues from which the virus can spread vertically to offspring. Horizontal transmission to other plant or animal hosts occurs following infection of salivary tissues and subsequently release of infectious virus from the glands in the salivary secretions that are injected into the host during feeding.

Vector competence (ability to transmit) is determined not only by the ability of the virus to replicate in the various tissues of the vector, but also by the ability of the virus to successfully enter and exit the tissues. The cellular barriers to transmission have been extensively studied and include a midgut infection barrier, which was first demonstrated for eastern equine encephalomyelitis alphavirus and has subsequently been demonstrated for other animal- and plant-infecting viruses. An active midgut infection barrier will effectively render the arthropod immune to the virus. Other barriers allow infection of the arthropod, but virus is not transmitted. A midgut escape barrier has been demonstrated for tomato spotted wilt tospovirus (*Bunyaviradae*) in the adult stage of the thrip vector. Virus can infect and replicate in midgut cells of both larval and adult thrips, but virus can only disseminate from larval midgut cells into other thrip tissues, therefore it must be acquired by the larval thrips to be transmitted. Wound tumor reovirus and sowthistle yellow vein rhabdovirus, both phytoarboviruses, are able to invade and replicate in several tissues of their leafhopper or aphid vectors, respectively. However, in nontransmitting individuals the viruses were not associated with the salivary glands. This suggests the existence of a salivary gland infection barrier, but does not rule out the possibility that the virus is not able to survive in the hemolymph or hemolymph-associated cells that would come in contact with the salivary glands. A salivary gland escape barrier has also been demonstrated for some arboviruses in their mosquito vectors, but has not been demonstrated for any phytoarbovirus. Similar to the situation described earlier for the circulative, nonpropagative luteoviruses, the specific barrier may differ for any combination of virus and vector and no generalities seem to be applicable.

The molecular and physiological basis for virus–vector interactions at these various barriers that regulate transmission are not well understood, but it is clear that genetic elements of the vector ultimately decide if a particular species or individual within a species of arthropod is able to vector a particular virus strain. Environmental or abiotic factors also play a role in determining virus–vector interactions, but in general these factors seem to influence the efficiency of the interaction rather than to determine the ability of the interaction to take place.

Virus transmission by vectors is not controlled solely by the vector: the virus also contributes to the overall process. Limited progress has been made in understanding the phytoarbovirus genes and gene products that influence vector transmission. A lack of stable cell lines from insect vector species and a difficulty in developing stable mutants with a transmission-deficient phenotype have contributed to the slow progress, but recently cell lines have been established for leafhopper vectors of reoviruses as well as for thrip vectors of tomato spotted wilt tospovirus.

Despite an absence of vector cell lines, progress on the identification of virus genes whose function is related to transmission has been made by studying

virus associations with whole insects. The animal-infecting La Cross bunyavirus glycoproteins have long been implicated in vector transmission and recently it was shown that the tomato spotted wilt bunyavirus glycoproteins interact with two different thrip proteins, one of which was associated with midgut tissues. The tissue association of the other was not determined. The glycoproteins are likely involved with cell attachment and virus entry.

Studies on whole, virus-infected insects have also identified differences in virus RNA and protein accumulation between the plant and insect hosts. A nonstructural protein of maize stripe tenuivirus and rice grassy stunt tenuivirus accumulates in maize and rice, but not in the leafhopper vector. The function of the nonstructural protein is unknown. Perhaps it serves as a plant virus movement protein or aids in the initial uptake of virus by the vector, but would not subsequently be needed to be produced by the vector. In contrast to the aforementioned studies, the RNAs encoding the two maize stripe virus glycoproteins are abundant in both insect and plant host cells, and all serologically detectable rice dwarf reovirus proteins are present in both insect and plant hosts. Similar findings of no qualitative differences in viral RNAs or proteins have been reported for the alphaviruses. However, there were differences in the post-translational processing of the viral proteins between the mosquito and vertebrate hosts.

Another major problem in understanding phytoarbovirus–vector interactions has been in developing or identifying stable mutants that have altered vector transmission phenotypes. The viruses have all remained recalcitrant to many of the modern molecular biology techniques. Infectious DNA clones have not yet been produced and therefore a directed mutational strategy is not possible, nor is a reverse genetic approach to identifying gene function. A limited number of transmission mutants have been obtained by repeated mechanical inoculation of plant hosts without going through the insect host. A strain of the rice dwarf reovirus maintained for 12 years in vegetatively propagated rice plants had lost the P2 outer capsid protein due to a point mutation that introduced a termination codon in the open reading frame. The P2-minus virus was able to infect plants, but was unable to infect the leafhopper vector and be transmitted to plants.

Vegetative propagation of wound tumor reovirus-infected plants also resulted in leafhopper transmission-defective virus isolates. This was later found to result from the generation of defective RNAs of four of the 12 genomic sequences. These defective viruses were not able to infect the leafhopper vector cells, but were able to maintain a near wild-type infection of the plant host. Presumably all of the virus functions necessary to infect the plant host were contained on the eight remaining genomic segments, whereas one or more of the four defective segments provided for, as of yet, undefined functions specific for infection of the insect host. The data that have been generated thus far point to the conclusion that outer capsid proteins of the phytoarboviruses are, as would be predicted, involved in the infection of insect cells, i.e. attachment proteins. These would not be necessary for plant hosts because of the cell wall, which must be breached by totally different mechanisms than the cell membrane of insect hosts.

There is very limited reference to the continued serial passage of phytoarboviruses in their insect vector. Sowthistle yellow vein rhabdovirus was mechanically passaged by injecting virus into the hemocele of aphid vectors. Continuous serial passage did give rise to virus isolates that were difficult to transmit to plants and also more pathogenic to the vector. The reasons for the low probability of transmission was not determined and may have been a deficiency in systemic plant infection or an inability to move through all the transmission barriers in the aphid. With the advances in technologies that now allow further characterization of such mutants, there should be advances in our understanding of virus–vector interactions despite the difficulties with vector cell cultures and a lack of infectious clones of the viruses.

Viruses with Fungal Vectors

The terminologies and concepts described in the previous sections were all developed for multicellular organisms that feed on plant tissues. These are not easily adapted to virus transmission by fungal vectors. Although the fungi are multicellular, the vector is the unicellular zoospore. Furthermore, the acquisition of virus by the fungus does not involve feeding and ingestion comparable to the arthropod or nematode vectors discussed previously. Therefore, different terminology has been applied to the transmission of viruses by fungi.

Properties of Fungal Vectors

The fungally transmitted viruses belong to at least seven plant virus genera contained within at least four families (**Table 1**). The fungi known to be involved in vector transmission are zoosporic obligate parasites of plant roots and include two species of Chytridiomycetes (*Olpidium bornovanus* (formerly *O. radicale*) and *O. brassicae*) and three species of Plasmodiophoromycetes (*Polymyxa graminis, P. be-*

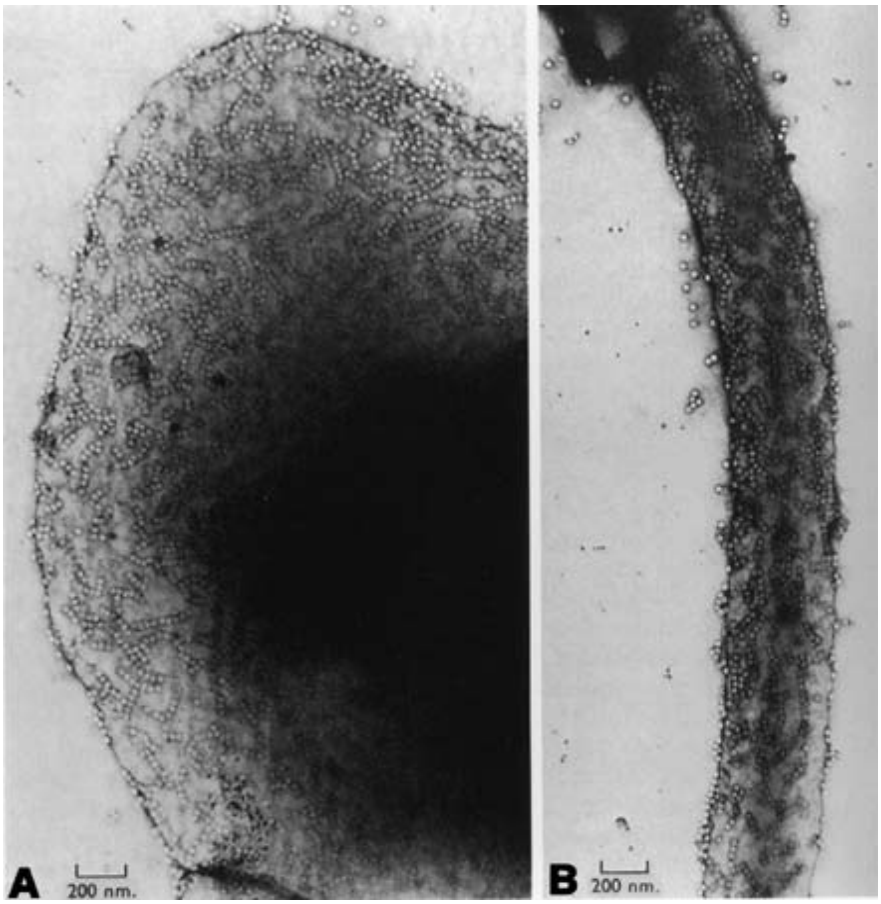


Figure 2 Electron micrograph showing binding of tobacco necrosis virus particles to the plasmalemma of the body (**A**) and to the axonemal sheath of the flagellum (**B**) of *Olpidium brassicae* zoospores. (Reprinted with permission from Temmink JHM, Campbell RM and Smith PR (1970) Specificity and site of *in vitro* acquisition of tobacco necrosis virus by zoospores of *Olpidium brassicae*. *J. Gen. Virol.* 9: 201–213.)

tae and *Spongospora subterannea*). The genera *Polymyxa* and *Olpidium* apparently cause little or no direct damage to crops but the viruses they vector can cause significant disease.

Most of the viruses vectored by *Olpidium* species are members of the family *Tombusviridae* and, as such, are small, spherical viruses containing single-stranded RNA genomes. Exceptions to this are the 'varicosaviruses' which are double-stranded RNA viruses with rod-shaped virus particles. Viruses with plasmodiophorid vectors are rod shaped and usually contain segmented single-stranded RNA genomes.

Olpidium spp. have zoospores which are uniflagellate, whereas *Polymyxa* and *Spongospora* spp. have biflagellate, heterokont zoospores. The developmental stages of these fungi are similar. Resting spores (or cystosori in the Plasmodiophoromycetes) produced in plant roots are released into the soil when roots decay. The resting spores germinate and produce motile zoospores which swim to roots and encyst when they encounter epidermal cells. Encystment involves withdrawal of the flagellum, attachment to root cell walls, and secretion of a thin cyst wall. The protoplasm of the zoospore is then released into the host cell and divides into a multinucleated plasmodium, which then develops into a sporangium. The sporangium liberates secondary zoospores, which are either released into the soil to infect other root cells, or which infect adjacent root cells. Vegetative sporangia produce the thick-walled resting spores. These are essential for survival of the fungus in the absence of a host and also permit survival of virus for extended lengths of time (>20 years for viruses transmitted in the *in vivo* fashion, see below).

Fungal Transmission Mechanisms

Two types of fungal–vector relationships are recognized: *in vitro* and *in vivo*. They are distinguished by the method of acquisition of the virus by the fungus and the location of the virus relative to the fungal resting spore (internal or external). In *in vitro*

transmission the virus is carried externally on the zoospores (**Fig. 2**) and virus is not found within resting spores. In *in vivo* transmission, virus is acquired during growth of the fungus in a virus-infected plant and is found within resting spores. The location of a virus particle relative to a resting spore can be determined by treating resting spores with strong chemicals that are known to inactivate virus exposed to them (e.g. 20% Na_3PO_4 or 5 mol l^{-1} HCl). A virus carried internally will remain transmissible following treatment, whereas a virus on the surface will not. Alternatively, mixing virus with zoospores obtained from virus-free plants in solution and then inoculating roots with the suspension can result in infection by *in vitro* transmitted viruses, but viruses transmitted *in vivo* will not be acquired by the zoospores in solution and cannot infect plants inoculated with the suspension. *Olpidium* spp. are the only vectors identified that transmit virus *in vitro*, whereas *in vivo* transmission occurs with *Olpidium* spp., *Polymyxa* and *Spongospora* vectors. There is no evidence for replication of fungally transmitted viruses in their vectors.

In vitro transmission

The mechanism of *in vitro* transmission has been best described using tobacco necrosis necrovirus (TNV) and its vector *O. brassicae*, but was also later demonstrated for cucumber necrosis tombusvirus (CNV) and *O. bornovanus*. The virus is adsorbed on to the surface of the zoospore (**Fig. 2**) as it moves through the soil following independent release of the fungus and virus into the soil. The source of zoospores can either be from vegetative sporangia or from resting spores. The source of virus can be from infected root material obtained during the growing season or from other decaying plant parts after the crop is harvested. The acquisition time is short, in the range of 5–15 min, and the virus is very stably adsorbed to the plasmalemma of the body and the axonemal sheath of the zoospore. Electron microscopy studies have shown that adsorption is very specific: TNV binds *O. brassicae* zoospores but not *O. bornovanus* zoospores; CNV binds *O. bornovanus* zoospores but not *O. brassicae* zoospores. In addition, there is intraspecific variation in transmission efficiency. Different *O. brassicae* isolates vary in their ability to transmit TNV and three categories of vector efficiency could be identified: efficient vectors, inefficient vectors and nonvectors. Virus is not absorbed on to zoospores of a nonvector isolate and inefficient vector isolates adsorb fewer particles than zoospores of efficient vectors. The ability or inability of particles to bind zoospores appears to play a major role in the specificity of transmission.

The manner in which bound virus enters a root cell following zoospore encystment has not been extensively studied. It has been suggested that virions enter the zoospore protoplasm as the flagellum is retracted during encystment. Virus then enters a root cell when the zoospore protoplasm is discharged.

The molecular basis of *in vitro* transmission is also not well understood. Work with CNV and its fungal vector *O. bornovanus* has shown that virus particles are required for transmission. Further work in which the coat protein (CP) gene of CNV was exchanged with that of the nontransmissible tomato bushy stunt virus showed that the CNV CP contains transmission-specificity determinants. Transmission-defective CNV mutants have been isolated by repeated mechanical passaging. The transmission deficiencies were found to be due to either a deleted CP gene, the inability to express CP or an altered CP. One characterized mutant contained a single amino acid substitution in the CP shell domain which decreased transmissibility to less than 20% that of wild-type virus without affecting particle stability, virus accumulation or infectivity. This mutant also showed a decreased ability to bind zoospores in an *in vitro* binding assay, providing further evidence that binding plays a critical role in the transmission process. In addition, binding of CNV to *O. bornovanus* zoospores has been found to be saturable, specific and pH dependent. These studies suggest that a specific zoospore receptor may be involved in mediating viral attachment.

In vivo transmission

Viruses such as beet necrotic yellow vein furovirus (BNYVV) and barley yellow mosaic bymovirus (BaYMV) are transmitted by the *in vivo* mechanism and have been visualized within zoospores and resting spores of their fungal vectors. The virus must be acquired by the fungus within an infected host plant, but the timing and mechanisms of acquisition are unknown. A majority of the research has focused on roles of virus-encoded proteins in the transmission process.

Similar to the luteoviruses described earlier, the furovirus capsid contains a predominant coat protein and lesser amounts of a coat protein readthrough. Repeated mechanical passage of BNYVV and soil-borne wheat mosaic furovirus (SBWMV) results in transmission-defective mutants, a trait that is associated with the accumulation of deletion mutations in the readthrough domain. These results suggest that the readthrough domain contains sequences which

can promote interactions between particles and fungus. Deletion analysis, along with alanine scanning mutagenesis, has identified a KTER amino acid motif near the C-terminus of the BNYVV readthrough as being important for the fungus transmission process. A similar sequence (KTEIR) is found in the readthrough domain of SBWMV.

Bymoviruses do not translate a coat protein readthrough. The repeated mechanical transfer of a BaYMV strain resulted in the loss of fungus transmission and a spontaneous approximate 1 kb deletion in RNA2. The deleted region is from a protein which is associated with crystalline inclusion bodies in infected tissue. Alignments of portions of the protein products of RNA2 of two bymoviruses and the readthrough portion of several furovirus capsids has revealed that certain amino acid combinations (ER or QR) are found consistently in all the viruses. These amino acids occur on the outside of the protein and therefore may be available for interaction with the fungus vector. In addition, certain conserved regions are absent in nontransmissible deletion mutants. These studies suggest that bymoviruses and furoviruses encode similar proteins for facilitating their transmission.

See also: **Luteovirus; Geminiviruses (*Geminiviridae*); Potyviruses (*Potyviridae*); Plant pararetroviruses (*Caulimoviridae*): Caulimoviruses: general features, Caulimoviruses: molecular biology; Tospoviruses (*Bunyaviridae*).**

Further Reading

Adams MJ (1991) Transmission of plant viruses by fungi. *Ann. Appl. Biol.* 118: 479.

Ammar ED (1994) Propagative transmission of plant and animal viruses by insects: factors affecting vector specificity and competence. In: Harris KF (ed.) *Advances in Disease Vector Research*, vol. 10, p. 289. New York: Springer-Verlag.

Brown DJF, Robertson WM and Trudgill DL (1995) Transmission of viruses by plant nematodes. *Annu. Rev. Phytopathol.* 33: 223.

Campbell RN (1996) Fungal transmission of plant viruses. *Annu. Rev. Phytopathol.* 34: 87.

Gergerich RC and Scott HA (1991) Determinants in the specificity of virus transmission by leaf-feeding beetles. In: Harris KF (ed.) *Advances in Disease Vector Research*, vol. 8, p. 1. New York: Springer-Verlag.

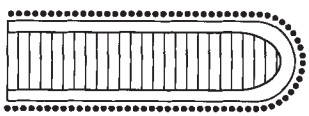
Gildow FE (1999) Luteovirus transmission and mechanisms regulating vector-specificity. In: *Luteoviridea*. Wallingford: CAB International.

Pirone TP and Blanc S (1996) Helper-dependent vector transmission of plant viruses. *Ann. Rev. Phytopathol.* 34: 227.

Venezuelan Equine Encephalitis Virus *see* **Equine Encephalitis Viruses**

Vesicular Exanthema Virus *see* **Caliciviruses**

VESICULAR STOMATITIS VIRUSES (*RHABDOVIRIDAE*)



Luis L Rodriguez, Plum Island Animal Disease Center, Agricultural Research Service, United States Department of Agriculture, Greenport, New York, USA

Stuart T Nichol, Special Pathogens Branch, Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, USA

History

Vesicular stomatitis viruses (VSVs) are rhabdoviruses which infect a wide range of wild and domestic animals, and several groups of insects. VSVs are best known as the cause of vesicular stomatitis (VS), a disease characterized by vesicular lesions in the mouth, tongue, udder teats and hoof coronary bands of cattle, horses and pigs. Clinical disease occurs every year in farming areas from northern South America to

VECTORS

Contents

Animal Viruses

Geoffrey Kitchingman and **J Victor Garcia**, Department of Virology and Molecular Biology, St. Jude Children's Research Hospital, Memphis, Tennessee, USA

Introduction

Researchers have exploited knowledge of the structure of viral genomes to insert DNA from foreign sources (transgenes) for over 20 years. Vectors, as such recombinant molecules are known, were initially generated from the genomes of the smaller DNA viruses (papovavirus and papillomavirus families). Knowledge of the genome structure of the larger DNA viruses (adenovirus, herpesvirus, poxvirus and baculovirus) and RNA viruses has expanded the numbers and types of backbones available for generating vectors. Each virus family, and vector backbone, has its own set of advantages and disadvantages that identify the types of operations for which they can optimally be used. Scientists have used these vector systems: (1) to study gene regulation; (2) in mRNA processing; (3) to achieve high-level expression of biologically active proteins; (4) to address questions of the structure–function relationship of specific polypeptides; (5) to investigate the immunobiology of specific pathogens; (6) to develop recombinant vaccine candidates and serodiagnostic reagents; and (7) to understand and treat cancer and genetic diseases in the clinic.

The salient features of many, but not all, RNA and DNA virus-based vectors will be discussed here. Not included are some of the vectors used infrequently, including the original vectors derived from papova- and papillomaviruses.

Adeno-associated Virus Vectors

Adeno-associated viruses (AAV) belong to the family *Parvoviridae*, genus *Dependovirus*. As indicated in their name, these viruses are replication defective and depend on other viruses for factors required for replication. In the laboratory, the factors are generally supplied by adenovirus or herpesvirus co-infection. The AAV vector genome consists of a single-stranded DNA of 4.7 kb, with 150 bp palindromic sequences or inverted terminal repeat sequences (ITRs) at both ends. These two ITRs are all that is needed for packaging and integration into the host genome. AAV vectors can be constructed with up to 5 kb of exogenous DNA between the two ITRs. The transgene inserted between the ITRs must have its own promoter, as these sequences do not function as promoters. During infection, a low percentage of the viral genomes will integrate into the host chromosome. Wild-type AAV has a propensity to integrate at a specific site in human chromosome 19, but the currently available AAV vectors have lost this property and integrate randomly into the genome of the target cell.

A number of systems have been developed for using the AAV DNA backbone as a vector for gene transfer, with varying degrees of success. Despite the relatively simple genomic structure of AAV, vector systems require three components: one that supplies the AAV capsid proteins, the transgene containing vector and the helper virus. The need for helper virus results in contamination of vector preparations with helper virus particles or with denatured viral proteins, even after extensive purification and heat inactivation of vector stocks. This contamination is undesirable for the implementation of AAV vectors in clinical applications. Identification of the adenovirus genes required for complementation of AAV has allowed the development of new schemes that use a non-replicating adenovirus genomic plasmid as a helper. When co-transfected with the AAV vector and the AAV helper construct, vector preparations free of replication-competent adenovirus and wild-type AAV can be produced at relatively high titers. However, this system is based on transient transfection of cell lines, which would make large-scale clinical implementation of protocols using AAV vectors difficult. Efforts to develop stable cell lines for the production of AAV vectors are still at an early stage, but their availability is likely to facilitate evaluation of this potentially useful vector system.

AAV vectors are being developed almost exclusively as vehicles for gene transfer and gene therapy in a clinical setting. Several features of AAV make it attractive for these uses. The virus is nonpathogenic

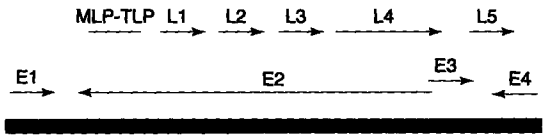


Figure 1 Adenovirus transcription map. Early (E) and late (L) transcription units are represented by solid lines relative to the 36 kb adenovirus genome. Arrowheads indicate direction of transcription. Each transcription unit gives rise to families of mRNAs derived by differential splicing events. The late mRNAs are generated by differential splicing and polyadenylation site utilization and all are initiated from the major late promoter (MLP) and contain the tripartite leader (TPL).

and has a broad host range and tissue tropism. A major advantage is its ability to infect nondividing cells, and its potential for targeted integration. In preclinical model systems the levels and duration of transgene expression have varied, so it is not entirely certain that therapeutic levels of expression can be achieved. Transduced cells do not elicit a cytotoxic T cell response, a common occurrence when using adenovirus vectors. Phase I clinical trials are underway to evaluate toxicity in humans.

Adenovirus Vectors

The human adenovirus (Ad) family consists of at least 49 members that have been classified into six subgroups based on a variety of biological and molecular characteristics. Two members of the subgroup C adenoviruses, Ad2 and Ad5, form the backbone for the majority of Ad vectors in use today. The viral genome is divided into five early and five late gene regions, with each region coding for a multiplicity of mRNAs and proteins (**Fig. 1**). All coding regions are by and large essential, with the exception of the early region 3 (E3), which is not necessary for growth in tissue culture. The viral genome is approximately 36 kb in length, and studies have shown that up to 105% of the viral genome can be packaged. High titer virus vector preparations are relatively easy to prepare and consequently they have found many uses, including the expression of large amounts of transgenes, in vaccine development, and especially in the area of gene therapy. Some of the types of adenovirus vectors used to date are discussed below.

There are two basic methods for inserting foreign genes (transgenes) into adenovirus vectors, methods that are also used for the larger DNA viruses. The first is through homologous recombination, which can be carried out in tissue culture cells, bacteria or yeast. The gene of interest is inserted into a shuttle vector containing about 2–4 kb of the viral genome, and it is co-transfected with DNA representing (at minimum) the remainder of the viral genome into the cells of choice. Recombinants can be selected by analyzing individual plaques, or by including marker genes in the shuttle plasmid that allow visual selection. The second method of construction of adenovirus vectors involves cloning of the whole genome in bacterial plasmids. An advantage of this method is that most of the characterization can be performed on the plasmid, and all plaques arising from transfection into human cells should contain the transgene. The maximal length of the transgene is directly dependent on the number of viral genes deleted. Most vectors are based on E1A- and E1B-deleted backbones, and can hold transgenes of up to 4 kb. Deletion of the nonessential E3 region brings the packaging capacity to about 7 kb, and deletion of additional early region genes further increases the packaging capacity. Every essential gene deleted has to be complemented, and cell lines expressing various complements of these genes are available. The promoter used to drive transgene expression will dictate the relative level of expression, and transgenes with and without introns are expressed equally well. With the exception of hematopoietic cells, adenovirus vectors efficiently infect most cell types.

Adenovirus vectors do not integrate, so expression of transgenes is limited to the time the vector DNA is maintained episomally in the cell. The viral episome is stable in cells, so when terminally differentiated cells are infected, the viral genome will last for essentially the life of the cell. *In vivo*, adenovirus causes significant immune responses, which is also true for adenovirus vectors, so this is potentially a limiting factor for their use in animals or humans.

Adenovirus, especially the attenuated virus produced when E1A and E1B are deleted, is generally innocuous for immunocompetent adults, and vaccines for a variety of human viruses are under development using Ad vectors for transgene delivery. Virus genes inserted into Ad vectors for vaccine development include genes from hepatitis B, human immunodeficiency virus (HIV), herpes simplex virus and pseudorabies, but to date no vaccine has come into common use. Humoral responses were elicited with the hepatitis B/Ad recombinant in chimpanzees, but they only partially protected against hepatitis B challenge. Other methods of gene delivery, including other viruses and direct injection of DNA, are finding their way into clinical trials, and it is likely that few adenovirus vectors will be used for vaccine development.

Adenovirus vectors have been used in numerous clinical trials to deliver therapeutic and/or toxic compound to cells. Use of Ad vectors for delivery of

therapeutic proteins has generally not been successful. The extent to which adults are already armed against adenovirus infections was underestimated in these trials. There are a number of physical and immunological barriers to adenovirus infection that diminish the duration and extent of transgene expression. These include: (1) physical barriers to infection; (2) virus removal from the blood through unknown mechanisms; and (3) the presence of a memory immune response to the virus. The initial failures with the subgroup C backbone-based Ad vectors has led to the development of a variety of vectors based on backbones from other subgroups to which the majority of the population has not been exposed. Vectors lacking all viral genes have also been developed, but have not yet been put to use in the clinic.

Adenovirus vectors that are potentially selectively toxic to tumor cells are now in clinical trials. Adenovirus mutants that replicate only in cells lacking p53 are being tested in clinical trials for their ability to specifically kill tumor cells (in combination with chemotherapy). Vectors carrying tumor suppressor genes such as p53 and Rb are being tested in numerous clinical trials for their efficacy in halting tumor growth. Vectors that carry transgenes for enzymes that convert prodrugs to their active forms are being developed. The transgene is expressed under the control of a promoter that is either tumor or tissue specific. Ad vectors with altered tropisms are being developed to target tumors or specific tissues. One problem with many of these approaches is that the virus is large and does not readily diffuse from the site of injection, and therefore killing can be quite localized. Thus, adenovirus vector-mediated therapy of cancer is likely to be an additional tool for the oncologist, to be worked into a regimen of more traditional chemotherapy and radiotherapy.

Adenovirus vectors are being developed for use in nonmalignant disease therapy. A vector containing the gene encoding vascular endothelial growth factor is being used to try to help ischemic hearts grow new blood vessels. Other vectors are being used to try to prevent the closure of blood vessels newly opened in angioplasty.

Adenovirus vectors are finding their way into many niches in medicine, but it is safe to say that researchers are still learning how to use the virus most effectively.

Poxvirus-based Vector Systems

Poxviruses are widespread in nature and tend to have a limited host range. They are large DNA viruses, with genomes of about 190 kb in length. They differ from other DNA viruses in that they replicate solely in the cytoplasm of cells. This requires the virus to encode nearly all of the enzymes involved in replication of the virus, many of which are found in the virus particle and enter a cell with the virus. Poxvirus particles can package DNA at least 25 kb larger than the wild-type genome, and deletion of nonessential genes can significantly increase the size of foreign DNA that can be inserted into the vector. Poxvirus vectors grow well in cells of the native host, and the virus can readily be purified to high titers. Vectors based on human, animal and avian poxviruses have been developed, each with a particular set of advantages for a given application.

Poxvirus DNA is not infectious because of the requirement for virus particle-associated enzymes, and the generation of recombinants requires the use of infectious virus. The favored technique is based on marker rescue, originally developed to map mutants in the viral genome. The technique is similar to the homologous recombination method used with adenovirus vectors, but cells are first infected with a virus whose genome is to be altered and then transfected with a plasmid containing the transgene between viral sequences normally contiguous in the genome. The relative level of homologous recombination is low, about 0.1%, so efficient selection methods have been developed. These include inactivating the viral thymidine kinase gene by insertion of the foreign gene within it, and the insertion of genes encoding visual marker proteins in the rescuing plasmid. Because poxvirus vectors replicate in the cytoplasm and use poxvirus-encoded enzymes, transgenes must be placed under the control of poxvirus promoters for expression (some exceptions are discussed below). There are three classes of poxvirus promoters, early, intermediate and late, and the timing and level of expression of any transgene can be regulated by the choice of promoter. Proteins produced in poxvirus expression systems are processed and transported normally, so relatively high levels of production of normal protein are possible in permissive and nonpermissive cell lines.

Vaccinia virus is the favored vector for a variety of applications. Many different variations on the straight transgene-containing vector have been developed. Systems are available for expression of proteins from recombinant plasmids introduced into vaccinia virus-infected cells. This type of transient expression system affords much higher levels of transgene expression than conventional transfection systems. Inducible expression systems based upon the bacteriophage T7 and T3 RNA polymerase promoter and the *Escherichia coli* lac operator–repressor systems have been developed. The RNA polymerase can either be constitutively expressed in the cell line, or be

brought in by a co-infecting virus. The gene to be expressed, under the control of the T3 or T7 promoter elements, is introduced into the cells either as an expression plasmid or as part of a vaccinia virus vector. These approaches allow for controlled expression, which is especially important when the protein product is cytotoxic.

Poxvirus expression systems have been used to express hundreds of proteins of biological and medical importance. The practical application of vaccinia virus vaccine candidates has been demonstrated in both the veterinary and human fields. Vaccinia was used successfully as a vaccine for the eradication of smallpox. Other examples of its use include vaccinia virus recombinants to protect raccoons against rabies, a capripoxvirus vector to protect cattle against rinderpest, a swinepox vector to protect pigs against pseudorabies, and fowlpox vectors to protect chickens against influenza virus, Newcastle disease virus and infectious bursal virus. Clinical trials with a vaccinia-based HIV-1(IIIB) *env* recombinant have been initiated in the USA. Results from these studies clearly demonstrated the ability of this recombinant to prime an immunological response in the recipients, although the response was suboptimal in some individuals. A vaccinia virus-based vector expressing the Epstein–Barr virus membrane glycoprotein has been used in China with some success in preventing natural infection.

New vectors have recently been developed that address some of the safety concerns of the original vectors. A strain of vaccinia virus with 18 genes implicated in the virulence and host range of the virus deleted has been developed. Another vector, based on a canarypoxvirus, replicates only in avian species. When this vector is introduced into nonavian cells, the transgene is expressed. Preliminary studies demonstrate that both are effective in generating high levels of transgene expression, and that in a vaccine setting they can both induce protective immunity. A canarypoxvirus-based vector expressing the rabies G glycoprotein has been assessed in phase I clinical trials in Europe and the USA, with encouraging results. In addition, vectors expressing the human cell surface protein CD80 are in clinical trials. This protein is involved in T cell recognition of target cells, so its expression in tumor cells potentially enhances the ability of the immune system to recognize cancer cells. This tumor vaccine approach is expanding, as poxvirus vectors encoding a variety of tumor antigens are currently undergoing preclinical testing. Given the large capacity of the vectors, genes encoding both tumor antigens and immune system recognition enhancing molecules could be inserted into a single virus.

Poxvirus vector-based systems have demonstrated their utility for expression of foreign genes for study in tissue culture, for use as a vaccine vehicle in both animals and humans, and they are beginning to be used for treating cancer. Because of the wide variety of available vectors, and the safety features that have been built into them, poxvirus vectors will increasingly become the vector of choice for a variety of applications.

Baculovirus Expression Vectors

Baculoviruses are pathogenic insect viruses that have been investigated for possible use as biological control agents for insect pests. Recently, these viruses have proven useful in the development of helper-independent vectors for overexpression of foreign genes in eucaryotic cells. Baculoviruses contain a double-stranded DNA genome of around 130 kb, and the DNA is infectious. The main attraction of this system is the ability to produce very large quantities of transgene protein, but until recently the system was limited to insect cells. Recent developments have considerably expanded the potential of the baculovirus vector system for use in mammalian cells.

Baculovirus vectors are generally produced by homologous recombination between a shuttle vector and a wild-type genome, using the viral polyhedrin gene as the recombination target. Shuttle vectors often contain the transgene of interest plus a selectable marker gene, although inactivation of the polyhedrin gene through transgene insertion gives rise to plaques that can be differentiated from those of wild-type virus. The polyhedrin gene of baculoviruses such as *Autographa californica* nuclear polyhedrosis virus (AcMNPV) is expressed at very late times of infection and produces a protein that surrounds the mature virus particle to form an occlusion body in insect larvae. Polyhedrin protein expression in infected tissue culture cells can continue for days following initial infection and often amounts to 50% of the total cell protein. The polyhedrin protein is not essential for replication of the virus in tissue culture, and transgenes placed under the control of its promoter can be expressed to levels approaching that of the polyhedrin itself. The virus has a rod-shaped capsid and has a large capacity for packaging foreign DNA. Baculoviruses do not productively infect vertebrate cells, but modifications to the system have allowed transgene expression in a variety of mammalian cell lines, further expanding the potential of the vector system.

Baculovirus vectors have found their greatest use in the production of large quantities of protein for purification. While insect cells can post-translation-

ally modify proteins correctly, there were concerns that they could not process high-mannose glycans like mammalian cells. However, recent reports on human plasminogen expressed in insect cells show that the enzymes necessary for processing high-mannose glycans can be activated with glycan processing identical to that found in the human-expressed protein. These are important considerations if baculovirus-expressed products are to have the same biological and immunological properties as that of the native protein. Baculovirus-expressed protein was used in the first human clinical trials for an acquired immune deficiency syndrome (AIDS) vaccine, using an HIV-1 (IIIB) *env* gene product purified from insect cells. Its general utility for production of purified proteins for clinical use remains to be demonstrated.

Herpesvirus Vectors

Two herpesviruses are emerging as alternatives for the development of gene transfer vectors: herpes simplex virus (HSV) and Epstein–Barr virus (EBV). HSV has a genome of approximately 155 kb in length, which is maintained as a concatemerized circular or linear episome in infected cells. It efficiently infects cells, including nondividing cells, from a wide variety of organisms, and is able to establish a persistent infection. Its genome can accommodate large amounts of foreign DNA, and a number of vector systems have been developed. The EBV-based systems have not been developed as extensively and currently employ replication-defective EBV strains. Unlike HSV, EBV vectors have a very limited tropism and their possible usefulness remains to be explored. The main difficulty using these two systems is the need for almost complete virus genomes that are very complex and large in size, making manipulations exceedingly difficult. The eventual usefulness of these systems will be dictated by further simplifications that render them more manageable in the laboratory and that facilitate vector production.

Helper-dependent and -independent systems have been developed for preclinical evaluation. The helper-independent systems have basically the complete viral genome with one or more transgenes substituted for nonessential viral genes. These vectors are rendered defective for growth by deletion of essential genes, permitting their growth only on complementing cell lines. So far these vectors have met with limited success in model systems because of leakiness in the system, which leads to cytotoxicity and cell death. The requirements for maintenance of gene expression during the latent state in neuronal cell types are also currently unknown, so prolonged transgene expression has been difficult to obtain. The second type of HSV vector is the helper-dependent system. The vectors contain only the sequences required for replication and packaging, but all the other functions must be supplied in *trans* from a helper virus. While such systems are capable of producing high-titer amplicon-containing virus, they are almost invariably contaminated with large quantities of the helper virus. Recent improvements in the amplicon system have allowed the generation of relatively high-titer amplicon preparations with much lower levels of contamination with helper virus. These systems hold promise for use in clinical situations, especially in gene transfer to neuronal cells, but further development of the system is required.

Retrovirus Vectors

Retroviruses are ubiquitous in nature and have a relatively simple RNA genome of around 9 kb in length. By reverse transcription, the RNA genome becomes a double-stranded DNA that integrates into the host chromosome and is stably transmitted to progeny cells. Specific aspects of the life cycle of retroviruses and their relatively simple genome have facilitated their development into gene transfer vectors. Most of the vectors currently used for basic science and for clinical applications are based on murine leukemia viruses. Because of the high efficiency with which retroviral vectors can transduce a variety of cell types from many different species, they have become a method of choice for gene transfer experiments.

Retrovirus vectors can be produced by either transient transfection of cells, or by the generation of stable producer cell lines. In both cases, the transcription units for the packaging functions for the virus particle and that for the transgene are on separate genetic elements. The packaging proteins are provided in *trans*, either by a co-transfected second plasmid that encodes the proteins needed for particle formation, reverse transcription and integration of the vector (transient production), or by a packaging cell line that contains these genes integrated into the chromosome (**Fig. 2**). Separation of the *trans* functions into several plasmids is important to minimize the chance that a wild-type replication competent virus may be generated by a recombination event. Packaging cell lines are able to generate large numbers of virus particles without a genome. These particles are empty because the packaging construct does not contain the information needed for specific incorporation of RNA into the virus particle. This is an important aspect of the system because it prevents the transmission of genomic information encoding virus proteins. The vector plasmid contains all of the *cis*

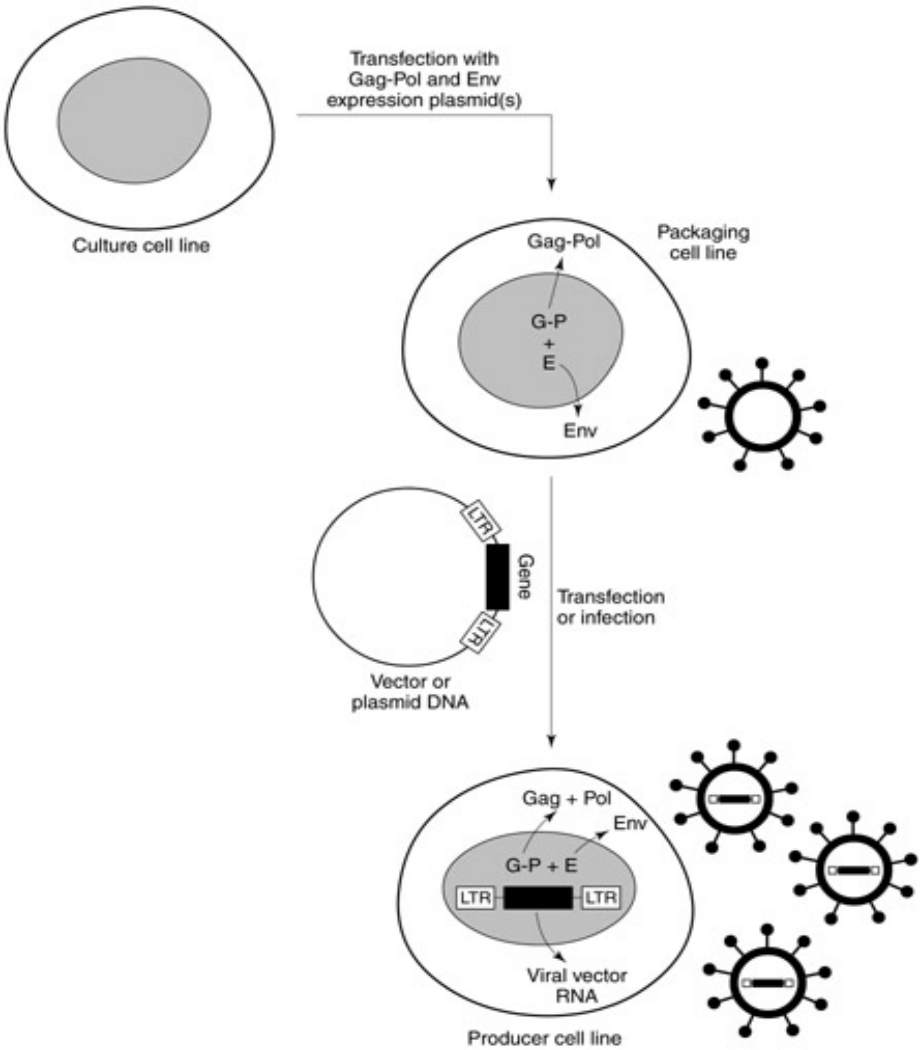


Figure 2 Construction of a retrovirus packaging cell line. Plasmids encoding the Gag-Pol polyprotein are transfected into cells. Clones expressing high levels of 'empty' virus particles in the supernatant are identified. An envelope expression construct is then transfected into the Gag-Pol-expressing clone and clones expressing high levels of both Gag-Pol and Env are identified. Finally, a retrovirus vector is introduced into the Gag-Pol-Env cell line and clones producing the highest levels of transducing particles are identified. Once cloned, these producer cell lines are stable and can be expanded to produce large amounts of vector for clinical or basic science experimentation. LTR, long terminal repeat.

elements necessary for transcription and polyadenylation of the transgene, and for reverse transcription and encapsidation (packaging) of the vector RNA. Once the vector that contains the packaging signal is introduced into the packaging cell, or cotransfected with a plasmid containing the genes for the packaging functions, particles are generated that contain the vector genome. The vector-containing particles are infectious and can introduce new genetic information into the genome of a target cell.

Producer cell lines can be made that express different envelope proteins, cell surface glycoproteins (*env*) needed for attachment and penetration into the target cell. By using *env* proteins from different retroviruses, the species specificity and tissue tropism of the vector can be altered. Recently, retrovirus vectors that use the vesicular stomatitis virus G protein in place of *env* have been produced. In addition to having a broad host range and tissue specificity, such vectors can be concentrated by centrifugation, which is not possible with *env*-containing vectors. Expression of the transgene in vectors can be under the control of the viral long terminal repeat promoter, or of an exogenous promoter such as the cytomegalovirus or SV40 early promoters. It is possible to express multiple genes under the control of a single promoter through the introduction of an element termed an internal ribosome entry site, which can be derived from an RNA virus such as encephalomyocarditis virus. Because

retrovirus vectors can be made free of wild-type virus, there is no expression of virus proteins in the transduced cell. Therefore, retrovirus vector-transduced cells do not induce virus-specific immune responses.

Advantages of the retrovirus gene transfer system include stable integration of the vector into the genome of target cells, the wide tropism of the available vectors, the relative ease of vector production, and the large number of vector and producer cell lines available for both basic research and clinical applications. Some disadvantages include: (1) integration of the vector into the genome of the host is random, which can potentially lead to gene mutation; (2) a limited packaging capacity; and (3) an inability to transduce nondiving cells. This last limitation has been resolved by using lentiviruses, such as HIV, instead of murine leukemia viruses to derive the packaging components and packaging cell lines. Lentivirus-based vectors have shown significant promise in early evaluations. Undoubtedly, lentivirus-based vectors are likely to complement the existing battery of vectors and possibly expand the usefulness of this system to nondividing targets such as liver, brain and the hematopoietic stem cell.

RNA Virus Vector Systems

Researchers are investigating the potential of RNA genome-containing viral vectors, such as poliovirus, alphaviruses, influenza virus and vesicular stomatitis virus (VSV). These viruses have the potential advantage of not having a DNA phase in their replication cycle, which provides a measure of safety for their use in humans. Negative-strand viruses such as influenza and VSV do not undergo measurable rates of homologous RNA recombination, which contributes to the stability and safety of these vectors. Expression of foreign proteins has now been shown for vectors derived from influenza, rabies, VSV, respiratory syncytial virus, SV5 and Sendai virus. The foreign proteins include antigens derived from other viruses, bacteria and parasites, and in general have represented attempts to derive vaccines for agents for which no adequate vaccine currently exists. The vectors produced thus far have coded for a single antigen, and we can expect that in the near future vectors encoding multiple antigens will become available. While the larger DNA viruses such as adenovirus, herpesvirus and the poxviruses code for proteins capable of downmodulating the immune response, no such proteins have been found in RNA viruses. Some of the RNA viruses have been shown to be excellent inducers of both cellular and humoral immune responses in humans.

Poliovirus, a positive-strand RNA virus, has been used to express a number of foreign genes. Poliovirus vectors expressing epitopes derived from hepatitis A virus, rhinovirus 14, human papillomavirus 16, foot-and-mouth disease virus and HIV have been produced. A poliovirus–HIV chimera has been shown to elicit anti-HIV antibodies in rabbits. Furthermore, this antiserum neutralized a wide range of American and African HIV-1 isolates. The major disadvantage of polio, relative to the negative-strand RNA virus vector systems, is that the systems that have been developed thus far have a limited capacity for foreign sequences.

Alphavirus (Sindbis virus and Semliki Forest virus)-based vector systems have also been introduced. The value of such systems is derived from the ability to use these vectors in a wide range of mammalian cells, as a plasmid, or a recombinant progeny virus. Noncytopathic Sindbis virus replicons that replicate in BHK cells and express large amounts of foreign protein have been produced. These vector systems are bipartite, contain a selectable marker for selection, and are capable of expressing the transgene protein at high levels for prolonged periods of time. They do not contain a complete complement of viral genes and thus no infectious particles can be produced. The vectors are probably minimally immunogenic for the parent viral components, as there is little of the parental viral RNA in the vector and structural proteins are not produced. Because of all of these features, the latest generation of alphavirus vectors have a number of safety advantages over some of the DNA virus-based vector systems, and could see applications soon not only in terms of vaccine development but also in the gene therapy arena. Since they replicate in the cytoplasm, and get distributed into the dividing cells without affecting the chromosomal make-up, many safety concerns could be satisfied. Use in the latter area is likely to be limited initially to the introduction of vectors into cells that can be grown in culture for periods of time to permit selection.

For vaccines, cytolytic virus vectors that stimulate an immune response to the virus infection may be useful in stimulating a response against a heterologous protein. Derivatives of Venezuelan equine encephalitis virus that show a self-limiting replication *in vivo* have been used to generate a protective immunity against influenza virus in animals.

Future Perspectives

Viral vectors have changed the way science is done, from the expression of single proteins in cells, to the generation of new vaccines and to the implementation

of new therapeutic strategies for the cure of genetic diseases and the treatment of cancer and other diseases. The variety of viral vectors continues to grow, and much is being learned about the advantages and disadvantages of each type. Practical application in the future will be based on extensive knowledge gained of the structures of viral genomes, their replication strategies and the response of immune systems to infection by such vectors. Such practical application will turn these causes of diseases into tools for curing disease.

See also: **Adenoviruses (*Adenoviridae*): Animal viruses, General features, Malignant transformation and oncology, Molecular biology; Baculoviruses (*Baculoviridae*): Granuloviruses, Nucleopolyhedrovirus; Herpes simplex viruses (*Herpesviridae*): General features, Molecular biology; Epstein-Barr virus (*Herpesviridae*): General features, Molecular biology; Human immunodeficiency viruses (*Retroviridae*): Molecular biology, Anti-retroviral agents, General features; Polioviruses (*Picornaviridae*): General features, Molecular biology; Papillomaviruses – human (*Papovaviridae*): General features, Molecular biology; Vaccinia virus (*Poxviridae*); Vesicular stomatitus viruses (*Rhabdoviridae*).**

Further Reading

Ertl HC and Xiang Z (1996) Novel vaccine approaches. *J. Immunol.* 156: 3579.

Moss B (1996) Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. *Proc. Natl Acad. Sci. USA* 93: 11341.

Garoff H and Li KJ (1998) Recent advances in gene expression using alphavirus vectors. *Curr. Opin. Biotechnol.* 9: 464.

Matsushita T, Elliger S, Elliger C *et al* (1998) Adeno-associated virus vectors can be efficiently produced without helper virus. *Gene Ther.* 5: 938.

Palese P (1998) RNA virus vectors: where are we and where do we need to go? (Commentary) *Proc. Natl Acad. Sci. USA* 95: 12750.

Paoletti E (1996) Applications of pox virus vectors to vaccination: an update. *Proc. Natl Acad. Sci. USA* 93: 11349.

Robbins PD and Ghivizzani SC (1998) Viral vectors for gene therapy. *Pharmacol. Ther.* 80: 35.

Rolph MS and Ramshaw IA (1997) Recombinant viruses as vaccines and immunological tools. *Curr. Opin. Immunol.* 9: 517.

Stavropoulos TA (1998) An enhanced packaging system for helper-dependent herpes simplex virus vectors. *J. Virol.* 72: 7137.

Plant Viruses

Thomas Hohn, Friedrich Miescher Institute, Basel, Switzerland

Rob Goldbach, Agricultural University, Wageningen, The Netherlands

Introduction

Virus vectors have become useful tools for introducing genes into a variety of organisms. The first successful virus vectors were derivatives of the bacteriophage λ, with the help of which the first homologous and heterologous gene libraries were established in *Escherichia coli*. Likewise, a number of animal viruses were used to introduce foreign genes into animal cells where they replicated and were expressed. The two most important types are based on the double-stranded (ds)DNA papovaviruses, e.g. bovine papillomavirus, and those based on retroviruses. While papovavirus vectors replicate as circular dsDNA, retroviral vectors mediate the incorporation of the passenger gene into the host chromatin. All of the cases mentioned above lead to relative stability of the passenger gene in the transfected cell, since their replication as dsDNA involves a proofreading mechanism.

Neither true dsDNA viruses nor viruses mediating DNA integration have yet been found amongst the large variety of plant viruses. Accordingly, experiments with plant virus vectors are based on single-stranded (ss)DNA viruses, pararetroviruses and RNA viruses; replication in all these cases can lead to genome rearrangements, because single-strand intermediates are involved on which proofreading mechanisms do not apply and where template switching could occur. If the design and use of the virus vector interferes with viral replication, expression or other vital functions, the passenger gene will be removed from the vector population within a few generations. The passenger gene will in any case be finally eliminated unless it provides some selective advantage. This instability can be also looked upon as a benefit, since it provides biological containment: hybrid plant virus derivatives released purposely or accidentally into the environment are unlikely to maintain a nonselective gene. Stable transformation of plants, on the other hand, can be achieved either with the help of *Agrobacterium*-derived vectors, which mediate integration of passenger DNA into the plant chromatin, or by direct gene transfer.

Another peculiarity of plant viruses is their mechanism of spreading within the host tissue. Spreading usually makes use of the plasmodesmata (cytoplasmic connections between individual cells) which are

of new therapeutic strategies for the cure of genetic diseases and the treatment of cancer and other diseases. The variety of viral vectors continues to grow, and much is being learned about the advantages and disadvantages of each type. Practical application in the future will be based on extensive knowledge gained of the structures of viral genomes, their replication strategies and the response of immune systems to infection by such vectors. Such practical application will turn these causes of diseases into tools for curing disease.

See also: **Adenoviruses (*Adenoviridae*): Animal viruses, General features, Malignant transformation and oncology, Molecular biology; Baculoviruses (*Baculoviridae*): Granuloviruses, Nucleopolyhedrovirus; Herpes simplex viruses (*Herpesviridae*): General features, Molecular biology; Epstein-Barr virus (*Herpesviridae*): General features, Molecular biology; Human immunodeficiency viruses (*Retroviridae*): Molecular biology, Anti-retroviral agents, General features; Polioviruses (*Picornaviridae*): General features, Molecular biology; Papillomaviruses – human (*Papovaviridae*): General features, Molecular biology; Vaccinia virus (*Poxviridae*); Vesicular stomatitus viruses (*Rhabdoviridae*).**

Further Reading

Ertl HC and Xiang Z (1996) Novel vaccine approaches. *J. Immunol.* 156: 3579.

Moss B (1996) Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. *Proc. Natl Acad. Sci. USA* 93: 11341.

Garoff H and Li KJ (1998) Recent advances in gene expression using alphavirus vectors. *Curr. Opin. Biotechnol.* 9: 464.

Matsushita T, Elliger S, Elliger C *et al* (1998) Adeno-associated virus vectors can be efficiently produced without helper virus. *Gene Ther.* 5: 938.

Palese P (1998) RNA virus vectors: where are we and where do we need to go? (Commentary) *Proc. Natl Acad. Sci. USA* 95: 12750.

Paoletti E (1996) Applications of pox virus vectors to vaccination: an update. *Proc. Natl Acad. Sci. USA* 93: 11349.

Robbins PD and Ghivizzani SC (1998) Viral vectors for gene therapy. *Pharmacol. Ther.* 80: 35.

Rolph MS and Ramshaw IA (1997) Recombinant viruses as vaccines and immunological tools. *Curr. Opin. Immunol.* 9: 517.

Stavropoulos TA (1998) An enhanced packaging system for helper-dependent herpes simplex virus vectors. *J. Virol.* 72: 7137.

Plant Viruses

Thomas Hohn, Friedrich Miescher Institute, Basel, Switzerland

Rob Goldbach, Agricultural University, Wageningen, The Netherlands

Introduction

Virus vectors have become useful tools for introducing genes into a variety of organisms. The first successful virus vectors were derivatives of the bacteriophage λ, with the help of which the first homologous and heterologous gene libraries were established in *Escherichia coli*. Likewise, a number of animal viruses were used to introduce foreign genes into animal cells where they replicated and were expressed. The two most important types are based on the double-stranded (ds)DNA papovaviruses, e.g. bovine papillomavirus, and those based on retroviruses. While papovavirus vectors replicate as circular dsDNA, retroviral vectors mediate the incorporation of the passenger gene into the host chromatin. All of the cases mentioned above lead to relative stability of the passenger gene in the transfected cell, since their replication as dsDNA involves a proofreading mechanism.

Neither true dsDNA viruses nor viruses mediating DNA integration have yet been found amongst the large variety of plant viruses. Accordingly, experiments with plant virus vectors are based on single-stranded (ss)DNA viruses, pararetroviruses and RNA viruses; replication in all these cases can lead to genome rearrangements, because single-strand intermediates are involved on which proofreading mechanisms do not apply and where template switching could occur. If the design and use of the virus vector interferes with viral replication, expression or other vital functions, the passenger gene will be removed from the vector population within a few generations. The passenger gene will in any case be finally eliminated unless it provides some selective advantage. This instability can be also looked upon as a benefit, since it provides biological containment: hybrid plant virus derivatives released purposely or accidentally into the environment are unlikely to maintain a nonselective gene. Stable transformation of plants, on the other hand, can be achieved either with the help of *Agrobacterium*-derived vectors, which mediate integration of passenger DNA into the plant chromatin, or by direct gene transfer.

Another peculiarity of plant viruses is their mechanism of spreading within the host tissue. Spreading usually makes use of the plasmodesmata (cytoplasmic connections between individual cells) which are

thought to become modified to allow passage of viral nucleic acid or particles. Since plant cells in culture lack these connections, their infection with viruses or viral genomes usually depends upon artificial methods, such as electroporation or polyethylene glycol (PEG) treatment, and further spread will not occur. In contrast, animal viruses have developed methods to both leave and enter cells, e.g. by exo- and endocytosis, and are, therefore, able to spread through host cell cultures after the initial infection.

Inoculation of Plants with Virus and Vectors

Many plant viruses, irrespective of whether their genome consists of DNA or RNA, can be inoculated mechanically on to plant leaves using an abrasive. In these cases, naked nucleic acid or manipulated derivatives can also usually be used as an inoculum. A further method is agroinoculation, which was first developed with CaMV (see **Table 1** for definitions of virus abbreviations) and potato spindle tuber viroid and makes use of the DNA-transfer machinery of *Agrobacterium tumefaciens*, the cause of crown gall disease in a number of dicotyledons. Using this method, multimers, or at least 'one-and-a-bit-mers', of the virus genome are cloned between the T-DNA borders of the *Agrobacterium tumefaciens* Ti-plasmid and this manipulated T-DNA is introduced into the plant upon bacterial infection. Once in the plant cell, probably in the nucleus, the viral genome can escape either by recombination or by the direct production of monomeric replicative intermediates, such as the CaMV 35S pregenomic RNA.

Table 1 Plant virus names and their abbreviations

Virus	*Abbreviation*	*Group*
African cassava mosaic[a]	ACMV	Bipartite gemini
Cassava latent[a]	CLV	
Alfalfa mosaic	AlMV	–
Beet curly top	BCTV	Monopartite gemini
Brome mosaic	BMV	Bromo
Beet necrotic yellow vein	BNYVV	Furo
Barley stripe mosaic	BSMV	Hordei
Barley yellow dwarf	BYDV	Luteo
Beet western yellows	BWYV	Luteo
Cauliflower mosaic	CaMV	Caulimo
Cowpea chlorotic mottle	CCMV	Bromo
Cucumber mosaic	CMV	Cucumo
Cucumber necrosis	CNV	Tombus
Cowpea mosaic	CPMV	Como
Cymbidium ringspot	CyRSV	Tombus
Digitaria streak	DSV	Monopartite gemini
Maize streak	MSV	Monopartite gemini
Odontoglossum ringspot	ORSV	Tobamo
Pea early browning	PEBV	Tobra
Plum pox	PPV	Poty
Potato X	PVX	Potex
Red clover necrotic mosaic	RCNMV	Diantho
Rice tungro bacilliform	RTBV	Badna
Tomato bushy stunt	TBSV	Tombus
Turnip crinkle	TCV	Carmo
Tomato golden mosaic	TGMV	Bipartite gemini
Tobacco mosaic	TMV	Tobamo
Tobacco rattle	TRV	Tobra
Tobacco vein mottling	TVMV	Poty
Turnip yellow mosaic	TYMV	Tymo
White clover mosaic	WCIMV	Potex
Wheat dwarf	WDV	Monopartite gemini

[a] The same virus.

Certain viruses and their nucleic acids are not infectious on mechanical inoculation. This group contains many viruses that are restricted to the phloem tissue, being directly introduced there by their insect vectors. Among these are many viruses that have members of the *Gramineae* as hosts, plants that are also not susceptible to crown gall disease. It was thus surprising that maize plants could be infected with MSV by agroinoculation. This success indicated that *Gramineae* are also susceptible to T-DNA transfer, and opened a route to the introduction of a number of different viruses that could not enter by other artificial means, e.g. WDV into wheat and RTBV into rice.

Pararetrovirus Vectors

Pararetroviruses, like retroviruses, use both the host nucleus and the cytoplasm for replication. In the nucleus, viral DNA is transcribed into terminally redundant RNA, in the cytoplasm the RNA is reverse transcribed into DNA, which is transported either directly or via new infections into the nucleus. Pararetrovirus DNA accumulates (but does not replicate) in the form of supercoiled circles, while retrovirus DNA is inserted in a terminally redundant form into the host chromatin. Plant pararetroviruses exist as two groups, the icosahedral caulimoviruses and the bacilliform badnaviruses. The genomes of these viruses are all around 8000 bp in length.

So far, only CaMV, a caulimovirus from this group, has been used as a vector. The seven open reading frames (ORFs) of this virus are arranged densely packed on the pregenomic 35S RNA, and it is thought that all but one of the ORFs are translated from this polycistronic messenger. Infection can be achieved in the laboratory by mechanical inoculation with virus particles or virus DNA and thus ORF 2, which codes for an aphid transmission factor, becomes dispensable. In view of this, an ORF-2-replacement vector was constructed and used to clone, express and spread systemically payloads through turnips. By this means, the ORFs for bacterial dihydrofolate reductase (DHFR; 240 bp), the Chinese hamster metallothionein (200 bp), human αD interferon (500 bp), and BNYVV ORF N (171 bp) were cloned, spread systemically and expressed in plants.

From these successful and other unsuccessful experiments, it was learned that sequences introduced into the CaMV genome are frequently lost, probably as a result of illegal template switching of the nascent minus or plus DNA strands as well as negative selection against the inserted DNA sequence. These recombination events could be minimized by avoiding homologies at the insertion site, and selection for the deletions could be minimized by avoiding whatever might interfere with the virus life cycle as follows:

1. The total size of the genome should not, or should only slightly, exceed the original viral genome length of 8000 bp. About 700 bp can be capitalized by deletion of ORF 2, as mentioned above, and ORF 7, which is also dispensable for virus growth. Allowing for overpackaging of 300 bp, a payload of about 1000 bp seems to be the upper limit. CaMV vectors to clone a larger payload might be developed from artificial bipartite CaMV genomes, which together contain all essential functions and lack recombination targets.
2. The tightly packed ORF organization should be maintained, probably because of the unusual CaMV translation mechanism. Experience has shown that noncoding sequences between CaMV and passenger ORFs diminish vector stability.
3. Inserted sequences should not interfere with CaMV gene expression and replication. For instance, the insertion of an RNA polyadenylation signal would interfere with the production of pregenomic RNA, insertion of a new promoter might occlude the original CaMV promoters and an intron would not survive the transcription/reverse transcription cycle for very long.
4. Sequences leading to gene products that interfere with virus replication either directly or by affecting the host cell must be avoided.

CaMV and its derived vectors do not proliferate efficiently in cell culture; while DNA replicative intermediates can be found in infected protoplasts, mature virus particles are absent or rare. This might be explained by developmental control of genome replication versus translation. Both processes depend on the same 35S RNA, and one can assume a control mechanism that switches between these two functions. In protoplasts and the derived cell cultures, the switch is apparently set to genome replication and particle formation is inhibited.

It is also likely that other plant pararetroviruses can be developed as gene vectors, collectively covering a large host range. This would also include the *Gramineae* if, e.g. RTBV is considered. Since this virus has a bacilliform capsid, overpackaging might be less of a problem.

Geminivirus Vectors

Geminiviruses (**Table 2**) contain 2.5–3 kb of circular ssDNA. They are characterized by twinned (geminate) icosahedral particles. Geminiviruses of dicotyledonous plants are usually, but not always, bipartite

Table 2 Geminivirus vectors

Virus	*Partite*	*Target*	*Host*	*Payload*
TGMV	Bi-	Plants	*Nicotiana tabacum*	CAT, GUS, NPT-II
ACMV	Bi-	Plants	*Nicotiana benthamiana*	CAT
MSV	Mono-	Protoplasts	*Zea mays*	NPT-II, GUS, DSI
WDV	Mono-	Protoplasts	*Triticum monococcum*	NPT-II, CAT, βGAL

and transmitted by white flies; geminiviruses of monocotyledonous plants are monopartite and transmitted by leafhoppers. Usually, component 'A' of the bipartite geminiviruses is sufficient for replication in single cells, while component B is required for systemic spread and needs component A for replication. With respect to their bidirectional expression strategy, geminiviruses resemble the animal polyomaviruses and simian virus 40 (SV40). Their intranuclear dsDNA forms contain two non-coding regions, one harboring the clockwise and counter-clockwise promoters and the other containing the two polyadenylators. Transcription in one direction (in consensus clockwise) produces RNAs coding for structural proteins, and transcription in the other direction gives RNAs required for DNA replication.

The coat proteins of geminiviruses are dispensable for genome replication but are required for insect transmission, and in the case of the monopartite viruses are also required for virus spread within the plant. Accordingly, mutants in the coat protein gene of bipartite geminiviruses can still replicate in single cells and spread through the host plant. Coat protein mutants of monopartite geminiviruses do not spread.

Geminivirus coat protein replacement vectors have been introduced into plants by either mechanical means or agroinoculation. Vectors based on component A DNA of bipartite geminiviruses can spread if component B is provided either as a transgene or as a co-inoculum. However, when transgenes of these vectors are used or transient expression in protoplasts or leaf disks is studied, component B is not required. Monopartite geminivirus vectors are so far restricted to transient expression experiments in localized patches of plant tissue disks, seed-derived embryos or cell cultures.

The following additional observations have been made.

1. In addition to the coat protein gene, the small ORF of unknown function located further upstream can also be replaced by the payload. Deletion or replacement mutants of the counter-clockwise coding regions, however, are defective in replication.
2. Systemically spreading vectors are unstable if they are either smaller or larger than wild-type virus; rearrangements occur which restore the original size. The reason for this size dependence in the absence of packaging is not known. Non-spreading vectors, on the other hand, can exceed normal genome size by up to 4 kb, for instance when they harbor the long β-glucuronidase (GUS) or neomycin phosphotransferase (NPT) II coding regions.
3. Geminivirus vectors also tolerate the insertion of additional promoters, e.g. the CaMV 35S promoter, which leads to a large increase in payload expression without affecting replication.
4. An *E. coli* replicon can also be incorporated into monopartite geminivirus genomes, creating shuttle vectors that replicate and express a payload gene in both bacteria and protoplasts of *Gramineae*.

RNA Viruses as Gene Vectors

Infectivity of cloned cDNA

To develop RNA viruses as gene vectors, it is essential that infectious RNA can be generated from cloned cDNA copies. This has been achieved for a growing number of plus-strand RNA viruses (but not minus-strand viruses) using four different approaches.

1. With a cDNA clone directly. Only limited results have been reported so far (excluding the viroids), namely for A1MV RNA3 and TMV RNA; in both cases, the cDNAs gave rise to virus at a very low level. The infectivity found here is probably due to transcription from unidentified promoters.
2. As cDNA fused to the CaMV 35S promoter. This results in more efficient production of progeny virus, as reported for BMV and BNYVV RNA 3 and RNA4.
3. By agroinoculation. This has been achieved for BWYV, a luteovirus, using a cDNA clone provided with a ribozyme sequence to enable *in vivo* run-off transcription.
4. As *in vitro* run-off transcripts, from linearized full-length cDNA clones, using phage SP6, T7, T3 or *E.*

coli RNA polymerase, in combination with their respective promoters. This is by far the most frequent approach, and infectious transcripts have been synthesized for more than 20 different RNA viruses belonging to 15 distinct taxonomic groups (**Table 3**). It is essential for infectivity that the transcripts are not only of full length but also (almost) identical to the natural viral RNAs. Some problems may arise here since most promoters extend into the transcribed region, resulting in transcripts that start with extra nonviral nucleotides. In general, it has been found that nonviral residues at the 3′ end are better tolerated than extra nucleotides at the 5′ end, which mostly lead to a significant drop in infectivity.

The best results have been obtained with *in vitro* transcription systems using SP6, T3 or T7 promoters and the corresponding specific polymerases. These systems are highly efficient (up to 20 μg of transcript from 1 μg of template) and can be designed to yield almost authentic termini. However, if the first viral nucleotide is not *a priori* a G residue, one extra G residue must be tolerated at the 5′ end. It should be noted that, in most cases studied, the extra nonviral nucleotides were deleted in progeny RNAs. In cases of virus with capped genomic RNAs, the addition of a cap structure at the 5′ end of the transcripts often enhances infectivity. For BSMV RNAs capping seems to be an absolute requirement. There is an additional complication with viruses belonging to the 'picornalike superfamily', e.g. the como-, nepo- and potyviruses. The genomic RNAs of these viruses possess a protein (denoted VPg) covalently linked to the 5′ end. Nevertheless, both capped and uncapped *in vitro* transcripts have been shown to be infectious.

Insertion and expression of foreign genes

Cloned full-length copies from which infectivity can be recovered by following one of the approaches discussed above provide a good starting point for constructing replicons that can be used as gene vectors (**Table 3**). Of course, insertion of foreign sequences should not interfere with any viral function or process (e.g. RNA replication, subgenomic mRNA synthesis, translation, encapsidation) required for (helper-independent) infectivity.

With respect to noncoding sequences, the terminal sequences should at least be retained for replication, while for some viral RNAs (e.g. BMV RNA3) internal noncoding sequences also appear to contain obligatory *cis*-acting replication signals.

With respect to the introduction of payloads into coding sequences ('replacement' vectors), the following points can be made.

Table 3 Plant viral cDNAs reported to generate infectious *in vitro* transcripts

Group	*Virus*
Alfalfa mosaic	A1MV (RNA3)
Bromo	BMV, CCMV (RNA1–3)
Carmo	TCV
Comovirus	CPMV (RNA1–2)
Cucumo	CMV (RNA1–3)
Diantho	RCNMV (RNA1–2)
Furo	BNYVV (RNA1–4)
Hordei	BSMV (RNA α, β, γ)
Luteo	BYDV, BWYV
Potex	PVX, WCIMV
Poty	PPV, TVMV
Tobamo	TMV
Tobra	PEBV, TRV (RNA1–2)
Tombus	CNV, CYRSV, TBSV
Tymo	TYMV

Genes encoding proteins involved in RNA replication

All plant RNA viruses so far studied possess one or more cistrons encoding replication proteins (e.g. putative helicase, viral polymerase). It is obvious that any manipulation of these genes will lead to non-replicating transcripts unless the functions can be provided in *trans*. This is feasible, e.g. by transformation of host plants with functional copies of the affected genes (transgenic tobacco plants expressing the RNA1 and RNA2 products of AIMV are able to support RNA3 replication) or by co-inoculation with helper virus (smaller TMV RNA-derived replicons co-replicate and spread systemically with TMV helper virus). In both cases, however, the viral vector has lost its truly independent character.

The movement protein gene(s)

A considerable number of plant RNA viruses encode a so-called 'movement protein' which is actively involved in cell-to-cell spread of the viral genome (e.g. tobamoviruses) or viral particle (e.g. comoviruses) through plasmodesmata. Its coding sequence should be retained if systemic spread of the viral vector is desired. This is true for TMV for the 30 kD protein, for BMV for the RNA3-encoded 32 kD protein and for CPMV for the M-RNA-encoded 58 kD/48 kD protein pair. For expression in protoplast systems or cultured cell suspensions, the movement protein cistron can be omitted and the site exploited.

Table 4 Heterologous gene expression from plant RNA viral genomes

Group	*Virus*	*Insertion site*	*Gene*	*Expression system*
Bromo	BMV	Coat protein gene	CAT	Protoplasts
Furo	BNYVV	25 kD gene (RNA3)	GUS	Inoculated leaf
Hordei	BSMV	βb gene (RNAβ)	LUC	Protoplasts
Tobamo	TMV	Coat protein gene	CAT	Inoculated leaf
		Coat protein gene	ENK	Protoplasts
		Extra site[a]	NPT-II	Whole plant
		Extra site[a]	DHFR	Whole plant

[a] From inserted ORSV coat protein promoter.

The coat protein gene(s)

The viral coat protein gene can, in some instances, be manipulated in order to express foreign sequences, though it always leads to lower fitness in terms of systemic spread. Although for some viruses the coat protein is dispensable for cell-to-cell movement (e.g. TMV), this structural protein cannot be omitted for long-distance transport of the inoculated vector (via the vascular system) throughout the whole plant. Therefore, replacement of coat protein genes by reporter genes (e.g. CAT, **Table 4**) always leads to naked, infectious RNA which is confined to the inoculated leaf. Such replacement vectors can, however, be efficiently expressed in protoplast systems (**Table 4**). The problem of limited spread has recently been circumvented for TMV by creating a viral vector (TB2) which contained a duplicated subgenomic promoter of the coat protein gene, enabling the expression of both the coat protein and the payload gene (NPT II, DHFR). It was found that the duplicated promoter should be cloned from a different tobamovirus with enough sequence divergency (i.e. ORSV) to avoid removal by homologous recombination.

Miscellaneous genes

Further genes with potential for exploitation as vectors exist in some viruses. Their function has usually not been resolved, although some of them have been shown to be dispensable for both viral RNA replication and systemic spread. For example, in the quadripartite RNA virus BNYVV, both RNA3 (specifying a 25 kD protein) and RNA-4 (specifying a 31 kD protein) have been shown to be dispensable and are in fact lost after serial mechanical inoculation. Large internal parts of both these RNAs can be deleted without affecting replication. Insertions of the bacterial GUS gene in a deletion-containing RNA3 cDNA has been shown to give rise to detectable GUS activity in the inoculated leaf (**Table 3**) and amplification of the transcript. A further example of successful replacement, this time with BSMV, is insertion of the firefly luciferase (LUC) coding sequence into the βb gene (located on β-RNA and encoding a 58 kD protein with a helicase motif), which resulted in infectious virus expressing high-level luciferase activity in both tobacco and maize protoplasts. However, luciferase activity was not detected in extracts of whole plants inoculated with BSMV RNAs α, β or the LUC-gene-containing RNA, which is consistent with the replaced βb protein being essential for multiplication in whole plants.

Independent versus 'disarmed' vectors

From the previous paragraph, it is clear that viral RNA vectors able to replicate and spread independently through whole plants should contain all *cis*- and *trans*-acting factors involved in RNA replication, encapsidation and spread. Two approaches have been shown to be successful for the development of such helper-independent vectors, i.e. the insertion of a ('recombination-proof') extra promoter, as shown for TMV, and the replacement of non- or less essential genes (e.g. BNYVV 25 kD gene).

As an alternative, 'disarmed' viral vectors could be constructed for foreign gene expression in plants, which lack one or more essential functions and are able to multiply only in target plants transformed with the required gene(s). The properly constructed transgenic plant which is required provides a bonus in the form of control over the spread of field-released genetically engineered virus. This approach is indeed feasible, as shown by the replication of engineered AlMV RNA3 molecules in tobacco plants transformed with the AlMV replicase genes, and the complementation of spread-deficient TMV strains in transgenic plants expressing the TMV 30 kD movement protein.

Plant Viruses as Vectors for Studying DNA and RNA Rearrangements

Virus vectors have also been used for purposes other than expression of a payload ORF.

Splicing

Cloning of introns in virus vectors allows precise measurement of the efficiency and accuracy of splicing in monocotyledons and dicotyledons. The precise excision of an intron introduced into a CaMV vector verified the intron and was a proof for reverse transcription of CaMV.

Recombination

Experiments can be designed which make use of essentially nonviable virus hybrids which become viable upon specific genome rearrangements. The most obvious examples, already mentioned in the course of discussing agroinoculation, are given by 'one-and-a-bit-mer' of manipulated caulimo- and geminivirus genomes which release viable virus upon recombination. If the redundant portions of these constructs are derived from different viral strains, questions can be answered concerning the recombination mechanism, the presence of recombination hot spots, the degree of homology required, etc. Escape of pregenomic virus RNA by transcription was observed if the promoter/polyadenylator region of CaMV constituted the terminal redundancy. If transgenic *Brassica napus* plants were produced containing CaMV one-and-a-bit-mers arranged such that CaMV could not escape by simple transcription, true recombination events could be scored by appearance of viral symptoms. Sequence analysis of recombinant molecules suggested that mismatch repair was linked to the recombination process. Intermolecular recombination could also be studied, i.e. between transgenic CaMV ORF 4 and supertransfected complementary virus sequences.

Infectious transcripts from cloned RNA viruses have been very useful for studying RNA recombination, e.g. in bromoviruses, and for analyzing and understanding the *cis*- and *trans*-acting factors in RNA replication.

Agrobacterium T-DNA transfer

A CaMV-based system was used to analyze independent *Agrobacterium* transfer DNA (T-DNA) transfer events. The complete T-DNA without the border sequences was replaced by the virus genome such that a viable replicon could be produced by circularization upon transfection of plants. Analysis of this replicon revealed rather conserved right border remnants, while sequences remaining at the left border were more variable. The presence of small direct repeats between some of the joined ends showed that linear T-DNA had been transported to the plant.

Transposition

Using WDV and MSV vectors that contained the maize transposable element *Ac* or its defective *Ds* derivatives, excision of the transposable element could be studied in protoplasts and whole maize plants. Excision of the *Ds* element was dependent on the presence of *Ac*. The junction sequences left on the viral genomes after excision revealed the typical footprints.

Conclusions

Based on the limited and (sometimes) rather preliminary results obtained so far, it may be concluded that plant viruses can be engineered into gene vectors able to express desired genes. Systems are described that allow expression in single plant cells; others allow replication, expression and spread through the plant, either independently or as 'disarmed vectors' in the presence of a helper virus, or within a transgenic host carrying the missing functions.

The advantages of the viral vector transfection systems are ease of handling, the short time periods required to obtain results and the replication of the vectors to high copy numbers which can result in expression levels much higher than in transgenic plants.

Because of the lack of any proofreading mechanism and the large number of replication cycles (even in a single cell), one might imagine that payload genes in RNA virus and pararetrovirus vectors would rapidly accumulate point mutations, leading to inactivation of these nonessential inserts. The (limited) experimental data obtained so far, however, indicate that at least for the duration of a single protoplast batch or plant infection, functional proteins can be obtained in desirable amounts. It is not excluded that viral vectors eventually lose their capacity to encode a functional protein upon serial passage, although this would merely provide an advantage in view of biological containment.

A second issue of concern is the risk of recombination involving viral vectors. Indeed, it has now been well documented that viral genomes, including those of RNA viruses, are frequently the subject of recombinational events. One obvious mechanism for recombination in all classes of plant viral vectors relies on template switches of the nascent DNA and RNA strands. On the one hand, nonviral inserts in viral vectors might become lost by recombination because of the lack of selective pressure; the con-

sequences seem only to be beneficial with respect to biological containment, since viral vectors will not survive for long in nature. On the other hand, new functional viral genomes may arise from recombinational events involving (disarmed) RNA vectors, co-infecting (helper-) viruses and transgenes, which could lead to undesired spread of pathogens. Although so far no evidence has been obtained for such events, it is clear that critical risk-assessment analyses should be performed prior to possible release of viral vectors in agricultural practice.

See also: **Bromoviruses (*Bromoviridae*); Geminiviruses (*Geminiviridae*).**

Further Reading

Ahlquist P and Pacha RF (1991) Gene amplification and expression by RNA viruses and potential for further application to plant gene transfer. *Physiol. Plant.* 79: 163.

Bakkeren G *et al.* (1989) Recovery of *Agrobacterium tumefaciens* T-DNA molecules in whole plants early after transfection. *Cell* 57: 847.

Beachy RN, Fitcher JH, Hein MB (1996) Use of plant viruses for delivery of vaccine epitopes. *Annals of The New York Academy of Sciences* 792: 43–9.

Brisson N and Hohn T (1988) Plant virus vectors: CaMV. In: Weissbach A and Weissbach H (eds) *Methods for Plant Molecular Biology*, p. 437. San Diego: Academic Press.

Donson J *et al.* (1991) Systemic expression of a bacterial gene by a tobacco mosaic virus-based vector. *Proc. Natl Acad. Sci. U.S.A.* 88: 7204.

Gray SM (1996) Plant virus proteins involved in natural vector transmission. *Trends in Microbiology* 4: 259–64.

Hohn B *et al.* (1987) Plant DNA viruses as gene vectors. *CIBA Found.* 133: 185.

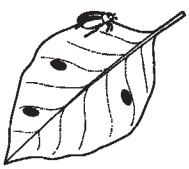
Joshi L, Joshi V and Ow DW (1991) BSMV genome mediated expression of a foreign gene in dicot and monocot plant cells. *EMBO J.* 9: 2663.

Matzeit V *et al.* (1991) Wheat dwarf virus vectors replicate and express foreign genes in cells of monocotyledonous plants. *Plant Cell* 3: 247.

Mushegian AR, Shepherd RJ (1995) Genetic elements of plant viruses as tools for genetic engineering. *Microbiological Reviews* 59: 548–78.

[This article is reproduced from the 1st edn (1994).]

VECTOR TRANSMISSION OF PLANT VIRUSES



Stewart M Gray, USDA, ARS and Department of Plant Pathology, Cornell University, Ithaca, New York, USA

D'Ann Rochon, Agriculture and Agri-Food Canada, Pacific Agri-Food Research Centre, Summerland, British Columbia, Canada

Introduction

It has been nearly 100 years since a leafhopper was confirmed as the vector of rice dwarf virus. Several hundred plant viruses have since been identified, a majority of which are dependent upon a vector for transmission between, and inoculation into, plant hosts. The plant viruses have evolved many interesting and biologically complex associations with their vectors, which include arthropods, nematodes and fungi. Although there is a great deal known about the general biology of most virus–vector interactions, we are only beginning to understand the molecular and cellular mechanisms that regulate the transmission processes and determine the efficiency of transmission.

General Mechanisms of Virus Transmission by Arthropods and Nematodes

In the early years viruses were said to be either mechanically transmitted or biologically transmitted by their vectors. Biological transmission was the specific association of a virus with a particular arthropod species or genus and the virus replicated in the vector. Mechanical transmission referred to the nonspecific transmission of viruses, usually by multiple vector species. The viruses did not replicate in the vector and transmission was thought to occur by the simple contamination of vector mouthparts. Most plant viruses do not replicate in their vectors, but it

can promote interactions between particles and fungus. Deletion analysis, along with alanine scanning mutagenesis, has identified a KTER amino acid motif near the C-terminus of the BNYVV readthrough as being important for the fungus transmission process. A similar sequence (KTEIR) is found in the readthrough domain of SBWMV.

Bymoviruses do not translate a coat protein readthrough. The repeated mechanical transfer of a BaYMV strain resulted in the loss of fungus transmission and a spontaneous approximate 1 kb deletion in RNA2. The deleted region is from a protein which is associated with crystalline inclusion bodies in infected tissue. Alignments of portions of the protein products of RNA2 of two bymoviruses and the readthrough portion of several furovirus capsids has revealed that certain amino acid combinations (ER or QR) are found consistently in all the viruses. These amino acids occur on the outside of the protein and therefore may be available for interaction with the fungus vector. In addition, certain conserved regions are absent in nontransmissible deletion mutants. These studies suggest that bymoviruses and furoviruses encode similar proteins for facilitating their transmission.

See also: **Luteovirus; Geminiviruses (*Geminiviridae*); Potyviruses (*Potyviridae*); Plant pararetroviruses (*Caulimoviridae*): Caulimoviruses: general features, Caulimoviruses: molecular biology; Tospoviruses (*Bunyaviridae*).**

Further Reading

Adams MJ (1991) Transmission of plant viruses by fungi. *Ann. Appl. Biol.* 118: 479.

Ammar ED (1994) Propagative transmission of plant and animal viruses by insects: factors affecting vector specificity and competence. In: Harris KF (ed.) *Advances in Disease Vector Research*, vol. 10, p. 289. New York: Springer-Verlag.

Brown DJF, Robertson WM and Trudgill DL (1995) Transmission of viruses by plant nematodes. *Annu. Rev. Phytopathol.* 33: 223.

Campbell RN (1996) Fungal transmission of plant viruses. *Annu. Rev. Phytopathol.* 34: 87.

Gergerich RC and Scott HA (1991) Determinants in the specificity of virus transmission by leaf-feeding beetles. In: Harris KF (ed.) *Advances in Disease Vector Research*, vol. 8, p. 1. New York: Springer-Verlag.

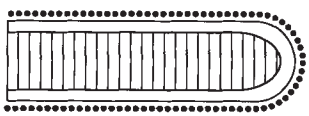
Gildow FE (1999) Luteovirus transmission and mechanisms regulating vector-specificity. In: *Luteoviridea*. Wallingford: CAB International.

Pirone TP and Blanc S (1996) Helper-dependent vector transmission of plant viruses. *Ann. Rev. Phytopathol.* 34: 227.

Venezuelan Equine Encephalitis Virus *see* **Equine Encephalitis Viruses**

Vesicular Exanthema Virus *see* **Caliciviruses**

VESICULAR STOMATITIS VIRUSES (*RHABDOVIRIDAE*)



Luis L Rodriguez, Plum Island Animal Disease Center, Agricultural Research Service, United States Department of Agriculture, Greenport, New York, USA

Stuart T Nichol, Special Pathogens Branch, Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, USA

History

Vesicular stomatitis viruses (VSVs) are rhabdoviruses which infect a wide range of wild and domestic animals, and several groups of insects. VSVs are best known as the cause of vesicular stomatitis (VS), a disease characterized by vesicular lesions in the mouth, tongue, udder teats and hoof coronary bands of cattle, horses and pigs. Clinical disease occurs every year in farming areas from northern South America to

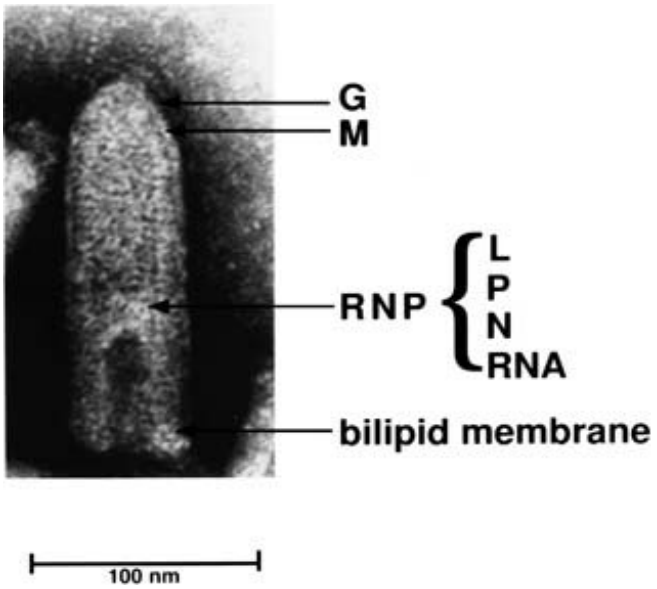


Figure 1 Electron micrograph of VSV-IN. Location of virion components is indicated. RNP is ribonucleoprotein.

southern Mexico where VSVs seem to have a stable natural cycle and are considered endemic. Humans living in these areas are often infected, and consequently develop flu-like symptoms. Early records of disease compatible with VS such as 'sore tongue' in pigs and 'hoof loss' in horses date back to the early 1800s in the Southeastern US and Central America. In 1862, a vesicular and febrile illness compatible with VS affected thousands of army horses in the US during the Civil War. The first large epizootic of VS to be described in detail occurred in 1916 in the US. The contagious, febrile and vesicular disease spread rapidly from Colorado to the East Coast, affecting large numbers of horses and mules and to a lesser extent cattle. Large epizootics have continued to occur in the US, on an approximately 10 year cycle, mostly in south western states.

Taxonomy and Classification

VSVs are classified in the order *Mononegavirales* (non-segmented-negative-sense RNA viruses), family *Rhabdoviridae*, genus *Vesiculovirus*. All rhabdoviruses share a characteristic bullet- or rod-shaped morphology and basic biochemical properties (**Fig. 1**). VSVs are grouped together on the basis of antigenic crossreactivity and genetic and biochemical relatedness. Two main serotypes, Indiana (IN) and New Jersey (NJ), have been defined based on cross-neutralization properties. The prototype strain of the IN serotype (VSV-IN1) was isolated in 1925 from an outbreak in cattle in Richmond, Indiana. A year later, the prototype virus strain of the NJ serotype (VSV-NJ) was isolated from cattle in New Jersey. Other VSVs serologically related to but distinct from VSV-IN1 have been found. Cocal virus, a virus isolated from mites in 1961 in Trinidad became the prototype strain of the VSV-IN type 2 (VSV-IN2). Another IN-related virus was isolated in 1964 from a mule in Alagoas, Brazil and became the prototype strain of VSV-IN type 3 (VSV-IN3). Many viruses serologically related to VSVs have been found, such as, Calchaqui in Argentina, Maraba and Carajas in Brazil, all isolated from insects, and Piry isolated from an opossum in Brazil.

Properties of the Virion

VSV particles are rod-shaped (hence the name rhabdo, from the Greek for rod) enveloped viruses of approximate dimensions 70 × 180 nm (**Fig. 1**). The virion core consists of a helical ribonucleoprotein (RNP) structure containing the RNA genome. The protein components of RNP cores consist of nucleocapsid (N) protein, the polymerase-associated phosphoprotein (P) and the large (L) polymerase protein. Based on studies using electron microscopy it has been estimated that there are 1258, 466 and 50 molecules of N, P and L, respectively, in each virion core of VSV-IN1. The matrix (M) protein is closely associated with the core structure, and provides structural support to the bullet-shaped helical RNP. The RNP is enveloped by a bilipid membrane derived from the host cell. Embedded in the membrane is the surface glycoprotein (G). The majority of the G molecule protrudes to the exterior of the viral particle and the short cytoplasmic tail contacts the underlying M protein.

Physical Properties

VS virions consist of approximately 74% protein, 20% lipid, 3% RNA and 3% carbohydrate. Infectivity is unstable at pH 3, but relatively stable in the pH range 5–10. It is rapidly inactivated at 56°C and by UV and X-ray irradiation. Infectivity is also sensitive to lipid solvents, detergents, formalin and various disinfectants such as bleach.

Properties of the Genome

The single-stranded non-segmented negative-sense RNA genome of VSV is approximately 11 kb in length. These linear RNA molecules possess 5′-phosphate and 3′-hydroxyl groups, and lack 5′ cap or 3′ poly(A) tail structures. The termini exhibit limited self-complementarity which may be involved in the circularization of the genome. The naked RNA

Table 1 Properties of proteins of vesicular stomatitis virus (NJ serotype)[a]

Protein	*Biochemical characteristics*	*Localization and function(s)*
Nucleocapsid (N)	422 aa; pI 5.8–6.3; 47–48 kDa	Encapsidates RNA to form nucleocapsid, essential component of transcription–replication complex
Phosphoprotein (P)	274 aa; acidic pI 4.2–4.3; 30–31 kDa; phosphorylated	Part of viral polymerase complex, mediates binding of L to nucleocapsid cores
Large (L)	2109 aa; basic pI 8.6–8.8; 241–242 kDa	Nucleocapsid, multifunctional enzyme, protein kinase, polymerase, capping, methylation and poly(A) addition
Matrix (M)	229 aa; highly basic pI 9.9; 26–27 kDa, phosphorylated	Forms scaffolding of nucleocapsids, promotes virus assembly, regulates genome transcription, inhibits host-cell gene expression, most abundant virion protein
Glycoprotein (G)	517 aa; 57–58 kDa class I membrane protein, N-glycosylated at 2 Asp residues	Major protein on viral envelope, attaches to cell receptor(s), mediates membrane fusion at low pH, induces cell toxicity, target of virus-neutralizing antibodies
C and C′	Coded in second ORF of P gene, 55 and 65 aa respectively, highly basic, arginine-rich	Nonstructural with unknown function

[a] Similar properties have been reported for VSV-IN1 proteins. aa, amino acids.

is not infectious. The nucleotide sequences of the complete genomes of VSV-IN1 and VSV-NJ have been determined. Furthermore, infectious virus has been recovered from plasmids containing the full-length genome of VSV-IN1. This has allowed extensive studies on the genome structure, transcription and replication strategies of VSV (see replication section).

Properties of the Proteins

VSV-NJ and VSV-IN1 code for seven proteins; five structural proteins (N, P, M, G, L) are coded in separate nonoverlapping open reading frames (ORFs) whereas two small highly basic nonstructural proteins (C and C′) are coded in a second ORF within the P gene. The following details relate to VSV-NJ proteins unless otherwise stated (for details see **Table 1**). The N protein encapsidates the viral RNA, it is an essential component of actively transcribing or replicating viral cores and functions in close association with the P protein. The P protein is a highly phosphorylated protein associated with viral polymerase activity. It mediates the binding of the L protein to the nucleocapsid core and facilitates access of the polymerase to the RNA template during transcription and replication. Phosphorylation of P protein seems to be necessary for optimal transcriptase activity of the RNP complex. C and C′ are nonstructural proteins of which the exact role in the life cycle of VSV is not clear. Engineered viruses that do not express C proteins are indistinguishable from wild-type virus in protein synthesis, virus production and host-protein synthesis shut off in tissue culture cells. The large L protein is a multifunctional enzyme required in only catalytic amounts and probably performs most of the polymerase-associated functions of the virus such as RNA synthesis, capping, methylation and poly(A) addition. It also has protein kinase activity which preferentially phosphorylates serine residues on the P protein. The matrix protein (M) is the most abundant protein of virions. M binds specifically to G protein monomers and promotes their trimerization, it also associates with the cellular lipid bilayer through the C-terminal domain to promote the condensation of viral RNP cores into tightly coiled helical structures that are subsequently enveloped and released as virions. The M protein, in tight association with RNPs, inhibits genome transcription and may play an important role in the correct regulation of viral genome transcription.

The G protein is a typical class I membrane-associated glycoprotein, with the N-terminal 90% of the molecule projecting from the surface of the virion or infected cell, a hydrophobic transmembrane domain anchoring the protein in the membrane, and a C-terminal 28-amino acid cytoplasmic domain projecting to the interior of the virion or infected cells. The G protein forms trimers that constitute the approximately 400 spikes on the virion envelope. The G protein plays a major role in attachment, penetration of VSV into susceptible cells and budding of virions from infected cells. It is the major target of VSV-specific neutralizing antibodies and is capable of inducing cell membrane fusion at low pH.

Replication Cycle

Unless otherwise stated, the following description of viral replication (**Fig. 2**) is that of VSV-IN1, although

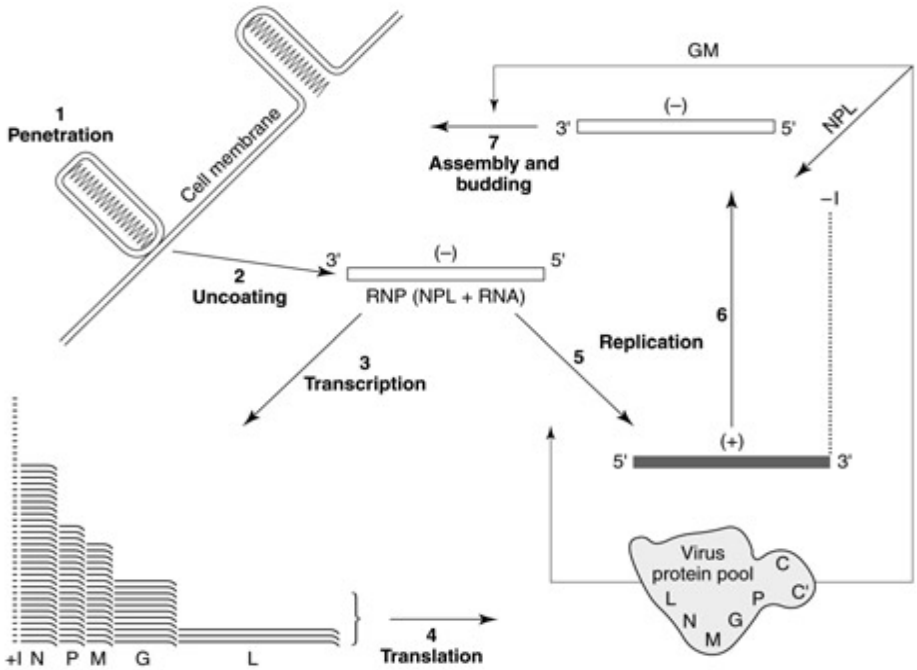


Figure 2 Model of VSV replication cycle. The major steps in the replication cycle of VSV are shown in diagram form. Open bars represent viral negative-sense RNA and closed bars represent viral positive-sense RNA. Refer to the text for discussion of steps 1–7.

it is likely to be representative of events occurring with the other VSVs. The infection cycle starts when the VSV G protein interacts with receptors on the host cell surface (such as phosphatidylserine). The virion penetrates by receptor-mediated endocytosis (**Fig. 2**, step 1). Once inside the cell, the G protein mediates the fusion of the cell and viral membranes under the low-pH conditions in the endosomes (**Fig. 2**, step 2). The viral ribonucleoprotein or nucleocapsid is released in the cytoplasm and the negative-sense viral RNA is transcribed by the virion-associated polymerase complex to produce six viral transcripts (**Fig. 2**, step 3). Active transcribing complexes consist of the template, made up of the RNA genome in tight association with the N protein, together with the polymerase, consisting of the phosphoprotein (P) and large (L) protein. The polymerase complex starts transcribing at a single entry site at the 3′ end of the genome and transcribes each gene in decreasing amounts as it moves away from the entry site. Therefore, the gene order provides an efficient way of regulating gene expression, where proteins necessary in larger amounts are located near the 3′ end and are transcribed in larger amounts and those needed in smaller amounts are located towards the 5′ end and are transcribed less frequently (i.e. 3′ N>P>M>G>L 5′). The complex initiates transcription by completing a 47-nucleotide transcript termed the leader RNA which is an exact copy of the genome 3′ end. This is followed by an untranscribed junctional sequence AAA (AAAA in VSV-NJ). The leader transcript which is neither capped nor polyadenylated, is transported to the nucleus where it inhibits host cell transcription. The leader transcript is followed by the N mRNA, which is capped during synthesis by the virion polymerase complex. At the end of the N gene, and indeed of all five viral genes, is the sequence 5′-AGUUUUUUUCAUA-3′ which signals the termination and polyadenylation of the mRNA. The mRNA poly(A) tract is probably synthesized by the viral polymerase slipping or chattering on the 7 U stretch. In an essentially identical manner, the polymerase completes the sequential synthesis of the remaining four mRNAs. Translation of the mRNAs is coupled to the transcription process and the protein amounts reflect the relative abundance of each mRNA (**Fig. 2**, step 4). Subsequently, after translation of viral proteins has occurred, a switch is made to synthesis of full-length positive-sense copies of the genome (**Fig. 2**, step 5). The switch mechanism is not fully understood but it requires protein synthesis and perhaps the participation of host cell factors. The critical step is the readthrough of the first junction encountered by the polymerase, i.e. the leader-N gene junction. The polymerase must be altered so as to no longer recognize the termination signal at this junction and continue down the full length of the genome. The

simplest model fitting most of the experimental data proposes that rising levels of encapsidation of the nascent positive-strand leader RNAs by N protein reaches a threshold whereby it prevents the polymerase complex from recognizing the termination signals. Once full-length positive-strand synthesis occurs, production of the virus negative-sense RNA genomes necessary for packaging into released virions commences (**Fig. 2**, step 6). A negative-sense leader RNA which represents a 46-nucleotide copy of the 3′ end of the positive-sense template can be found in infected cells. Its function is not known but it is probably synthesized in a manner similar to the positive sense leader RNA, i.e. in the presence of limiting levels of N protein the nascent negative-sense RNA synthesis terminates after the leader RNA transcript. In the presence of optimal levels of N protein the efficient encapsidation of the nascent strand pushes the polymerase through the termination signal and allows the polymerase complex to complete the synthesis of the entire negative-sense RNA genome. Such a proposed mechanism is attractive as it closely couples the production of virion RNA with the availability of viral proteins necessary for virion production.

The five viral mRNAs are transcribed in the cytoplasm of infected cells and their translation is directly coupled to the transcription process. Four of the viral mRNAs, N, P, M and L, are translated on free polysomes in a manner essentially analogous to cellular mRNAs. The G mRNA is translated on membrane-bound polysomes, the N-terminus of the newly emerging G protein has a 16 amino acid hydrophobic signal peptide which targets the protein to the rough endoplasmic reticulum (ER). The signal peptide is cleaved in the lumen of the ER. The protein is glycosylated as it is transported through the Golgi complex and is eventually expressed on the cell surface membrane in a trimeric form.

Assembly and Release

The glycoprotein forms patches on the surface of the infected cells. The M protein appears to form a bridge between the G protein membrane patches and virion RNP cores and promotes the tight condensation of the cores and their assembly into mature virion particles. These are then released by budding from the surface of the cell (**Fig. 2**, step 7). Considerable virus release occurs prior to the eventual disruption and death of the host cell. VSVs are generally highly cytopathic causing rapid cell death (in less than 12 h in some cases) in susceptible cells. Both M and G proteins and the viral inhibition of cell macromolecular synthesis have been shown to be involved in the cytotoxicity and death of infected cells.

Growth in Tissue Culture

The growth of VSVs is supported by a large number of vertebrate and insect tissue culture cells. VSV grows to high titers (8–9 $\log_{10}$) and produces dramatic cytopathic effect (CPE) in vertebrate cells such as cell lines and primary cultures of hamster, bovine and primate origin. In contrast, CPE is less obvious or absent and titers are lower in insect cells including several cell lines from mosquitoes (*Aedes albopictus* and *Aedes aegypti*).

Geographic and Seasonal Distribution

VSVs have been isolated almost exclusively from New World mammals and insects. Disease epizootics have been reported as far north as Canada, and as far south as Argentina. According to the intervals between outbreaks of clinical disease, there are areas of high, moderate or low VS activity with intervals of less than 1 year, 2–10 years or more than 10 years, respectively (**Fig. 3**). VSV-NJ infections are the most common, and have the widest distribution, with isolations as far north as Canada and as far south as Peru. VSV-IN1 has a similar wide geographical distribution but is less frequently encountered. VSV-IN2 (Cocal) viral infections have been detected in Trinidad, Brazil and

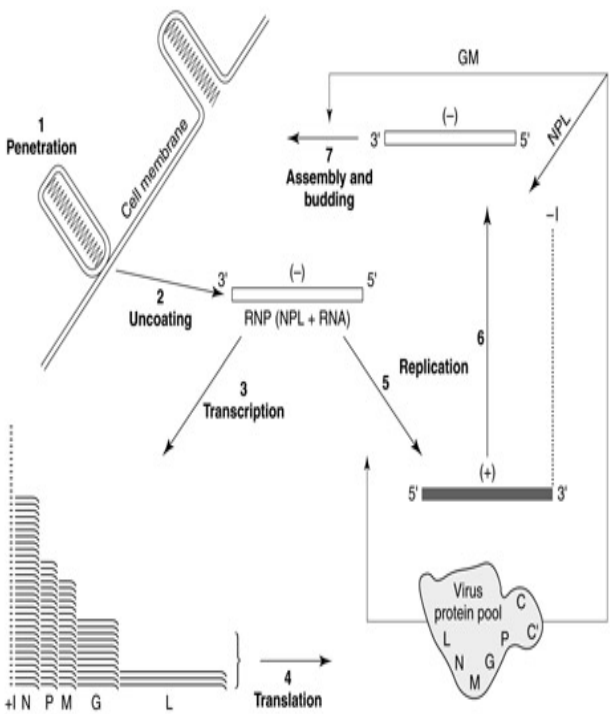


Figure 3 Geographical distribution of VSV serotypes. Areas are shaded according to the approximate interval between outbreaks of clinical vesicular stomatitis: □, never reported; ■, > 10 years; ■, 2–10 years; ■, 1 year.

Argentina. The last reported outbreaks caused by VSV-IN 2 date back to 1979 in Brazil. VSV-IN3 (Alagoas) is the main cause of VS outbreaks in Brazil. VS outbreaks often follow natural features of the land (e.g. spreading throughout river valley systems rather than along road systems or trucking routes) and tend to have a marked seasonality. In temperate regions, they begin in the late spring, peak in the late summer and cease with the first frosts of late fall/early winter. In more tropical regions, the epizootics frequently appear at the cessation of rainy seasons. In both regions, the seasons of high VSV activity tend to correlate with high insect population levels.

There are only two reports of suspected VS epizootics outside of the New World. The first was an apparent VS outbreak in horses and mules in Transvaal, South Africa in the late nineteenth century. The second was a VS outbreak in France during World War I which was initiated by a shipment of infected US army horses. There is no evidence of VSV activity persisting for prolonged periods in either of these continents. VS is a mandatory reportable disease in most countries, either because of it being exotic in those countries or for the similarity of its clinical signs to those caused by foot-and-mouth disease virus, a virus exotic or under eradication in most parts of the world.

Natural History

VSVs seem to infect a wide range of hosts in endemic areas, with evidence of viral infections in wild small mammals such as: cotton rats (*Sigmodon* spp.), rice rats (*Oryzomis* spp.) and field mice (*Peromyscus* spp. and *Reithrodontomys* spp.); arboreal mammals such as bats (many species) and howler monkeys (*Alloata palliatta*); and large wild mammals such as white tail deer (*Odocoileus virginianus*) and feral swine (*Sus scrofa*). Infections of wild animals seem to be asymptomatic. Insects are also infected by VSVs, with isolations reported from sand flies (*Lutzomyia* spp.), black flies (*Simulium* spp.), midges (*Culicoides* spp.), mosquitoes (*Culex* spp., *Aedes* spp.), eye gnats (*Hippelates* spp.), mites (*Gigantolaelaps* spp.) and domestic flies (*Musca domestica*). No disease symptoms are observed in insects and their importance in the maintenance and transmission of VS disease is unclear. However, the ability of VSVs to replicate and be transovarially transmitted in sand flies and black flies and the seasonal appearance of clinical disease, suggests a role for these insects in the natural cycle of VSV. Since transovarial transmission of VSV in insects is inefficient it has been proposed that natural reservoirs, in which the virus induces high titers in the blood, are necessary for hematophagous insects to maintain the virus in nature. However, to date such wild or domestic natural reservoir(s) remain elusive.

One of the best documented VSV endemic foci is on Ossabaw Island, a barrier island off the coast of Georgia in southeastern US. On this island, the local feral swine population is infected every year by VSV-NJ. Several lines of evidence indicate that the sand fly (*Lutzomyia shannoni*) is the vector responsible for VSV-NJ transmission to feral swine. First, *L. shannoni* is the only species of sand fly on the island. Second, VSV-NJ has been isolated not only from hematophagous females but also from nonhematophagous male sand flies which indicates that transovarial transmission must have occurred in these flies. Third, the population peak of sand flies on the island precedes by about a month the peak in seroconversion rates of sentinel pigs, and fourth, it was found that the virus is transmitted most often to sentinel pigs in those habitats of the island where the sand flies are abundant, such as maritime live oak forests. However, the reservoir that feeds this apparent insect–swine cycle in Ossabaw remains unknown, since neither deer nor feral swine from the island were capable of producing detectable viremias after experimental inoculation with the local strain of VSV-NJ. Other studies in well-documented endemic areas in Costa Rica have shown that the presence of clinical VS in dairy cattle is associated with the presence of forested areas near the affected farms, where wild animals including rodents, primates and bats have high antibody prevalence to VSV, and also where sand flies and black flies are present. The role of these wild animals and insects in the natural cycle of VSV is to be determined.

Evolution

RNA-dependent RNA-polymerase of VSVs has an estimated error rate of $1–4 \times 10^{-4}$ substitutions per base incorporated. As a consequence of this high error rate, every time the genome is replicated new variants arise producing a heterogeneous virus progeny referred to as a 'quasispecies'. VSV uses this genetic variability to adapt and persist in its natural niche. Despite the high mutation rate of VSV *in vitro*, naturally occurring VSVs show a clear pattern of genetic stability (**Fig. 4**). There is good correlation between viral phylogeny and the location of virus isolation, indicating that many lineages and sublineages are maintained in infection foci of limited geographical distribution (**Fig. 4**). There is no correlation between lineages and host species, i.e. there is not a distinct VSV of any particular host species. Viruses isolated from mammals and insects in the same endemic area usually have identical se-

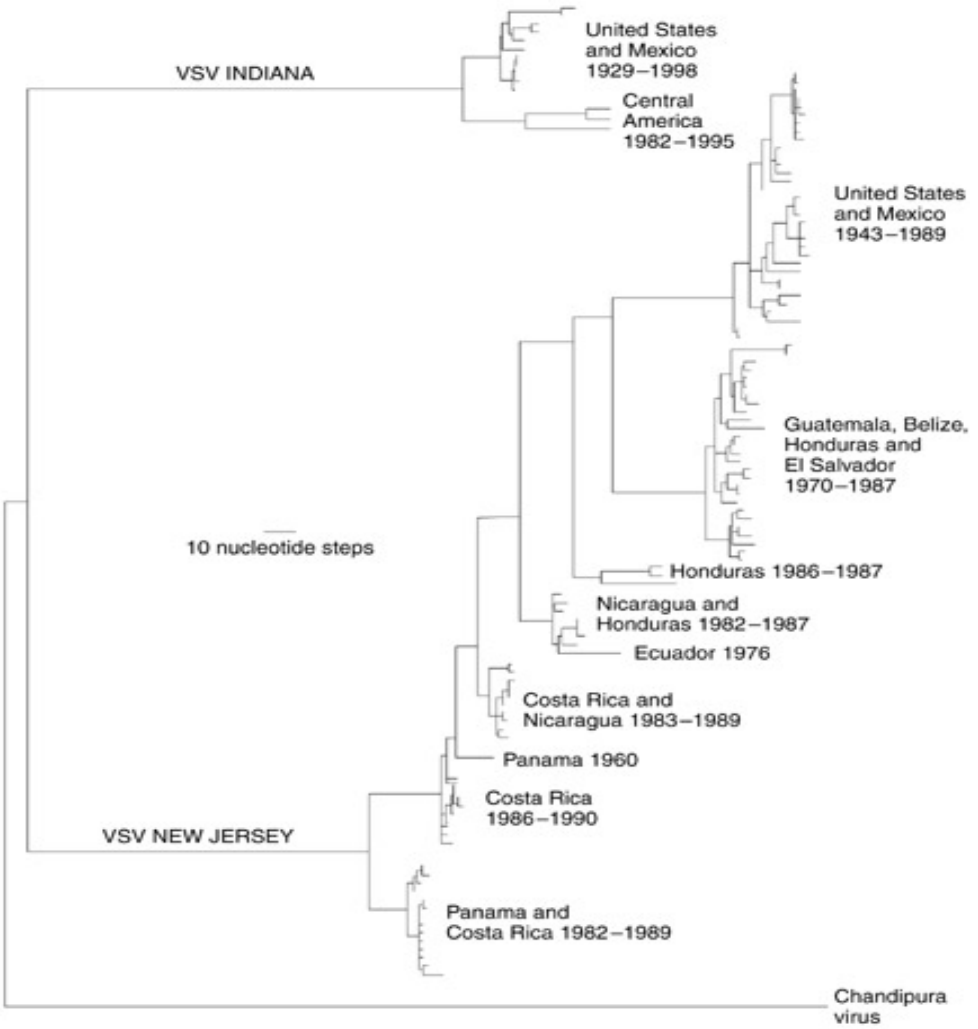


Figure 4 Evolutionary tree topology for VSV-NJ and VSV-IN1 from North and Central America, obtained by maximum parsimony analysis of partial phosphoprotein sequences from 122 VSV isolates. Genetic distance is proportional to the length of horizontal lines. Vertical distances are for graphic display only.

quence, furthermore, viruses isolated in these areas 30 years apart are more closely related than viruses from the same year but from different areas. The absence of a molecular clock contrasts with the evolutionary pattern of other RNA viruses such as human influenza A viruses which show a clear relationship between year of isolation and position within a phylogenetic tree. The evolutionary pattern of influenza virus A is driven mainly by the immune response of the infected hosts, where new antigenic variants capable of escaping the immune response have a selective advantage. In contrast, sequence analysis of VSVs causing clinical disease in Central and North America suggest that ecological factors rather than immune selection are the major forces driving VSV evolution (**Fig. 4**). For instance, viruses originating less than 25 km apart but from different ecological zones had different sequences, whereas viruses originating over 800 km apart but from areas of similar ecological characteristics had identical sequences. The ancestral origin of the VSVs is currently unclear. A relatively conserved gene order and limited sequence similarity with other nonsegmented negative-strand RNA animal viruses suggest a common, albeit very ancient, ancestor.

Serological Relationships and Variability

All VSVs share common N protein epitopes which are group specific and are usually detected by complement fixation assay. The two major VSV serotypes, IN and NJ, have been defined by crossneutralization assays using antibodies to the surface glycoprotein (G). Limited antigenic variation has been observed

among natural virus isolates of VSV-NJ. Greater antigenic diversity is seen among VSV-IN with at least three distinct types identified. Antisera raised to virus of one IN type will neutralize virus of another type but to a lesser degree. Although some antigenic variation can be found, there is no evidence of distinct antigenic drift of VSVs in nature (see Evolution section).

Epidemiology

The epidemiological characteristics of VS outbreaks depend to a large extent on whether the outbreak occurs in an endemic or a nonendemic area. Endemic areas are located in tropical and subtropical areas of the Americas where the interval between clinical outbreaks is less than one year. An epidemiologic study carried out in an endemic area of dairy production in Costa Rica reported clinical cases starting in November, peaking in December and January and continuing until March (the dry season in this area begins in November and continues through May). Most clinical cases occurred in adult lactating cattle (average age 5.4 years) and were caused by VSV-NJ (90%) and to a lesser extent IN 1 (10%). The majority of the adult population, including those that became clinically ill, had significant titers of neutralizing antibodies to VSV-NJ (93%) and VSV-IN1 (25%). Interestingly, there was no evidence for genetic drift in the viral glycoprotein gene in viruses recovered from these cases, despite the fact that clinically affected animals had high titers of neutralizing antibodies prior to clinical disease. In these endemic areas many animals, particularly young animals (less than 2 years) become seropositive to VSV during periods of clinical activity without showing any signs of clinical disease.

VSV causes periodic large-scale disease epizootics of cattle, horses and swine in non-endemic areas with peaks of activity approximately every 10 years. Clinical cases appear in many premises in a short period of time, generally during the summer months, and the disease disappears with the first frosts. However, in 1982–83 a large outbreak of VSV-NJ in western US persisted well into the winter months. After the 1982/83 outbreaks, VS has occurred in Southwestern US in 1995, 1997 and 1998. All cases in 1995 were caused by VSV-NJ, however, during the 1997 and 1998 outbreaks, VSV-IN1 was the major cause of clinical cases. Interestingly, this was the first report of VSV-IN1 in the US since 1966. It is not known what the sources of VSVs are for the US outbreaks. Nucleotide sequencing of virus isolates from US in 1982/83 showed that these viruses were very close to each other, they differed greatly from viruses from the only documented endemic focus in the US in Ossabaw Island, Georgia, but were related to VS strains circulating in Mexico during the same years. VSV-IN type 2 and type 3 cause outbreaks mainly in South America. Little is known regarding the epidemiology of these two virus types.

Transmission and Tissue Tropism

There is little information regarding transmission and tissue tropism of VSVs. Presumably domestic animals are infected by bites from insect vectors. Viral activity is most obvious in cattle, horses and pigs because of their clinical signs. However, it is likely that these animals play little or no role in the maintenance and transmission of the virus in nature, since infected animals do not show detectable viremias. There is no virus shedding in milk, feces or urine. Fluid from vesicular lesions contains large amounts of infectious virus ($>10^{10}$ PFU ml^{-1}) which can serve as a source for mechanical transmission from animal to animal. However, attempts to reproduce animal-to-animal transmission of VSV have met with only occasional success. Experimental data would suggest that the epidermal layer probably needs to be broken or abraded for successful direct viral transmission. Several lines of evidence suggest that VSVs are insect viruses and the infection of animals may be incidental. Tissue tropism of VSVs in insects has not been studied extensively, although the midgut and fat body have been shown to be major sites of VSV-NJ viral replication in sand flies. Serological surveys of humans in areas enzootic for VSVs suggest that human infection can be relatively common. Numerous cases of infection of laboratory workers, veterinarians and animal handlers have been reported. These are usually associated with viral entry through breaks in the skin or exposure to high virus titer aerosols such as infected animals sneezing in the faces of susceptible individuals. Human to human transmission has never been reported.

Tissue tropism of the virus in naturally infected animals is poorly understood. It seems likely that the majority of viral replication is found close to the site of entry of the virus and remains relatively localized. However, cases in which lesions appear in multiple sites (hoof, mouth and teats) in the same animal might suggest that generalization occurs through blood circulation. The localized nature of the vesicular lesions in the mouth (tongue, gum, lips), coronary band of the hoof and the skin of the teats or prepuce, suggest a tropism to tissues in these areas. It has been shown that the virus replicates in the *stratum spinosum* layer of the skin. However, the physiological basis of this tropism remains unknown.

Pathogenicity

The majority of VSV natural infections of domestic animals appear to be asymptomatic, as seroconversion in the absence of obvious clinical symptoms is frequently observed. Data from epidemiologic studies have showed that physiological state (e.g. lactation, pregnancy, age) are significant risk factors for clinical VS, suggesting that host, rather than viral factors likely determine the clinical outcome of infections. However, tissue-culture-grown virus appears to quickly lose the ability to produce clinical disease when reintroduced into animals. This might be related to loss in fitness of the viral quasi-species by repeated passage in tissue culture (see Evolution section).

Clinical Features of Infection

VS disease in cattle, horses and swine is characterized by the appearance of vesicular lesions on the mouth (tongue, lips, gums), teats, or hoof coronary band epithelium layer approximately 2–4 days after inoculation of the virus. Animals rarely exhibit lesions at more than one site, but when it happens it is usually in serologically naive animals introduced to endemic areas or infected during large epizootics. Depression, lameness, fever and excessive salivation are often seen before vesicles are detected. Alterations in hepatic enzyme levels suggest that VSV infection may also affect the liver in cattle and humans. In dairy cattle, milk production virtually ceases and weight loss can be as much as 300 pounds (140 kg) in beef cattle. This is especially true if mouth lesions become too sore for animals to eat, or they become too lame to get to food and water. Mastitis is a common consequence of the infection due to milk retention (caused by pain during milking) and secondary infections. In severe cases the udder can be lost to gangrenous mastitis. In most cases healing of lesions occurs within 7–10 days. Human VSV infections are characterized by an influenza-like illness after 24–48 h incubation. The majority of cases develop a biphasic fever accompanied by malaise, myalgia, headache, photophobia and chills. Vesicles appear on the tongue, oral and pharyngeal mucosa, lips or nose in a minority of cases. There is one report of VSV causing severe encephalitis in a child.

Pathology and Histopathology

The main lesions associated with clinical VSV infections are the vesicles that can appear in the tongue, the mouth, the hoof coronary bands or on the skin of the teat or prepuce. Vesicles begin as small blanched areas, the epithelium separates from the basal layer and forms a vesicle filled with clear-yellowish fluid. Vesicles rupture easily leaving a red surface which usually heals in 1–2 weeks if they are kept free of secondary bacterial infections. Necropsy reports of infected animals are rare since VSV does not cause mortality. No gross lesions of internal organs have been reported in experimentally infected animals. Histopathological examination of vesicular lesions reveals that lesions develop in the *stratum germinativum* where intercellular edema is observed. Cells become necrotic later which results in the separation of the epithelial layers above the *stratum basale* and accumulation of vesicular fluid.

Immune Response

Little is known about the immune response to VSV in domestic animals or wild natural hosts. However, VSV has been used extensively to study the immune response to viruses in laboratory animals, particularly mice. There are three major components in the immune response to VSV: the nonspecific immunity (interferon and nitric oxide); humoral immunity (antibodies); and cellular immunity (T cells). Interferon seems to play a role in survival of laboratory mice inoculated with VSV. Naturally occurring VSV strains differ in their capacity to induce interferon *in vitro*; in general VSV-NJ strains induce higher interferon responses than VSV-IN1. However, there is no clear correlation between interferon induction capacity of VSV strains and their pathogenesis *in vivo*. Neutralizing antibodies directed against VSV glycoprotein play an important role in protection of laboratory animals against VSV. The exact mechanism of protection is not clear, since virus–antibody complexes are still capable of binding to susceptible cells but do not initiate infection. The majority of cattle, horses and pigs living in endemic areas have neutralizing antibodies to VSV. However, it seems that the presence of neutralizing antibodies is not sufficient to prevent clinical disease in these animals. This may be explained by the majority of viral replication being localized in the epithelium.

Experiments in cattle and swine have demonstrated proliferative responses of peripheral blood mononuclear (PBM) cells to VSV antigens which could be detected at 3 weeks postinoculation in swine or postvaccination in cattle. In both cases, these responses could still be detected 6 months later. However, the role of the cellular immune response in protection against VSV is questionable since laboratory animals devoid of direct and indirect cytotoxicity, but capable of humoral response, survive VSV infections.

Prevention and Control of VSVs

The first step in controlling VS is rapid detection of clinical cases. Differential diagnosis particularly to distinguish VS from foot-and-mouth-disease is important. Diagnostic methods currently used include complement fixation, fluorescent antibody and isolation in cell culture. Other detection methods include antigen-capture ELISA and reverse-transcriptase-polymerase chain reaction (RT-PCR). Epithelium or vesicular fluid from fresh lesions are ideal samples for submission. In premises affected with VS in non-endemic areas, quarantine is usually established to avoid further spread of the disease. Within the affected premises, control measures include insect control and cleaning and disinfection with bleach of feed and water troughs, milking equipment and any utensils that could transfer the virus between animals. Since it has been suggested that scarification plays a role in virus entry, rough hay or overgrown pastures should be avoided.

Several inactivated vaccines containing both serotypes NJ and IN1 have been used in South and Central America. Although their effectiveness has not been rigorously tested, a recent report indicates that an oil-based bivalent vaccine decreased the incidence of clinical disease by as much as 25-fold among vaccinated cattle in endemic areas of Colombia. Other vaccine approaches including subunit vaccines and Vaccinia vectors containing G protein have had limited success in laboratory trials using domestic animals and have not been tested in the field. International control of VS is by means of import/export control on the movement of animals with significant neutralizing antibody titers to VSVs.

Future Perspectives

Despite significant efforts made during the last 30 years, many of the questions about the natural cycle of VSV remain unanswered. There is compelling evidence indicating that VSVs are transmitted to domestic animals by blood-sucking insects. However, despite the testing of many species of wild and domestic mammals, none has been found capable of producing the sustained viremias necessary to be a source of virus for insects. The genetic stability of VSV in endemic areas and the specific ecological characteristics associated with different VSV genotypes, indicate that there could be different natural cycles of VSV under different ecological conditions. The fact that VSV activity can be traced to very specific ecological conditions and even to specific forest types in endemic areas, should be taken into consideration in designing future studies on the natural cycle of VSV.

The ability to obtain infectious VSV-IN from cDNA clones has opened a new chapter in VSV research, since by using reverse genetics it is now possible to dissect the role that individual genes and genome sequences play in the life cycle of VSV, both within the cell and also within the animal and insect hosts. The availability of VSV cDNA clones has also made possible the inclusion of foreign genes in the VSV genome, which are expressed and incorporated in the virion along with other viral structural proteins, providing the potential to use VSV as a vector for vaccine delivery. One of the most interesting applications of this technology is the incorporation of the human immunodeficiency virus 1 (HIV-1) cell receptor CD4 and coreceptor CXCR4 in the VSV virion. This virus is unable to infect normal cells but infects and kills those expressing HIV-1 membrane fusion protein on their surface, making it a 'guided' killer for HIV-1-infected cells only.

See also: **Rabies virus (*Rhabdoviridae*); Rabies-like viruses (*Rhabdoviridae*).**

Further Reading

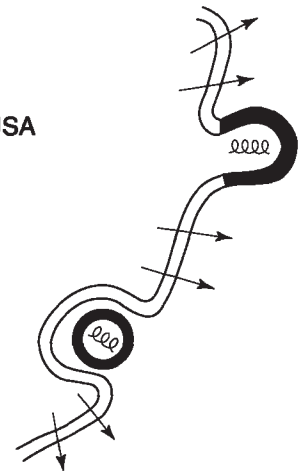
Banerjee AK and Barik S (1992) Gene expression of vesicular stomatitis virus genome RNA. *Virology* 188: 417.

Dietzschold B, Rupprecht CE, Zhen FF and Koprowski H (1996) In: Fields BN, Knipe DM, Howley PM *et al* (eds) *Fields Virology* 3rd edn, p. 1137. Philadelphia: Lippincott-Raven.

Wagner RR and Rose JK (1996) In: Fields BN Knipe DM, Howley PM *et al* (eds) *Fields Virology* 3rd edn, p. 1121. Philadelphia: Lippincott-Raven.

VIRAL MEMBRANES

John Lenard, Department of Physiology and Biophysics, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Piscataway, USA



Introduction

Viruses of many kinds possess lipids as integral components of their structure. Lipid-containing, or enveloped, viruses include *Corona-*, *Orthymyxo-*, *Paramyxo-*, *Bunya-*, *Rhabdo-*, *Toga-*, *Retro-*, *Herpes-*, *Baculo-* and *Poxviridae*. Despite the great diversity of these viruses in regard to structure, replicative strategy, host range and pathogenicity, the function of the lipid is the same in all of them: to form a membrane surrounding the encapsidated viral genome. In all these viruses the lipids form a continuous bilayer that functions as a permeability barrier protecting the viral nucleocapsid from the external milieu. Embedded in the bilayer are numerous copies of a limited number (usually one or two) of virally encoded transmembrane proteins that are required for virus entry into a host cell. These proteins must mediate two essential functions: attachment of the virion to the cell surface; and fusion of the viral envelope with a cell membrane.

The membrane is acquired during viral assembly within an infected cell. Membrane acquisition generally occurs by budding of the viral nucleocapsid through a particular cellular membrane, which is characteristic for each enveloped virus. Many of the viruses mentioned above bud through the plasma membrane. However, bunyaviruses bud through the Golgi apparatus, coronaviruses take their membranes chiefly from the endoplasmic reticulum and herpesviruses bud from the nuclear membrane. Poxviruses, which are among the largest and most complex animal viruses, are unique in not acquiring membrane by simple budding. They acquire several membranes through a series of interactions with different elements of the intracellular membrane transport system.

Viruses that lack lipids often possess capsids or shells consisting only of viral protein. These structures perform the same functions as viral membranes, i.e. protection of the genome, attachment to a suitable host cell and facilitation of its entry into the host cell. Increasingly, the actions of these proteins are being shown to resemble those of the glycoproteins of enveloped viruses.

Viral Bilayer

Knowledge of the structure of viral membrane bilayers has come chiefly from the study of a few viruses that are easily grown in large quantities in the laboratory, namely the orthomyxo-, paramyxo-, rhabdo- and togaviruses. In these, the bilayer arrangement of the lipids has been directly demonstrated using physical methods, and the lipid composition of various viruses grown under different conditions has been described in detail. Since all of these viruses acquire their bilayer by budding through the host cell's plasma membrane, the viral membrane contains the lipids present there. Wide variations of lipid composition are tolerated, and most of the lipids display the properties characteristic of lipids in a bilayer, not those of protein-bound lipids. The precise content of each individual phospholipid or glycolipid in a viral membrane does not always reflect the bulk composition of the host cell membrane from which it was derived, however. This difference may arise from interactions of lipids with the viral membrane proteins, or from inhomogeneity in the host cell membrane.

Intact virions are impermeant to proteases and other enzymes. Indeed, virions can swell and shrink in response to changes in osmolarity, showing that the viral membrane is impermeant to small molecules and ions as well as large proteins. This property indicates that the viral membrane consists of an intact bilayer, completely surrounding the encapsidated viral gen-

ome. It is generally assumed that intact bilayers are characteristic of all enveloped viruses, and not just for those few for which this property has actually been demonstrated.

Viral Membrane Proteins

The proteins of viral membranes, like those of other membranes, may be classified as either integral or peripheral. Integral proteins are those that span the membrane one or more times, and thus cannot be solubilized without disrupting the bilayer, e.g. with detergents. Peripheral proteins do not cross the membrane, and can be removed from it and solubilized by treatment with aqueous salts or chaotropic agents, which do not destroy the bilayer.

Integral membrane proteins of enveloped viruses generally span the membrane only once; an exception is the E1 protein of coronavirus, which spans the membrane three times. Each membrane-spanning, or transmembrane, or anchoring, domain is a sequence of 18–27 predominantly hydrophobic amino acid residues. Transmembrane sequences are inherently insoluble in water, so that integral membrane proteins require the presence of detergents to be soluble. In the absence of detergents or lipids, membrane proteins tend to aggregate as rosettes, with the transmembrane sequences clustered together at the center of the rosette, in order to minimize contact with water. Viral membrane proteins can be reinserted into lipid bilayers of defined composition by mixing detergent-solubilized proteins and lipids together, then removing the detergent by dialysis or centrifugation. These reconstituted viral membranes often possess biological activity.

As much as 90% of the polypeptide chain of a viral membrane protein may be external to the bilayer, where it is accessible to degradation by added proteases. In some favorable cases, nearly the entire external domain can be recovered intact and correctly folded after limited proteolysis, facilitating crystallization and structural analysis. The best example is the influenza virus HA protein. The external portions of viral membrane proteins generally possess oligosaccharide side chains, identical to those of cellular proteins in attachment position and structure. Often, they also possess disulfide bonds. These post-translational modifications reflect the viral proteins' synthesis at, assembly within and translocation through, the cell's rough endoplasmic reticulum (see Membrane Synthesis below).

Peripheral proteins are attached to the viral membrane by a combination of electrostatic and hydrophobic interactions. Although they may penetrate the bilayer to some extent, they do not cross it as the integral proteins do. Viral peripheral proteins include the M1 protein of influenza, the M proteins of paramyxo- and rhabdoviruses, and the MA proteins of retroviruses.

Attachment of Viruses to Host Cells

The first step in infection, attachment of the virus to the outer surface of the host cell, is performed by the membranes of enveloped viruses. Each virus recognizes a unique feature of its host cell membrane. Thus, the nearly total specificity of human immunodeficiency virus (HIV)-1 for cells expressing CD4 protein is conferred by the affinity of the viral envelope protein gp120 for this cell surface 'receptor'. Other enveloped viruses bind to different specific cell surface proteins to initiate infection. An emerging generalization is that, even when viruses bind to true cell surface receptors that normally initiate complex intracellular responses such as phosphorylation cascades, these responses are not essential for viral infectivity. The virus is simply using a characteristic surface landmark to identify and attach to its appropriate host cell.

Orthomyxo- and paramyxoviruses have a broader receptor specificity than was discussed above. Their hemagglutinin (HA and HN, respectively) glycoproteins bind to sialic acid residues attached to various cell surface proteins and lipids. Sialic acids are bound to the host cell membrane through several different kinds of glycosidic linkages, and different virus strains show some preference for sialic acid residues in particular linkages. The most nonspecific of the enveloped viruses may be the rhabdoviruses, represented by vesicular stomatitis virus (VSV) and rabies, which bind indiscriminately to clusters of negative charges, whether created by lipids, proteins or oligosaccharides. This nonspecific binding property helps to account for the extremely broad host range of these viruses.

Viral Fusion

Before viral transcription and replication can commence, the viral genome must cross the barriers presented by both the viral envelope and the cell membrane. Fusion of the viral membrane with a cellular membrane accomplishes this, introducing the viral genome into the host cell cytoplasm. Fusion, like cell attachment, is a property conferred upon each virus by one (or perhaps, in some cases, more) envelope glycoprotein. In most well-studied cases (notably the orthomyxo-, paramyxo-, rhabdo- and togaviruses) fusion does not require the participation of cell proteins, as virions and reconstituted viral

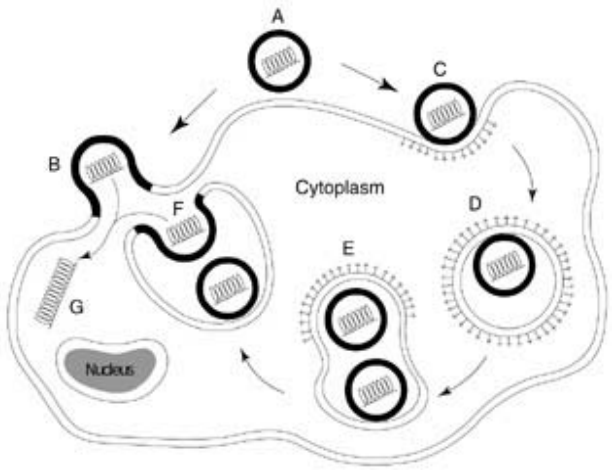


Figure 1 The two major pathways for cellular penetration and uncoating of enveloped viruses. Uncoating begins with attachment of the virion to the cell surface (A), through the binding of an integral viral membrane protein to a 'receptor' on the cell surface, which may be a specific cell protein, an oligosaccharide or a patch of charged lipids. Attachment is followed by fusion, mediated by a viral protein which may or may not be of the same type as the attachment protein. If the viral fusion protein is active at neutral pH, fusion can occur directly with the plasma membrane (B). Alternatively, if fusion requires an acidic pH, the virion must first be endocytosed via the coated pit–coated vesicle pathway (C,D). The viral fusion protein is activated in the acidic endosomes (E,F). Both pathways result in the introduction of the viral nucleocapsid, containing the viral genome, into the cytoplasm (G). For viruses that undergo transcription and replication in the nucleus (such as orthomyxoviruses and most DNA viruses), uncoating is followed by transport of the nucleocapsid through the nuclear pores into the nucleus by unrelated processes.

membranes (often called 'virosomes') fuse readily with protein-free liposomes and planar lipid bilayers.

The fusion proteins of the paramyxoviruses differ from those of the orthomyxo-, rhabdo- and togaviruses in regard to the pH at which they act. While the former are active at neutral pH, the latter require a more acidic environment, usually below pH 6, for fusion. This difference has profound consequences, as it reflects two distinctly different modes of viral entry (**Fig. 1**). The paramyxoviruses, and others capable of fusing at neutral pH, can fuse with the cells' plasma membranes under normal conditions, i.e. at the neutral pH of extracellular fluid or culture medium (**Fig. 1B,G**). Those viruses that fuse only at acidic pH, on the other hand, must first be internalized from the cell surface into specialized vacuoles, the endosomes. Although this process brings the virus inside the cell, the viral genome is still separated from the cell cytoplasm by the same two membranes as before. The endosomes are acidic, however, which activates the viral fusion protein and allows fusion between the viral and endosomal membranes (**Fig. 1C–G**). The pH dependence of viral fusion activity, which is conveniently measured as virus-induced hemolysis, or by a variety of direct fusion assays, serves to distinguish between viruses that enter the cell by the two distinct routes shown in **Fig. 1.**

The two different routes of cell entry shown in **Fig. 1** are differentially inhibitable by specific compounds. A variety of membrane-permeant or 'lysosomotropic' amines (notably chloroquine, ammonia and methylamine, but also including a variety of local anesthetics, tranquilizers and other commonly used pharmaceuticals) possess the property of being able to diffuse across membranes only in their unprotonated, uncharged form. The protonated, charged form of these compounds thus accumulates in acidic compartments such as the endosomes, raising the endosomal pH and preventing the activation of acid-dependent viral fusion proteins. Lysosomotropic amines generally have no effect on the entry of those enveloped viruses that fuse at neutral pH.

Viral fusion proteins are generally glycoproteins which possess a single transmembrane domain, and which assemble into multimers, usually homotrimers. In the last few years considerable progress has been made in understanding the molecular events that occur during viral fusion. Fusion mediated by the influenza fusion protein, HA, has been the most thoroughly studied and is the best understood, since the three-dimensional structure of its proteolytically derived extracellular portion has been determined by x-ray crystallography. The fusion-competent form of HA arises from proteolytic cleavage of an inactive precursor, HA0, to yield HA1 and HA2. The single transmembrane domain is in HA2, to which HA1 is attached by disulfide bonds. HA1 is primarily concerned with cell attachment, while HA2 chiefly mediates the fusion reaction, which relies on three structural features of the molecule:

1. *The fusion peptide,* situated at the N-terminus of HA2. This hydrophobic sequence was recognized early as a conserved feature between influenza and paramyxovirus fusion proteins. Its release from constraint by the proteolytic cleavage that creates HA2 is an essential element in the proteolytic activation of HA.
2. *The three-stranded coiled-coil* that comprises the stem of the HA trimer at neutral pH. The low pH-mediated activation of HA for fusion consists of a rearrangement and extension of this coiled-coil. This repositions the fusion peptide, from a sequestered location close to the viral membrane to an exposed position at the extreme end of the newly elongated coil. This enables the fusion

peptide to contact and penetrate the target membrane (**Fig. 2**).

3. *The transmembrane domain of HA2.* An essential role of this domain was demonstrated through the use of a mutant from which it had been deleted. When the entire external portion of HA was anchored through a lipid only into the outer leaflet of the membrane bilayer, it was no longer capable of mediating fusion. However, it could still catalyze half the reaction, or hemifusion, a process in which only the outer leaflets of the two membrane bilayers become mixed.

Using influenza HA protein as the model, viral fusion may be considered to occur in a series of discrete steps following virus-receptor binding (**Fig. 3**):

1. *Activation of the fusion protein* by conformational rearrangement. For influenza HA this is induced by acid, and consists of the extension of the coiled-coil stalk and exposure of the fusion peptide (**Fig. 2**). For fusion proteins that act at neutral pH, other activation processes operate. Some paramyxovirus fusion proteins (named F) may be activated by interaction with a partner viral protein, the cell attachment protein HN. For the HIV-1 fusion protein, activation is thought to occur by interaction with one of several chemokine receptors present on the surface of host cells (part of step 1, **Fig. 3**).
2. *Penetration of the fusion peptide* into the target bilayer, thus linking the viral bilayer with the target bilayer (step 1, **Fig. 3**).
3. *Relocation of the extended coiled coil stem* so as to bring the two linked membranes into close apposition (part of step 3, **Fig. 3**).
4. *Hemifusion* between the outer leaflets of the viral membrane and the target membrane. This may be mediated by the fusion peptide. Hemifusion is initiated by formation of a *stalk*, a lipid structure of very high negative curvature that provides continuity between the two outer leaflets. Mixing of outer leaflet lipids, but not those of the inner leaflet, characterizes hemifusion (step 3, **Fig. 3**).
5. *Formation of fusion pores.* The transition from the structure present in the hemifused state (the *hemifusion diaphragm*, a small area consisting of a single bilayer composed of the inner leaflets of the two reacting membranes; **Fig. 3**) to the fusion pore is energetically favorable. This transition requires participation of the transmembrane domain of the viral fusion protein (step 4, **Fig. 3**).
6. *Enlargement of the fusion pore.* Initially, fusion pores allow only flickering electrical contact between the aqueous compartments. The pore eventually widens out to permit transfer of the viral genome or other large molecules, thus completing the fusion process (step 5, **Fig. 3**).

It should be noted that the mechanism by which the viral fusion protein catalyzes (3)–(6) above is not yet understood. The major recognized fusion intermediates – the stalk, the hemifusion diaphragm and the fusion pore – are predominantly lipidic in nature, and have been defined in pure lipid systems. None the less, it has been estimated that HA-mediated fusion requires the concerted action of at least three HA trimers (step 2, **Fig. 3**). It seems likely that all viral fusion reactions proceed through a very similar series of steps. A more detailed understanding of the mechanisms of viral fusion would be of fundamental importance, and might also suggest new avenues for antiviral therapeutic intervention.

Membrane Synthesis

Viruses in general make maximal use of mechanisms already in place in the infected cell to perform their functions. Hence, viral protein synthesis is carried out on host cell ribosomes. Synthesis of viral membrane proteins occurs on membrane-bound ribosomes, from

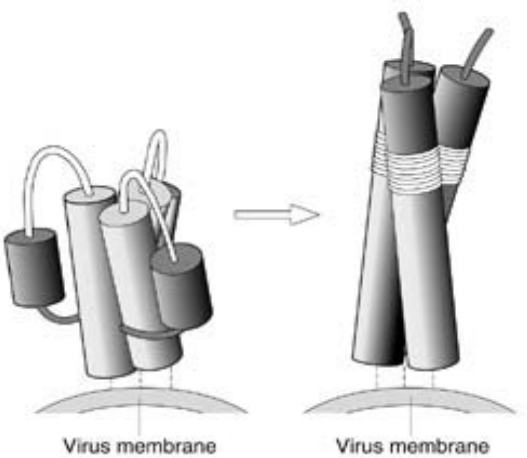


Figure 2 How influenza HA protein is activated for fusion by low pH. *Left:* A simplified representation of the trimeric structure of HA2 at neutral pH, as revealed by x-ray crystallography. Cylinders represent α helices; the light colored cylinders interact to form a three-stranded coiled-coil stem. The 'fusion peptide', which extends from the short, dark cylinders, is sequestered close to the viral membrane. *Right:* Upon exposure to low pH (e.g. inside an endosome), the previously unordered region shown in white becomes helical and extends the coiled-coils, which are still further extended by incorporation of the darkly colored region as shown. This repositions the fusion peptide to the end of the coiled-coils, enabling it to insert into a target membrane. (Modified with permission from Carr CM and Kim PS (1993) *Cell* 73: 823–832.)

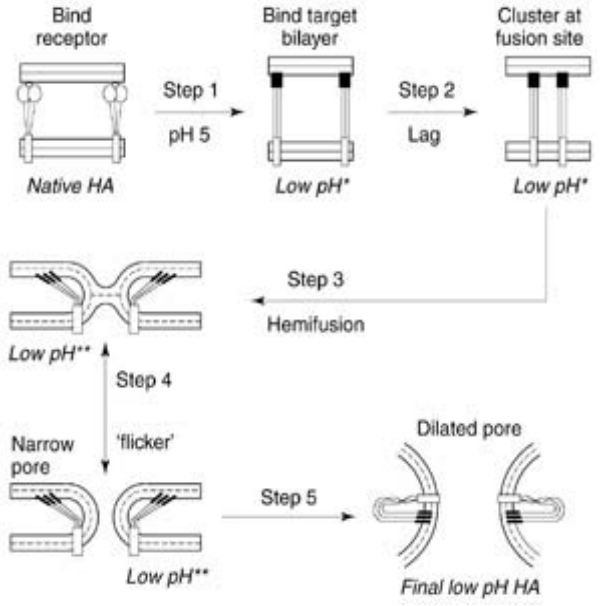


Figure 3 Sequence of events during HA-mediated virus fusion. See text for details. (Reproduced with permission from Hernandez *et al* (1996).) 'Low pH*' and 'Low pH**' are successive low pH-mediated conformations postulated by Hernandez *et al* (1996) to mediate the successive fusion events.

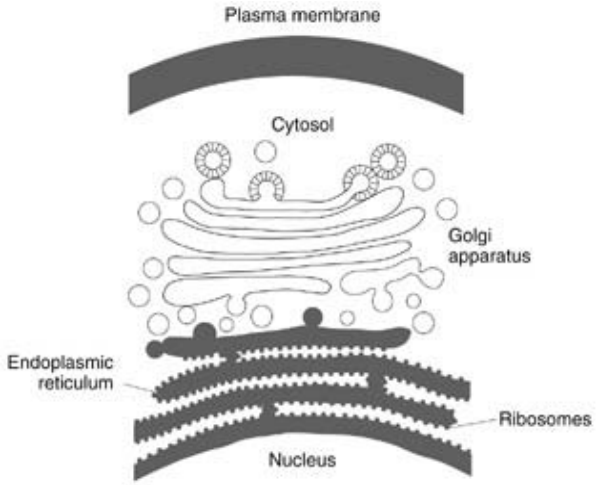


Figure 4 Endoplasmic reticulum–Golgi–plasma membrane system of a cell. All viral and cellular integral membrane proteins are synthesized by ribosomes bound to the endoplasmic reticulum membrane. Proteins destined for the plasma membrane then undergo vesicular transport to the nearest lamella of the Golgi apparatus (the *'cis'* face). A series of sequential vesicular transport steps then carries these proteins through the Golgi to the *'trans'* face and out to the plasma membrane. In polarized cells, further sorting steps target the viral protein to the apical or basolateral plasma membrane. Assembly and budding of different enveloped viruses occurs at characteristic points within this membrane system.

which they are inserted, always in the correct orientation, into the endoplasmic reticulum membrane. There they are glycosylated and assembled into multimeric form. The influenza HA protein, for example, is assembled into the trimers that form the 'spikes' seen in electron micrographs of the surface of influenza virions. The viral glycoproteins may then be further processed through the Golgi and on to the plasma membrane (**Fig. 4**). In fact, the membrane proteins of VSV, influenza, and several other enveloped viruses have provided valuable tools for the study of these transport processes. This is because many of these viruses possess only one major membrane protein, which is expressed in infected cells at very high levels. Further, host cell protein synthesis is inhibited by both VSV and influenza infection, so large amounts of a single membrane protein are produced and correctly processed in infected cells.

Viral proteins are targeted to specific cellular locations by the same mechanisms that cells use for their own membrane proteins. Viral proteins, in fact, are widely used in the study of these targeting mechanisms. Newly synthesized VSV G protein, for example, is targeted to the basolateral plasma membranes of polarized cells. Newly synthesized influenza HA protein, on the other hand, is delivered to the apical plasma membranes of the same polarized cells. Similarly, the retention of coronavirus glycoproteins by the endoplasmic reticulum, and of bunyavirus glycoproteins by the Golgi, are thought to reflect the operation of the same cellular mechanisms that retain resident cellular proteins in these organelles. The localization of viral membrane proteins is of particular importance, as it determines the location of viral assembly and budding.

As described above, the lipids of the viral membrane are taken from the host cell membrane during budding. No new lipids are specifically synthesized in response to viral infection, and viruses seem to tolerate wide variations in their lipid composition. Alterations in cellular lipid metabolism have been reported to result from some viral infections in cultured cells, but these are most likely secondary to other cytopathic effects; there is no indication that they play an important role in the progress of the infection.

Virus Assembly

The budding process consists of the wrapping of a specific piece of membrane around the previously assembled nucleocapsid, which contains the viral genome. The process is shown diagrammatically in **Fig. 5.** The specificity of the process is remarkable in

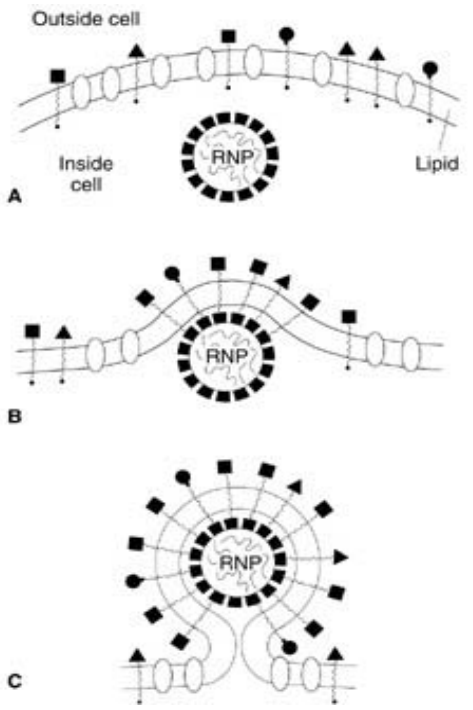


Figure 5 One kind of virus budding. Viral glycoproteins, inserted into the cellular membrane at the endoplasmic reticulum (**Fig. 4**), associate with the assembled viral nucleocapsid. The direct association pictured here is characteristic of togaviruses. For other viruses, possessing helical nucleocapsids, the association is mediated by a peripheral membrane protein. Cellular membrane proteins are excluded from the envelope of the mature virion. This may occur during assembly, as pictured, or by prior formation of a viral membrane patch, before the nucleocapsid arrives at the membrane.

that the completed viral envelope contains viral proteins and host cell lipids almost exclusively, with host cell membrane proteins being almost completely excluded.

Viruses can bud anywhere in the endoplasmic reticulum–Golgi–plasma membrane pathway shown in **Fig. 4.** While the parmyxo-, orthomyxo-, rhabdo- and togaviruses (and many others) generally bud from the plasma membrane, they have also been shown to bud intracellularly under certain conditions. Other viruses normally bud intracellularly, from the endoplasmic reticulum or Golgi apparatus, e.g. coronaviruses and bunyaviruses, respectively. In these cases the nucleocapsid, assembled in the cytoplasm, buds into the lumen of the appropriate organelle. The assembled virus is often seen inside vesicles in electron micrographs, producing a double-shelled appearance. Eventually, the newly formed virion may be secreted out of the cell through the normal secretory pathway, although this does not always occur efficiently.

In many cases viral assembly occurs at the plasma membrane. Some retroviruses assemble there, while others do not; this has provided a classical basis for distinguishing between different types of retroviruses. In orthomyxo-, paramyxo- and rhabdoviruses, budding is mediated by a peripheral membrane protein (called M). Surprisingly, this protein does not interact specifically with the corresponding viral membrane glycoproteins; in fact, successful budding has been observed in the complete absence of viral glycoproteins. This lack of specificity has made possible the creation of pseudotype viruses, possessing the encapsidated genome of one virus and the membrane glycoproteins of another. Since the membrane proteins determine host range and host cell specificity (see above), pseudotypes have proven useful in redirecting specific viral genomes to alternate host cells.

In contrast, togaviruses, which lack any M protein, possess an icosahedral nucleocapsid, which interacts directly with the viral membrane protein. Completed virions contain an equal number of nucleocapsid and membrane protein molecules. Both are in a similar geometric arrangement, so in this case specific interaction between the two proteins appears likely.

See also: **Human immunodeficiency viruses (*Retroviridae*): Molecular biology, Anti-retroviral agents, General features; Influenza viruses (*Orthomyxoviridae*): General features, Molecular biology, Structure of antigens; Pathogenesis: Animal viruses; Replication of viruses; Sendai virus (*Paramyxoviridae*); Virus structure: Atomic structure, Principles of virus structure; Viral receptors.**

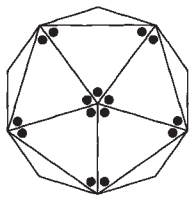
Further Reading

Hernandez LD, Hoffman LR, Wolfsberg TG and White JM (1996) Virus–cell and cell–cell fusion. *Ann. Rev. Cell Dev. Biol.* 12: 627.

Lenard J (1996) Negative-strand virus M and retrovirus MA proteins: all in a family? *Virology* 216: 289.

Lodish H, Baltimore D, Berk A *et al* (1995) Synthesis of plasma membrane, secretory and lysosomal proteins. In: *Molecular Cell Biology* 3rd edn, p. 669. New York: Freeman.

VIRAL RECEPTORS



Horacio U Saragovi, Gordon J Sauvé and **Mark I Greene**, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA

Introduction

Virus–host interactions are complex processes that span binding to cells, entry, dissemination and finally lytic or persistent infection. The first step, binding to host molecules that serve as viral receptors, is critical in all subsequent events. However, host cell alterations can occur in the virtual absence of entry and infection. These effects are due to viral binding to the cellular receptor and the activation of second messenger systems linked to the receptor.

Several viruses reportedly exploit cellular molecules for binding (see **Table 1**). Two interesting examples are the human immunodeficiency virus (HIV) and the vaccinia virus. HIV gp120 appears to bind to two distinct molecules, CD4 in T cells and galactosylceramide in brain. No homologies have been reported between gp120 and Class II major histocompatibility complex (MHC) molecules which are the biological ligand of CD4. Furthermore, it seems that the sites of CD4–Class II interaction are distinct from sites of CD4–gp120 interaction. This example emphasizes the evolution of the gp120 molecule as an effective binding agent for two completely different receptors. In contrast, the vaccinia VGF protein appears to bind to the epidermal growth factor (EGF) receptor. Vaccinia virus VGF is partially homologous to EGF which may explain its binding to EGF receptors. Therefore, some viruses may mimic the biological ligand of the cellular receptor.

Viruses have been proven to be invaluable tools in the molecular dissection of cellular processes and immune regulation. We have been studying the cellular receptor for reovirus type 3 and the role of this receptor in growth and development. Reoviruses provide an excellent model for the study of viral binding which includes biological effects. Furthermore, the as yet unknown structure of the reovirus receptor and its putative biological ligand provides an interesting system in which receptor biological function can be analyzed. In the following section we describe the role of reovirus type 3 cellular receptor in growth and differentiation of a variety of tissue types. New findings also point to the development of novel vaccines and drugs to treat viral infections.

Table 1 Cell surface molecules that serve as viral receptors

Virus	*Putative receptor*
Vaccinia	EGF receptor
Sendai	Gangliosides
Epstein–Barr	C3d receptor
Lactic dehydrogenase	Ia and Fc receptors
Rabies	Acetylcholine receptors
Rhinovirus	ICAM-I adhesion molecules
HIV (in T cells)	CD4
HIV (in brain)	Galactosylceramide

Abbreviations: EGF, epidermal growth factor; HIV, human immunodeficiency virus.

The Reovirus Type 3 Cellular Receptor (Reo3R)

Reo3R was initially analyzed by Fields and colleagues when mammalian lymphocytes and neurons were recognized to express a molecule utilized by the reovirus as its attachment site. Initially, cellular tropism by reovirus type 3 was linked to the expression of the viral hemagglutinin (HA) encoded by the $\sigma 1$ viral gene. Later, receptor-ligand assays using murine cells and labeled virus demonstrated that reovirus type 3 binds to lymphoid cells and fibroblasts with similar affinity. In contrast, reovirus type 1 or a recombinant construct of reovirus type 3 expressing type 1 HA was found to bind to fibroblast L cells but did not bind to lymphocytes (**Table 2**). The viral HA determines both the serotype specificity and the ability of reovirus to bind to cells. Thus, the HA type 1 (HA1) and HA type 3 (HA3) appear to recognize and bind to different and nonoverlapping structures on cell surfaces. These cell surface molecules bound by viral HA are exploited as cellular receptors for reovirus.

Table 2 Binding affinity of *Reoviridae* to murine cell lines

Cell type	*K_d for reovirus type 3 (nM)*	*K_d for reovirus type 1 (nM)*
R1.1 thymoma	0.6	Not detected
Fibroblast cells	0.8	0.4

The R1.1 murine thymoma cells express ~50 000 Reo3R molecules per cell and the murine L fibroblasts express ~100 000 Reo3R molecules per cell.

Table 3 Sequence comparison of MAb 87.92.6 variable heavy (V_H) and light (V_L) chains with HA3

87.92.6 V_H	43	**Q**	**G**	**L**	**E**	**W**	**I**	**G**	R	I	D	P	A	N	G			56
Reovirus HA3	317	**Q**	**S**	**M**	**-**	**W**	**I**	**G**	I	V	S	**Y**	**S**	**G**	**S**	**G**	**L**	N	332
87.92.6 V_L	39	K	P	G	K	T	N	K	L	L	I	**Y**	**S**	**G**	**S**	**T**	**L**	Q	55
									I			Reo3R binding						I	

Amino acid sequence similarity of the reovirus type 3 σ1 protein and MAb 87.92.6 light chain CDR2 is shown using the single-letter code. Amino acid numbers of the proteins are shown, and the regions within HA3 and V_L required for Reo3R binding are indicated. Bold letters indicate amino acid homology between viral protein and mAb.

To analyze Reo3R in detail, we obtained a murine monoclonal antibody (MAb) directed against the receptor. The anti-Reo3R MAb 87.92.6 was raised against MAb 9BG5 which binds and neutralizes HA3. Thus, the interactions of MAb 9BG5 with MAb 87.92.6 or HA3, and the interactions of HA3 and MAb 87.92.6 with the cellular receptor, can be studied. An important feature of this model is that binding to Reo3R is accomplished by a structure that is shared by MAb 87.92.6, the HA3 and their analogues. It has been demonstrated that the light chain variable region (V_L) of MAb 87.92.6 (specifically the complementarity determining region 2; CDR2) bears the internal image of HA3 (**Table 3**), emphasizing the relevance of structural domains in binding to Reo3R. Conversely, it has been hypothesized that Reo3R may bear the internal image of MAb 9BG5. Work in progress towards the molecular cloning of Reo3R cDNA will answer this question.

Given the primary sequence and structural similarity between HA3 and MAb 87.92.6, it is not surprising that the MAb prevents reovirus type 3 attachment to cells and primes mice to develop humoral or cellular immunity to the virus. Most importantly, binding of MAb 87.92.6 or its peptide analogues to Reo3R elicits receptor-mediated biological effects identical to those elicited by binding of inactivated virus to cells. Functional effects include the inhibition of mitogen-induced proliferation of T cells, maturation of oligodendrocytes and demyelination of neurons. Peptide analogues derived from V_L amino acids 45–55, as well as a synthetic nonamino-acid-based β-loop structure which mimics this region, also bind to Reo3R and elicit functional responses, indicating that receptor ligation generates the biological effects. Thus the technology developed using the Reo3R system might lead to the development of new drugs to modulate immune responses and affect neural development.

Effects mediated upon Reo3R binding are so profound and significant that an important role for the cellular receptor is suggested, resulting in an increased interest in understanding the cognate function of this surface molecule. Unlike other viral receptors the biological functions of which are known (e.g. CD4 is the HIV receptor and its biological ligands are Class II MHC gene products), it is not known whether Reo3R is also a true receptor with a true ligand. We will discuss evidence addressing this question in the next section.

Functional Studies of Reo3R

Reoviral infection takes place primarily in the gut epithelium, and can be spread to neutral tissue by an as yet unclear mechanism. The uncoupling of viral entry and replication processes is emphasized by the fact that, while productive infection occurs in the epithelium, latent chronic infection can take place in lymphoid cells. Obviously Reo3R must be expressed on epithelial, neural and lymphoid tissues for viral binding, and studies with MAb 87.92.6 have determined that the viral receptor is the same for these three cell types. However, the varied effects manifested upon receptor ligation suggest that expression of Reo3R has different repercussions for different tissues. Therefore, in order better to understand the function of Reo3R, we have studied its expression and the functional consequences of receptor engagement with the use of anti-Reo3R MAb 87.92.6.

Lymphoid cells

Most (80%) freshly isolated murine splenic B cells express Reo3R. In contrast, only a small proportion (5–15%) of resting splenic T cells and thymocytes express Reo3R. Interestingly, activation of T cells with concanavalinA (ConA) mitogen induces expression of Reo3R to detectable levels after 24h and optimally after 36h. Therefore, Reo3R is constitutively expressed in splenic B cells, but is inducible in T-cells, suggesting that differential regulation of transcription or translation occurs. The kinetics of expression in T cells parallel those of other inducible T-cell activation markers such as interleukin 2 (IL-2) receptor p55 (α) subunit and transferrin receptor, suggesting that Reo3R plays an important role in cellular immunity. After expression of Reo3R has been induced in T cells, binding by inactivated virus,

MAb 87.92.6, peptides derived for the MAb V_L domain or synthetic compounds that mimic these structures to the cell surface leads to a dramatic inhibition of proliferation even in the presence of the powerful stimulator ConA.

Litle is known about the mechanism of inhibition, but it does not involve cell death. Alternative possibilities include (i) blocking of an as yet unidentified biological ligand to the receptor or (ii) induction of receptor-mediated inhibitory signals. An exciting recent development has been the demonstration that inhibitory effects require ligation of Reo3R at the cell surface. In contrast to the negative growth regulation induced by Reo3R extracellular ligation, intracellular ligation has no effect on T cell proliferation. The data, however, do not clarify which of the possible mechanisms for inhibition may be operational.

Central nervous system (CNS)

The CNS is an important target of reovirus type 3 infection. Mature oligodendrocytes and both type 1 and 2 astrocytes, but not glial progenitor cells, express Reo3R. Surface Reo3R appears at an early stage of development prior to expression of myelin basic protein (MBP).

The effects of MAb 87.92.6 and its peptides in neural tissue have been tested *in vitro* and *in vivo*. In both systems neural tissue biology is altered and demyelination is induced, suggesting that Reo3R normally plays an important role in oligodendrocyte differentiation. Thus, it may be possible to create analogues of MAb 87.92.6 that stimulate myelin synthesis *in vivo* leading to the development of new drugs to modulate immune responses and affect neural development. A recent advance in this area has shown that synthetic analogues of MAb 87.92.6 bind Reo3R and induce functional effects, emphasizing the usefulness of viruses in analyzing receptor biology.

Future studies will reveal the structure and function of Reo3R, define the true physiological ligand(s) and provide insights into the signal transducing pathways by which this receptor exerts its important effects.

See also: **Cell structure and function in virus infections; Human immunodeficiency viruses (*Retroviridae*): General features; Pathogenesis: Animal viruses; Reoviruses (*Reoviridae*): General features; Replication of viruses; Virus–host cell interactions.**

Further Reading

Cohen JA *et al* (1989) In: Sercarz E (ed.) *Antigenic Determinants and Immune Regulation,* vol 46, p. 126. Basel: Karger.

Holmes KV, Tresnan DB and Zelus BD (1997) Virus-receptor interactions in the enteric tract. Virus-receptor interactions. *Adv. Exp. Med. Biol.* 412: 125.

Miller AD (1996) Cell-surface receptors for retroviruses and implications for gene transfer. *Proc. Natl Acad. Sci. USA* 93(21): 11407.

Moore JP, Trkola A and Dragic T (1997) Co-receptors for HIV-1 entry. *Curr. Opin. Immunol.* 9(4): 551.

Norkin LC (1995) Virus receptors: implications for pathogenesis and the design of antiviral agents. *Clin. Microbiol. Rev.* 8(2): 293.

Williams WV *et al* (1990) *Trends Biotechnol.* 8: 256.

[This article is reproduced from the 1st edn (1994).]

VIROIDS

Robert A Owens, Molecular Plant Pathology Laboratory, Beltsville Agricultural Research Center, Beltsville, Maryland, USA

Introduction

Viroids are the smallest known agents of infectious disease – small (246–375 nucleotides), highly structured, circular, single-stranded RNAs which lack both a protein capsid and detectable messenger RNA activity. Whereas viruses have been described as 'obligate parasites of the cell's translational system' and supply some or most of the genetic information required for their replication, viroids can be regarded as 'obligate parasites of the cell's transcriptional machinery'. Thus far, viroids are known to infect only plants.

History

While scientific investigation of plant diseases now known to be caused by plant viruses did not begin until the late nineteenth century, the earliest written records of plant viral disease date to the eighth century AD. The first viroid disease to be studied by

MAb 87.92.6, peptides derived for the MAb V_L domain or synthetic compounds that mimic these structures to the cell surface leads to a dramatic inhibition of proliferation even in the presence of the powerful stimulator ConA.

Litle is known about the mechanism of inhibition, but it does not involve cell death. Alternative possibilities include (i) blocking of an as yet unidentified biological ligand to the receptor or (ii) induction of receptor-mediated inhibitory signals. An exciting recent development has been the demonstration that inhibitory effects require ligation of Reo3R at the cell surface. In contrast to the negative growth regulation induced by Reo3R extracellular ligation, intracellular ligation has no effect on T cell proliferation. The data, however, do not clarify which of the possible mechanisms for inhibition may be operational.

Central nervous system (CNS)

The CNS is an important target of reovirus type 3 infection. Mature oligodendrocytes and both type 1 and 2 astrocytes, but not glial progenitor cells, express Reo3R. Surface Reo3R appears at an early stage of development prior to expression of myelin basic protein (MBP).

The effects of MAb 87.92.6 and its peptides in neural tissue have been tested *in vitro* and *in vivo*. In both systems neural tissue biology is altered and demyelination is induced, suggesting that Reo3R normally plays an important role in oligodendrocyte differentiation. Thus, it may be possible to create analogues of MAb 87.92.6 that stimulate myelin synthesis *in vivo* leading to the development of new drugs to modulate immune responses and affect neural development. A recent advance in this area has shown that synthetic analogues of MAb 87.92.6 bind Reo3R and induce functional effects, emphasizing the usefulness of viruses in analyzing receptor biology.

Future studies will reveal the structure and function of Reo3R, define the true physiological ligand(s) and provide insights into the signal transducing pathways by which this receptor exerts its important effects.

See also: **Cell structure and function in virus infections; Human immunodeficiency viruses (*Retroviridae*): General features; Pathogenesis: Animal viruses; Reoviruses (*Reoviridae*): General features; Replication of viruses; Virus–host cell interactions.**

Further Reading

Cohen JA *et al* (1989) In: Sercarz E (ed.) *Antigenic Determinants and Immune Regulation,* vol 46, p. 126. Basel: Karger.

Holmes KV, Tresnan DB and Zelus BD (1997) Virus-receptor interactions in the enteric tract. Virus-receptor interactions. *Adv. Exp. Med. Biol.* 412: 125.

Miller AD (1996) Cell-surface receptors for retroviruses and implications for gene transfer. *Proc. Natl Acad. Sci. USA* 93(21): 11407.

Moore JP, Trkola A and Dragic T (1997) Co-receptors for HIV-1 entry. *Curr. Opin. Immunol.* 9(4): 551.

Norkin LC (1995) Virus receptors: implications for pathogenesis and the design of antiviral agents. *Clin. Microbiol. Rev.* 8(2): 293.

Williams WV *et al* (1990) *Trends Biotechnol.* 8: 256.

[This article is reproduced from the 1st edn (1994).]

VIROIDS

Robert A Owens, Molecular Plant Pathology Laboratory, Beltsville Agricultural Research Center, Beltsville, Maryland, USA

Introduction

Viroids are the smallest known agents of infectious disease – small (246–375 nucleotides), highly structured, circular, single-stranded RNAs which lack both a protein capsid and detectable messenger RNA activity. Whereas viruses have been described as 'obligate parasites of the cell's translational system' and supply some or most of the genetic information required for their replication, viroids can be regarded as 'obligate parasites of the cell's transcriptional machinery'. Thus far, viroids are known to infect only plants.

History

While scientific investigation of plant diseases now known to be caused by plant viruses did not begin until the late nineteenth century, the earliest written records of plant viral disease date to the eighth century AD. The first viroid disease to be studied by

plant pathologists was potato spindle tuber disease. In 1923, its infectious nature and ability to spread in the field led Schultz and Folsom to group potato spindle tuber disease with several other 'degeneration diseases' of potatoes. These maladies, long attributed to senility, 'reversion', or loss of vigor caused by prolonged asexual reproduction, are now known to be caused by infection with conventional RNA viruses. Nearly 50 years were to elapse between the first published descriptions of potato spindle tuber disease and Diener's 1971 demonstration of the fundamental differences between the structure and properties of its causal agent, potato spindle tuber viroid (PSTVd), and those of conventional plant viruses.

Growers and plant pathologists are unlikely to have simply overlooked diseases with symptoms as severe as those of chrysanthemum stunt or cucumber pale fruit, two other viroid diseases first reported after World War II. Thus, certain aspects of modern agricultural practice seem to have favored the appearance of viroid diseases in the twentieth century. Large-scale monoculture of genetically identical crops and the commercial propagation/distribution of many cultivars are two comparatively modern developments which can facilitate the development of serious disease problems following the chance transfer of viroids from wild hosts to cultivated plants.

Genome Structure

Efforts to understand how viroids replicate and cause disease without the assistance of any viroid-encoded polypeptides have prompted detailed analysis of their structure. Viroids possess rather unusual properties for single-stranded RNAs (e.g. a pronounced resistance to digestion by ribonuclease and a highly cooperative thermal denaturation profile), leading to an early realization that they might have an unusual higher-order structure. Their small size also made viroids tempting objects for detailed structural investigation.

To date, the complete sequences of more than 20 distinct viroid species plus a large number of sequence variants have been determined (**Table 1**). All known viroids are single-stranded circular RNAs which contain 246–399 unmodified nucleotides and lack any unusual 2,′5′-phosphodiester bonds or 2′-phosphate moieties. Electron microscopy, optical melting and other physicochemical studies, and theoretical calculations of their lowest free-energy secondary structure all indicate that PSTVd and related viroids assume a highly base-paired, rod-like conformation *in vitro* (**Fig. 1**). Pairwise sequence comparisons of PSTVd with several related viroids suggest that the series of short double helices and small internal loops which comprise this so-called 'native' structure are organized into five domains whose boundaries are defined by sharp changes in sequence similarity.

As implied by its name, the 'conserved central domain' is the most highly conserved viroid domain and is believed to contain the site where multimeric viroid RNAs are cleaved and ligated to form circular progeny. The 'pathogenicity domain' contains one or more structural elements which modulate symptom expression, and the relatively small 'variable domain' exhibits the greatest sequence variability between otherwise closely-related viroids. The two 'terminal domains' appear to play an important role in viroid replication and evolution. Although these five domains were first identified in members of the PSTVd viroid group, ASSVd and related viroids also contain a similar domain arrangement. (See **Table 1** for explanation of abbreviations.) Certain viroids such as *Columnea* latent viroid appear to be 'mosaic molecules' formed by exchange of domains between two or more viroids infecting the same cell. Individual plants of the *Coleus blumei* cultivar 'Ruhm von Luxemburg' may contain as many as three viroid species, one of which (i.e. CbVd 2) represents a fusion product of the right half of CbVd 1 and the left half of CbVd 3. RNA rearrangement/recombination can also occur within individual domains, thereby leading, in the case of CCCVd, to duplication of the right terminal domain plus part of the variable domain, as disease progresses. The presence of domains in ASBVd and other ribozyme-containing viroids remains uncertain pending identification of additional group members.

Much less is currently known about viroid tertiary structure, especially *in vivo* where these molecules accumulate as ribonucleoprotein particles. Although the extended, rod-like nature of the 'native' structure might suggest that viroids lack significant tertiary structure, the ability of UV irradiation to crosslink certain nucleotides within the conserved central domain of PSTVd provided the first definitive evidence for such tertiary interactions. Similar UV-sensitive structural elements have also been discovered in a number of other RNAs, including 5S ribosomal RNA, adenovirus VAI RNA, and the viroid-like domain of the hepatitis delta virus genome. Studies of the spontaneous self-cleavage of various ASBVd-related RNAs as well as the nuclease-dependent conversion of multimeric PSTVd RNAs into monomers have provided additional evidence for the functional importance of viroid tertiary structure.

Classification

Viroids of known sequence have recently been assigned to one of two taxonomic families based

upon differences in the structural and functional properties of their genomes (**Table 1**). Members of the *Pospiviroidae* (type member, potato spindle tuber viroid) have a rod-like secondary structure that contains five structural–functional domains. Two members of the *Avsunviroidae* (type member, avocado sunblotch viroid), in contrast, appear to adopt a branched conformation, and multimeric RNAs of all three known family members undergo spontaneous self-cleavage *in vitro*. Groups of independently replicating sequence variants that show >90% sequence homology in pairwise comparisons have been arbitrarily defined as viroid species.

Phylogenetic evidence for an evolutionary link between viroids and other viroid-like subviral RNAs has been presented by Elena *et al* (**Fig. 2**). Among several subviral RNAs possibly related to viroids is carnation small viroid-like RNA, a 275 nt circular molecule with self-cleaving hammerhead structures in both its plus and minus strands. This novel retroviroid-like element shares certain features with both viroids and a small RNA transcript from newt.

Host Range and Transmission

All viroids are mechanically transmissible, and most are naturally transmitted from plant to plant by humans and their tools. Nevertheless, individual viroids vary greatly in their ability to infect and replicate in different plant species. PSTVd can replicate in about 160 primarily solanaceous hosts, while only two members of the Lauraceae are known

Table 1 Viroids of known nucleotide sequence (1998)

Family[a]	*Genus*[a]	*Name*	*Abbreviation*	*Nucleotides*[b]
Pospiviroidae	*Pospiviroid*	Chrysanthemum stunt	CSVd	354–356
		Citrus exocortis	CEVd	368–375 (463)
		Columnea latent	CLVd	370–373
		Iresine	IrVd	370
		Mexican papita	MPVd	359–360
		Potato spindle tuber	PSTVd	358–361 (341)
		Tomato apical stunt	TASVd	360–363
		Tomato planta macho	TPMVd	360
	Cocadviroid	Citrus viroid IV	CVd-IV	284
		Coconut cadang-cadang	CCCVd	246–247 (287–301)
		Coconut tinangaja	CTiVd	254
		Hop latent	HLVd	256
	Hostuviroid	Hop stunt[c]	HSVd	294–303
	Apscaviroid	Apple dimple fruit	ADFVd	306
		Apple scar skin[d]	ASSVd	329–334
		Australian grapevine	AGVd	369
		Citrus bent leaf	CBLVd	315, 318
		Citrus viroid III	CVd-III	294, 297
		Grapevine yellow speckle 1	GYSVd 1	336–388
		Grapevine yellow speckle 2	GYSVd 2	363
		Pear blister canker	PBCVd	315
	Coleviroid	*Coleus blumei* 1	CbVd 1	248–251
		Coleus blumei 2	CbVd 2	301
		Coleus blumei 3	CbVd 3	361–364
Avsunviroidae	*Avsunviroid*	Avocado sunblotch	ASBVd	246–251
	Pelamoviroid	Chrysanthemum chlorotic mottle	CChMVd	398, 399
		Peach latent mosaic	PLMVd	336–337

[a] Classification follows scheme proposed by Flores *et al* (see Seventh Report of the International Committee on Taxonomy of Viruses). The nucleotide sequences of apple fruit crinkle, burdock stunt, eggplant latent, *Nicotiana glutinosa* stunt, pigeon pea mosaic mottle and tomato bunchy top viroids are currently unknown; thus, these viroids have not been assigned to specific genera.

[b] Sizes of variants containing insertions or deletions arising *in vivo* are shown in parentheses.

[c] Includes cucumber pale fruit, citrus cachexia, peach dapple and plum dapple viroids.

[d] Includes pear rusty skin and dapple apple viroids.

[e] Formerly named grapevine viroid 1B.

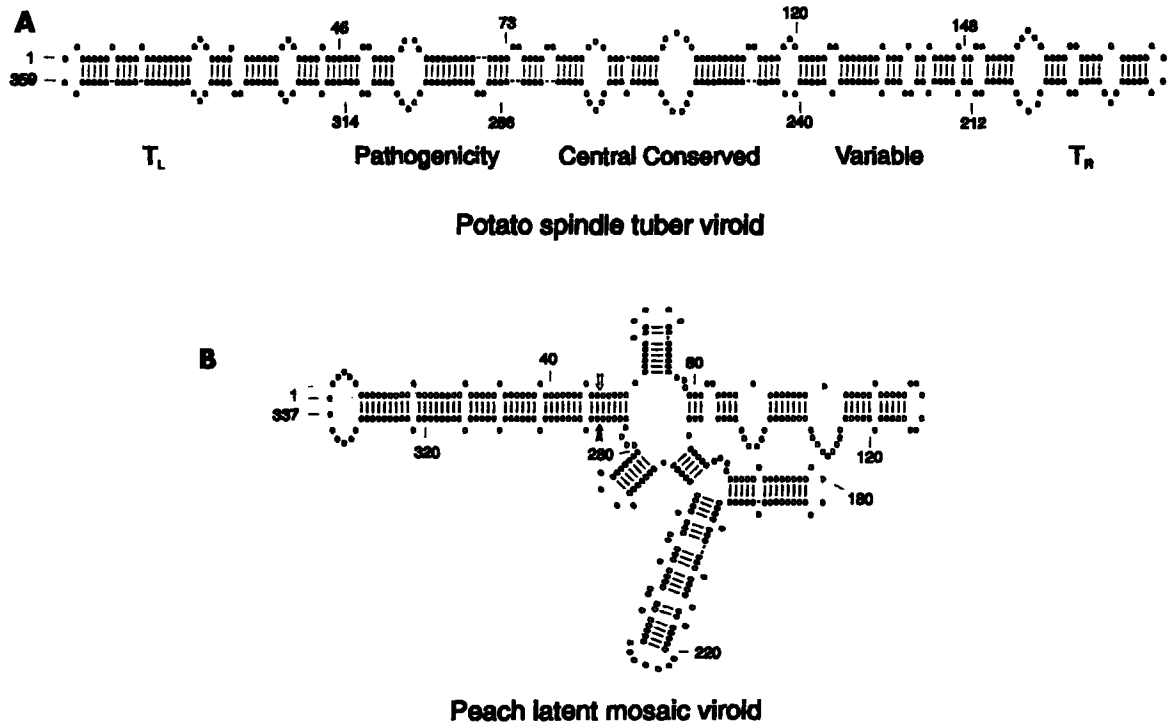


Figure 1 Secondary structures of potato spindle tuber and peach latent mosaic viroids. (**A**) A rod-like secondary structure for PSTVd is supported by a variety of physical studies as well as chemical and enzymatic mapping data. Boundaries of the terminal-left (T_L), pathogenicity, central conserved, variable and terminal-right (T_R) domains are indicated by vertical lines. (**B**) Proposed lowest-free-energy structure of PLMVd. Predicted self-cleavage sites in the plus and minus strands are indicated by filled and open arrows, respectively. (Redrawn from Hernandez and Flores (1991) *Proc. Natl. Acad. Sci. USA* 89: 3711.)

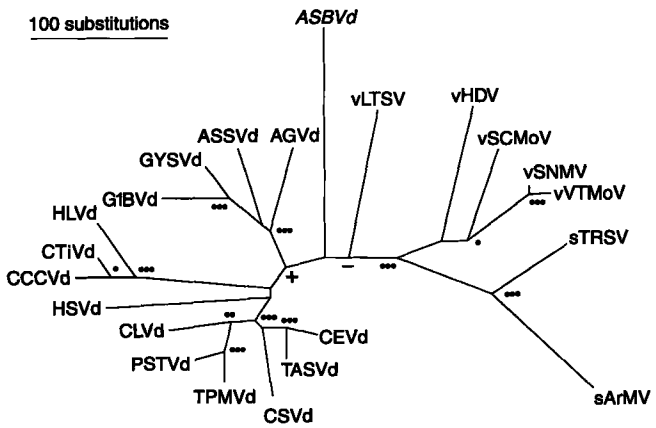


Figure 2 Consensus phylogenetic tree containing 22 viroids, viroid-like satellite RNAs and the viroid-like domain of hepatitis delta virus RNA. ASBVd has been taken as outgroup. ***, Group monophyletic in all 1000 bootstrap replicates; **, monophyletic in more than 99%; *, in more than 95%; +, in more than 90%; and −, in more than 80% of all replicates. From ASBVd to the left of the figure groups are considered as being within the viroid family, and from ASBVd to the right (including the viroid-like domain of HDV RNA) as within the satellite family. For example, satellite tobacco ringspot virus (sTRSV) and satellite *Arabis* mosaic virus (sArMV) (satellite family) or CCCVd, CTiVd and HLVd (viroid family) conformed to two well-defined monophyletic groups in all bootstrap replicates. G1BVd, grapevine viroid 1B; LTSV, Lucerne transient streak virus; ScMoV, subterranean clover mottle virus; SNMV, *Solanum nodiflorum* mosaic virus; VTMoV, velvet tobacco mottle virus. Other abbreviations as in **Table 1**. (From Elena SF *et al* (1991) *Proc. Natl. Acad. Sci. USA* 88: 5631, with permission.)

to support ASBVd replication. HSVd has a particularly wide host range which includes several herbaceous species as well as woody perennials (e.g. grapes, citrus and various *Prunus* spp.). Many natural hosts of viroids are either vegetatively propagated crops, such as potato and chrysanthemum, or those that are subjected to repeated grafting or pruning operations. PSTVd, ASBVd and CbVd can all be vertically transmitted through pollen and/or true seed, but the significance of this mode of transmission in the natural spread of disease is unclear. PSTVd can be encapsidated by potato leafroll luteovirus (PLRV) as well as velvet tobacco mottle sobemovirus, and epidemiological surveys suggest that PLRV can facilitate viroid spread under field conditions.

Commonly used techniques for the experimental transmission of viroids include the standard leaf abrasion methods developed for use with conventional viruses, various 'razor slashing' methods in which phloem tissue in the stem or petiole is inoculated via cuts made with a razor blade previously dipped into the inoculum, and, in the case of CCCVd, high-pressure injection into folded apical leaves. PSTVd and HSVd have also been experimentally transmitted by 'Agroinoculation', a technique in which a modified *Agrobacterium tumefaciens* Ti plasmid is used to introduce full-length viroid cDNA into the potential host cell. In both cases, Agroinoculation was able to overcome a marked host resistance to mechanical inoculation. Identification of the molecular mechanism(s) which determine host range remains an important research goal.

Symptomatology

Viroids and conventional plant viruses induce a very similar range of macroscopic symptoms (and presumably metabolic changes) in their hosts. Symptom expression is usually optimal at the same relatively high temperatures that promote viroid replication (i.e. 30–33°C). Prominent among metabolic changes are dramatic alterations in growth regulator levels. Stunting and leaf epinasty (a downward curling of the leaf lamina resulting from unbalanced growth within the various cell layers) are often considered the classic symptoms of viroid infection, and other commonly observed symptoms include vein clearing, veinal discoloration or necrosis, and the appearance of localized chlorotic/necrotic spots or mottling in the foliage. Viroid infections only rarely kill the host (**Figs 3** and **4**).

Viroid infections are also accompanied by a number of cytopathic effects – various chloroplast and cell wall abnormalities, the formation of membranous structures (so-called 'plasmalemmasomes' or 'paramural bodies') in the cytoplasm, and the accumulation of electron-dense deposits in both chloroplasts and cytoplasm. A combination of subcellular fractionation and *in situ* hybridization studies has shown that the plus and minus strands of PSTVd and related viroids both accumulate in the nucleolus. Precise partitioning between the nucleoplasm and nucleolus remains to be established, but this nucleolar localization may have important implications for replication and pathogenicity. ASBVd, in contrast, appears to be associated with chloroplast thalykoid membranes rather than the nucleoli of infected cells, thereby raisng the possibility that a host-encoded enzyme other than RNA polymerase II may be responsible for its replication (see below).

Geographic Distribution

Viroids such as PSTVd, HSVd, CEVd and ASBVd are widely distributed throughout the world, while others have never been detected outside the areas where they were first reported. Several factors may contribute to this variation in distribution pattern. Among the crops most affected by viroid diseases are a number of valuable woody perennials such as grapes, citrus, various pome and stone fruits, and hops. Propagation and distribution of improved cultivars is becoming increasingly commercialized, with the result that many are now grown worldwide. The international exchange of plant germplasm has also continued to increase at a rapid rate. In both instances, the large number of latent (asymptomatic) hosts facilitates viroid spread. Also, several newly-discovered viroids affect either tropical or subtropical crops. The combination of the generally high temperature optimum for viroid replication and an increased interest in diseases affecting tropical crops is likely to cause a continued shift in the known geographic distribution of viroid diseases.

Epidemiology and Control

While many viroids were first detected in ornamental or crop plants, most viroid diseases are thought to be the result of their chance transfer from endemically-infected wild species to susceptible cultivars. Several lines of circumstantial evidence are consistent with this hypothesis:

- The experimental host ranges of several viroids include many wild species, and these wild species often tolerate viroid replication without the appearance of recognizable disease symptoms.
- Although coevolution of host and pathogen is often accompanied by appearance of gene-for-gene ver-

Figure 3 Characteristic symptoms of viroid infection in citrus. (**A**) Dwarfing of sweet orange induced by citrus viroid(s). Note the difference in height between the infected tree (foreground) and uninfected trees. (**B**) Leaf epinasty in 'Etrog' citron induced by severe (left) and mild (center) isolates of citrus exocortis viroid. The plant on the right is an uninfected control. (**C, D**) Additional symptoms of viroid infection in 'Etrog' citron: Midvein and petiole browning (arrows) and petiole wrinkling induced by citrus viroid III. (Photographs courtesy of S.M. Garnsey and L. Levy.)

tical resistance, no useful sources of resistance to PSTVd has been identified in the cultivated potato.

- Viroids and/or viroid-related RNAs closely related to TPMVd and CCCVd have been detected in weeds and other wild vegetation growing near fields containing viroid-infected plants.

Viroid diseases may also arise by transfer between cultivated crop species. Studies conducted in the People's Republic of China have shown that pears provide a latent reservoir for ASSVd; likewise, while there is no obvious correlation between disease status and the presence of HSVd in grapes, this viroid is known to cause severe disease in hops. In both instances, the two crops are often grown in close proximity.

All viroid diseases pose a potential threat to agriculture, and several are of considerable economic importance. Coconut cadang-cadang has killed over 30 million palms in the Philippines since it was first recognized in the early 1930s, and estimates of the resulting loss in copra production are in the range of US $80–100 per tree (1987 estimate). Ready transmission of PSTVd by vegetative propagation, foliar contact and true seed or pollen poses a potentially serious threat to potato production, germplasm collections and breeding programs. For many plant viruses, the preferred method of prevention involves incorporation of genetic resistance into the genomes of commercially desirable cultivars. Unfortunately, no useful sources of natural resistance to viroid disease are known, and thermotherapy and/or mer-

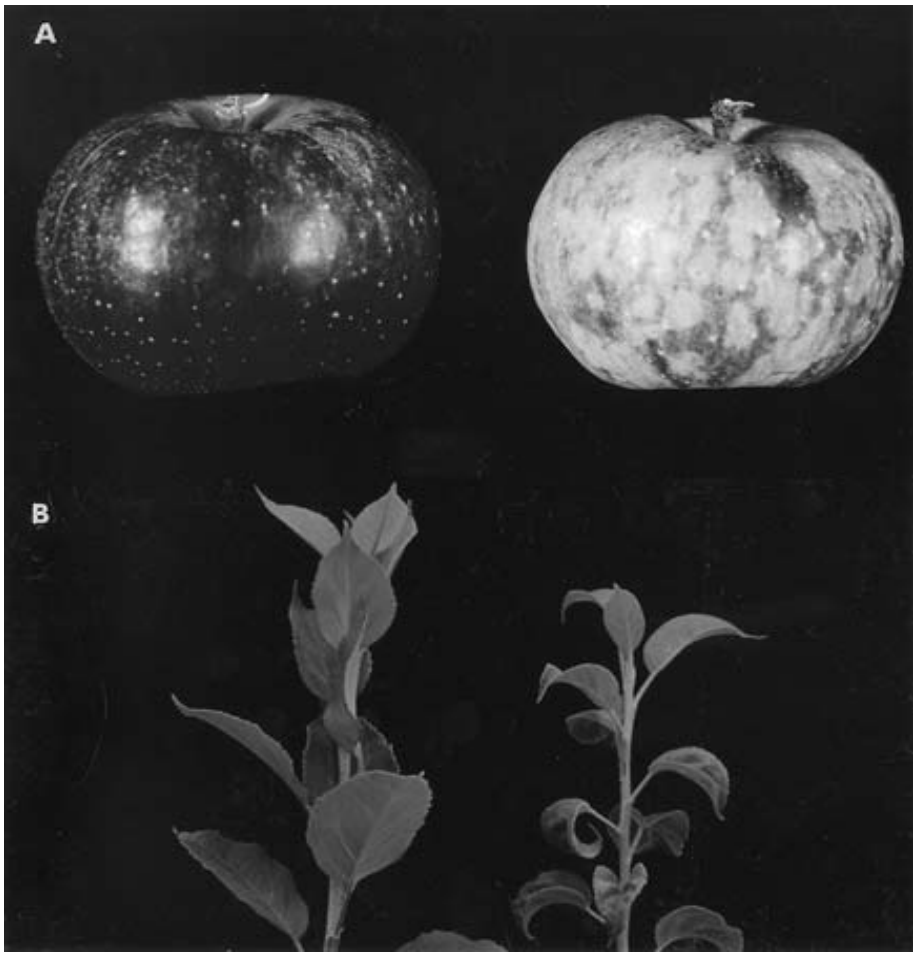


Figure 4 Characteristic symptoms of viroid infection in apple. (**A**) Normal (left) and dappled (right) fruit from 5-year-old orchard-grown 'Lord Lambourne' trees. The tree producing dappled fruit was infected with apple scar skin viroid. (**B**) Leaf epinasty induced by apple scar skin viroid in the variety 'Stark's Earliest'. The plant on the left is an uninfected control. (Photographs courtesy of E.V. Podleckis.)

istem culture protocols have not been widely adopted. Thus, suitable diagnostic tests for the rapid, specific and reliable detection of viroids continue to play a prominent role in disease control efforts.

Tests based upon their unique physical or chemical properties have largely supplanted biological assays for viroid detection. Problems associated with viroid bioassays include the often extended period of time required for completion (weeks to months) and difficulties in detecting mild or latent strains of the pathogen. Several rapid (1–2 day) protocols involving two-dimensional or bidirectional polyacrylamide gel electrophoresis have been developed which take advantage of the circular nature of viroids. Using these protocols, subnanogram amounts of viroid can be unambiguously detected without the use of radioactive isotopes, but neither bioassay nor electrophoretic assays are well suited for the routine analysis of large numbers of samples. Because viroids lack a protein capsid, antibody-based diagnostic techniques are not applicable.

In recent years, diagnostic procedures based upon nucleic acid hybridization or the polymerase chain reaction have become widely used. The simplest methods involve the hybridization of a highly radioactive viroid-complementary DNA or RNA probe to viroid samples that have been bound to a solid support, followed by autoradiographic detection of the resulting DNA–RNA or RNA–RNA hybrids. Such conventional 'dot blot' assays can detect picogram amounts of viroids using clarified plant sap rather than purified nucleic acid as the viroid source, but sample preparation is often a significant stumbling block. Polymerase chain reaction-based protocols are finding increasing acceptance in those cases where either this level of sensitivity is inadequate or a number of closely-related viroids are present in the same sample.

Molecular Biology

Although apparently devoid of messenger RNA activity, viroids replicate autonomously and induce disease in a wide variety of plant species. The many gaps in our present understanding of the biological properties of these unusual molecules have been aptly summarized by Diener as a series of questions:

- What molecular signals do viroids possess (and cellular RNAs evidently lack) that induce certain host enzyme(s) to accept them as templates for the synthesis of complementary RNA molecules?
- What are the molecular mechanisms responsible for viroid replication? Are these mechanisms also operative in uninfected cells? If so, what are their functions?
- How do viroids induce disease? In the absence of viroid-specified proteins, disease must arise from direct interaction(s) of viroids (or their complementary RNA molecules) with as yet unidentified host cell constituents.
- What are the molecular determinants of viroid host range? Are viroids restricted to higher plants, or do they have counterparts in animals?
- How did viroids originate?

Over the past several years, considerable information has accumulated concerning the molecular biology of viroid replication, pathogenesis and host range determination. The precise nature of the molecular signals involved nevertheless remains elusive.

Replication

Viroid replication is believed to proceed via a 'rolling circle' mechanism involving the synthesis of a minus-strand RNA template. A variety of multimeric plus- and minus-strand viroid RNAs have been detected by nucleic acid hybridization. ASBVd replication appears to utilize a symmetric replication cycle in which the multimeric minus strand is first cleaved to unit-length molecules and circularized before serving as template for the synthesis of multimeric ASBV plus strands. PSTVd and related viroids appear to utilize an asymmetric cycle in which the multimeric minus strand is directly copied into a multimeric plus-strand precursor.

A variety of host-encoded enzymes have been implicated in different aspects of viroid replication. Low concentrations of α-amanitin specifically inhibit the synthesis of both PSTVd plus and minus strands in nuclei isolated from infected tomato, strongly suggesting the involvement of RNA-dependent RNA polymerase II in the replication of PSTVd and related viroids. Localization of both the mature viroid and the replicative intermediates within the nucleolus would seem to argue against the involvement of RNA polymerase II in their synthesis, but this contradiction may be more apparent than real. One or more host-encoded nuclease activities appear to be required for the specific cleavage of multimeric PSTVd plus strands, while both plus- and minus-strand ASBVd RNAs transcribed *in vitro* undergo a spontaneous self-cleavage to form linear monomers. The final step in viroid replication is the ligation of linear monomers to form mature circular progeny. Plant cells are known to contain RNA ligase activities which can act upon the 5′-hydroxyl and 2′,3′-cyclic phosphate termini formed during either cleavage pathway, and ribonuclease T1 is able to generate circular RNA molecules from PSTVd-specific RNA transcripts by cleavage and intramolecular ligation *in vitro*.

During replication, the rod-like native structure of PSTVd and related viroids must rearrange to assume one or more as-yet-undefined alternative conformations. How a viroid 'switches' between these different conformations remains to be determined. As shown in **Fig. 5**, there is compelling biochemical and molecular genetic evidence for the ability of dimeric ASBVd minus-strand RNA to undergo spontaneous *in vitro* cleavage via two different (but related) structures. The preferred pathway for enzymatic processing of longer-than-unit-length PSTVd plus-strand RNAs seems to involve a cleavage site formed by rearrangement of the conserved central domain, but other less efficient sites can also be used *in vivo*.

Pathogenicity

Both viroid cDNAs and their RNA transcripts are infectious when inoculated on to susceptible plants, a fact which provides a unique opportunity to relate sequence (and hence structural) variation to pathogenicity. Infectivity studies with chimeras constructed by exchanging the pathogenicity domains of naturally occurring mild and severe strains of CEVd have clearly shown that the pathogenicity domain contains important determinants of symptom expression. Sequence variation within the variable domain of CEVd also influenced viroid titer in infected plants. Application of a similar experimental strategy to TASVd revealed the presence of a third pathogenicity determinant located in the left terminal loop.

The ability of novel viroid chimeras to replicate and move normally from cell-to-cell implies certain basic similarities between their structures *in vitro* and *in vivo* but provides no information about the nature of the molecular interactions responsible for symptom development. Viroid infections are accompanied by quantitative changes in a variety of host-encoded

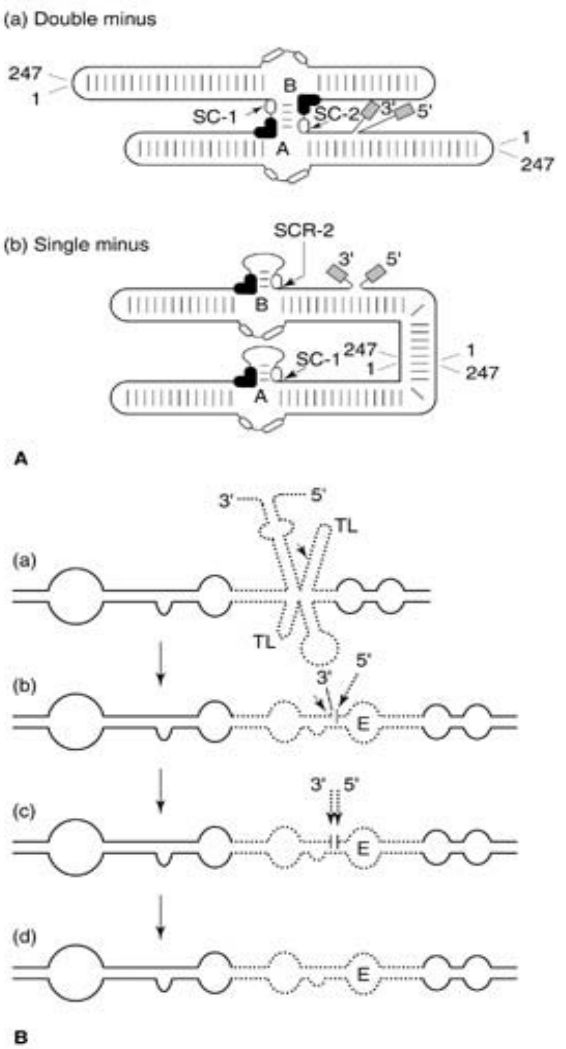


Figure 5 Cleavage of multimeric viroid RNAs requires rearrangement of the native structure. (**A**) A dimeric minus ASBVd RNA transcribed *in vitro* from a *Bst*NI dimeric cDNA clone can fold to produce a structure containing either a double- (**a**) or single- (**b**) hammerhead. Self-cleavage sites, labeled SC-1 and SC-2, are indicated by arrows; stippled boxes, vector sequences at 5′ and 3′ ends; closed boxes, conserved GAAAC sequences labeled A and B; open boxes, remaining conserved nucleotides. Base-pairing is represented by lines between RNA strands. (From Davies C *et al* (1991) *Nucl. Acids Res.* 19: 1893, with permission.) (**B**) Processing of a longer-than-unit-length plus PSTVd RNA transcript in a potato nuclear extract. The central conserved region of the substrate for the first cleavage reaction (**a**) contains a tetraloop (denoted TL). (**b**) After dissociation of the 5′ segment from the cleavage site, the new 5′ end refolds and is stabilized by formation of a UV-sensitive loop E, while the 3′ end partially base-pairs with the lower strand. Single-stranded nucleotides at the 3′ end are then cleaved between positions 95 and 96 (**c**), and ligation of the 5′ and 3′ termini (**d**) results in formation of mature circular progeny. (From Baumstark T *et al* (1997) *EMBO J.* 16: 599, with permission.)

proteins. Certain of these proteins may be 'pathogenesis-related' proteins whose synthesis or activation is part of a general host reaction to both biotic and abiotic stresses; others, such as a 140 kDa protein whose accumulation requires the presence of a replicating low molecular weight viroid or viral satellite RNA, may be more specific. In tobacco, PSTVd infection results in the preferential phosphorylation of a host-encoded 68 kDa protein that is immunologically related to an interferon-inducible, double-stranded RNA-dependent mammalian protein kinase of similar size. Diener and coworkers have presented evidence for the differential activation of the human kinase by PSTVd strains of varying pathogenicity. Although major differences have been reported in the overall three-dimensional conformations of the pathogenicity domain from a series of PSTVd strains, precise cause and effect relationships for the role of protein kinase(s) in viroid pathogenicity remain to be established.

Host range

Perhaps as a result of its involvement in the cleavage/ligation of multimeric plus-strand progeny RNA, a variety of evidence indicates that the central conserved region of PSTVd and related viroids also plays an important role in determining host range. For example, a severe isolate of PSTVd known as as KF 440-2 replicates very poorly in tobacco, but a single nucleotide substitution in the so-called 'loop E' portion of the central conserved region results in a dramatic increase in the rate of replication and systemic movement. The biological properties of Columnea latent viroid also suggest that this domain contains one or more host range determinants. As described above, CLVd appears to be a natural mosaic of sequences present in other viroids; phylogenetic analysis (**Fig. 2**) suggests that it can be considered to be a PSTVd-related viroid whose conserved central domain has been replaced by that of HSVd. Like HSVd (but unlike other PSTVd-related viroids), CLVd is able to replicate and cause disease in cucumber.

Origin and Evolution

As discussed by Matthews, available evidence suggests three possible origins for viruses: (1) descent from molecules present in a prebiotic RNA world; (2) descent from normal host RNAs; and (3) degeneration from simple obligate cellular parasites. Present-day plant viruses form four clusters (i.e. single-stranded positive-sense RNA viruses (three superfamilies); plant reoviruses, rhabdoviruses and bunyaviruses; caulimoviruses; and geminiviruses) and

the existence of these clusters may reflect their different origins. Much of the early speculation about viroid origin involved their possible origin as 'escaped introns' (i.e. descent from normal host RNAs). More recently, however, viroids have been proposed to represent 'living fossils' of a precellular RNA world that assumed an intracellular mode of existence sometime after the evolution of cellular organisms.

According to Diener, the chief proponent of this view, the inherent stability of viroids and viroid-like RNAs which arises from their small size and circularity would have enhanced the probability of their survival in primitive, error-prone RNA self-replicating systems and assured their complete replication without the need for initiation or termination signals. Most viroids (but not satellite RNAs or random sequences of the same base composition) also display structural periodicities with repeat units of 12, 60 or 80 nucleotides. The high error rate of prebiotic replication systems would be expected to have favored the evolution of polyploid genomes, and the mechanism of viroid replication (i.e. rolling-circle transcription of a circular template) provides an effective means of genome duplication.

Viroids and viroid-like satellite RNAs all possess efficient mechanisms for the precise cleavage of their oligomeric replication intermediates to form monomeric progeny. As discussed earlier, PSTVd and related viroids appear to require proteinaceous host factor(s) for cleavage, but others (the self-cleaving viroids and viroid-like satellite RNAs) function as self-cleaving RNA enzymes far smaller and simpler than those derived from introns. Thus, ASBVd and the other self-cleaving viroids may represent an evolutionary link between viroids and satellite RNAs. No viroid is known to code for protein, a fact that is consistent with the possibility that viroids are phylogenetically older than introns. It is conceivable that introns may be 'captured' viroids, rather than viroids 'escaped' introns.

See also: **Hepatitis Delta virus; Diagnostic techniques: Detection of viral antigens, nucleic acids and specific antibodies; Epidemiology of viral diseases; Pathogenesis: Plant viruses; Plant resistance to viruses: Natural resistance; Satellite RNAs and Satellite viruses; Ribozymes.**

Further Reading

Diener TO (1979) *Viroids and Viroid Diseases*. New York: Wiley Interscience.

Diener TO (ed.) (1987) *The Viroids*. New York: Plenum Press.

Flores R, Di Serio F and Hernàndez C (1997) Viroids: the noncoding genomes. *Semin.Virol.* 8: 65.

Matthews REF (1991) *Plant Virology*, 3rd edn. New York: Academic Press.

Semancik JS (ed.) (1987) *Viroids and Viroid-Like Pathogens*. Boca Raton: CRC Press.

Symons RH (ed.) (1990) Viroids and related pathogenic RNAs. *Semin. Virol.* 1: 75.

VIRUS SPECIES

Marc HV Van Regenmortel, UPR 9021 IBMC CNRS, Strasbourg, France

Introduction

The question of what is a virus species is part of the general problem of how the diversified world of viruses should be organized to achieve a coherent scheme of distinct and easily recognizable viral entities. Virus taxonomy suffers from a bad image among virologists, the subject often being equated with arcane debates about proposed names for newly discovered viruses or theoretical discussions about possible phylogenetic relationships of little relevance to practising virologists. This is unfortunate since virus classification and the demarcation of virus species are subjects of fundamental importance for clarifying the nature and identity of the objects studied by virologists and for allowing virology to develop into a mature scientific discipline.

The first internationally organized attempt to introduce some order in the bewildering variety of viruses took place at the International Congress of Microbiology held in Moscow in 1966. A committee was created, later called the International Committee on Taxonomy of Viruses (ICTV) which was given the task of developing a single, universal taxonomic scheme for all the viruses infecting animals (vertebrates, invertebrates and protozoa), plants (higher

the existence of these clusters may reflect their different origins. Much of the early speculation about viroid origin involved their possible origin as 'escaped introns' (i.e. descent from normal host RNAs). More recently, however, viroids have been proposed to represent 'living fossils' of a precellular RNA world that assumed an intracellular mode of existence sometime after the evolution of cellular organisms.

According to Diener, the chief proponent of this view, the inherent stability of viroids and viroid-like RNAs which arises from their small size and circularity would have enhanced the probability of their survival in primitive, error-prone RNA self-replicating systems and assured their complete replication without the need for initiation or termination signals. Most viroids (but not satellite RNAs or random sequences of the same base composition) also display structural periodicities with repeat units of 12, 60 or 80 nucleotides. The high error rate of prebiotic replication systems would be expected to have favored the evolution of polyploid genomes, and the mechanism of viroid replication (i.e. rolling-circle transcription of a circular template) provides an effective means of genome duplication.

Viroids and viroid-like satellite RNAs all possess efficient mechanisms for the precise cleavage of their oligomeric replication intermediates to form monomeric progeny. As discussed earlier, PSTVd and related viroids appear to require proteinaceous host factor(s) for cleavage, but others (the self-cleaving viroids and viroid-like satellite RNAs) function as self-cleaving RNA enzymes far smaller and simpler than those derived from introns. Thus, ASBVd and the other self-cleaving viroids may represent an evolutionary link between viroids and satellite RNAs. No viroid is known to code for protein, a fact that is consistent with the possibility that viroids are phylogenetically older than introns. It is conceivable that introns may be 'captured' viroids, rather than viroids 'escaped' introns.

See also: **Hepatitis Delta virus; Diagnostic techniques: Detection of viral antigens, nucleic acids and specific antibodies; Epidemiology of viral diseases; Pathogenesis: Plant viruses; Plant resistance to viruses: Natural resistance; Satellite RNAs and Satellite viruses; Ribozymes.**

Further Reading

Diener TO (1979) *Viroids and Viroid Diseases*. New York: Wiley Interscience.

Diener TO (ed.) (1987) *The Viroids*. New York: Plenum Press.

Flores R, Di Serio F and Hernàndez C (1997) Viroids: the noncoding genomes. *Semin.Virol.* 8: 65.

Matthews REF (1991) *Plant Virology*, 3rd edn. New York: Academic Press.

Semancik JS (ed.) (1987) *Viroids and Viroid-Like Pathogens*. Boca Raton: CRC Press.

Symons RH (ed.) (1990) Viroids and related pathogenic RNAs. *Semin. Virol.* 1: 75.

VIRUS SPECIES

Marc HV Van Regenmortel, UPR 9021 IBMC CNRS, Strasbourg, France

Introduction

The question of what is a virus species is part of the general problem of how the diversified world of viruses should be organized to achieve a coherent scheme of distinct and easily recognizable viral entities. Virus taxonomy suffers from a bad image among virologists, the subject often being equated with arcane debates about proposed names for newly discovered viruses or theoretical discussions about possible phylogenetic relationships of little relevance to practising virologists. This is unfortunate since virus classification and the demarcation of virus species are subjects of fundamental importance for clarifying the nature and identity of the objects studied by virologists and for allowing virology to develop into a mature scientific discipline.

The first internationally organized attempt to introduce some order in the bewildering variety of viruses took place at the International Congress of Microbiology held in Moscow in 1966. A committee was created, later called the International Committee on Taxonomy of Viruses (ICTV) which was given the task of developing a single, universal taxonomic scheme for all the viruses infecting animals (vertebrates, invertebrates and protozoa), plants (higher

plants and algae), bacteria, fungi and archaea. Since 1971 the ICTV, operating on behalf of the world community of virologists, has produced six reports describing the current state of virus taxonomy; a seventh one is due for publication in 1999. The current system of virus classification uses the classical hierarchical levels of order, family, genus and species. At present 184 genera have been recognized and of these, 161 are classified in 54 families.

Although the species is the most fundamental unit in all biological classifications, it took many years before an internationally agreed definition of virus species applicable to all viruses became generally accepted and was ratified by the ICTV. The virologists who study viruses that infect plants had been particularly reluctant to admit that the species concept could be used in virology. Some of these virologists argued that the only legitimate definition of species was that of *biological species*, used for sexually reproducing organisms but which is clearly not applicable to entities such as viruses that replicate by clonal means. However, a great variety of species concepts exists and some of them may be applicable to viruses. Unfortunately, there is no general agreement among biologists on what counts as a good species concept. Before outlining the concept of virus species, it will be helpful, therefore, to define a few terms and to analyze some of the key concepts used in biological classification.

What is a Virus?

A virus is an elementary biosystem that possesses some of the properties of living systems such as having a genome and being able to adapt to changing environments. However, viruses cannot capture and store free energy and they are not functionally active outside their host cells. Although viruses are pathogens, they should not be equated with pathogenic microorganisms.

A virus has both *intrinsic properties* (e.g. its size) and *relational properties* (e.g. its host), the second type of property existing only by virtue of a relation with other objects. These properties are either *resultant properties* already possessed by the components of the virus (e.g. the mass of the virion equals the sum of the mass of its parts) or *emergent properties* that are only possessed by the system as a whole and are not present in its constituent parts (e.g. the viral replication cycle or the viral ecological niche). It should be stressed that only cells and multicellular systems possess the emergent property of being alive and that this property is not present in subcellular components or individual molecules. A virus becomes part of a living system only after its genome has been integrated in the host cell and viral replication is made possible through the metabolic activity of the cell. Viruses are thus not organisms and they lead only a kind of borrowed life.

It is also important to clearly distinguish between the entity called a virus and a single, discrete virus particle or virion. The virion possesses intrinsic biochemical and structural properties which, incidentally, include as one of the phenotypic characters the composition and sequence of the viral genome (although this is generally called the genotype). A virus, on the other hand, possesses in addition a number of relational and emergent properties that become actualized, for instance during the viral replication cycle, i.e. when the virus forms an integrated whole with its host cell. A virus can thus not be reduced to the physical constituents and chemical composition of a virion and it is necessary to include in its description the functional activity it possesses inside its host, as well as a variety of other biotic interactions. Furthermore, the hereditary material of a particular virus cannot be described in terms of the unique genomic sequence present in a single virion. The genome of a virus is not a single molecular species but must be viewed as a dynamic population consisting of the thousands of viral mutants that are always present in a given viral clone. This population, which is the target of selection, is referred to as a viral quasi-species (see below).

Classes, Fuzzy Sets and Species

One of the difficulties in defining species is that the term is used in many different ways which unfortunately are not always clearly distinguished. For instance, the term species is often used to designate groups of real organisms studied by taxonomists, in which case it refers to concrete objects, i.e. material things that are located in time and space. One popular taxonomic theory views species as concrete individuals in the sense that they correspond to historical entities related by common descent. The thesis that species should be regarded as individuals instead of classes has led to vigorous debate, but this has not solved the species problem in biology.

Classes

A second meaning of the term species is that of a class, i.e. a conceptual construct or abstraction that does not exist on its own in the absence of someone conceiving of the idea. Properties and classes are related abstract entities. Whatever is said about a thing is seen as ascribing a property to it, and the thing thereby becomes a member of a particular class. If a virus has a positive-strand RNA genome, it

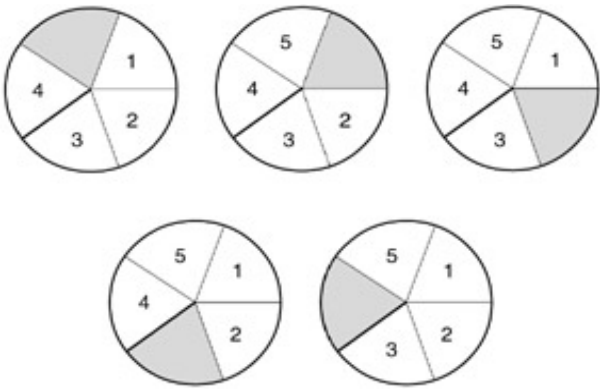


Figure 1 Five members of a polythetic class characterized by five properties, 1–5. Each member possesses several of these properties, but no single property is present in all the members of the class. The missing property in each case is represented by the hatched sector.

becomes automatically a member of the class of positive-strand RNA viruses. Such a class is called a universal class because it is defined by a single property, or a combination of properties, necessarily present in all the members of the class.

A biological classification is a conceptual construction made up of classes with a hierarchical structure, the ranks being the species, genus, family, order and phylum. It must be emphasized that the classes or taxonomic categories used for building up a classification are conceptual constructions that do not correspond to groups of real organisms with a spatiotemporal location. It is thus impossible for a biologist ever to encounter an abstract genus or species when handling organisms. It is odd that many biologists who readily accept that genera, families or orders are conceptual constructions of the mind, i.e. abstractions, insist that species have a real existence in space and time and are not abstract classes. It seems that species are more readily perceived as populations of real organisms rather than as abstractions. On the other hand, higher ranks such as genera and families, are more easily accepted as universal classes defined by one or more properties present in every member of the class. One problem with the species class is that its members, i.e. living organisms, undergo continuous developmental and evolutionary changes in time, and are therefore always endowed with intrinsic variability. This seems to contradict the notion that such organisms could belong to a universal class, seen as immutable and timeless. It also seems problematic to speak of the origin of species if one conceives of them as abstract concepts with neither beginning nor end in time. However, these difficulties disappear when species are viewed, not as classical, universal classes, but as polythetic classes, the members of which share only a certain number of characteristic attributes which can change with time. Whereas a traditional class is defined by the necessary presence of at least one property in all its members, the members of a polythetic class need not have a single defining property in common (**Fig. 1**). When species are viewed as polythetic classes, it is possible to accommodate individuals that lack one or other character normally considered typical for the species. The concept of polythetic species is thus particularly suited for dealing with replicating biological entities endowed with intrinsic variability and undergoing continual evolutionary changes. When viewed in this way, species correspond to fuzzy sets with hazy boundaries, and it is not possible to draw sharp boundaries between them as is done with classical sets and universal classes.

Fuzzy sets

The use of vagueness or fuzziness as a descriptor of reality has a long history in Western philosophy. Vagueness stems from a continuum with innumerable steps and is exemplified in the well-known sorites paradox of the heap described by Greek philosophers. This paradox arises because it is impossible to say how many grains of sand can be removed from a heap before it stops being a heap. This implies that the concept of heap cannot be defined in a precise manner. In a similar way, fuzzy sets have no sharp boundaries, and since the set is defined by a vague predicate, membership of a fuzzy set is not an all-or-nothing matter. This means that the law of excluded middle of classical logic (a swan is either white or nonwhite) does not apply, as in some cases it may not be possible to ascertain if an object is a member of the set or not. Handling fuzzy sets, therefore, falls outside the scope of classical, bivalent logic. In a biological classification, it may sometimes be necessary to allocate an organism to a particular species purely as a matter of convention or convenience rather than logical necessity.

Science is based on empirical observations, the precision of which is always limited. Scientific evidence, therefore, can never be assigned the absolute truth value possessed by certain types of statement in formal logic that lack factual reference. Scientific knowledge always remains approximate and incomplete and it would be counterproductive to look for certainty and for absolutely precise boundaries where none exist. When fuzziness is accepted as an unavoidable ingredient of species taxa, spurious problems of definition disappear and it becomes possible to describe species in terms of continuums devoid of artificial sharp edges. In a similar way, colors can be

distinguished conceptually in spite of the continuous nature of the spectrum of electromagnetic waves, and mountain peaks are given names although there are no sharp boundaries in geological rock formations.

Species

The traditional view of species is that they correspond to groups of similar organisms that can breed among themselves and produce fertile offspring. Mayr's classical definition of biological species states that 'species are groups of interbreeding natural populations that are reproductively isolated from other such species'. This definition has been criticized because if defines species as a population instead of a class; furthermore it applies only to organisms that reproduce sexually and it ignores the phenomenon of interspecies hybridization that is widespread in the plant kingdom. Since groups of plants lie on a continuum from completely interfertile to completely reproductively isolated, the criterion of infertility for demarcating species is very often inapplicable and the choice of what constitutes a significant breeding discontinuity is rather arbitrary. In order to make it applicable to asexual organisms, Mayr subsequently modified his definition and stated that 'a species is a reproductive community of populations, reproductively isolated from others, that occupies a specific niche in nature'.

As biologists became increasingly committed to evolutionary theory, several authors introduced the notion of evolutionary species corresponding to a time-slice in an evolving lineage. By including in the definition the idea of ancestry and descent, the concept of species acquired an internal cohesion which was absent when species were defined on a morphological or phenetic basis, i.e. only in terms of similarity. However, the evolutionary species concept remains a highly theoretical one as it gives no indication of how individual time-slices of evolving lineages segregate into separate species. Transition from one species to another during evolution occurs within an uninterrupted chain of replicating nucleic acid molecules and ancestral-descendant organisms and there are no criteria for deciding how far back in time a species can be traced. It is thus as difficult to demarcate boundaries in time in order to identify evolutionary species as it is to define clear-cut breeding discontinuities for identifying biological species.

For most practical purposes, biologists today still tend to distinguish between different species on a phenetic or morphological basis. As noted before, the genomic sequence of an organism is also a phenotypic trait and it seems that the overall degree of phenotypic difference observed between organisms, which also includes genomic divergence, is roughly proportional to the amount of evolutionary distance from a common ancestor. This is the reason why phenetic species defined only in terms of overall similarity are often very similar to the species defined as lineages of ancestral-descendant populations.

What is a Virus Species?

The rationale for using the species concept in viral taxonomy is that viruses are biological entities and not simply chemicals. Viruses possess genes, replicate, evolve and are adapted to particular biotic habitats and ecological niches. Like all biological entities that possess the ability to replicate and reproduce themselves, viruses are endowed with an intrinsic variability derived from the error-prone process of nucleic acid replication. Whereas the molecules of a compound studied by a chemist are all identical, the virus particles in a clone always include thousands of mutants which constitute a so-called quasi-species population. This built-in variability allows any biological system to become adapted through selection and in the end guarantees its survival.

In 1981, the ICTV proposed the following definition of virus species: 'A virus species is a concept that will normally be represented by a cluster of strains from a variety of sources, or a population of strains from a particular source, which have in common a set of correlating stable properties that separate the cluster from other clusters of strains.' This definition does not explain what a strain is and it proposes grouping viruses purely on a phenetic basis without considering the cohesive forces present in ancestral-descendant populations. In 1989, the author proposed a definition of virus species which took into account that a species is a polythetic class, the members of which are united by relational properties of descent and by occupation of a particular biotic niche. In 1991, the ICTV endorsed this definition, which states that: 'A virus species is a polythetic class of viruses constituting a replicating lineage and occupying a particular ecological niche.'

Virus species as polythetic class

As shown in **Fig. 1**, there is no single attribute in a polythetic class that can be used to indicate that a virus qualifies as a member of a particular species. A polythetic class consists of members that have several properties in common, although there is no single defining property which is present in all the members of a particular species and absent in the members of other species. A single discriminating character, for instance a particular host reaction or a certain degree

of genome sequence dissimilarity, cannot be used as an absolute criterion for differentiating two species within the same genus. Attempts to use a single discriminating character for distinguishing species would fail because of the inherent variability of members of the species, and furthermore it would contradict the notion that species are not universal classes definable by a single property. The situation is different with higher taxa, such as genera and families, which are universal classes and consist of members that share one or more defining properties that are both necessary and sufficient for class membership. As far as virus species are concerned, it is always a combination of statistically covariant properties that provides the rationale for deciding that a particular virus should be considered a member of a species.

Virus species as a replicating lineage

This part of the definition acknowledges that a virus species, in addition to being a polythetic class, is made up of members that represent an evolving lineage. The membership of a virus species varies over time but all its members share descent from a common ancestor. It should be noted that shared descent is a property that also links different species and different genera.

The genomic plasticity inherent in any viral replicating lineage leads to continual phenotypic variation that makes the virus species a polythetic rather than a universal class. It should be stressed that a single criterion, such as the degree of genome sequence divergence or the potential for genetic reassortment, cannot serve as a criterion for species demarcation. Classifying viral genomes should not be confused with classifying viruses. Genome comparisons cannot by themselves justify taxonomic placements that would disregard the biotic and phenotypic properties which are the main reason why virologists want to classify viruses.

Ecological niche occupancy

The ecological niche refers to biotic properties of members of a virus species, such as host range, tissue tropism, virulence, pathogenesis, host responses, vectors and habitat. The ecological niche does not simply refer to a location in three-dimensional space but is a functional concept based on relational properties of the virus. The niche is not a property of the environment but a property of the virus and there can therefore be no vacant or empty niches but only unoccupied habitats or geographical spaces. In the absence of the virus, its ecological niche property is also absent and the notion of a vacant niche is thus meaningless. A niche provides the needs that must be met for the virus to replicate and to survive and it is restricted to the relations that have a positive biological advantage for the virus.

Taxonomic Polythetic Species Versus Molecular Quasi-species

As mentioned above, the genome of a virus cannot be defined by a unique sequence corresponding to a so-called wild type but consists of a distribution of mutant sequences, each one differing in one or a few nucleotide positions from the consensus sequence of the clone. This consensus sequence, identified by sequencing a clone, may give the impression of a stable, unique structure, although it corresponds in fact to the average of a large number of different individual sequences. Since RNA viruses have genomes that replicate in the absence of repair mechanisms, they evolve very rapidly, with a mutation frequency per nucleotide site in the genome of 10^{-3}–10^{-5}. The genome of an RNA virus therefore consists of a master sequence corresponding to the most fit genome sequence under a given environment, together with thousands of competing mutants. Such a population is usually referred to as a quasi-species, a somewhat unfortunate term as it may seem to imply that the virus corresponds to some sort of imperfect species, as opposed to a 'true' or genuine species that would possess a single, invariant genome sequence. Such idealized species, of course, do not exist. The term quasi-species was introduced by Eigen and his colleagues to describe self-replicating RNA molecules which, because of mutation, do not consist of a unique molecular species. In this context, the term species refers to a purely chemical entity, i.e. a species of molecule, and not to the taxonomic concept of virus species as a variable biological entity. Whereas all the members of a chemical species are identical molecules, the members of a virus species are not. Taxonomic species are thus automatically quasi-species in the molecular sense.

Virus Species Demarcation

Although the acceptance of a definition of virus species by the international virological community was an important step for establishing a unified virus classification system based on the traditional taxonomic categories, it should be stressed that such a definition is of little use for deciding if a particular virus isolate is a member of a certain species or not. The reason for this is that definitions apply only to abstract concepts such as the notion of species taken as a category or class used in classification. Individual viruses located in time and space cannot be 'defined'

Table 1 Characters that would demarcate virus isolates as distinct species in the families *Potyviridae* and *Geminiviridae* (Van Regenmortel *et al*, 1997).

Character	*Potyviridae*	*Geminiviridae*
Genome features		
	—	Different numbers of genome components
	—	Different organization of genes in the genome
	—	No transcomplementation of gene products
	—	No pseudorecombination between components
Genome sequence		
	< *c.* 85% identical over whole sequence	< *c.* 90% identical in coat protein sequence
	< *c.* 75% identical in 3′ noncoding region	—
Protein features		
	Different polyprotein cleavage sites	—
	Virions react differently with key antibodies	Virions react differently with key antibodies
	< *c.* 90% identical in coat protein sequence	< *c.* 90% identical in coat protein sequence
Transmission		
	Different vector species	Different vector species
	Different seed transmissibility	—
Effects in infected tissue		
	Different inclusion body morphology	—
	No crossprotection effects	—
	—	Different tissue tropism
Host range		
	Different in key species	Different in key species

but can only be identified by means of so-called diagnostic properties. The difference between definition and identification can be clarified by the following analogy. It is possible to define the concept of a human family in terms of an ancestral-descendant population comprising parents, grandparents, children, siblings, etc., but such a definition of the family concept would be of no help whatsoever for recognizing the members of the Smith family who have gathered for the annual school concert and for distinguishing them from members of the Brown family. What is required is a set of characters and diagnostic properties that can be used for identifying individual members of a family or species. It is thus necessary to reach an agreement about which diagnostic properties are most useful for identifying the members of a virus species.

The identification of a virus isolate is a comparative process based on a number of different characters that will indicate the extent of relationship of the isolate with members of an established species. Since species are polythetic, the comparison must involve several characters rather than the presence or absence of a single key feature. For species diagnosis it is of course essential not to use characters that are present in all the members of a genus or family, as these obviously will not permit species demarcation within the group. Characters such as virion morphology, genome organization, method of replication and the number and size of structural and nonstructural viral proteins are family- or genus-defining properties that are of little value for identifying individual species. The following characters are useful for discriminating between virus species within the same genus:

- genome sequence relatedness,
- natural host range,
- cell and tissue tropism,
- pathogenicity and cytopathology,
- mode of transmission,
- physicochemical properties,
- antigenic properties.

A list of characters that have been used to decide if two virus isolates belonging to the plant virus families *Potyviridae* or *Geminiviridae* are different species or not are listed in **Table 1.** Some criteria are the same for the two families, others are qualitatively the same but quantitatively different and some criteria do not apply to viruses in both families. No one criterion has an absolute supremacy over others; some are more

informative and discriminatory than others, but it is the sum total of the information that is gathered which allows reliable species demarcation. Once different species have been established on this basis in the classification scheme, it may be sufficient to check for the presence of a few characters in a particular isolate to be able to allocate it to a particular species.

In each genus, one species for which considerable knowledge is available is designated as the type species. However, this designation does not imply that the properties of the type species are most typical and representative of the properties of all species in the genus.

See also: **Diagnostic techniques: Detection of viral antigens, nucleic acids and specific antibodies, Isolation and identification by culture and microscopy; Lysogeny and prophage; Phage ecology, evolution and speciation; Phage taxonomy and classification; Quasispecies; Recombination of viruses; Taxonomy and classification – general; Taxonomy of viruses (Quantitive).**

Further Reading

Claridge MF, Dawah HA and Wilson MR (eds) (1997) *Species. The Units of Biodiversity*. London: Chapman & Hall.

Gibbs A, Calisher CH and Garcia-Arenal F (eds) (1995) *Molecular Basis of Virus Evolution*. Cambridge: Cambridge University Press.

Mayo MA and Pringle CR (1998) Virus taxonomy 1997. *J. Gen. Virol.* 79: 649.

Murphy FA, Fauquet CM, Bishop DHL *et al* (eds) (1995) *Virus Taxonomy*. Sixth Report of the International Committee on Taxonomy of Viruses. Vienna: Springer.

Van Regenmortel MHV (1990) Virus species, a much overlooked but essential concept in virus classification. *Intervirology* 31: 241.

Van Regenmortel MHV, Bishop DHL, Fauquet CM *et al* (1997) Guidelines to the demarcation of virus species. *Arch. Virol.* 142: 1505.

VIRUS STRUCTURE

Contents

Atomic Structure

Ming Luo, Center for Macromolecular Crystallography, The University of Alabama, Birmingham, Alabama, USA

Architecture of Viruses

Viruses have two essential components: protein and nucleic acid. A closed capsid may be formed by one type or a few types of proteins to encapsidate the nucleic acid genome. The protein capsid can have a helical (filamentous virus) or icosahedral (spherical virus) symmetry. The symmetry allows a small protein unit to assemble into a large particle. The helical symmetry is described by the diameter d, the pitch P, and the number of subunits per turn. There are as many capsid proteins as necessary for completely covering the nucleic acid genome. The icosahedral symmetry is defined by six fivefold axes, ten threefold axes, and 15 twofold axes. A number T, called the *triangulation number*, indicates how many quasisymmetrical subunit interactions there are within one asymmetrical region of the icosahedron. There are a total of $60T$ copies of proteins in one icosahedral capsid. In some viruses, there is a membrane envelope wrapped around the protein–nucleic acid core. There are proteins on the surface of the envelope.

Methods of Structure Determination

X-ray diffraction is the common technique used for studying the atomic structure of proteins and nucleic acids. When X-rays strike electrons of the atoms in a stationary specimen, a diffraction pattern of different intensities is generated and recorded. By analysis of the diffraction pattern and the intensities, a three-dimensional electron density map (EDM) can be calculated by Fourier transformation. A three-dimensional chemical structure could be built based on the interpretation of the EDM. Two types of X-ray diffraction experiments are useful for virus structure studies: fiber diffraction (for filamentous viruses) and

informative and discriminatory than others, but it is the sum total of the information that is gathered which allows reliable species demarcation. Once different species have been established on this basis in the classification scheme, it may be sufficient to check for the presence of a few characters in a particular isolate to be able to allocate it to a particular species.

In each genus, one species for which considerable knowledge is available is designated as the type species. However, this designation does not imply that the properties of the type species are most typical and representative of the properties of all species in the genus.

See also: **Diagnostic techniques: Detection of viral antigens, nucleic acids and specific antibodies, Isolation and identification by culture and microscopy; Lysogeny and prophage; Phage ecology, evolution and speciation; Phage taxonomy and classification; Quasispecies; Recombination of viruses; Taxonomy and classification – general; Taxonomy of viruses (Quantitive).**

Further Reading

Claridge MF, Dawah HA and Wilson MR (eds) (1997) *Species. The Units of Biodiversity*. London: Chapman & Hall.

Gibbs A, Calisher CH and Garcia-Arenal F (eds) (1995) *Molecular Basis of Virus Evolution*. Cambridge: Cambridge University Press.

Mayo MA and Pringle CR (1998) Virus taxonomy 1997. *J. Gen. Virol.* 79: 649.

Murphy FA, Fauquet CM, Bishop DHL *et al* (eds) (1995) *Virus Taxonomy*. Sixth Report of the International Committee on Taxonomy of Viruses. Vienna: Springer.

Van Regenmortel MHV (1990) Virus species, a much overlooked but essential concept in virus classification. *Intervirology* 31: 241.

Van Regenmortel MHV, Bishop DHL, Fauquet CM *et al* (1997) Guidelines to the demarcation of virus species. *Arch. Virol.* 142: 1505.

VIRUS STRUCTURE

Contents

Atomic Structure

Ming Luo, Center for Macromolecular Crystallography, The University of Alabama, Birmingham, Alabama, USA

Architecture of Viruses

Viruses have two essential components: protein and nucleic acid. A closed capsid may be formed by one type or a few types of proteins to encapsidate the nucleic acid genome. The protein capsid can have a helical (filamentous virus) or icosahedral (spherical virus) symmetry. The symmetry allows a small protein unit to assemble into a large particle. The helical symmetry is described by the diameter d, the pitch P, and the number of subunits per turn. There are as many capsid proteins as necessary for completely covering the nucleic acid genome. The icosahedral symmetry is defined by six fivefold axes, ten threefold axes, and 15 twofold axes. A number T, called the *triangulation number*, indicates how many quasisymmetrical subunit interactions there are within one asymmetrical region of the icosahedron. There are a total of $60T$ copies of proteins in one icosahedral capsid. In some viruses, there is a membrane envelope wrapped around the protein–nucleic acid core. There are proteins on the surface of the envelope.

Methods of Structure Determination

X-ray diffraction is the common technique used for studying the atomic structure of proteins and nucleic acids. When X-rays strike electrons of the atoms in a stationary specimen, a diffraction pattern of different intensities is generated and recorded. By analysis of the diffraction pattern and the intensities, a three-dimensional electron density map (EDM) can be calculated by Fourier transformation. A three-dimensional chemical structure could be built based on the interpretation of the EDM. Two types of X-ray diffraction experiments are useful for virus structure studies: fiber diffraction (for filamentous viruses) and

crystallography (for spherical viruses and globular viral proteins).

Atomic Structure of Helical Viruses

The disc of the tobacco mosaic virus (TMV) coat protein has been crystallized and its atomic structure resolved by X-ray crystallography. The intact TMV structure containing the nucleic acid could only be determined by X-ray fiber diffraction experiments, as could that of Pf2 phage. The coat proteins of TMV and Pf2 contain mainly α-helices and the nucleic acid interacts with the coat protein with one base (Pf2) or three bases (TMV) per protein unit. The axis of the coat protein helix coincides with that of the nucleic acid. The coat proteins of TMV have many aggregation forms, depending on pH or ionic strength. The TMV RNA is inserted into the coat protein helix in the growing virus particle. The coat protein of Pf2 was added one by one to the DNA helix emerging from the membrane.

Atomic Structure of Spherical Viruses

Spherical viruses without a membrane envelope form large single crystals under proper conditions. Their atomic structure can be determined by X-ray crystallography with the aid of supercomputers and synchrotron X-ray sources. Since 1978, numerous atomic structures of viruses have been reported. These include the plant RNA viruses tomato bushy stunt virus (TBSV), southern bean mosaic virus (SBMV), satellite tobacco necrosis virus (STNV), bean pot mottle virus (BPMV), and cow pea mosaic virus (CPMV); the animal RNA viruses human rhinovirus, poliovirus, black beetle virus (BBV), Mengo virus, foot-and-mouth disease virus (FMDV), Theiler's murine encephalomyelitis virus (TMEV), coxsackievirus, and tetravirus; and the animal DNA viruses canine parvovirus (CPV), simian virus 40 (SV40), bluetongue virus and adenovirus hexon; as well as the bacteriophages MS2 and ϕx174.

Most capsid proteins of these viruses contain an antiparallel, eightstranded β-barrel folding motif. The motif has a wedge-shaped block with four β-strands (BIDG) on one side and another four (CHEF) on the other. There are also two conserved α-helices (A and B), one is between βC and βD, the other between βE and βF. In animal viruses, there are large loops inserted in between the β-strands. These loops form the surface features of individual viruses. The wedge shape is best suited to making a concealed icosahedral shell.

A virus capsid may contain multiple copies of the β-barrel fold with the same amino acid sequence (such as $T = 3$ TBSV or $T = 1$ CPV) or with different amino acid sequences (such as pseudo $T = 3$ rhinovirus). In some cases there are two β-barrel folds in a single polypeptide (such as CPMV and adenovirus hexon). Capsid proteins of spherical viruses can have other motifs such as plane β-sheet in MS2 and α-helices in bluetongue virus.

Atomic Structure of Viral Proteins

There are many functional viral proteins that do not have any symmetrical quaternary structure in virus particles. Therefore, their atomic structure has to be studied by crystallizing isolated proteins. Crystal structures have been determined for the hemagglutinin (HA) and the neuraminidase (NA) of enveloped influenza virus, and the protease and reverse transcriptase of human immunodeficiency virus (HIV) and other retrovirus, matrix and capsid proteins (influenza virus and HIV/SIV), a fragment of the HIV glycoprotein gp41, the protease of picornaviruses, the protease and the thymidine kinase of herpesvirus, the protease of hepatitis C virus, the receptor binding domain of adenovirus fiber and the envelope glycoprotein of tick-borne encephalitis virus.

The HA has two domains in the subunit and the functional molecule is a trimer. The domain extending from the membrane contains α-helices and β-sheets. This domain forms the base interacting with the membrane envelope. The distal domain has an eight-stranded β-barrel fold similar to that seen in the spherical viruses. This domain bears the binding site for sialic acid, the receptor for influenza virus on the cell surface. The membrane fusion peptide at the N terminus of HA_2 is located in the membrane-interacting domain.

The NA is a tetrametric molecule and its subunit contains six sheets of four β-strands each. The six β-sheets are arranged like the blades of a propeller. The enzymatic site is at a hydrophobic depression in the center of the β-sheets. The antibody recognition site has been defined on the external surface near the enzymatic site by the atomic structure of the NA complexed with Fab fragments.

The HIV protease is an aspartic acid protease with two β-sheets in each subunit. The enzyme has to dimerize before it becomes active. This activation mechanism has an important role in HIV assembly. The virus assembly complex attached to the membrane will not proceed during maturation until all the necessary components are present to initiate dimerization of the protease.

The matrix protein of influenza virus contains at least two α-helix domains and works as a dimer in the

virion. The N-terminal domain has a hydrophobic surface which can bind membrane, while the middle domain has a positively charged surface that interacts with the RNA. This bifunctional protein can simultaneously bind membrane and RNA. The matrix protein and the capsid protein of HIV have similar folds as the matrix protein of influenza virus when compared with each domain. In HIV, the two proteins are initially covalently linked, as are the two domains in the matrix protein of influenza virus. After being cleaved during maturation, the matrix protein remains bound to membrane and the capsid protein binds to the RNA nucleocomplex.

Nucleic Acid–Protein Interaction

The viral nucleic acid genome is always packaged inside the protein capsid. Usually the structure of the nucleic acid cannot be observed in a single crystal X-ray diffraction experiment because of the random orientation of the icosahedral particles in the crystal. However, in rare cases, the nucleic acid might assume icosahedral symmetry by interacting with the protein capsid. Fragments of the complete genome make the same conformation, although with different nucleotide sequences, at locations related by icosahedral symmetry. Such structures have been seen in BPMV (RNA virus) and CPV (DNA virus). The bases are stacked either as in A-type RNA helix (BPMV) or to form a coiled conformation to fit the interactions with the protein capsid. These viruses readily form empty virus particles and have a hydrophobic pocket on the interior surface of the capsid. The nucleic acid generally interacts nonspecifically with the protein.

Evolution

The highly conserved β-barrel motif of the viral capsid protein indicates that many viruses must have evolved from a single origin. The unique three-dimensional structure of this motif is required for capsid assembly and it is generally conserved over a longer period of time than the amino acid sequence. The superposition of the capsid proteins from different viruses can be used to estimate the branch point in the evolutionary tree for each virus group. The structural alignment not only relates plant viruses to animal viruses, RNA viruses to DNA viruses, but also viruses to other proteins like concanavalin A, which has a similar fold and competes with poliovirus for its cellular receptor. The evolutionary relationship of these viruses is supported by amino acid sequence alignment of more conserved viral proteins, such as the viral RNA polymerase. The structural similarities of the matrix and capsid proteins, as well as the surface glycoproteins of influenza virus and HIV, further support the idea of a common evolutionary origin among enveloped viruses.

Assembly

The icosahedral capsid is assembled from smaller units made of several protein subunits. In small animal RNA viruses, a protomeric unit is first formed with one copy of each polypeptide after translation. The termini of the subunits are intertwined with each other to hold the subunits together in the protomer. The protomers are then associated as pentamers, which in turn form the complete icosahedral virion while encapsidating the viral RNA. In $T = 3$ or $T = 1$ plant RNA viruses, the pentamers are formed by dimers of the capsid proteins. In adenovirus and SV40, the capsid proteins form hexon units (three polypeptides, each has two β-barrels) or pentamers before they assemble into an icosahedral shell. The assembly of enveloped viruses like influenza virus is directed by the matrix protein, which initiates the assembly at the cellular membrane.

Host Receptor Recognition Site

Animal viruses have to recognize a specific host cellular receptor for entry during infection. Host receptor binding is the initial step in the viral life cycle and could be an effective target for preventing viral infection. Based on the atomic structure of animal viruses, it was found that the receptor recognition site is located in an area surrounded by hypervariable regions of the antigenic sites. Usually the area is in a depression (called the 'canyon') on the viral surface that is protected from recognition by host antibodies. This structural feature is present in rhinovirus, and the active site of influenza virus NA. The receptor-binding site on influenza virus HA does not have a deep depression, but it is surrounded by antigenic sites.

Antigenic Sites

Antibodies are the first line of defense by the immune system against a viral infection. The epitopes combined with the neutralizing antibodies are mapped on a few isolated locations on the surface of viral proteins. The structure of the influenza virus NA complexed with Fab fragments showed that the antibody makes contact with an area about 6 nm^2 and the epitope spans four discontinuous polypeptides. Therefore, an effective vaccine usually needs to include a complete viral protein or a large fragment. The binding of the antibodies does not change the

structure of the antigen. The exact mechanism by which antibodies neutralize antigens is still unclear.

Antiviral Agents

Viral infectious diseases can be cured if an agent can be administered to stop viral infection. Such agents have been synthesized and shown to bind to the capsid of rhinovirus in the crystal structure. The compounds were inserted into the hydrophobic pocket within the β-barrel of the major capsid protein VP1. Binding of the compounds stops uncoating of the virion and the receptor binding, which results in the failure of release of the viral RNA into the cytoplasm. These compounds inhibit infections of several other RNA viruses and may be effective against other viruses after modification, as the β-barrel structure exists in many viruses.

The most successful antiviral drugs are the HIV protease inhibitors, which are developed based on the atomic structure of the protease. Through interactive cycles of computer modeling, chemical synthesis and structural studies of the protein–inhibitor complexes, a panel of clinically effective drugs has been brought to the market and shown benefits to patients. Inhibitors of influenza virus NA are also under development by the same method.

For an illustration of protein crystallography used to determine protein structure, see **Color Plate 30.**

See also: **Virus structure: Principles of virus structure; Viral receptors; Influenza viruses (*Orthomyxoviridae*): General features, Molecular biology, Structure of antigens.**

Further Reading

Fields BN, Knipe DM, Howley PM *et al* (eds) (1996) *Fields Virology*, 3rd edn, pp 59–99. Philadelphia: Lippincott-Raven.

Jones I and Stuart D (1996) Journey to the core of HIV. *Nat. Struct. Biol.* 3: 818.

Rossmann MG and Johnson JE (1989) Icosahedral RNA virus structure. *Annu. Rev. Biochem.* 58: 533.

Principles of Virus Structure

John E Johnson and **Jeffrey A Speir**, Department of Molecular Biology, The Scripps Research Institute, La Jolla, California, USA

Introduction

The virion is a nucleoprotein particle designed to move the viral genome between susceptible cells of a host and between susceptible hosts. An important limitation on the size of the viral genome is its container, the protein capsid. The virion has a variety of functions during the virus life cycle (**Table 1**); however, the principles dictating its architecture result from the need to provide a container of maximum size with a minimum amount of genetic information. The universal strategy evolved for the packaging of viral nucleic acid requires multiple copies of one or more protein subunit types arranged symmetrically about the genome. The assembly of these subunits into nucleoprotein particles is, in many cases, a spontaneous process that results in a minimum free energy structure under intracellular conditions. The two broad classes of symmetric virions are helical rods and spherical particles.

The nucleoprotein helix can, in principle, package a genome of any size. Extensive studies of tobacco mosaic virus (TMV) show that protein subunits will continue to add to the extending rod as long as there is exposed RNA. Protein transitions required to form the TMV helix from various aggregates of subunits are now understood at the atomic level. It is clear that subunits forming the helix display significant polymorphism in the course of assembly; however,

Table 1 Functions of the virus capsid in simple RNA viruses

Assembly	Subunits must assemble to form a protective shell for the RNA
Package	Subunits must specifically package the viral RNA
Infection	The capsid may actively participate in virus infection processes.
Binding to receptors	Binding to receptors and mediating cell entry (animal)
Transport	Virion transport within the host (plant)
Mutation	Capsid protein mutation to avoid the immune system
Replication	Some capsid proteins function as a primer for viral RNA replication

structure of the antigen. The exact mechanism by which antibodies neutralize antigens is still unclear.

Antiviral Agents

Viral infectious diseases can be cured if an agent can be administered to stop viral infection. Such agents have been synthesized and shown to bind to the capsid of rhinovirus in the crystal structure. The compounds were inserted into the hydrophobic pocket within the β-barrel of the major capsid protein VP1. Binding of the compounds stops uncoating of the virion and the receptor binding, which results in the failure of release of the viral RNA into the cytoplasm. These compounds inhibit infections of several other RNA viruses and may be effective against other viruses after modification, as the β-barrel structure exists in many viruses.

The most successful antiviral drugs are the HIV protease inhibitors, which are developed based on the atomic structure of the protease. Through interactive cycles of computer modeling, chemical synthesis and structural studies of the protein–inhibitor complexes, a panel of clinically effective drugs has been brought to the market and shown benefits to patients. Inhibitors of influenza virus NA are also under development by the same method.

For an illustration of protein crystallography used to determine protein structure, see **Color Plate 30.**

See also: **Virus structure: Principles of virus structure; Viral receptors; Influenza viruses (*Orthomyxoviridae*): General features, Molecular biology, Structure of antigens.**

Further Reading

Fields BN, Knipe DM, Howley PM *et al* (eds) (1996) *Fields Virology*, 3rd edn, pp 59–99. Philadelphia: Lippincott-Raven.

Jones I and Stuart D (1996) Journey to the core of HIV. *Nat. Struct. Biol.* 3: 818.

Rossmann MG and Johnson JE (1989) Icosahedral RNA virus structure. *Annu. Rev. Biochem.* 58: 533.

Principles of Virus Structure

John E Johnson and **Jeffrey A Speir**, Department of Molecular Biology, The Scripps Research Institute, La Jolla, California, USA

Introduction

The virion is a nucleoprotein particle designed to move the viral genome between susceptible cells of a host and between susceptible hosts. An important limitation on the size of the viral genome is its container, the protein capsid. The virion has a variety of functions during the virus life cycle (**Table 1**); however, the principles dictating its architecture result from the need to provide a container of maximum size with a minimum amount of genetic information. The universal strategy evolved for the packaging of viral nucleic acid requires multiple copies of one or more protein subunit types arranged symmetrically about the genome. The assembly of these subunits into nucleoprotein particles is, in many cases, a spontaneous process that results in a minimum free energy structure under intracellular conditions. The two broad classes of symmetric virions are helical rods and spherical particles.

The nucleoprotein helix can, in principle, package a genome of any size. Extensive studies of tobacco mosaic virus (TMV) show that protein subunits will continue to add to the extending rod as long as there is exposed RNA. Protein transitions required to form the TMV helix from various aggregates of subunits are now understood at the atomic level. It is clear that subunits forming the helix display significant polymorphism in the course of assembly; however,

Table 1 Functions of the virus capsid in simple RNA viruses

Assembly	Subunits must assemble to form a protective shell for the RNA
Package	Subunits must specifically package the viral RNA
Infection	The capsid may actively participate in virus infection processes.
Binding to receptors	Binding to receptors and mediating cell entry (animal)
Transport	Virion transport within the host (plant)
Mutation	Capsid protein mutation to avoid the immune system
Replication	Some capsid proteins function as a primer for viral RNA replication

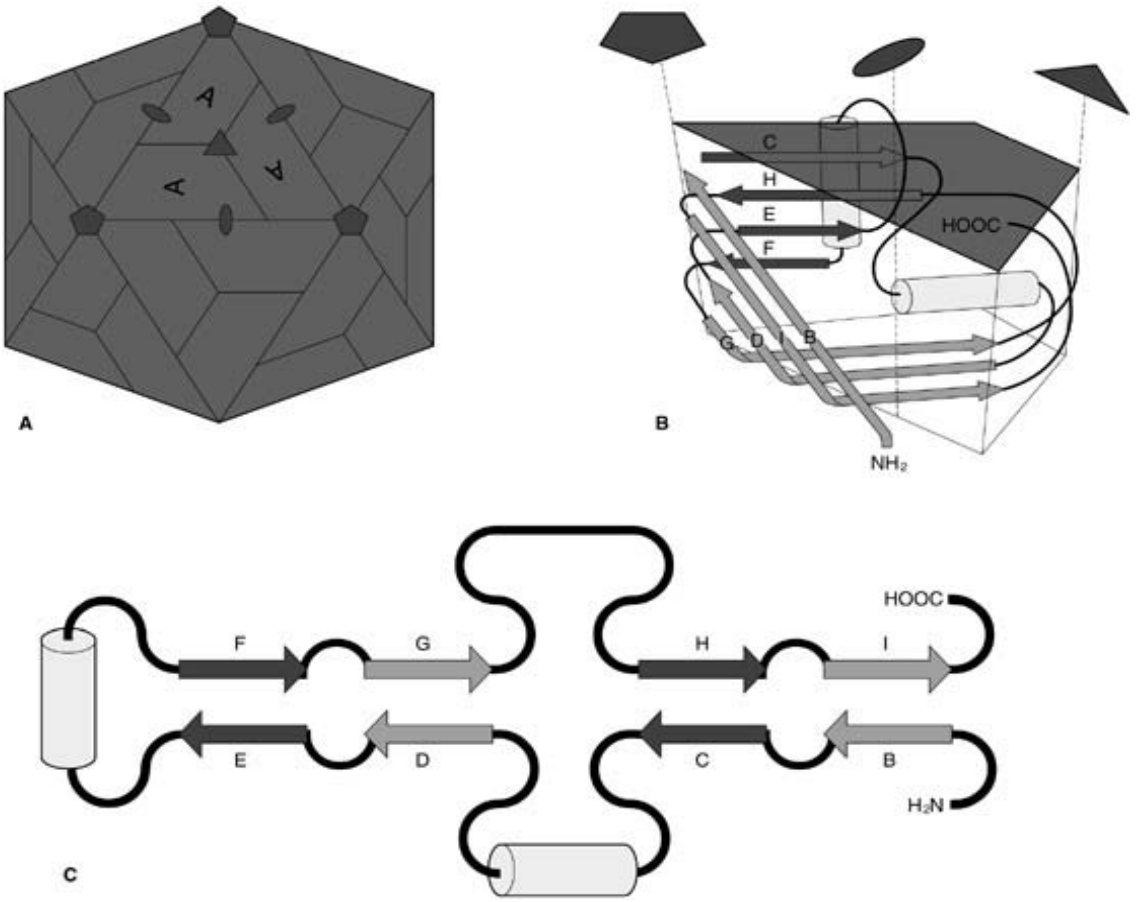


Figure 1 (**A**) The icosahedral capsid contains 60 identical copies of the protein subunit (blue) labeled A. These are related by fivefold (yellow pentagons at vertices), threefold (yellow triangles in faces) and twofold (yellow ellipses at edges) symmetry elements. For a given sized subunit this point group symmetry generates the largest possible assembly (60 subunits) in which every protein lies in an identical environment. (**B**) A schematic representation of the subunit building block found in many RNA and some DNA viral structures. Such subunits have complementary interfacial surfaces which, when they repeatedly interact, lead to the symmetry of the icosahedron. The tertiary structure of the subunit is an eight-stranded β-barrel with the topology of the jellyroll (see (**C**), β-strand and helix coloring is identical to (**B**). Subunit sizes generally range between 20 and 40 kDa with variation among different viruses occurring at the N-and C-termini and in the size of insertions between strands of the β-sheet. These insertions generally do not occur at the narrow end of the wedge (B–C, H–I, D–E and F–G turns). (**C**) The topology of viral β-barrel showing the connections between strands of the sheets (represented by yellow or red arrows) and positions of the insertions between strands. The green cylinders represent helices that are usually conserved. The C–D, E–F and G–H loops often contain large insertions. (**For color references see Color Plate 31.**)

excluding the two ends of the rod, all subunits are in identical environments in the mature helical virion. This is the ideal protein context for a minimum free energy structure. In spite of these packaging and structural attributes the helical virion must be deficient in functional requirements that are common for animal viruses because they are found only among plant and bacterial viruses. Even among plant viruses only seven of the 25 recognized groups are helical. The large majority of all viruses are roughly spherical in shape.

The architectural principles for constructing a 'spherical' virus were first articulated by Crick and Watson in 1956. They suggested that identical subunits were probably distributed with the symmetry of Platonic polyhedra (the tetrahedron, 12 equivalent positions; the octahedron, 24 equivalent positions; or the icosahedron, 60 equivalent positions). Subunits distributed with the symmetry of the icosahedron (**Fig. 1A**) provide the maximum sized particle in which all copies of a subunit will lie in identical positions. The repeated interaction of chemically complementary surfaces at the subunit interfaces leads naturally to such a symmetric particle. The 'instructions' required for assembly are contained in the tertiary structure of the subunit (**Fig. 1B, C**). The actual assembly of the protein capsids is a remarkably accurate process. The use of subunits for

the construction of organized complexes places strict control on the process and will naturally eliminate defective units. The reversible formation of noncovalent bonds between properly folded subunits leads naturally to error free assembly and a minimum free energy structure.

Crystallographic studies of more than 40 unique viruses have demonstrated that there are a limited number of folds utilized in forming viral capsids. **Figure 2** shows schematically the folds of subunits from viruses infecting vertebrates, insects and bacteria. By far the most common fold is the eight-stranded antiparallel β sandwich shown schematically in **Fig. 1B**. Other folds include one that is closely similar to the protease enzyme chymotrypsin.

Early ideas explaining spherical virus architecture were extended on the basis of physical studies of small spherical RNA plant viruses. The large yields and ease of preparation made them ideal subjects for investigations requiring substantial quantities of material. Protein subunits forming virus capsids of this type are usually 20–40 kDa. An example of a virus consistent with the Crick and Watson hypothesis is satellite tobacco necrosis virus (STNV) which is formed from 60 identical 25 kDa subunits. The particle outer radius is 80 Å and the radius of the internal cavity is 60 Å, providing a volume of 9×10^5 Å^3 for packaging RNA. A single hydrated ribonucleotide in a virion will occupy on average roughly 600 to 700 Å^3. The STNV volume is adequate to package a genome of only 1200–1300 nucleotides. STNV is a satellite virus and the packaged genome codes for only the coat protein. Proteins required for RNA replication are supplied by the 'helper virus', tobacco necrosis virus. Most simple ribovirus genomes contain coding capacity for at least two proteins, roughly 1200 nucleotides for the capsid protein and 2500 nucleotides for a RNA-directed RNA polymerase. The inner radius required to package a minimal genome is 90 Å. Consistent with this requirement were experimental studies showing that the vast majority of simple spherical viruses had outer radii of at least 125 Å which corresponds to inner radii of roughly 100 Å. Such particles had to be formed from more than 60 subunits, yet X-ray diffraction patterns of crystalline tomato bushy stunt virus (TBSV) and turnip yellow mosaic virus (TYMV) were consistent with icosahedral symmetry. Although a number of investigators developed hypotheses explaining the apparent inconsistent observations, in 1962 Caspar and Klug derived a general method for the construction of icosahedral capsids that contained multiples of 60 subunits. The method for systematically enumerating all possible quasi-equivalent structures was similar to that used by Buckminster Fuller in constructing geodesic domes. The quasi-equivalent theory of Caspar and Klug has explained the distribution of morphological units (features identifiable at low resolution by electron microscopy often corresponding to hexamer, pentamer, trimer or dimer aggregate of the subunits) on all structures observed to date, but the results from high resolution crystallographic studies have shown some remarkable inconsistencies with the microscopic principles upon which the theory is based.

Quasi-equivalence is best visualized graphically. Formally, subunits forming quasi-equivalent structures must be capable of assembling into both hexamers (which are conceptually viewed as planar) and pentamers (which are convex because one subunit has been removed from the planar hexamer and yet similar (quasi-equivalent) contacts are maintained). If subunits assembled as all hexamers, the result would be a sheet of hexamers and a closed shell could not form (**Fig. 3A**). The rules of quasi-equivalence described a systematic procedure for inserting pentamers into the hexagonal net in such a way as to form a closed shell with exact icosahedral symmetry. **Figure 3** illustrates this principle and the selection rules for inserting pentamers. **Figure 4** illustrates how the morphogenesis of such an assembly may occur using the crystallographic structure of cowpea chlorotic mottle virus (CCMV) as inspiration. CCMV, although solved only recently, was the first virus structure that agreed in detail with the predictions of Caspar and Klug.

The quasi-equivalence theory has been universally successful in describing surface morphology of spherical viruses observed in the electron microscope and, prior to the first high resolution crystallographic structure of a virus, it was assumed that the underlying assumptions of Caspar and Klug were essentially correct. The structure of TBSV determined at 2.9 Å resolution revealed an unexpected variation from the concept of quasi-equivalence which was defined as 'any small nonrandom variation in a regular bonding pattern that leads to a more stable structure than does strictly equivalent bonding'. Unlike CCMV, the structure of TBSV showed that differences occurring between pentamer interactions and hexamer interactions were not small variations in bonding patterns, but almost totally different bonding patterns. **Figure 5A** shows diagrammatically the subunit interactions in the shell of TBSV, southern bean mosaic virus (SBMV), BBV and TCV. These high resolution structures revealed that the mathematical concept of quasi-equivalence predicted surface lattices with high fidelity, but *not* for the reasons expected. Bonding contacts between quasi-threefold related subunits are maintained with little deviation

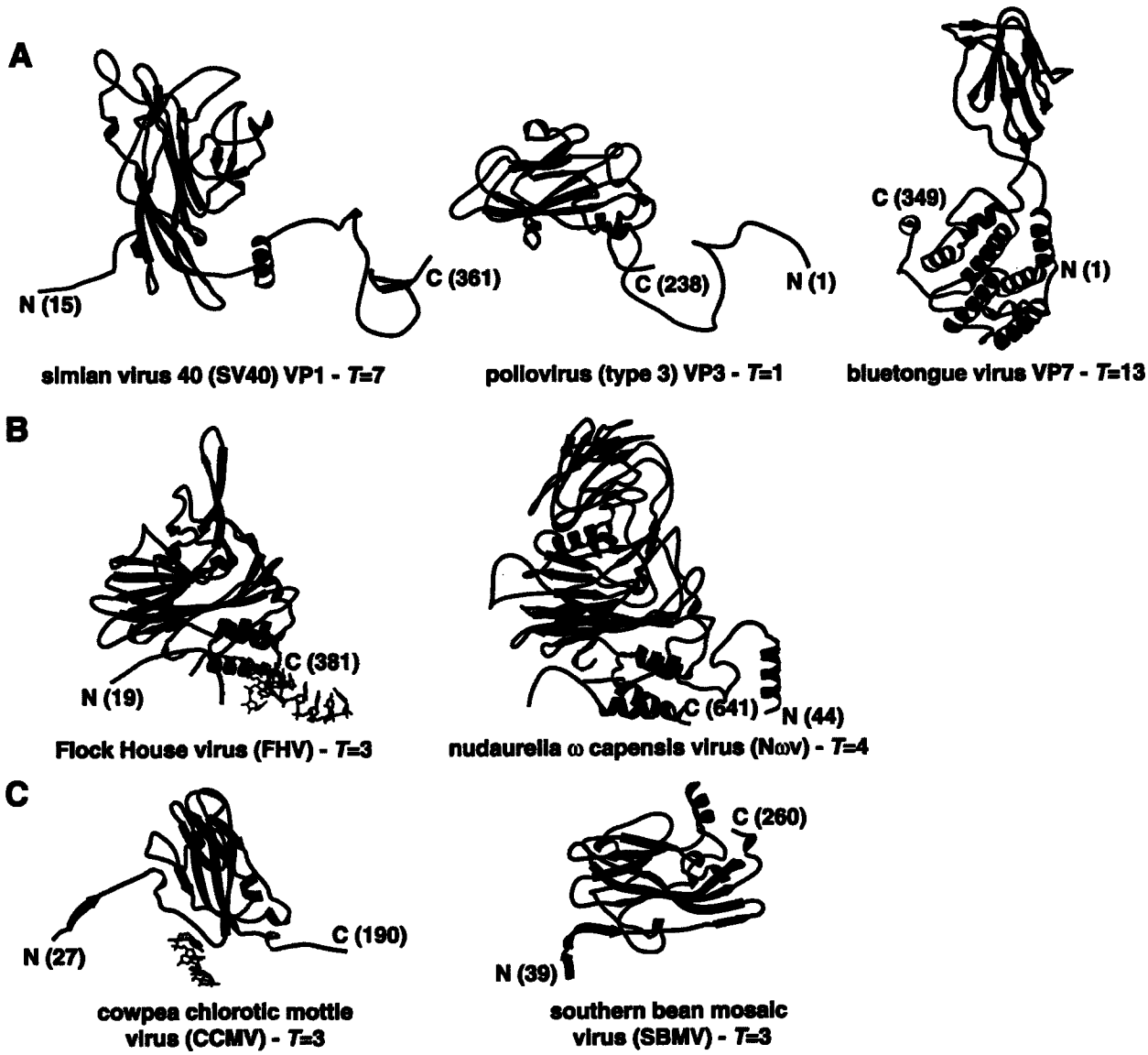
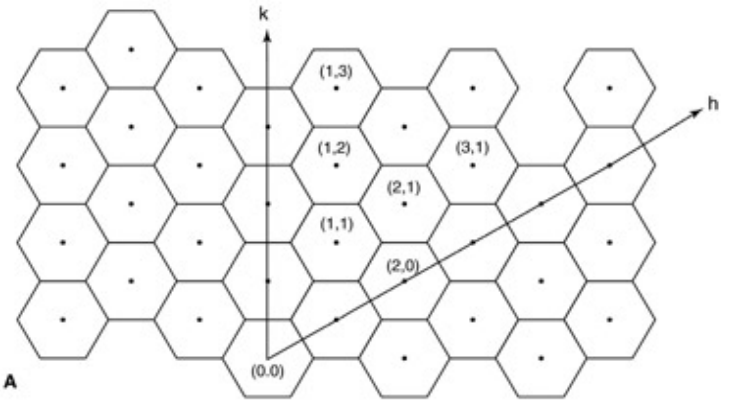
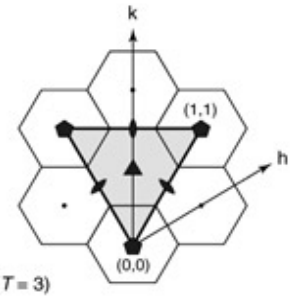


Figure 2 Structure of (**A**) vertebrate, (**B**) insect and (**C**) plant virus protein subunits that assemble into icosahedral shells. The name of the virus appears below the corresponding protein subunit along with the capsid triangulation number *T* (explained in **Fig. 3**). The N- and C-termini are labeled with the residue numbers in brackets. Many virus subunit structures determined to near atomic resolution have the β-barrel fold and/or insertions with nearly all β-secondary structure (colored red, see **Fig. 1B, C**). Multiple copies (from 180 to 780) of the single subunit shown for each virus, except for that of poliovirus, form the entire icosahedral protein shell. Assembly of icosahedral virus particles with more than 60 subunits (e.g. see **Fig. 1A**) requires quasi-symmetric interactions (nonidentical interactions between neighboring identical subunits, discussed in detail later in this chapter, see **Figs 3** and **4**) often involving subtle to extensive differences in structure at the subunit N- and C-termini. The subunit regions involved in quasi-symmetric interactions critical to virion structure and assembly are colored green (only a single variation is shown for each virus). The 'switch' in structure between identical subunits is a response to differences in the local chemical environment, defined the number of subunits forming the icosahedral shell, in order to maintain similar bonding between neighboring subunits. The structural variations include the presence or absence of highly ordered RNA structure (green stick models) in FHV and CCMV. Poliovirus utilizes multiple copies of two additional subunits highly similar to VP3 to form a complete virion. Thus, there is no quasi-symmetry in poliovirus (note the absence of any green highlights) since neighboring subunits are different proteins. **(For color references see Color Plate 32.)**

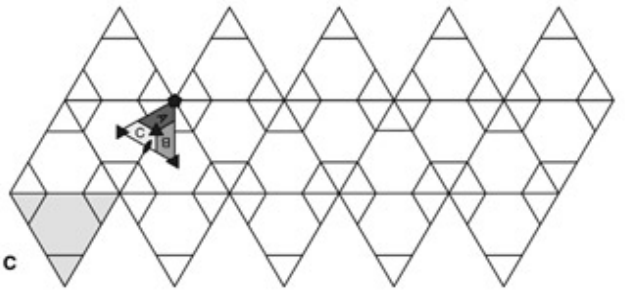
from exact symmetry, while quasi-twofold contacts and icosahedral twofold contacts (which are predicted to be very similar) are quite different. The hexamer quasi-symmetry is better described as a trimer of dimers in the TBSV and related structures. Unlike the conceptual model and the CCMV capsid



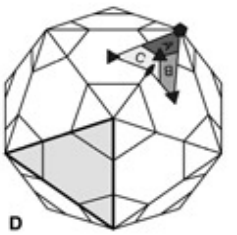
A



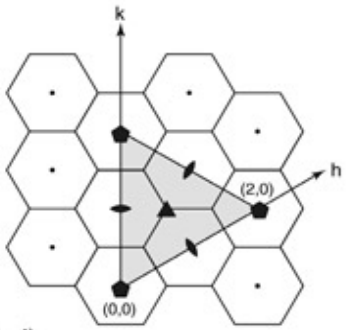
B ($T = 3$)



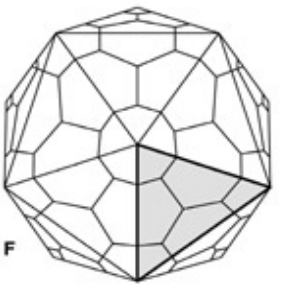
C



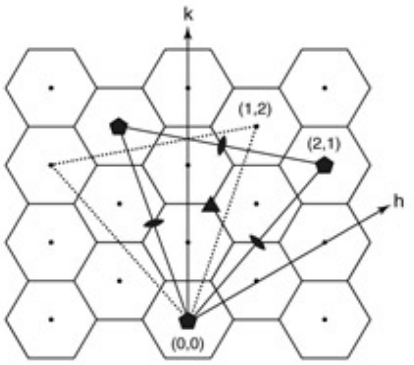
D



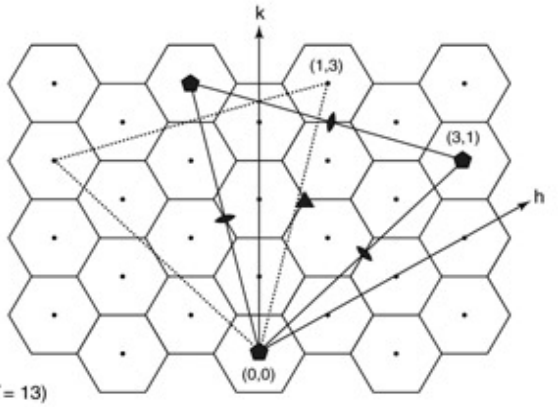
E ($T = 4$)



F



G ($T = 7$)



H ($T = 13$)

(Fig. 5B), the particle curvature in TBSV results from both pentamers and hexamers.

The high resolution $T = 3$ structures showed that the overall features of the quasi-equivalent theory were correct, but that the underlying concepts of quasi-equivalent bonding had to be revised. The first low (22.5 Å) resolution structure of a $T = 7$ virus required an even greater conceptual adjustment to the underlying principles of quasi-equivalence. Rayment, Baker and Caspar reported, in 1982, that the polyomavirus capsid contained 72 capsomeres, as previously reported from electron microscopy studies, but that all the capsomeres were pentamers of protein subunits even though they were located at hexavalent lattice points. The $T = 7$ surface lattice predicts 12 pentamers and 60 hexamers, thus the prediction of the number and position of the morphological units is correct, but the fine structure of the morphological units is incorrect. Although the result was highly controversial when first reported, additional electron microscopy studies and the 3.5 Å resolution X-ray structure of polyomavirus have fully confirmed the all-pentamer structure. This result clearly shows the limits of theory in predicting virus structure and indicates that further understanding of capsid structure will come only from experimental studies. The structure of the polyomavirus and its relatives illustrates an important concept when considering surface lattice formation. The important feature is not the symmetry of the morphological unit positioned on the hexamer sites, but only its ability to accommodate six neighbors (it is a hexavalent position). Normally this is accomplished by morphological units with sixfold symmetry, but here, rather acrobatic molecular switching has permitted a pentamer of subunits to accommodate six neighbors. Although the all-pentamer capsid has been observed for the $T = 7$ structure of papillomaviruses, cauli-

Figure 3 Geometric principles for generating icosahedral quasi-equivalent surface lattices. These four constructions show the relation between icosahedral symmetry axes and quasi-equivalent symmetry axes. The latter are symmetry elements that hold only in a local environment. (**A**) It is assumed in quasi-equivalence theory that hexamers and pentamers can be interchanged at a particular position in the surface lattice. Hexamers are initially considered planar (an array of hexamers forms a flat sheet as shown) and pentamers are considered convex, introducing curvature in the sheet of hexamers when they are inserted. Inserting 12 pentamers at appropriate positions in the hexamer net generates the closed icosahedral shell, composed of hexamers and pentamers. The positions at which hexamers are replaced by pentamers are defined by the indices **h** and **k** measured along the labeled axes. The values of (**h, k**) used in the following examples are labeled. To construct a model of a particular quasi-equivalent lattice, one face of an icosahedron (equilateral triangles colored orange in (**B–F**) is generated in the hexagonal net. The origin (0,0) is replaced with a pentamer, and the (**h,k**) hexamer is replaced by a pentamer. The third replaced hexamer is identified by threefold symmetry (i.e. complete the equilateral triangle). Each quasi-equivalent lattice is identified by a number $T = \mathbf{h}^2 + \mathbf{hk} + \mathbf{k}^2$ where **h** and **k** are the indices used above. T indicates the number of quasi-equivalent units in the icosahedral asymmetric unit (a hexamer contains six units and a pentamer contains five units). For the purpose of these constructions it is convenient to choose the icosahedral asymmetric unit as one-third of an icosahedral face defined by the triangle connecting a threefold axis to two adjacent fivefold axes. Other asymmetric units can be chosen such as the triangle connecting two adjacent threefold axes and an adjacent fivefold axis (see (**C**) and **Fig. 5**). The total number of units in the particle is $60T$, given the symmetry of the icosahedron. The number of pentamers must be 12 and the number of hexamers is $(60T - 60)/6 = 10(T - 1)$. (**B**) One face of the icosahedron for a $T = 3$ surface lattice is identified by the orange triangle with the bold outline (this corresponds to a face of the icosahedron in **Fig. 1A**). The yellow symmetry labels are the same as those defined in **Fig. 1**. The hexamer replaced has coordinates $\mathbf{h} = 1$, $\mathbf{k} = 1$. The icosahedral asymmetric unit is one-third of this face and it contains three quasi-equivalent units (two units from the hexamer coincident with the threefold axis and one unit from the pentamer). (**C**) Arranging 20 identical faces of the icosahedron as shown can generate the three-dimensional model of the quasi-equivalent lattice. Three quasiequivalent units labeled A (blue), B (red) and C (green) are shown. These correspond to the three quasi-equivalent units defined in **Figs 4** and **5** rather than the alternative definition used in (**A**) and (**B**). (**D**) The folded icosahedron is shown with hexamers and pentamers outlined. The orange face represents the triangle originally generated from the hexagonal net. The $T = 3$ surface lattice represented in this construction has the appearance of a soccer ball. The trapezoids labeled A, B and C identify quasi-equivalent units in one icosahedral asymmetric unit of the rhombic tri-icontahedron discussed in **Fig. 5**. (**E**) An example of a $T = 4$ icosahedral face ($\mathbf{h} = 2$, $\mathbf{k} = 0$). In this case the hexamers are coincident with icosahedral twofold axes. (**F**) A folded $T = 4$ icosahedron with the orange face corresponding to the face outlined in the hexagonal net. Note that folding the lattice has required that the hexamers have the curvature of the icosahedral edges. (**G**) A single icosahedral face generated from the hexagonal net for a $T = 7$ lattice. Note that there are two different $T = 7$ lattices ($\mathbf{h} = 2$, $\mathbf{k} = 1$ in bold outline; and $\mathbf{h} = 1$, $\mathbf{k} = 2$ in dashed outline). These lattices are the *mirror* images of each other. To fully define such a lattice, the arrangement of hexamers and pentamers must be established as well as the enantiomorph of the lattice. (**H**) A single icosahedral face for a $T = 13$ lattice is shown. The two enantiomorphs of the quasi-equivalent lattice ($\mathbf{h} = 3$, $\mathbf{k} = 1$ – bold; and $\mathbf{h} = 1$, $\mathbf{k} = 3$ – dashed) are outlined. The procedure for generating quasi-equivalent models described here does not exactly correspond to the one described by Caspar and Klug (1962). Caspar and Klug distinguish between different icosadeltahedra by a number $P = \mathbf{h}^2 + \mathbf{hk} + \mathbf{k}^2$ where **h** and **k** are integers that contain no common factors but 1. The deltahedra are triangulated to different degrees described by an integer f that can take on any value. In their definition $T = Pf^2$. The description in this figure has no restrictions on common factors between h and k, thus $T = \mathbf{h}^2 + \mathbf{hk} + \mathbf{k}^2$ for all positive integers. The final models are identical to those described by Caspar and Klug. **(For color references see Color Plate 33.)**

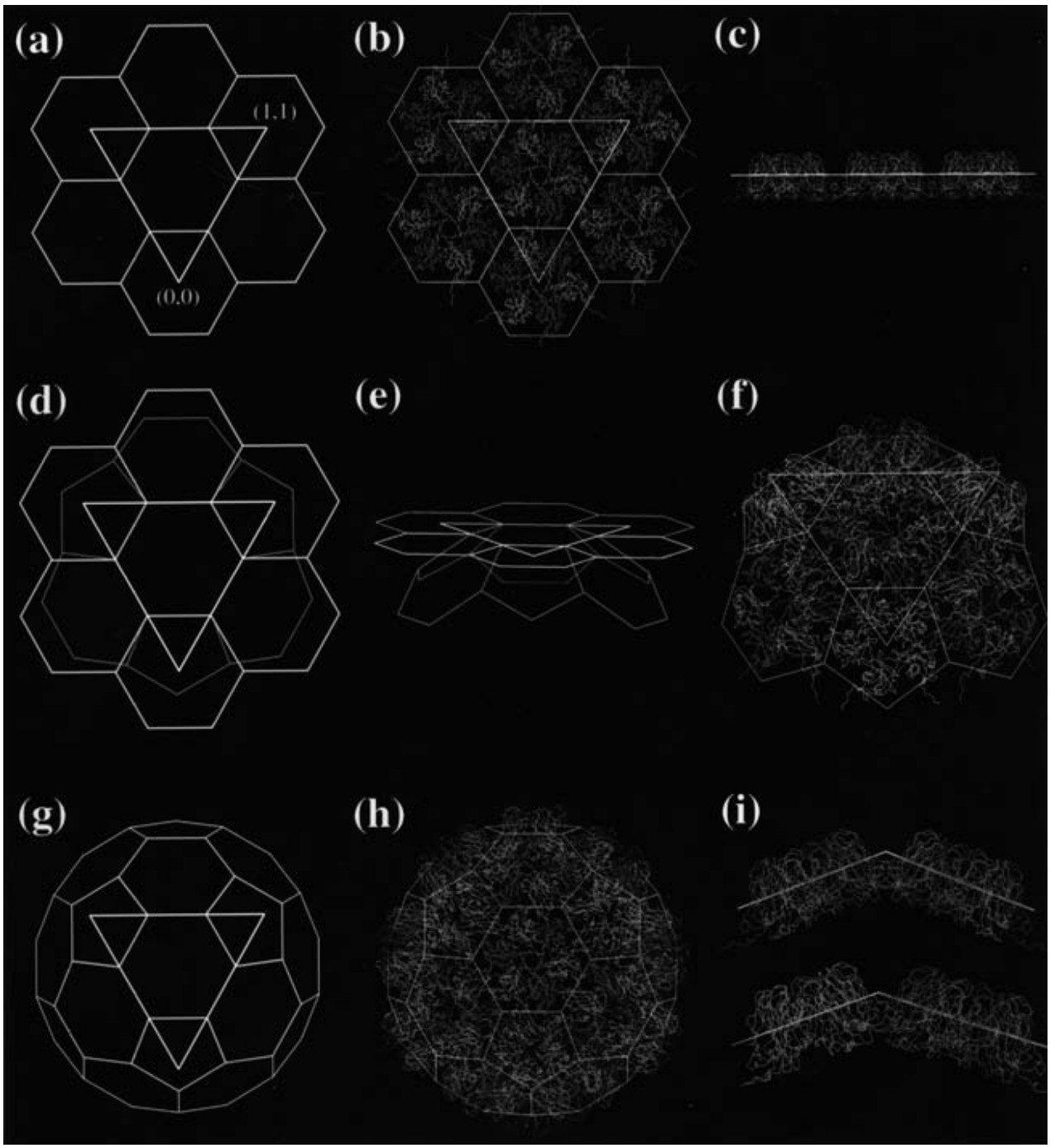


Figure 4 Molecular graphics construction of a $T = 3$ quasi-equivalent icosahedron. (**a**) Hexagonal sheet overlaid with the triangular coordinates (white) for a theoretical $T = 3$ quasi-equivalent icosahedron (**h** = 1, **k** = 1, see **Fig. 3B**). The sheet has true sixfold rotational symmetry about axes passing through the hexamer centers, which are normal to the sheet. (**b**) Copies of the hexamer coordinates from the CCMV X-ray structure (colored by asymmetric unit position, see **Fig. 5**) can be positioned in the sheet by simple translations. (**c**) A side view of the modeled sheet demonstrates its planarity. (**d**) Hexamers at the corners of the white (**h** = 1, **k** = 1) triangle become pentamers. The planar sheet (yellow model) takes on curvature to maintain contacts between the polygons (green model). (**e**) The magnitude of the pentamer-induced curvature is displayed in the side view of the partial polyhedron. (**f**) Coordinates of the CCMV X-ray structure fit this construction without any manipulation. (**g**) A completed $T = 3$ icosahedral model. The 12 pentamers generate curvature that closes the structure. This cage (a truncated icosahedron) accurately describes the geometric morphology of CCMV (**h**) which is composed of modular, planar pentamers (12) and hexamers (20). Angular pentamer–hexamer and hexamer–hexamer interfaces (**i**) stabilize curvature in the absence of convex pentamers used to construct the soccer ball of **Fig. 3D** (see also **Fig. 5**). (**For color references see Color Plate 34.**)

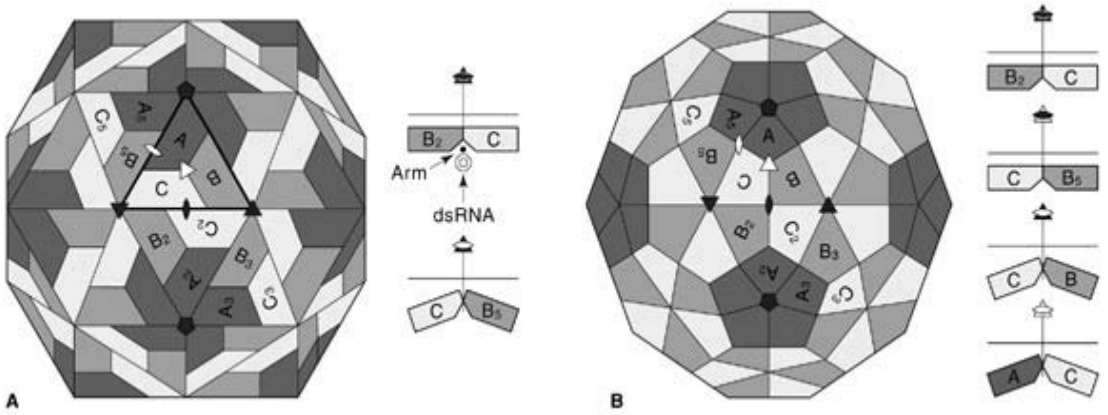


Figure 5 Although quasi-equivalence theory can predict, on geometrical principles, the organization of hexamers and pentamers in a viral capsid, the detailed arrangement of subunits can only be established empirically. High-resolution X-ray structures of $T = 3$ plant and insect viruses show that the particles are organized like the icosahedral rhombic tri-icontahedron or truncated icosahedron (**Fig. 4**). A convenient definition of the icosahedral asymmetric unit for both geometrical shapes is the wedge defined by icosahedral threefold axes left and right of the particle center and an icosahedral fivefold axis at the top. The icosahedral asymmetric unit contains three subunits labeled A (blue), B (red) and C (green) (see **Fig. 3C, D**). The asymmetric unit polygons represent chemically identical protein subunits that occupy slightly different geometrical (chemical) environments as indicated by differences in their coloring. Polygons with subscripts are related to A, B, and C by icosahedral symmetry (i.e. A to A_5 by fivefold rotation). The shapes of the $T = 3$ soccer ball model in **Fig. 3D**, truncated icosahedron in **Fig. 4** and rhombic tri-icontahedron are all different; however, the quasi-symmetric axes are in the same positions relative to the icosahedral symmetry axes for all three models. Quasi-threefold and quasi-twofold axes are represented by the white symbols. The quasi-sixfold axes are coincident with the icosahedral threefold axes in $T = 3$ particles as shown in **Figs 3B–D** and **4**. **(A)** The rhombic tri-icontahedron is constructed by placing rhombic faces perpendicular to icosahedral twofold symmetry axes (yellow ellipse). Thus, the A, B and C polygons are coplanar within each asymmetric unit. The shape of the subunit in $T = 3$ plant and insect viruses is nearly identical to the shape of the subunit in the $T = 1$ virus and they pack in a very similar fashion. The $T = 1$ subunits in one face (**Fig. 1A**) are related by an icosahedral threefold axis, while the $T = 3$ subunits in one face are related by a quasi-threefold axis. The dihedral angle between subunits C and B_5 (juxtaposed across quasi-twofold axes) is 144° and is referred to as a bent contact (bottom right image), while the dihedral angle between subunits C and B_2 (juxtaposed across icosahedral twofold axes) is 180° and is referred to as a flat contact (top right image). Two dramatically different contacts between subunits with identical amino acid sequences are generated by the insertion of an extra polypeptide from the N-terminal portion of the C subunit into the groove formed at the flat contact. This polypeptide is called an 'arm'. The flat contact can also be upheld by insertion of nucleic acid structure into the same groove. The N-terminal arms of the A and B subunits are disordered, and nucleic acid structure has not been observed in the groove across the quasi-twofold axis; thus, C and B_5 are in direct contact as in, for example, the X-ray structure of FHV. **(B)** A truncated icosahedron achieves curvature at different interfaces compared to the rhombic tri-icontahedron. Interactions between B_2–C and between C–B_5 polygons are both defined by 180° dihedral angles (side view at top right) whereas bends similar in magnitude occur within the asymmetric unit at the B–C and C–A polygon interfaces (138° and 142°, respectively; side view at bottom right). This creates the planar pentamer and hexamer morphological units characteristic of the truncated icosahedron and the CCMV X-ray structure (**Fig. 4h**). **(For color references see Color Plate 35.)**

flower mosaic virus appears to have the hexamer/pentamer distribution predicted by quasi-equivalent theory, as do the $T = 7$ capsids of the λ-like bacteriophage, HK97. A substantial number of complex virus structures have been determined by cryoelectronmicroscopy and the surface lattices agree well with the predictions of quasi-equivalence. Thus there is considerable confidence in the lattice assignments, but the capsomere and therefore number of subunits must be carefully confirmed.

A number of viral capsids are constructed with pseudo $T = 3$ symmetry. These structures contain β-barrel subunits (**Fig. 1B**) in the quasi-equivalent environments formed in $T = 3$ structures, but each of the three β-barrels in the asymmetric unit has a unique amino acid sequence. Rather than 180 identical subunits, the $P = 3$ particles contain 60 copies each of three different subunits (**Fig. 6**). These structures do not require quasi-equivalent bonding because each unique interface will have different amino acids interacting, rather than the same subunits forming different contacts. The animal picornaviruses have capsids of this type. Animal virus capsids undergo rapid mutation to avoid recognition by the circulating immune system. Capsids composed of three subunit types could mutate in one subunit without affecting the other two. This would be less likely to affect assembly or other functions of the particle in $P = 3$ shells than it would in $T = 3$ shells. At least one plant virus group displays $P = 3$ shells,

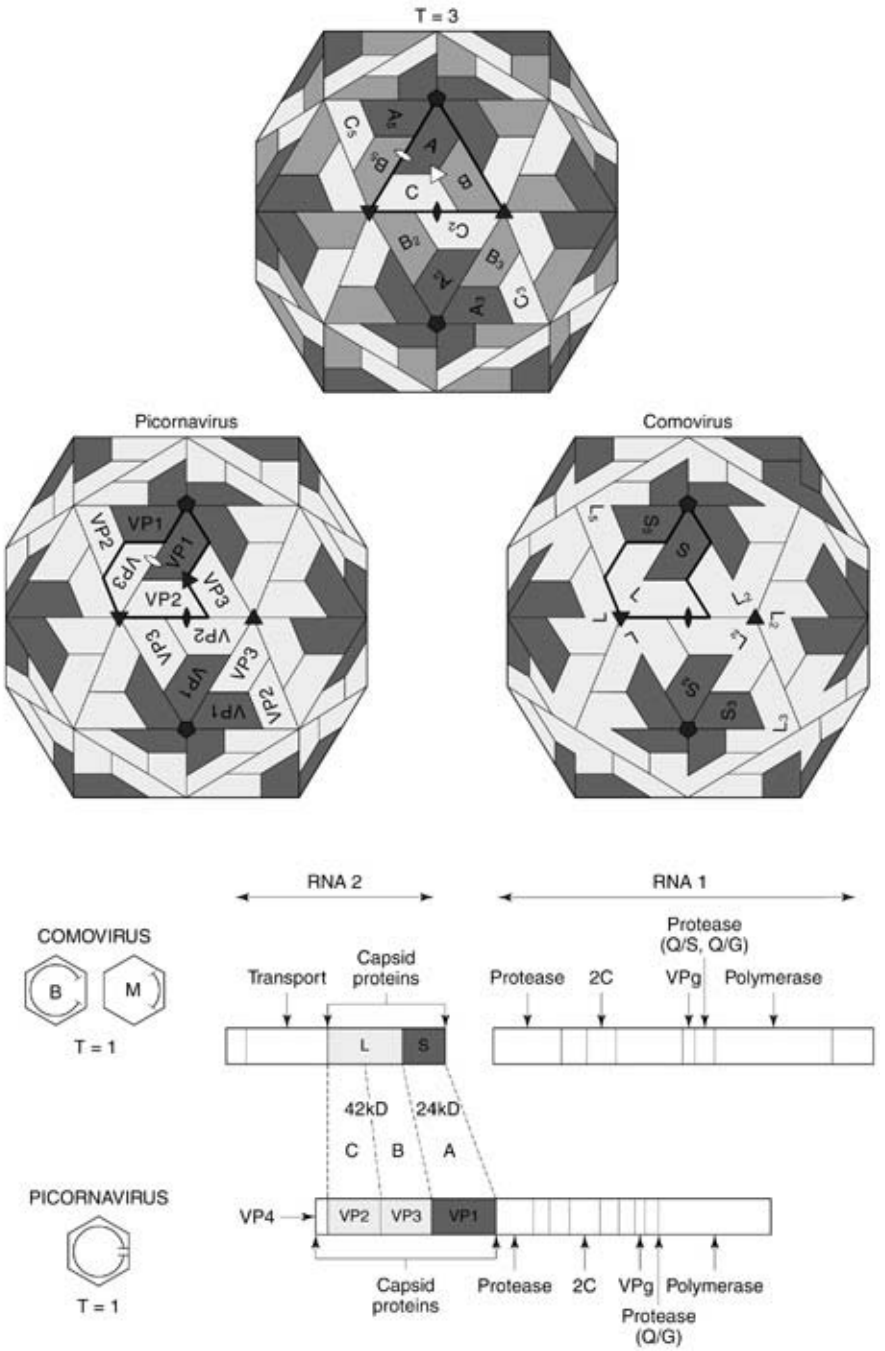


Figure 6 A comparison of $T = 3$, picornavirus and comovirus capsids. In each case, one trapezoid represents a β-barrel and the icosahedral asymmetric units are outlined in bold. The icosahedral asymmetric unit of the $T = 3$ shell contains three identical subunits labeled A, B and C (see **Fig. 5**). The asymmetric unit of the picornavirus capsid contains three β-barrels, but each has a characteristic amino acid sequence labeled VP1, VP2 and VP3. The comovirus capsid is similar to the picornavirus capsid except that two of the β-barrels (corresponding to the green VP2 and VP3 units) are covalently linked to form a single polypeptide, the large protein subunit (L), while the small protein subunit (S) corresponds to VP1 (note the similar color shading). The individual subunits of the comovirus and picornavirus capsids are in identical geometrical (chemical) environments (e.g. VP1 and S are always pentamers) making these $T = 1$ capsids. Comoviruses and picornaviruses have a similar gene order, and the nonstructural 2C and polymerase genes display significant sequence homology. The relationship between the capsid subunit positions in these viruses and their location in the genes is indicated by color coding and the labels A, B and C in the gene diagram. **(For color references see Color Plate 36.)**

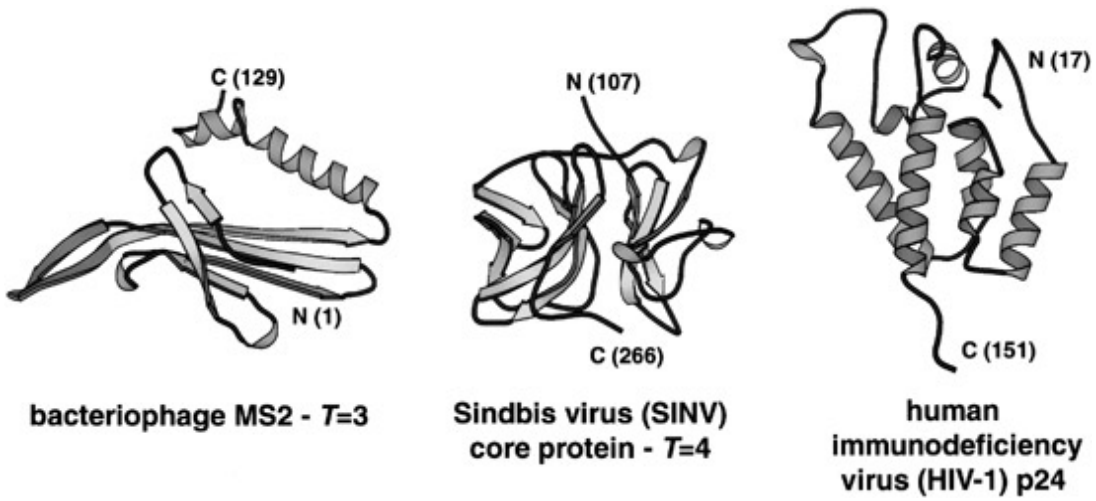


Figure 7 Viral capsid subunits having a fold other than the β-barrel. The MS2 and SINV subunits assemble into icosahedral shells with the *T* numbers shown. The SINV core is further surrounded by a lipid bilayer and glycoprotein spikes to form the complete virus particle. HIV-1 p24 does not form a capsid with icosahedral symmetry. Together with other products from Gag protein cleavage by the viral protease, p24 forms a conical core structure that encloses the HIV nucleocapsid protein–RNA complex.

the comoviruses. An interesting variation occurs in these capsids when compared with the picornaviruses. Two of the domains forming the shell are contained in a single polypeptide chain. This phenomenon is readily understood in the context of the synthesis of the subunits in picornaviruses and comoviruses. In both cases the proteins are synthesized as a polyprotein that is subsequently cleaved into functional proteins by a virally encoded protease. Clearly one of the cleavage sites in picornaviruses is missing in the comoviruses, resulting in these two domains being still a 'polyprotein'.

Other icosahedral shells containing more than one type of protein subunit have been investigated by high resolution electron microscopy and X-ray crystallography.

Subunit Tertiary Structure

The dominant tertiary fold observed in high resolution X-ray structures determined to date is the eight-stranded β-barrel illustrated in **Fig. 1B**. This fold has been observed in a wide range of viruses. The wedge shape is ideally suited to form pentamers and hexamers, as it does in $T = 3$ virus and $P = 3$ viruses. In other viruses, however, the wedge is not found in this favorable geometric environment. Canine parvovirus, the phage ϕX174, the DNA tumor virus SV40 and adenovirus hexons have the β-barrel fold, but its geometrical environment in each of these viruses is very different from that observed in the $T = 3$ and $P = 3$ viruses.

In all cases the residues in the β-barrel form most of the contiguous shell, while insertions between strands of the barrel project outward. In some cases these insertions can be up to 100 residues or more, creating additional domains of the protein. Extensions at the N-terminus are generally on the interior of the shell, and in many plant and some animal viruses this portion will be extremely basic. These regions are generally not visible in X-ray structures because they are assuming different structures and do not obey icosahedral symmetry. Extensions at the C-terminus are generally external. In the case of TBSV and TCV an entire protruding domain is created by the 90 residues following the polypeptide that forms the contiguous shell (**Fig. 2**).

Although infrequent, other tertiary structures have been seen in viral capsid subunits (**Fig. 7**). The RNA phage MS2 was found to have an entirely different fold than the β-barrels observed in other $T = 3$ structures. What is more, like CCMV, the quaternary structure of MS2 is more closely related to the quasi-equivalence of subunit interactions originally envisioned by Caspar and Klug. The difference between quasi and icosahedral twofold axes are extremely small and the difference in contacts at the pentamers and hexamers seems to be regulated by a loop between two strands that is extended at hexamer axes and folded down at pentamer contacts.

Another tertiary structure found is in the core protein of Sindbis virus. In this case subunits were purified and crystallized because the intact nucleoprotein core was not stable enough to crystallize. The fold found was that of chymotrypsin. It was

previously known that this subunit functions as an enzyme because it cuts itself out of a polyprotein after synthesis. Generally the structure has shorter loops than found in chymotrypsin, but the topology and active site are conserved. The C-terminal tryptophane, where the cleavage occurs, is still in the active site of the subunit. These subunits form $T = 4$ shells with the viral RNA. In this case a protein fold with a totally different function has been adapted to form shells. The N-terminal region of this protein (residues 1–100) is composed predominately of basic residues and is not visible in the crystal structure.

The principles of virus structure discussed reflect the level of current understanding for a few relatively simple virus systems. More than 40 unique virus structures have been determined at moderate to high resolution (35 nm or higher) by X-ray crystallography and coordinates are available for these capsids in the Brookhaven Protein Data Bank. This structural information has allowed an understanding of virus assembly and disassembly at the chemical level and this has led to the rational design of antiviral agents that effect particle stability. Approximately 30 virus structures, many with dimensions greater than 80 nm, have been determined at moderate resolution (~2 nm) with cryo-electron microscopy and image reconstruction and these structures have revealed organizational principles of exceptional complexity. The large icosahedral viruses extend the principles of quasiequivalence to multisubunit capsid types, but it remains a unifying theme over a remarkable range of virus particle size.

Complex Virus Structures

Many viruses are composed of complex particles with specific functions associated with different structural elements. Complex bacteriophages, for example, have been a subject of study for decades, and the details of their morphogenesis and low resolution structure have been determined. A variety of viruses contain multiple copies of different subunits in their capsids, and the structural roles of each subunit type are still being determined. In most cases these particles are too large to be analyzed by crystallography, but some have been successfully examined by high resolution electron microscopy. Many viruses of medical importance are enveloped by a membrane that contains functionally important proteins. Usually a quasi-symmetric nucleoprotein particle assembles in the cytoplasm and the membrane and associated proteins are acquired when the particle buds through the plasma membrane of the host cell. In the paramyxoviruses, two proteins in the membrane (neuraminidase and hemagglutinin) have been purified, crystallized and analyzed at high resolution. There are a number of examples in which envelopes have been removed and nucleoprotein cores have been analyzed by cryoelectronmicroscopy and image analysis. Their structures display a variety of T numbers, and they are not significantly different from nonenveloped protein capsids. The largest structure ever determined by crystallography is the core of the nonenveloped, double-stranded RNA bluetongue virus that is delivered to the cytoplasm of infected cells. The particle is over 600 Å in diameter, has a $T = 13$ outer capsid and an inner capsid with $T = 1$ symmetry formed of 120 copies of the same gene product. The subunit that forms the outer $T = 13$ shell is shown in **Fig. 2**.

The continued rapid development of single crystal X-ray diffraction, cryo-electron microscopy and associated image processing techniques virtually guarantees rapid progress in understanding the structure and function of large complex viruses. These complex structures will certainly provide insights for broader biological mechanisms as well.

See also: **Virus structure: Atomic structure; Comoviruses (*Comoviridae*); Human immunodeficiency viruses (*Retroviridae*): Molecular biology, Antiretroviral agents, General features; Picornaviruses – insect (*Picornaviridae*); Polioviruses (*Picornaviridae*): General features, Molecular biology; Sindbis and Semliki Forest viruses (*Togaviridae*); Single-stranded RNA phages (*Leviviridae*).**

Further Reading

Caspar DLD (1980) Movement and self-control in protein assemblies. Quasi-equivalence revisited. *Biophys. J.* 32: 103.

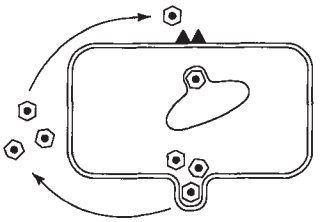
Caspar DLD and Klug A (1962) Physical principles in the construction of regular viruses. *Cold Spring Harbor Symp. Quant. Biol.* 27: 1.

Grimes J, Burroughs J, Gouet P *et al* (1998) The atomic structure of the bluetongue virus core. *Nature* 395: 470.

Johnson JE (1996) Functional implications of protein–protein interactions in icosahedral viruses. *Proc. Natl Acad. Sci. USA* 93: 27.

Johnson J and Speir J (1997) Quasi-equivalent viruses: a paradigm for protein assemblies. *J. Mol. Biol.* 269: 665.

VIRUS–HOST CELL INTERACTIONS



Patricia Whitaker-Dowling and **Julius S Youngner**, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA

Introduction

The topic of virus–host cell interactions spans all of virology and provides some of the most important insights into this field. Since viruses are intracellular parasites, they rely on their host cells for the energy, macromolecular synthesis machinery and the work benches for genome replication and particle assembly. Because of this dependence, viruses have evolved a myriad of mechanisms for exploiting normal host cell functions. Often this exploitation is associated with damage to the host cell which may be one of the major factors in the pathology and disease caused by viruses. The material in this entry is confined to model systems of virus–host cell interactions that involve the infection by animal viruses of cells in culture.

The past few decades have witnessed a dramatic expansion of our knowledge of animal viruses. These advances have provided a detailed understanding of the structure and composition of the viral genome and the virus particle as well as insight into the replication strategies used by viruses and the regulation of viral gene expression during infection. Development of an understanding of the virus growth cycle has proved easier than a clear comprehension of the interaction of the virus with the host cell. Owing to the complexity of the cell, many of the effects of virus infection on the host occur by mechanisms yet to be determined.

Types of Virus Infections

When a virus infects a cell, the outcomes that may occur can be grouped into several general categories which are determined by the particular virus involved, as well as by the type of cell and its functional state. Productive infections result in the formation of progeny virus and usually cause the destruction of the host cell. In some cases the host cells are not all destroyed, leading to persistent infections in which the surviving cells multiply and continue to produce progeny viruses. When persistent infections occur in which the viral genome is present but no infectious virus is produced, these infections are referred to as latent infections. In such infections some level of viral gene expression is usually detectable although virions are not produced. When genetic information of the virus is integrated as DNA into the host cell genome or is carried as episomal DNA, transforming infections may take place. Such infections can cause an oncogenic alteration of the growth properties of the cell. Abortive infections occur when viruses infect cells that are nonpermissive or only partially permissive. In this instance, the virus is able to enter the cell but because some step essential for viral replication is absent, the replication cycle does not go to completion and no progeny are produced. Such abortive infections may or may not cause cell death.

A few examples follow which demonstrate that different outcomes of infection are dependent on the particular virus and host cell involved, as well as on the state of the host cell. For example, influenza A virus causes a productive, cytolytic infection of a line of canine kidney cells (MDCK). However, when the same virus infects the L cell line of mouse fibroblasts an abortive infection occurs because of a block at the level of virion RNA replication. The same mouse L cell line supports a productive, cytolytic infection by vesicular stomatitis virus (VSV). However, if the L cells are pretreated with interferon, the functional state of the cells is altered; the VSV replication cycle is blocked at the level of protein synthesis and an abortive infection results. When VSV infects insect cell lines derived from *Aedes* or *Drosophila*, productive noncytolytic infections occur. Continuous passage of the insect cell lines reveals that they have become persistently infected and continuously produce infective virus without any signs of cytopathology. Adeno-associated virus (AAV), a parvovirus, is capable of a productive or a latent infection depending upon whether or not the host cells are co-infected with a helper virus such as adenovirus. In some host cells, AAV can cause a latent infection by integrating its DNA into the host cell genome.

There are instances in which infection with a second unrelated virus can dramatically alter the type of infection produced by certain viruses. As mentioned in the preceding paragraph, adeno-associated viruses are capable of productive, cytolytic infections only in cells co-infected with adenovirus. Human adeno-viruses can multiply in monkey cells only in the presence of SV40, a simian papovavirus that supplies a helper function that permits translation of adenovirus mRNAs. In rabbit corneal cells, co-infection with vaccinia or some other poxviruses can convert nonproductive infections with VSV into cytolytic infections.

Effects of Virus Infection on the Host Cell

Effects on host cell morphology and viability

The most readily recognized effects of viruses on host cells are those that involve morphologic changes or cell death. Enders defined viral cytopathogenicity as 'the capacity to induce any demonstrable departure from the normal either in the morphological or functional properties of cells'. The space available for this entry precludes a comprehensive survey of all the cytopathic effects induced by infection with the various families of animal viruses. However, one of the most striking observations that emerges from an overview of the effects of viruses on host cells is how little is known of the mechanisms by which viruses induce cytopathology. The production of cytopathic effects has been observed with most families of viruses and in many cases the viral gene(s) involved or implicated in these morphological changes has been defined. However, in most cases the mechanisms responsible for cell destruction have not been identified. It is fair to say that one of the most fundamental questions of virology, namely, how viruses kill cells, remains for the most part unanswered.

Effects on host cell macromolecular synthesis

Many of the investigations of the effect of virus infection on the host cell have centered on virus-induced alterations of host cell macromolecular synthesis. While these studies are important to an understanding of the viral growth cycle and have yielded significant insights into the control of host cell gene expression, there is no direct evidence that inhibition at this level is the direct cause of visible cytopathology or cell death. In fact, treatment of host cells with drugs such as actinomycin D and cycloheximide, which inhibit nucleic acid and protein synthesis, does not mimic the morphological changes produced by virus infection. Nevertheless, viruses do employ a variety of strategies to affect the host cell at the level of gene expression.

Effects on host cell DNA and RNA synthesis A variety of DNA and RNA viruses are capable of affecting gene expression by directly altering the host cell genome. For example, the host cell DNA is degraded after infection with poxviruses. Herpes-, picorna- and reoviruses cause a displacement of the cellular chromatin, while an inhibition of host DNA synthesis has been reported following infection with herpes-, pox-, adeno-, picorna-, reo-, alpha- and rhabdoviruses. This inhibition of host DNA synthesis may be a direct effect of a virus factor in the nucleus or a secondary consequence of the inhibition of host cell protein synthesis.

Viral products can directly affect the activity of cellular RNA polymerases and cause an inhibition of host RNA synthesis. Such an inhibition has been seen with VSV and polioviruses. In the case of VSV, both a small viral-encoded RNA molecule (leader RNA) and a viral protein (the matrix M protein) have been implicated in the inhibition of the cellular polymerases at the level of RNA synthesis initiation. Another mechanism to inhibit host RNA synthesis is employed by polioviruses. These agents encode a protease that is capable of cleaving transcription factors required by host RNA polymerases II and III. Reo- and alphaviruses also block host RNA synthesis but the mechanism of this inhibition is not known. Synthesis is not the only level at which viruses can affect host mRNA. Infection with herpes- and poxviruses increases the rate of host mRNA degradation. A unique effect on host cell mRNA is produced by influenza viruses. These agents cleave the cap structure and the first 10–13 nucleotides from the 5′ ends of newly synthesized host mRNAs, and utilize this oligomer as a primer for viral mRNA synthesis. Another mechanism that affects host RNA is seen with adenoviruses; in this instance, infection inhibits the transport of host mRNA out of the nucleus.

Effects on host cell protein synthesis Although much effort has been directed at understanding the effect of virus infection on host protein synthesis, it is unlikely that an inhibition of host protein synthesis is required for successful virus replication. Many viruses, such as paramyxo-, papova- and retroviruses do not normally inhibit host protein synthesis during their replication. Furthermore, mutant viruses that are defective in their ability to shut down host protein synthesis are not necessarily defective for virus growth. In fact, VSV mutants selected during a persistent infection have a reduced ability to inhibit the host's translational machinery; nevertheless, these mutants grow to higher titer during a lytic growth cycle than the parental wild-type virus. With several virus families, infection causes a selective inhibition of the translation of host cell mRNA. Such viruses include picorna-, pox-, herpes-, adeno-, rhabdo-, reo- and orthomyxoviruses. In many cases this inhibition is accompanied by a decrease in the overall rate of protein synthesis in the infected cell. It is likely that this overall inhibition occurs at the level of initiation of protein synthesis since, where it has been examined, the average size of the polysomes in the infected cells is reduced.

The most clearly defined case of virus-induced damage to the translational machinery of the host cell

is the effect of poliovirus on one of the translation initiation factors. Following infection with poliovirus, the cap binding complex responsible for recognition of the capped 5′ end of cellular mRNA is inactivated by a proteolytic cleavage of the p220 component of the complex. It has been speculated that the destruction of the p220 protein confers a selective advantage on the translation of poliovirus messages which are uncapped. Infection with poliovirus also causes the release of host mRNA from the cytoskeleton.

Virus-mediated inactivation of other initiation factors for protein synthesis has also been reported. Translational extracts prepared from VSV-infected cells are deficient in eucaryotic initiation factor 2 (eIF-2) activity in one report and eIF-3 in another, while infection with reoviruses impairs the function of eIF-2. It has recently been shown that vaccinia virus, a poxvirus, encodes a small protein which has significant homology to the α subunit of eIF-2. This protein may function as a replacement initiation factor since there is evidence that it may be important in making the virus resistant to inhibition by interferon.

Another viral strategy to inhibit host protein synthesis involves a direct competition of viral and host RNAs. VSV and reoviruses compete successfully with the host for the translational machinery through sheer abundance of viral transcripts. Mengovirus, a picornavirus, produces mRNA which initiates translation more efficiently than the bulk of the host message and, in addition, synthesizes a factor that causes an overall inhibition of protein synthesis in infected cells.

It has been suggested that selective translation of viral mRNA may also occur following changes in intracellular ion concentrations during infection. Increased plasma membrane permeability is a common cytopathic effect of virus infection which can alter the intracellular levels of sodium and potassium ions. Under conditions that cause increased intracellular sodium ion concentrations, the translation of viral mRNAs may be unimpaired while the translation of host mRNAs is severely reduced. Such a differential effect on virus and host protein synthesis has been reported for cells infected with poliovirus, encephalomyocarditis virus, VSV, reovirus and Sindbis virus. In the case of Sindbis virus, the shutdown of host protein synthesis following infection has been correlated temporally with an increase in permeability of the plasma membrane.

Effects on host cell membranes and cytoskeleton

In addition to altering membrane permeability, virus infection can cause other changes in the membranes of the host cell. Insertion of viral proteins into the plasma membrane can induce syncytia formation by fusing infected cells with neighboring uninfected cells. This fusion can be induced either from without by input virions or from within by newly synthesized viral fusion protein made during infection. The ability to fuse cells, which is characteristic of the paramyxovirus family, is also seen with herpes-, flavi-, lenti-, pox- and coronaviruses. Flaviviruses can also affect internal membranes by causing the proliferation of the rough endoplasmic reticulum, a site associated with the assembly of viral particles. Reo-, picorna- and alphavirus infections frequently produce a significant increase in vesicle formation in the cytoplasm.

Cytolytic virus infections generally cause a progressive loss of integrity of the lysosomal membranes. Two phases of damage are recognized. In the first phase, which in some cases is reversible, the lysosomes become permeable to small molecules and are able to concentrate dyes such as neutral red. Visible evidence of this phenomenon is seen with a mutant strain of Newcastle disease virus, a paramyxovirus, which produces red plaques when assayed using an agar overlay containing neutral red. Concentration of this vital stain in lysosomes can also be detected in cells infected with certain strains of influenza A virus. In this instance a ring of darkly staining cells surrounds a clear area of unstained dead cells. In the second phase of lysosomal damage the membrane becomes so permeable that lysosomal enzymes are released into the cytoplasm. As a rule, this release occurs late in the replicative cycle. The release of lysosomal enzymes into the cytoplasm has been described for a wide variety of viruses such as picorna-, pox-, herpes-, orthomyxo-, paramyxo-, corona-, adeno- and papovaviruses. The mechanism responsible for this type of virus-induced cytopathology and the role it plays in cell death have not been clearly defined.

One of the most common signs of virus-induced cytopathology is cell rounding, a morphological change which has been correlated with alterations in the cytoskeleton. Disruption of one or more of the elements of the cytoskeleton has been described after infection with several viruses. Early gene products of herpes, vaccinia and SV40 viruses produce a disassembly of the actin-containing microfilaments, while infection with polio- and reoviruses causes an alteration and reorganization of the virimentin-containing intermediate filaments of the cytoskeleton. Microtubules, another element of the cytoskeleton, are depolymerized following infection with herpes simplex virus 1 (HSV-1), canine distemper virus and frog virus-3. It has been reported that infection with VSV causes a sequential disassembly of all three filament

components of the cytoskeleton. The mechanisms by which virus infections disrupt the cytoskeleton are not known and it is not clear whether these morphologic changes are a direct effect of some virus product or a secondary consequence of some other aspect of virus-induced cytopathology. It is interesting to note that in normal cells polyribosomes are closely associated with the cytoskeleton, and on the basis of this association it is possible to speculate that some of the effects of virus infection on the host translational apparatus may be caused by virus-induced changes in the integrity of the cytoskeleton.

Viruses also use the structural elements of the cytoskeleton as the work benches for virion assembly and for transport of viral products within the cell. Examples of this function of the cytoskeleton include adenoviruses which appear to use the microtubules for movement within the infected cell; Newcastle disease virus, the viral products of which are associated with actin filaments; and reoviruses which produce inclusion bodies found in association with microtubules and are the site of viral RNA synthesis and virion assembly. It has also been suggested that, in VSV infections, assembly of nucleocapsids occurs in close association with the cytoskeleton.

Inclusion bodies

Another commonly recognized form of virus-induced alteration of the infected host cell is the formation of intracellular masses called inclusion bodies. It should be noted that at the beginning of this century the discovery of a characteristic cytoplasmic inclusion, the Negri body, in cells infected with rabies virus provided an effective diagnostic test for this disease. Depending upon the virus, these intracellular masses may consist of either virions or unassembled viral products. Inclusion bodies may occur in the cytoplasm, as in cells infected with pox-, paramyxo-, orthomyo-, reo-, rubella or rabies viruses, or may be found in the nucleus in cells infected with adeno- and herpesviruses.

Transformation of host cells

In addition to producing various forms of cell destruction, some families of animal viruses are capable of inducing cell transformation. In most cases, transformation is associated with integration of the viral genome into the host cell DNA or maintenance of viral DNA in an episomal state. Only one family of RNA viruses, the retroviruses, is capable of transforming cells. This family of viruses induces transformation through the action of a variety of oncogenes that are cellular in origin and that are not part of or necessary to the virus replicative cycle. There are several families of DNA viruses that are the cause of or are associated with tumor induction in animals and cell transformation in cultured cells. These include polyoma-, adeno-, herpes-, papilloma-, hepadna- and poxviruses. In contrast to the RNA viruses, the genes of DNA viruses responsible for transformation are viral in origin and required for virus replication.

Is It Murder or Suicide?

It is clear from the information reviewed above that the mechanisms responsible for cell death following virus infection have not been clearly defined. Perhaps the reason it has been so difficult to explain how viruses kill cells is that they do not do this directly. An alternative to a direct cell killing is the induction by viruses of a suicide function in infected cells. It would be advantageous for a cell, as part of a metazoan, to induce an apoptosis-like function in response to viral infection rather than to continue on as a factory producing a constant stream of progeny virus. Some recent evidence has appeared that lends support to this possibility. The cytopathic effect of human immunodeficiency virus (HIV) infection has been associated with apoptosis; and in another report, a noncytopathic latent infection of B cells with Epstein–Barr virus (EBV) has been associated with an inhibition of the apoptosis function. In this connection, it is interesting to note that latent infection with EBV blocks the killing of B cells by VSV with little or no effect on the replicative ability of this RNA virus. These observations provide some basis for suggesting that virus-associated cell killing may involve the induction of apoptosis or some other suicide function in the infected cell.

Resistance of Cells to Virus Infection

The major determining factor of the susceptibility of a cell to a particular virus is the ability of the viral attachment proteins to recognize and interact with specific receptors on the cell surface. In many cases, cells are resistant to infection by a particular virus simply because of the lack of appropriate surface receptors. A dramatic example of this type of resistance is seen when chicken fibroblast cells, which lack specific receptor molecules on their plasma membranes, are exposed to poliovirus. Infection does not take place because the viruses cannot adsorb to the cell membrane. However, the avian cells are fully able to support the growth of poliovirus if transfected with the virion RNA rather than infected with intact virions. In addition to cell surface viral receptors, the host range of some viruses can be determined by other

factors such as host cell transcriptional regulators. There is evidence that suggests that viruses from the herpes-, polyoma-, retro- and hepadnavirus families can replicate only in cells that express the appropriate factors that permit recognition of the viral enhancers.

Although viruses have an adaptive advantage in terms of genetic plasticity, cells are not totally powerless to mount a defensive response to viral infection. The best characterized defense that cells have evolved for protection against viral infection is the interferon system. The interferon family of proteins that is produced in response to viral infection promotes the development of an antiviral state through the induction of a second group of proteins. Two of these proteins, the 2′-5′ A synthetase and the protein kinase, have been well characterized and evidence has accumulated that demonstrates their role in the development of the interferon-mediated antiviral state. Perhaps the best evidence to suggest that these proteins are actually involved in the interferon-induced antiviral state comes from the fact that several families of viruses have evolved factors that are capable of blocking the activity of the 2′-5′ A synthetase (herpes- and poxviruses) and the protein kinase (herpes-, pox-, adeno-, reo- and orthomyxoviruses).

Viruses as Tools for Probing the Host Cell

Many of the crucial discoveries concerning cellular processes were offshoots of investigations into the replication cycle of viruses or derived from the use of viruses as model systems. This is particularly true for understanding the mechanisms involved in gene expression. It is apparent that all viruses must use the host cell translational apparatus for the synthesis of viral proteins and that many DNA viruses depend on the host transcriptional and DNA replication machinery as well. Consequently, investigation of the intricacies of viral gene expression has led to the discovery of nearly all identified host factors involved in host genome replication, RNA splicing, enhancer sequences, the scanning model for the initiation of protein synthesis, the use of translational frameshifting for gene expression, and the manner in which proteins are targeted within the cell. This list, which is far from exhaustive, will surely be expanded in the future.

It would be difficult to overestimate the impact of the study of tumor viruses on our understanding of the mechanisms involved in transformation and the nature of the cancer cell. In spite of the fact that most naturally occurring cancers of humans and animals are not caused by viruses, investigation of transforming viruses, and retroviruses in particular, has led to an understanding of the major mechanisms and cellular genes responsible for transformation. A detailed review of this subject can be found elsewhere in this volume.

See also: **Cell structure and function in virus infections; Enteroviruses (*Picornaviridae*): Human enteroviruses (serotypes 68–71); Host genetic resistance; Influenza viruses (*Orthomyxoviridae*): General features; Interferons: Therapy of aids and cancer; Pathogenesis: Animal viruses; Persistent viral infection; Polioviruses (*Picornaviridae*): General features; Replication of viruses.**

Further Reading

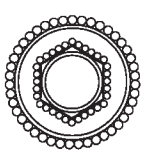
Frankel-Conrat H and Wagner RR (eds) (1984) *Comprehensive Virology,* Vol. 19, *Viral Cytopathology.* New York: Plenum Press.

Knipe DM (1990) Virus–host cell interactions. In Fields BN *et al.* (eds) *Virology,* 2nd edn, p. 1091. New York: Raven Press.

Norkin LC (1995) Virus receptors: implications for pathogenesis and the design of antiviral agents. *Clin. Microbiol. Rev.* 8(2): 293.

[This article is reproduced from the 1st edn (1994).]

VISNA-MAEDI VIRUSES (*RETROVIRIDAE*)



Opendra Narayan, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

History

Visna and maedi are Icelandic terms for two sheep diseases characterized by wasting paralysis and progressive labored breathing respectively. These diseases broke out in epizootic proportions among Icelandic sheep following introduction of European sheep into the local flocks. The newly introduced

factors such as host cell transcriptional regulators. There is evidence that suggests that viruses from the herpes-, polyoma-, retro- and hepadnavirus families can replicate only in cells that express the appropriate factors that permit recognition of the viral enhancers.

Although viruses have an adaptive advantage in terms of genetic plasticity, cells are not totally powerless to mount a defensive response to viral infection. The best characterized defense that cells have evolved for protection against viral infection is the interferon system. The interferon family of proteins that is produced in response to viral infection promotes the development of an antiviral state through the induction of a second group of proteins. Two of these proteins, the 2′-5′ A synthetase and the protein kinase, have been well characterized and evidence has accumulated that demonstrates their role in the development of the interferon-mediated antiviral state. Perhaps the best evidence to suggest that these proteins are actually involved in the interferon-induced antiviral state comes from the fact that several families of viruses have evolved factors that are capable of blocking the activity of the 2′-5′ A synthetase (herpes- and poxviruses) and the protein kinase (herpes-, pox-, adeno-, reo- and orthomyxoviruses).

Viruses as Tools for Probing the Host Cell

Many of the crucial discoveries concerning cellular processes were offshoots of investigations into the replication cycle of viruses or derived from the use of viruses as model systems. This is particularly true for understanding the mechanisms involved in gene expression. It is apparent that all viruses must use the host cell translational apparatus for the synthesis of viral proteins and that many DNA viruses depend on the host transcriptional and DNA replication machinery as well. Consequently, investigation of the intricacies of viral gene expression has led to the discovery of nearly all identified host factors involved in host genome replication, RNA splicing, enhancer sequences, the scanning model for the initiation of protein synthesis, the use of translational frameshifting for gene expression, and the manner in which proteins are targeted within the cell. This list, which is far from exhaustive, will surely be expanded in the future.

It would be difficult to overestimate the impact of the study of tumor viruses on our understanding of the mechanisms involved in transformation and the nature of the cancer cell. In spite of the fact that most naturally occurring cancers of humans and animals are not caused by viruses, investigation of transforming viruses, and retroviruses in particular, has led to an understanding of the major mechanisms and cellular genes responsible for transformation. A detailed review of this subject can be found elsewhere in this volume.

See also: **Cell structure and function in virus infections; Enteroviruses (*Picornaviridae*): Human enteroviruses (serotypes 68–71); Host genetic resistance; Influenza viruses (*Orthomyxoviridae*): General features; Interferons: Therapy of aids and cancer; Pathogenesis: Animal viruses; Persistent viral infection; Polioviruses (*Picornaviridae*): General features; Replication of viruses.**

Further Reading

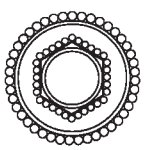
Frankel-Conrat H and Wagner RR (eds) (1984) *Comprehensive Virology*, Vol. 19, *Viral Cytopathology*. New York: Plenum Press.

Knipe DM (1990) Virus–host cell interactions. In Fields BN *et al.* (eds) *Virology*, 2nd edn, p. 1091. New York: Raven Press.

Norkin LC (1995) Virus receptors: implications for pathogenesis and the design of antiviral agents. *Clin. Microbiol. Rev.* 8(2): 293.

[This article is reproduced from the 1st edn (1994).]

VISNA-MAEDI VIRUSES (*RETROVIRIDAE*)



Opendra Narayan, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

History

Visna and maedi are Icelandic terms for two sheep diseases characterized by wasting paralysis and progressive labored breathing respectively. These diseases broke out in epizootic proportions among Icelandic sheep following introduction of European sheep into the local flocks. The newly introduced

Karakul rams had gone through a long pre-importation quarantine period and had come from flocks with no history of the type of disease that broke out in Iceland. These animals were intended to provide a new gene pool for native Icelandic animals which had been maintained in isolation on the island for several centuries. The new diseases spread rapidly among the Icelandic sheep, involving as many as 50% of the animals in some flocks. Maedi was the predominant disease with visna occurring mainly as a complication. The disease complex was finally eradicated from the islands during the 1960s by slaughter of all sheep on the farms that had affected animals. The farms were then restocked with sheep from other parts of the island that had had no contact with the foreign sheep or local sheep with the disease syndromes. Virus obtained from sick animals was the origin of the prototype visna-maedi virus. In 1974, a virus genetically and serologically related to visna-maedi virus was obtained from goats affected with arthritis and encephalitis in Washington State. This virus was named caprine arthritis encephalitis virus (CAEV).

Clinical and Pathological Criteria

Clinical signs of visna are gradual weight loss and gradual weakening of the hind legs followed by ataxia. These signs progress slowly during a period of months to cachexia and paralysis of the hindlimbs. Rarely, forelimbs are also involved in the paralytic syndrome. Signs of maedi are characterized by weight loss, labored breathing, a dry cough and inability to keep up with the rest of the flock. Both of these diseases occur in adult animals, 2–3 years old, and the disease syndromes last 6–8 months. Arthritis synovitis also occurs in some sheep and is the hallmark of the infection in goats. Similar to visna-maedi, this disease appears in adult life of the animals. Clinically, signs of swelling of the carpal joints ('knees') appear first and this progresses to arthritis and eventual lameness. The encephalitis in goats is confined to kids and becomes apparent a few weeks after birth. This progresses to paralysis and eventually death. In all cases, the animals have normal blood counts. They do not develop fever and all have normal appetites. They do not succumb to opportunistic infections.

Pathology

Histologically, all of the lesions in sheep and goats are characterized by infiltration and proliferation of mononuclear cells consisting of macrophages, B lymphocytes and T lymphocytes in the affected tissues. In the brain, this inflammation is accompanied by demyelination and necrotizing changes in the neural parenchyma. In the lungs, these mononuclear cells infiltrate into the interalveolar interstitial areas of the lung causing consolidation of the organ and physical obliteration of the alveoli. The arthritis consists of thickening and infiltration of mononuclear cells into the synovial lining of the joint and accumulation of large amounts of synovial fluid. This is followed later by degeneration and calcification of the articular facets of the joint and eventually degeneration in the bone. Massive lymphadenopathy accompanies these organ-specific diseases.

There are only a few reports of visna from other parts of the world but pulmonary lesions in sheep similar to maedi had been described previously in the USA and subsequently in several other countries except Australia and New Zealand. In the Netherlands the disease was called Zwoegerziekte, in South Africa, Graaf Reinet, in France, La Bouhite, in the USA, Montana lung disease and/or progressive pneumonia, etc. This provides a clear indication at the pathological level that visna-maedi viruses are present in sheep throughout the world. Modern diagnostic procedures have confirmed this, but unlike the experience in Iceland, the disease in most sheep populations occurs mainly sporadically and usually only in adult animals. The caprine disease complex has also been reported in most of the industrialized countries of the world, including New Zealand and Australia. The infection is rare in most underdeveloped countries where herds are small and isolated.

Host Range and Epizootiology

Visna-maedi virus replicates best in sheep and CAEV in goats. However, reciprocal infection has been documented. Further, some strains of ovine virus such as those found in the USA are biologically more similar to CAEV than Icelandic visna-maedi virus. These viruses are infectious for other small ruminant animal species. Such cross-over infections have been documented in zoos. The viruses are spread mainly by colostrum, milk and respiratory exudates. Under field conditions, viral transmission from mother to offspring is very efficient but localized unless the milk is used for feeding other animals. Virus is also disseminated by poor management practices and this has led to local epizootics. A high rate of transmission occurs in poorly ventilated barns during winter months and this is exacerbated further during intercurrent respiratory infections (caused by other agents) that cause increased production of respiratory exudates. Minimal spread occurs during summer months while animals are on pasture. Infected macrophages in colostrum milk and respiratory exudates are a source of the infection. Intercurrent mastitis causes

increased numbers of infected macrophages in the milk and this increases the efficiency of transmission. The major mechanism of dissemination of CAEV has been the dairy husbandry practice by which all kids were fed pooled milk. This guaranteed infection in all kids when only a single infected lactating female may have been present in the herd.

Taxonomy and Classification

By the early 1960s, Icelandic investigators had established that the visna-maedi syndrome was caused by a new virus which replicated productively in stationary ovine cell cultures and caused acute cytopathic effects characterized by fusion of the cells into multinucleated giant cells. Paradoxically, sheep inoculated with the virus at that time remained clinically well for several months. Onset of clinical disease occurred insidiously and progressed slowly but inexorably, leading to death. These findings led to the definition of the viruses as 'slow viruses', the forerunner of the present term, lentiviruses. CAEV had similar biological properties. Some 10 years after the discovery of reverse transcriptase in retroviruses by Temin and Baltimore, the ovine and caprine viruses were both shown to be retroviruses. As lenti-retroviruses, these new agents were distinct from the oncogenic retroviruses which require dividing cells for replication and the oncogenic and transforming properties of which are associated with defective helper virus-dependent replication. The viruses also differ from spuma retroviruses which are not associated with disease. This new ungulate lentivirus, visna, was shown to share similar genetic and biological properties with the lentiviruses of horses, cattle, cats, macaques and humans. Its genome has approximately 9.5 kb of nucleotide sequences and its reverse transcriptase enzyme requires Mg^{2+} instead of Mn^{2+} to catalyze transcription of viral RNA to DNA. In addition to the long terminal repeat (LTR)-gag-pol-env-LTR arrangement of the proviral DNA of retroviruses, visna virus and other lentiviruses also have open reading frames that encode regulatory genes important for replication of the virus. *Tat* and *rev* are examples of these genes.

Variability

The lentiviruses of sheep are genetically and biologically highly heterogeneous. The high mutation rate of the virus has been attributed in part to mistake-prone reverse transcription of viral RNA to DNA. The high mutation rate may be more apparent than real, however, because many mutations in the *env* gene of the virus are viable and viruses with distinct biological properties can be selected by various host systems. Mutant viruses with slow and fast replication rates have been selected by various tissues. Virus-neutralizing antibodies select for neutralization-escape variants (antigenic drift viruses) and macrophages in various tissues such as the brain, lung, synovium and mammary glands select for specific viral phenotypes that replicate optimally in these tissues.

Immune Responses

The envelope proteins of the lentivirus elicit biological responses of greatest importance in the infection. Neutralization epitopes in the envelope consist of either linear peptides or conformational structures and induce antibodies that inhibit replication of the virus. Other epitopes in the viral envelope induce antibodies that bind viral particles but do not neutralize infectivity. These antibodies thus bind to viral particles and enhance entry into target macrophages by Fc receptor-mediated endocytosis. Such antibodies comprise the so-called enhancing antibodies. Cytotoxic CD8 T lymphocytes are also induced by the virus. While these cells, along with neutralizing antibodies, probably lower the virus load *in vivo*, they are incapable of curing the infection, because the virus usually persists for the lifetime of the animal. Part of the failure of the immune responses to eliminate the virus may be due to the integrated unexpressed viral genome in precursor cells in the animal.

Cell Biology and Pathogenesis

The main cell type that supports visna replication *in vivo* is the macrophage-lineage cell, and virus-laden macrophages can be found in all tissues with lesions such as the encephalitic brain, the pneumonic lung, the arthritic joint and mastitic mammary gland. Intense viral replication in local macrophage populations along with local overproduction of cytokines lead to worsening of the inflammatory lesion and more viral replication. Hence, the progressive nature of the lesion and the disease. In animals with organ-specific disease, there is also a high level of infection in precursor cells in bone marrow and in dendritic cells in the blood. In subclinically infected animals that do not have organ-specific lesions, the virus is found at a low rate in the bone marrow and in rare cells in blood but not in tissues. Factors causing the upregulation of viral replication in specific macrophages in tissues have not yet been identified. Studies on viral replication in monocytes (immature macrophages) and in mature macrophages have shown that the viral life

cycle is incomplete in the monocytes and mature viral particle formation occurs only in the mature macrophages. The linkage between the life cycle of the virus to that of the cell is probably due in part to the requirement for DNA binding proteins c-Fos and c-Jun (present only in mature cells) to activate viral transcription in the virus LTR. The LTR of the proviral DNA has AP-1 and AP-4 sites which specifically interact with these proteins in mature cells and result in increased transcription of viral RNA. Linkage of the viral life cycle to the physiology of the macrophage as the cell begins its life cycle in the bone marrow and ends in the tissues has relevance in both the disease state in the animals and the mechanism of transmission of the virus in body fluids to other animals.

Prevention and Control

The most effective mechanism for control of lentiviruses in sheep and goats has been the prevention of infection. This has been achieved by removal of infected animals and their young from flocks, prevention of consumption of colostrum and correction of management practices that facilitate enhancement of the spread of virus in respiratory exudates and milk. The sexual route is not a major mechanism for transmission of these viruses. Lentivirus proteins are poor at inducing protective immunity, and such mechanisms have not been investigated thoroughly.

Future Perspectives

These viruses are agricultural pathogens with effects that are in general too subtle to grasp the attention of regulatory agencies. They provide excellent models of human immunodeficiency virus (HIV) infection since they represent an example of natural lentivirus infection in which the host usually wins the battle. More studies in the future may be directed to investigations on mechanisms by which these hosts keep their viruses in check and mechanisms by which virulent strains of these viruses cause disease.

See also: **Bovine immunodeficiency virus (*Retroviridae*); Feline immunodeficiency virus (*Retroviridae*); Human immunodeficiency viruses (*Retroviridae*): Molecular biology; Pathogenesis: Animal viruses; Simian immunodeficiency viruses (*Retroviridae*).**

Further Reading

Clements JE, Hu L, Lindstrom L, Powell A, Rexroad C and Zink MC (1996) Molecular studies of visna virus gene expression: analysis of envelope gene expression in transgenic sheep. *AIDS Research & Human Retroviruses* 12: 42–3.

Haase AT (1988) Pathogenesis of lentivirus infections. *Nature (London)* 322: 130.

Narayan O and Clements JE (1989) Biology and pathogenesis of lentiviruses. *J. Gen. Virol.* 70: 1617.

Narayan O and Cork LC (1985) Lentiviral diseases of sheep and goats: chronic pneumonia, leukoencephalomyelitis and arthritis. *Rev. Infect. Dis.* 7: 89.

Pepin M, Vitu C, Russo P, Mornex JF and Peterhans E (1998) Maedi-visna virus infection in sheep: a review. *Veterinary Research* 29: 341–67.

[This article is reproduced from the 1st edn (1994).]

W

WAIKAVIRUSES (*SEQUIVIRIDAE*)

Donald T. Gordon, The Ohio State University, Wooster, Ohio, USA

History

Maize chlorotic dwarf was first recognized in the early 1960s in the southeastern USA. Initially, the disease was thought to be incited by the maize dwarf mosaic potyvirus, and in the late 1960s by the Ohio corn stunt agent (CSA-OH). The maize chlorotic dwarf virus (MCDV) was initially characterized in 1972. MCDV and CSA-OH were subsequently shown to be identical. MCDV infections were also frequently associated with symptoms of another similar disease, the Mississippi corn stunt.

Tungro, Philippine for 'degenerated growth', was similarly first recognized in the early 1960s. It was observed at the International Rice Research Institute (IRRI) in the Philippines. The disease was known by other names in countries of South and Southeast Asia and as early as 1859 in Indonesia. Initially, spherical or isometric particles, 30 nm in diameter, were associated with the tungro disease and the virus was named rice tungro virus (RTV). Later, small bacilliform virus particles were also associated with the disease and both spherical and bacilliform particles were observed frequently in rice (*Oryza sativa*) plants expressing tungro symptoms. The virus with the isometric particles was renamed rice tungro spherical virus (RTSV), and the second virus rice tungro bacilliform virus (RTBV). RTSV is also named rice tungro waikavirus. The rice waika disease was discovered in 1971 in southwestern Japan. Initially, the disease was known as the 'waisei' or stunting phenomenon in Japanese. In 1973, the disease became known as 'rice waika' (stunting) and the virus was named the rice waika virus (RWV). RWV is confined to Japan.

Anthriscus yellows virus (AYV) was first isolated in 1968 from *Anthriscus sylvestris*. AYV is the least agriculturally important of the waikaviruses and has been the least studied. Interest in AYV comes from its role as a helper virus in the transmission of parsnip yellow fleck *sequivirus* (PYFV). In the following sections, where nothing is known for AYV reference to the virus is omitted.

Taxonomy and Classification

The genus *Waikavirus* was approved in 1995 by the International Committee on Taxonomy of Viruses (ICTV). Previously, the genus was referred to as the maize chlorotic dwarf virus group by the ICTV (1993). Species of the *Waikavirus* include the RTSV, which is the type species, MCDV, RWV and AYV. The genus is classified in the family *Sequiviridae*. The latter is not classified at upper taxonomic levels of class and order.

The waikaviruses are differentiated from the sequiviruses, the other genus of the *Sequiviridae*, by genome organization and size with the genome of PYFV, the *Sequivirus* type species, being smaller, about 10 kb in length, and lacking a 3′ poly(A) tract. The amino acid sequences of the conserved motifs of the waikavirus polyprotein most closely resemble those of the *Comoviridae* and *Picornaviridae*, and the genome organization that of the *Picornaviridae*. The waikaviruses belong to the picornavirus superfamily.

Geographic and Seasonal Distribution

The geographical distributions of MCDV and RTSV are the western and eastern hemispheres, respectively, where they occur in temperate and subtropical regions. MCDV occurs in 18 states in the southeastern quadrant of the USA, from the east coast to eastern Texas and north to southern Missouri and the Ohio river valley and in Pennsylvania and Arizona and Mexico. MCDV occurrences are delimited by the overlapping distributions of Johnson grass (*Sorghum halepense*), the overwintering host, and the principal vector, *Graminella nigrifrons*.

Tungro, and presumably RTSV, occurs in the Philippines, Malaysia, Indonesia, Thailand, India, Bangladesh, China, Nepal, Pakistan, Sri Lanka and

Vietnam and RWV in Japan. AYV occurs in Scotland and possibly England and other northern European countries where the transmission-dependent PYFV occurs.

During the growing season, MCDV occurs in maize (*Zea mays*) and a few crop and weed grass species. Over winter, when maize is not present, the virus survives in perennial grass species, most notably in the rhizomes of Johnson grass. For RTSV seasonal occurrence is principally in rice, during the growing season, and rice stubble, ratoons and volunteer plants between rice crops. RTSV infects various weed grass species. When long rice-free periods occur between rice plantings, the virus may occur primarily in weed hosts. RWV occurs in rice and in several wild rice species during the growing season. Between seasons the virus survives in rice ratoons and in the roots of rice stubble.

Host Range and Virus Propagation

Hosts of MCDV, RTSV and RWV are species within the *Poaceae*. The principal cultivated host of MCDV is maize, in which economic yield loss occurs. Several other cultivated species including sorghum (*Sorghum bicolor*) are susceptible but no important disease caused by MCDV has been recorded for these species. The principal natural host is Johnson grass, a perennial grass species and the overwintering host of MCDV. Other perennial and grass hosts are known but have no known role in the epidemiology of the virus. Maize is the principal propagation host.

Rice is the principal host of RTSV. In addition to cultivated rice, wild rice, weed grasses and other crop plants are alternate hosts of the tungro viruses. Hosts of RWV include rice and wild rice species. Rice is the propagation host of RTSV and RWV.

Hosts of AYV are *Anthriscus sylvestris* (anthriscus), *A. cerefolium* (chervil) and *Corandrum sativium* (coriander). Coriander is the preferred propagation host.

Genetics

The genomes of MCDV and RTSV contain a large single component, single-stranded (ss), linear, positive-sense, 3′ polyadenylated RNA consisting of one major open reading frame (ORF) which encodes a large polyprotein. The latter is presumably cleaved by a self-encoded protease(s) to yield individual functional proteins, the total number of which is unknown. Further, it is not known whether the 5′ end of the genome has a 7-methylguanosine or a VPg, although a VPg is probable. The sizes of the sequenced genomes are 11 786 nt (MCDV-OH), 11 813 nt (MCDV-TN) and 12 433 nt (RTSV-PH1). The genomes of other RTSV isolates may be 253 nt shorter. The 5′ untranslated leader sequence ranges in length from 435 to 514 nt and is followed by a large ORF which encodes a polyprotein of 3342–3473 amino acids. The 5′ leader contains extensive secondary structure and multiple AUG triplets before the large ORF internal initiation AUG. Immediately downstream of the noncoding 5′ leader is P1, a putative polypeptide of *c.* 78 kDa or 644 amino acids in length. Downstream of the putative P1 protein are the encoding sequences of the capsid proteins CP2, CP3 and CP1 of MCDV or CP1, CP2 and CP3 of RTSV. Electrophoretically estimated M_rs for CP1 (MCDV) and CP3 (RTSV), which are equivalent, are 30–35 kDa; CP2 (MCDV)/CP1 (RTSV), 22.5–27.1 kDa; and CP3 (MCDV)/CP2 (RTSV), 18–24.5 kDa. The three CPs of each virus are immunologically distinct. Polyprotein sequences downstream of the structural proteins contain domains characteristic of proteins with nucleoside triphosphate (NTP) binding, picornaviral serine-like 3C protease and RNA-dependent RNA polymerase (RdRP) domains. A serine-like proteinase, with similarity to the cysteine proteinase of cowpea mosaic *comovirus*, appears to be involved in the cleavage of the individual proteins from the polyprotein. The C-terminal portion of the RTSV polyprotein is processed into a penultimate 35 kDa protease and a C-terminal 68 kDa RdRP. The protein(s) between the C-terminus of CP3 and the C-terminal protease and RdRP has not been identified but contains the NTP-binding domain. The 3′ terminal noncoding region (NCR) sequence ranges from 956 to 1240 nt in length, which is unusually long for a plant virus genome and is a distinctive characteristic of the waikavirus genome. A small ORF (ORF 1) occurs in the 5′ NCR of MCDV-TN and another (ORF 3) in the 3′ NCR. Two small ORFs (ORF 2 and 3) occur in 3′ NCR of RTSV. Subgenomic RNAs but not proteins have been detected for the RTSV small ORFs, whereas neither have been detected for MCDV-TN.

The AYV genome is a single species of RNA with an electrophoretically estimated $10\,600 \pm 70$ nt. Virions contain four electrophoretic CP species of M_rs 35.0, 28.4, 24.3 and 22.3 kDa, respectively.

Evolution

Waikaviruses appear to have an evolutionary relationship with the plant and animal picornaviruses. Waikaviruses resemble the animal picornaviruses in having: (1) a large polyadenylated unsegmented (+)-sense ssRNA genome with a 3′ poly(A) tract; (2) an isometric virion containing three similarly sized CPs;

Table 1 Predicted amino acid sequence identities between portions and the entire polyprotein of MCDV-TN and of other waikaviruses, a sequivirus and plant and animal picornaviruses

Virus	*CP1*	*CP2*	*CP3*	*NTP-binding*	*Proteinase*	*Replicase*	*Entire polyprotein*
MCDV OH or T	60	71	68	86	76	80	60
RTSV	35	41	45	69	38	65	37
PYFV	23	18	24	37	19	41	22
Cowpea mosaic *comovirus*[a]	—	—	—	32	—	33	—
Tomato black ring *nepovirus*[a]	—	—	—	—	—	30	—
Hepatitis A virus[b]	—	—	—	31	—	24	—
Polio virus[b]	—	—	—	24	—	—	—

[a] A plant picornavirus.
[b] An animal picornavirus.
Reproduced from Reddick BB, Habera LF and Law MD (1997) Nucleotide sequence and taxonomy of maize chlorotic dwarf virus within the family *Sequiviridae*. *J. Gen. Virol.* 78:1165–1174, with permission of the senior author and the journal.

(3) location of the CP encoding sequences upstream of the putative genome replication proteins; (4) polyprotein expression; and (5) a polyprotein processing involving a 3C-type virus-encoded protease. Preliminary comparisons of the waikavirus sequences, principally the amino acid sequences of the CP2, NTP-binding region and replicase, with those of the plant and animal picornaviruses support an evolutionary relationship. The greater similarity of the genome organization of the waikaviruses with that of the animal picornaviruses suggests a closer evolutionary relationship between the waikaviruses and the animal picornaviruses than between the latter and the other plant picornaviruses of the *Comoviridae* and *Potyviridae*. This greater similarity suggests that the waikaviruses may be more closely related to the ancestral virus of the plant picornaviruses that may have evolved from the animal picornaviruses.

Serologic Relationships and Variability

MCDV strains include the severe, white stripe (WS), type (T) or Ohio (OH), and M1. MCDV-M1 differs from MCDV-T in that its CP2 and CP3 are slightly larger. Also full, partially full and empty virus particles occur *in situ* and *in vitro* for M1, whereas for T and WS mainly full particles occur. Also, the CP1s of M1 and T show no serological relationship, whereas T and WS are serologically indistinguishable. Infection of maize by individual strains causes only mild symptoms, with M1 causing the mildest symptoms, whereas co-infection by T and M1 incites severe symptoms. MCDV-M1 and MCDV-T show a 54% sequence similarity for a corresponding region of 839 nt at the 3′ end of their genomes. Based on predicted amino acid sequences of the polyprotein, MCDV-TN has a sequence identity of 60% to MCDV-OH. It is less closely related to other picornaviruses (**Table 1**).

The waikaviruses share many characteristics with the animal picornaviruses (see Evolution). Differences include the putative P1 protein, which is larger than the leader (L) proteins of the two *Picornaviridae* genera with L proteins and which is absent for the remaining three genera. Also, MCDV and RTSV lack a CP equivalent to the *Picornaviridae* VP4.

Like MCDV, some RTSV isolates show symptomatological differences. A virulent RTSV strain from the Philippines, Vt6, readily infects rice cultivar TKM6, which is highly resistant to the IRRI strain A. RTSV isolates from India also show differences in virulence on TKM6. RTSV isolates from Thailand, Malaysia and India are serologically indistinguishable from the Philippines isolate when tested with antisera to the individual CPs of the latter. However, CP3 of the Indian RTSV isolate has a slightly higher electrophoretic mobility, which allows it to be differentiated from Southeast Asian isolates. Nucleotide and predicted amino acid sequences of part of the CP1 gene also show that Indian and Bangladeshi RTSV isolates are different from the Philippine and Malaysian isolates, suggesting the existence of two strain groups. Isolates from Thailand and Nepal may be mixtures of the two groups.

RTSV isolates also show variability in their sequences and organization in the 3′ terminus downstream of the large ORF. Significant sequence differences exist in ORF 2 of isolates from the Philippines (RTSV-PH-2), Thailand and India compared to ORF 2 of RTSV-PH-1. Also, a repeating nucleotide sequence in the 3′ NCR of RTSV-PH-1 is absent in the sequences of the other isolates. Because RTSV causes indistinct symptoms on many rice varieties and antisera to the virion or individual CPs

do not differentiate isolates, sequence analysis will be required to differentiate additional RTSV variants.

In Japan two strains of RWV occur: the common or C strain and a more virulent strain S. Since RWV or RTSV assists in the transmission of RTBV by the leafhopper *Nephotettix virescens* and symptoms incited by RWV in combination with RTBV are similar to those of the tungro virus complex (RTBV + RTSV), RWV is believed to be identical or closely related to RTSV.

Epidemiology

The epidemiology of the waikaviruses depends principally on virus and vector sources for early infections. MCDV survives winters in the USA in the rhizomes of infected Johnson grass. Its principal leafhopper vector, *G. nigrifrons*, is one of the most common leafhopper species in the eastern half of the USA and frequently the most abundant leafhopper on maize. In the southern USA, *G. nigrifrons* overwinters as adults and eggs on winter grasses, grains and grazing crops which allow survival in the absence of maize. Most of these overwintering hosts are not virus hosts. In the northern USA, where winter temperatures are probably too low for leafhopper survival, *G. nigrifrons* may be introduced at the beginning of each growing season by migration from the southern overwintering areas. *G. nigrifrons* presumably acquires MCDV from virus-infected Johnson grass early in the season and initiates the disease anew in seedling maize.

Outbreaks of tungro are unpredictable and appear suddenly over large areas, frequently followed by disease disappearance in the next season. Virus sources for these outbreaks include infected rice stubble and volunteer rice ratoons, which may act as vector sources as well, especially in asynchronously planted areas. Weed grasses generally are relatively poor virus sources. Rice nursery beds may also serve as a virus source. In single-crop areas with a long rice-free period, virus may be introduced by long distance migration of viruliferous leafhoppers from virus endemic areas and locally by leafhoppers from infected weeds in and near rice plantings. *N. virescens*, the dominant and most efficient vector, is monophagous and prefers rice, whereas the relatively inefficient *N. nigropictus* prefers weeds. In areas of overlapping rice plantings, *N. virescens* may be of greater importance in virus spread and survival, whereas in single-cropping areas, *N. nigropictus* may be important in transmitting the virus among weed hosts in fallow fields.

The primary source of RWV for leafhopper acquisition is infected rice plants, rice stubble and spring ratoons. Winter host plants of *N. cincticeps*, the principal vector, are restricted to several grass species that are not virus hosts. Since rice plants do not survive over winter in most areas of Japan where RWV occurs owing to cold temperatures, migration of *N. virescens* from the disease-free islands, where it overwinters, to the areas of virus occurrence and then to areas where the virus does not overwinter may perpetuate RWV in the latter.

Transmission and Tissue Tropism

The waikaviruses are obligately vector transmitted in nature. The principal vectors of MCDV, RTSV and RWV are the leafhopper species *G. nigrifrons, N. virescens* and *N. cincticeps*, respectively. AYV is transmitted by the aphid *Cavariella aegopodii*. These vectors acquire and inoculate the viruses following a 10–15 min to 2 h acquisition access period and a 2 min to 2 h inoculation access period, respectively, and usually lose inoculativity within up to 4 days following acquisition and always following a molt. Preacquisition starvation has no effect on transmission efficiency and there is no detectable latent period in vectors. The viruses are not transovarially transmitted in their vectors. Leafhopper nymphal instars as well as adults transmit the viruses with similar efficiencies. The virus–vector relationship is semipersistent and transmission presumably involves a virus-encoded helper component (HC) protein.

RTSV is required for transmission of RTBV, which occurs only when the leafhopper has previously or simultaneously fed on RTSV-infected plants from which it acquires the putative RTSV HC. RTSV can be transmitted independently. AYV also acts as a helper virus for the transmission of PYFV, presumably by providing an HC for the transmission of PYFV.

Vectors acquire the viruses while ingesting from phloem cells and retain the viruses at sites on the lining of the vector's foregut. The particles are surrounded by a lightly or densely staining matrix, which in turn is embedded in a lightly staining material (M material) overlying the lining of the foregut. The matrix presumably allows virus retention within the vector for transmission. The viruses are released from these sites to infect phloem cells when the vectors extravasate during subsequent phloem feeding. The viruses are mainly phloem limited in infected plants and presumably move cell-to-cell via plasmodesmata.

Pathogenicity

Maize chlorotic dwarf and rice tungro are incited by

co-infections of MCDV and RTSV plus other viruses, respectively. For maize chlorotic dwarf, the severe symptoms are caused by synergistic co-infections of MCDV-T and MCDV-M1, which individually cause mild symptoms. However, not all field occurrences of the disease involve co-infections by isolates of the two viruses, making the disease etiology problematic.

Rice tungro is caused by co-infections of RTSV and RTBV. RTBV alone causes symptoms of moderate stunting and dark green and transitory yellow-orange discoloration of leaves, whereas RTSV alone causes no conspicuous symptoms. RWV occurs alone unassociated with RTBV.

AYV may occur alone or with PYFV.

Clinical Features and Infection

The principal symptoms incited by MCDV on susceptible maize are a reduction in plant height, chlorotic clearing or banding of tertiary veins and red or yellow discoloration of upper leaves. Symptoms of plant stunting or dwarfing and red or yellow leaf discoloration are often associated with infections of maize by the corn stunt spiroplasma or maize bushy stunt phytoplasma. MCDV infections can be differentiated from infections by the latter two pathogens by the diagnostic chlorotic vein clearing or banding of tertiary veins.

RTSV incites almost no symptoms and infected plants appear green with only mild stunting. Moderately resistant cultivars may exhibit recovery from tungro symptoms incited by RTSV plus RTBV, whereas stunting and leaf discoloration persist on susceptible cultivars. Tungro symptoms may show similarity to feeding damage caused by *N. virescens*, vector of both viruses, and symptoms caused by leafhopper feeding may be mistaken for those caused by the viruses and *vice versa*. Both the tungro virus (RTSV plus RTBV) and *N. virescens* cause foliar discoloration (orange or reddish-brown), reduction in tiller numbers, plant stunting and plant death when large numbers of leafhoppers feed on rice plants during tillering. Plants recover from leafhopper- but not virus-caused stunting.

RWV causes stunting and yellowing of leaves, but symptoms are less severe than those of RTSV plus RTBV. Leaf discoloration ranges from green to yellow and is transitory, not appearing on subsequently emerging leaves. Stunting symptoms persist.

AYV is associated with no conspicuous symptoms for infections of *Anthriscus sylvestris*, its principal host, but causes stunting and reddish purple leaf discoloration on *A. cerefolium*.

Pathology and Histopathology

Waikaviruses, which have 30 nm diameter isometric particles, are mostly phloem-limited in sieve tubes, companion cells and phloem parenchyma and cause distinctive inclusions detected by both light and electron microscopy. For MCDV, these inclusions include electron-dense granular and fibrous, fibriform or striated sheet inclusions. The former resemble the currant-bun inclusions of AYV and the viroplasms of RTSV and RWV. The fibrous inclusions are unique to MCDV infections. For the M1 and WS strains of MCDV, accumulations of virus-like particles occur within the dense granular inclusions, whereas with type strain isolates most of these inclusions contain no or very few virus-like particles. Individual virus-like particles are rarely found in the surrounding cytoplasm. In maize leaf cells infected with the WS isolate, the chloroplasts are deformed; and in stunted plants doubly infected with MCDV-T and MCDV-M1, some phloem cells degenerate. Histological changes include plugging of xylem but not phloem, reduced thickness of the leaf cuticular layer, and reduction of the diameter of the sieve elements in the rachis.

In RTSV-infected cells, inclusions include amorphous X bodies and lattice or tubular structures as well as viroplasms. Viroplasms are not membrane bound and appear as masses of electron-dense bodies. Virus particles are mostly scattered in the cytoplasm or vacuoles. Small vesicles, containing fibers and occasionally RTSV particles, are found in the cytoplasm, usually along the cell wall. RWV particles present in vacuoles are often in crystalline arrays. Phloem necrosis is observed with RWV infection. AYV-infected chervil petioles show phloem cells containing clusters of isometric virus-like particles embedded in amorphous densely staining material.

Prevention and Control

The recommended control for MCDV, RTSV and RWV is the cultivation of virus-resistant or -tolerant cultivars. For MCDV, no maize hybrids are immune and only a few are highly resistant. Other means of MCDV control include early planting, herbicide eradication of Johnson grass, and leafhopper vector control using the systemic insecticide carbofuran. Of these, widespread Johnson grass eradication and vector control are effective but problematic, being costly and potentially harmful to the environment. Cultivation of resistant or tolerant hybrids in combination with Johnson grass eradication and early planting is sufficient to limit yield loss due to the virus.

While RTSV causes only mild to inconspicuous

symptoms and presumably little or no yield loss, its control is important because of its role in RTBV transmission by the leafhopper vector and in inciting the severe symptoms of tungro when co-infecting with RTBV. While control of tungro is possible by use of vector resistance, the latter has been short-lived due to its breakdown after a few seasons. Resistance in rice is available for RTSV, but not for RTBV, and can provide an effective control of tungro in large fields, as plants infected with RTBV do not serve as sources for secondary spread of the virus in the absence of RTSV. However, RTSV resistance may be ineffective in small plots where high numbers of viruliferous vectors migrate from neighboring fields. Other means of tungro control include: (1) eradication of rice stubble and volunteer seedlings; (2) early transplanting; (3) large scale synchronous rice planting followed by a sustained fallow period; and (4) application of insecticides, especially carbofuran, for vector control. Control of RWV involves use of insecticides and cultivation of resistant varieties.

Future Perspectives

To complete knowledge of the waikavirus genome organization and protein functions, determination of the function(s) and the numbers and sizes of proteins is needed for the following portions of the polyprotein: the putative P1 protein downstream of the 5′ leader sequence and the sequences downstream of the structural proteins which contain an NTP-binding domain. Information is also needed on whether an RTSV-like C-terminal protease and RdRP are also present in the C-terminus of the MCDV polyprotein. Also, it is not known whether the cysteine-like C-terminal protease alone cleaves the viral polyproteins to release all the functional proteins. Functions are also unknown for the unusually long 5′- and 3′-terminal NCR and the small ORFs of the terminal NCRs of several waikavirus genomes. The designations of the three CPs (CP1, CP2 and CP3) need to be uniform among the waikaviruses. Finally, the relationship of the encapsidated small RNAs, found in slower sedimenting particles of some waikaviruses, to their associated viruses is unresolved.

Among the unresolved taxonomic questions is the relationship between MCDV-OH and MCDV-TN, which seem sufficiently distinct based on sequence identities to consider them as distinct waikaviruses. MCDV-M1 may also be a distinct species, but the similarity of its sequence to that of MCDV-TN is unknown. Also, nucleotide sequence information for the RWV genome would resolve its relationship with RTSV.

Other matters concerning the waikaviruses in need of demonstration are the HC protein, the role of MCDV-OH and -M1 in inciting the severe symptoms of maize chlorotic dwarf, and biological differences among waikavirus isolates differentiated by sequence and sometimes serological characteristics.

Finally, several characteristics of AYV raise the question of whether it is properly classified as a waikavirus. These characteristics are its apparent smaller genome, the presence of four rather than three CP species, an aphid rather than a leafhopper vector, and a dicot rather than monocot host range. The sequence of the AYV genome is needed to resolve this question.

See also: **Sequiviruses (*Sequiviridae*); Plant virus disease – economic aspects; Pathogenesis: Plant viruses.**

Further Reading

Anjaneyulu A, Satapathy MK and Shukla VD (1995) *Rice Tungro*. Lebanon, NH: Science Publishers, Inc.

CAB International (1999) Maize chlorotic dwarf waikavirus [original text by D. T. Gordon]. In: *Crop Protection Compendium*. Wallingford, UK: CAB International.

Hull R (1996) Molecular biology of rice tungro viruses. *Ann. Rev. Phytopathol.* 34: 275.

Thole V and Hull R (1998) Rice tungro spherical virus polyprotein processing: identification of a virus-encoded protease and mutational analysis of putative cleavage sites. *Virology* 247: 106.

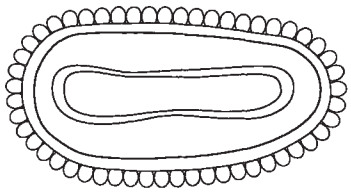
Wesselbron Virus *see* **Encephalitis Viruses**

West Nile Encephalitis Virus *see* **Encephalitis Viruses**

Western Equine Encephalitis Virus *see* **Equine Encephalitis Viruses**

Y

YABAPOX AND TANAPOX VIRUSES (*POXVIRIDAE*)



HA Rouhandeh, Torrey Pines Cancer/Aids Research Institute, San Diego, California, USA

History

The disease produced by Yabapox virus is characterized by formation of tumors that regress slowly. The disease was first observed in 1958 in captive Rhesus monkeys housed in open pens in Yaba, Nigeria. At first the disease was confined to Asian monkeys and one baboon. Later one species of African monkey (*Cercopithecus aethiops tantalus*) became infected with the virus. In 1969 a spontaneous outbreak of the disease occurred in monkeys housed in Roswell Park Memorial Institute in Buffalo, New York. Infection of a laboratory worker reported in 1963 showed the virus to be pathogenic for humans.

The disease produced by Tanapox virus is characterized by a mild febrile illness in humans with one or two pock-like lesions developing on the upper part of the body. Two outbreaks occurred in 1957 and 1962 among the indigenous population living in the Tana River Valley in Kenya. In 1966 outbreaks occurred in monkey colonies in the USA and in humans having contact with the animals. Although Tanapox virus was first isolated from humans during an outbreak of the disease, it, like Yabapox virus, primarily causes a disease of macaque monkeys.

Taxonomy and Classification

The International Committee for Taxonomy of Viruses (ICTV) has proposed a classification of *Yatapoxviruses* as the genus name for Yabapox virus and Tanapox virus (as well as Yabavirus-like disease) within the *Poxviridae*.

Evolution

Both Tanapox and Yabapox viruses have been identified within the last 40 years. As far as is known, both viruses originated in monkeys. Human infections of Tanapox are believed to have originated from monkeys through the mosquito as a vector.

Properties of the Virion

Yabapox virus measures 250–280 μm in the long axis. The surface consists of thread-like structures which are randomly formed around the viral particle. Both Yabapox and Tanapox viruses are symmetrical and dumbbell-shaped; both contain a core with lateral bodies in the concavities and a double lipoprotein bilayer envelope comparable to other poxviruses.

Properties of the Genome

Yabapox and Tanapox viruses contain linear double-stranded DNA; the number of segments of 3′ terminal poly(A) tracts present or absent is presently unknown for these viruses. The size of both DNAs is estimated to be approximately 145 kbp with a molecular mass of 119×10^3 kD. The G+C content is $32.5 \pm 0.5\%$.

Properties of Virus Proteins

Tanapox virus contains approximately 55 proteins ranging in size from about 200 to 11 kDa. Yabapox virus contains approximately 44 proteins of which 21 are core-associated, ranging in molecular mass from 220 to 10 kDa. Four enzymatic activities have been identified with purified Yaba virions: DNase with pH optimum at 5.0; DNase with pH optimum at 7.8; RNA polymerase; and nucleotide phosphohydrolase.

Physical Properties

Yabapox viral DNA has a density of 1.6905 g ml^{-1} in CsCl and its T_m value in 0.015 M citrate in saline is 82.3°C.

Replication of Yabapox Virus

Nucleic acid

Viral DNA is detected 3 h after infection. At 6 h postinfection 7–10S RNA is detected which increases in amount after 12 h. At 24 h after infection, 14–15S RNA as well as 7–10S RNA is detected. The first and largest peak of mRNA synthesis occurs between 11 and 12 h postinfection and a second slightly smaller peak occurs between 21 and 23 h after infection. Late in the infection cycle, viral DNA is present in the host cell nucleus.

Protein synthesis

Yabapox viral proteins are synthesized at different times after infection and can be grouped into two classes, early and late. Early proteins are synthesized before the onset of viral DNA replication (3 h postinfection). Some of the proteins in this group are structural and continue to be synthesized in the presence of DNA inhibitor. Late viral proteins are detected at 6 h postinfection and continue to increase in number during the infection period. Viral infection does not inhibit host protein synthesis.

Virus synthesis

Yabapox virus is synthesized in the cytoplasm. At 35°C, the minimum length of replicative cycle is 35 h; however, maximum virus yields are not obtained until 75 h postinfection. Synthesis of at least two viral structural antigens occurs in the presence of the DNA inhibitor, cytosine arabinofuranoside, indicating potential transcription and translation of these antigens from parental DNA. The first progeny DNA is completed after 20 h postinfection, but is not detected in infectious form until 35 h postinfection. The maximum rate of progeny DNA synthesis occurs between 20 and 30 h postinfection. Viral DNA synthesis continues until 45–50 h after infection.

Morphogenesis

Within 3 h postinfection, the adsorption and phagocytosis of Yabapox virus particles by the cells can be seen by electron microscopy. This is followed by the disruption of the phagocytic vacuole membrane, with the release of viral DNA into the cytoplasm. At 24 h postinfection, large cytoplasmic inclusions termed 'factories' are observed. A typical factory contains a large number of viral particles, particulate glycogen, DNA-containing electron dense material, and small membranous spherical structures (40 nm in diameter) designated as 'micelles'.

Transformation

Various cell lines of monkey kidney cells have been transformed with UV-irradiated Yabapox virus. Two different cloned restriction enzyme fragments can also transform CV-1 monkey kidney cells. Transformed cells do not produce virus but exhibit biological characteristics typical of transformed cells. These include increased saturation density, reduced serum requirements for growth, and ability to grow in soft agar. The morphological alterations of transformed cells are similar to Yabapox virus-induced tumor cells and are characterized by loss of contact inhibition, multinucleated cells, and cytoplasmic lipid droplets. Southern blot hybridization showed that sequences homologous to low molecular weight viral DNA (5.1, 4.8 and 3.9 kbp) are present in transformed cells. Virus-specific antigens detected by immunofluorescence assays are found in the cytoplasm of transformed cells. Four virus-specific proteins, with molecular masses of 160, 140, 108 and 74 kD, are contained in transformed cells immunoprecipitated with sera from tumor-bearing monkeys.

Serological Relationships and Variability

Serologically Tanapox and Yabapox viruses show minimal to moderate cross-reactivity. The presence of type-common and type-specific antigens has been demonstrated. Yabapox viral infection fully protects primates against Tanapox virus challenges. In monkeys infected with Tanapox virus then challenged with Yabapox virus, symptoms were delayed in developing, and histiocytomas were smaller.

Complement fixing (CF) and complement fixing inhibiting (CFI) antibodies were demonstrated in the clinical and convalescent stages, respectively, of Rhesus monkeys infected with either Tanapox or Yabapox viruses. The persistence of CF antibody in monkeys infected with Tanapox virus is from 10 to 12 weeks; in monkeys infected with Yabapox virus CF antibody persists up to 35 weeks postinfection.

Epidemiology

The epidemiology of Tanapox virus is believed to be a reservoir of monkeys in the wild in Kenya from which the natives of the Tana River Valley are occasionally infected as a result of mosquito transmission of the virus. An outbreak occurred in monkey colonies in California, Oregon and Texas in 1965 and 1966 and spread to men in contact with the housed diseased animals. Tanapox of man is essentially a zoonosis. Yabapox is believed to be epidemic in scope in African and Asian monkeys. Infection in man occurs only through injection of the virus.

Transmission and Tissue Tropism

Yabapox and Tanapox viruses are both believed to be transmitted by insect vectors. Yabapox virus transforms fibrocytes of the dermis and subcutaneous cells to pleomorphic polygonal cells. The histiocyte gives rise to the tumor. In monkeys inoculated with Yabapox virus, histiocytes migrate into the infected area by 48 h postinoculation. After 3–5 days, the histiocytes undergo striking morphologic alterations and proliferate rapidly, leading to tumor formation. Intravenous inoculation results in many tumors in the heart, lungs, muscles and subcutaneous tissues of susceptible monkeys.

Tanapox viral infection is histiologically distinct from Yabapox. It affects the epidermis almost exclusively, resulting in hypertrophy and thickening of the epithelial layers of the skin with swelling and ballooning of the deeper epithelial cells; there is little cellular infiltration into the underlying dermis.

Pathogenicity

Because of the lack of studies on the prevalence of either Yabapox or Tanapox disease in the population of monkeys in the wild, little is know about pathogenicity. However, both viruses are of low pathogenicity in housed monkey populations. Pathogenicity from monkeys to humans is probably nonexistent in the case of both viruses in that each depends upon a vector or artificial means of transmission.

Clinical Features of Infection

In humans, Tanapox starts with a short febrile illness lasting 3–4 days and sometimes a severe headache, backache and pronounced prostration also occur. During the course of the febrile illness, one or two pock-like lesions appear on the upper part of the body, usually on the upper arm, face, neck and trunk. The lesion resembles a modified smallpox lesion in a vaccinated individual, except there is no pustulation in the Tanapox lesion. The illness is nonfatal and of short duration.

In monkeys, Yabapox is characterized by tumors on the hairless areas of the face, on the palms and interdigital areas and on the mucosal surfaces of the nostrils, sinuses, lips and palate. The benign tumors develop 5 days after inoculation, grow to 25–45 mm in diameter and project up to 25 mm in diameter. Tumor growth proceeds steadily, reaching a maximum in 6 weeks, after which regression occurs and is completed by 12 weeks postinoculation.

Pathology and Histopathology

In Yabapox infection tumor cells which develop are characterized by the appearance of multinucleated cells, cytoplasmic granulation, nuclear enlargement, nucleolar hypertrophy and the formulation of numerous lipid vacuoles in the cytoplasm. Granular inclusions in the cytoplasm stain positively for DNA with acridine orange.

Tanapox virus infection is characterized by hypertrophy and thickening of the epithelial layers with swelling and ballooning of the deeper epithelial cells, which show vacuolation of cell nuclei and eosinophilic cytoplasmic inclusions.

Immune Response

In Yabapox, circulating neutralizing antibody is ineffective in preventing growth of established tumors. Immunity to superinfection is present when tumors are present or regressing, but after total regression of tumors, re-infection results in new tumor formation.

In Tanapox infection immunity persists in monkeys for at least nine months following healing of the skin lesions.

Prevention and Control

Prevention of Tanapox in the human population is thought to be controllable by controlling the mosquito population and avoidance of mosquito-infested areas where the disease is epidemic in monkeys. Control of both diseases in housed monkeys has been accomplished by control of insects and by strict isolation of infected animals.

Future Perspectives

The control of Tanapox in the human population presents a serious problem because of the highly isolated and rare epidemiology of the disease and its non-serious nature. More studies of wild monkey populations where the disease is epidemic are needed in order to determine for certain how the disease is spread. If both diseases are spread by mosquitoes as postulated, complete control is unlikely to be achieved.

See also: **Smallpox and monkeypox viruses (*Poxviridae*); Zoonoses.**

Further Reading

Amano H, Ueda Y and Miyamura T (1995) Identification and characterization of the thymidine kinase gene of Yaba virus. *J. Gen. Virol.* 76 (Pt 5): 1109.

Knight C *et al.* (1989) Studies on Tanapox virus. *Virology* 172: 116.
Rouhandeh H (1988) Yaba virus. In: Darai G (ed.) *Virus Diseases in Laboratory and Captive Animals*. Boston: Martinus Nijhoff Publishers.
[This article is reproduced from the 1st edn (1994).]

YEAST RNA VIRUSES (*TOTIVIRIDAE*)

Lionel Benard, Herman Edskes, Juan Carlos Ribas and **Reed B Wickner**, Laboratory of Biochemistry and Genetics, National Institutes of Health, Bethesda, Maryland, USA

History

Some strains of the yeast *Saccharomyces cerevisiae* secrete a protein toxin that kills other strains, but to which the secreting strain is itself immune. Toxin production ability is inherited as a non-mendelian trait. This finding (in 1963) by Makower and Bevan led to the discovery of viral particles containing various double-stranded RNAs (dsRNAs), one of which, called M, was correlated with the killer phenomenon. Studies of this area have taken two directions, one concerned with the virology of the system, and the other with the processing, secretion and action of the toxin and the mechanism of immunity.

Classification

Yeast RNA viruses belong to the genus *Totivirus* of the *Totiviridae* family. Other genera in the family are *Giardiavirus* and *Leishmaniavirus*.

Yeast RNA Replicons

Four completely distinct RNA to RNA replication systems have been found in various strains of *S. cerevisiae*: L-A and its satellites (including M), L-BC, 20S RNA and 23S RNA. The L-A and L-BC systems are encapsidated, whereas the 20S and 23S RNAs apparently replicate naked in the cells (**Table 1**). None of the viruses are known to have a natural extracellular infectious cycle, although they can be introduced into spheroplasts along with transforming DNA plasmids. All are efficiently transmitted from cell to cell by the cytoplasmic mixing that occurs on mating. Perhaps because mating is a very frequent event for yeasts in nature, these viruses are widespread and most strains have more than one. The best studied is the L-A virus (**Fig. 1**) and its satellite viruses, M_1, M_2, M_3 and M_{28}, encoding different killer toxins and immunity functions. Defective interfering deletion mutants of L-A and M_1 have been described. Yeast strains also contain at least four families of retroviruses. Recently, brome mosaic virus and flockhouse virus have been found able to replicate in yeast, a result that promises further

Table 1 RNA replicons in *Saccharomyces cerevisiae*

	kb	*Proteins encoded*	*Comments*
dsRNA viruses			
L-A	4.6	76 kDa major coat protein = Gag 170 kDa Gag-Pol fusion protein	Single segment ribosomal frameshifting no 5′ cap or 3′ poly(A) Gag has decapping activity
M_1, M_2, M_{28},...	1.0–1.8	Preprotoxin-immunity protein	Satellites of L-A
L-BC	4.6	78 kDa major coat protein = Cap 175 kDa Cap-Pol fusion protein	
ssRNA replicons			
20S RNA	2.8	90 kDa RNA-dependent RNA polymerase	W dsRNA is RF; circular form seen
23S RNA	3.2	RNA-dependent RNA polymerase	T dsRNA is RF supported by cDNA clones for segs. 1 and 2
Brome mosaic V. segment 3		*URA3*	

Knight C *et al.* (1989) Studies on Tanapox virus. *Virology* 172: 116.
Rouhandeh H (1988) Yaba virus. In: Darai G (ed.) *Virus Diseases in Laboratory and Captive Animals*. Boston: Martinus Nijhoff Publishers.
[This article is reproduced from the 1st edn (1994).]

YEAST RNA VIRUSES (*TOTIVIRIDAE*)

Lionel Benard, Herman Edskes, Juan Carlos Ribas and **Reed B Wickner**, Laboratory of Biochemistry and Genetics, National Institutes of Health, Bethesda, Maryland, USA

History

Some strains of the yeast *Saccharomyces cerevisiae* secrete a protein toxin that kills other strains, but to which the secreting strain is itself immune. Toxin production ability is inherited as a non-mendelian trait. This finding (in 1963) by Makower and Bevan led to the discovery of viral particles containing various double-stranded RNAs (dsRNAs), one of which, called M, was correlated with the killer phenomenon. Studies of this area have taken two directions, one concerned with the virology of the system, and the other with the processing, secretion and action of the toxin and the mechanism of immunity.

Classification

Yeast RNA viruses belong to the genus *Totivirus* of the *Totiviridae* family. Other genera in the family are *Giardiavirus* and *Leishmaniavirus*.

Yeast RNA Replicons

Four completely distinct RNA to RNA replication systems have been found in various strains of *S. cerevisiae*: L-A and its satellites (including M), L-BC, 20S RNA and 23S RNA. The L-A and L-BC systems are encapsidated, whereas the 20S and 23S RNAs apparently replicate naked in the cells (**Table 1**). None of the viruses are known to have a natural extracellular infectious cycle, although they can be introduced into spheroplasts along with transforming DNA plasmids. All are efficiently transmitted from cell to cell by the cytoplasmic mixing that occurs on mating. Perhaps because mating is a very frequent event for yeasts in nature, these viruses are widespread and most strains have more than one. The best studied is the L-A virus (**Fig. 1**) and its satellite viruses, M_1, M_2, M_3 and M_{28}, encoding different killer toxins and immunity functions. Defective interfering deletion mutants of L-A and M_1 have been described. Yeast strains also contain at least four families of retroviruses. Recently, brome mosaic virus and flockhouse virus have been found able to replicate in yeast, a result that promises further

Table 1 RNA replicons in *Saccharomyces cerevisiae*

	kb	*Proteins encoded*	*Comments*
dsRNA viruses			
L-A	4.6	76 kDa major coat protein = Gag 170 kDa Gag-Pol fusion protein	Single segment ribosomal frameshifting no 5′ cap or 3′ poly(A) Gag has decapping activity
M_1, M_2, M_{28},...	1.0–1.8	Preprotoxin-immunity protein	Satellites of L-A
L-BC	4.6	78 kDa major coat protein = Cap 175 kDa Cap-Pol fusion protein	
ssRNA replicons			
20S RNA	2.8	90 kDa RNA-dependent RNA polymerase	W dsRNA is RF; circular form seen
23S RNA	3.2	RNA-dependent RNA polymerase	T dsRNA is RF supported by cDNA clones for segs. 1 and 2
Brome mosaic V. segment 3		*URA3*	

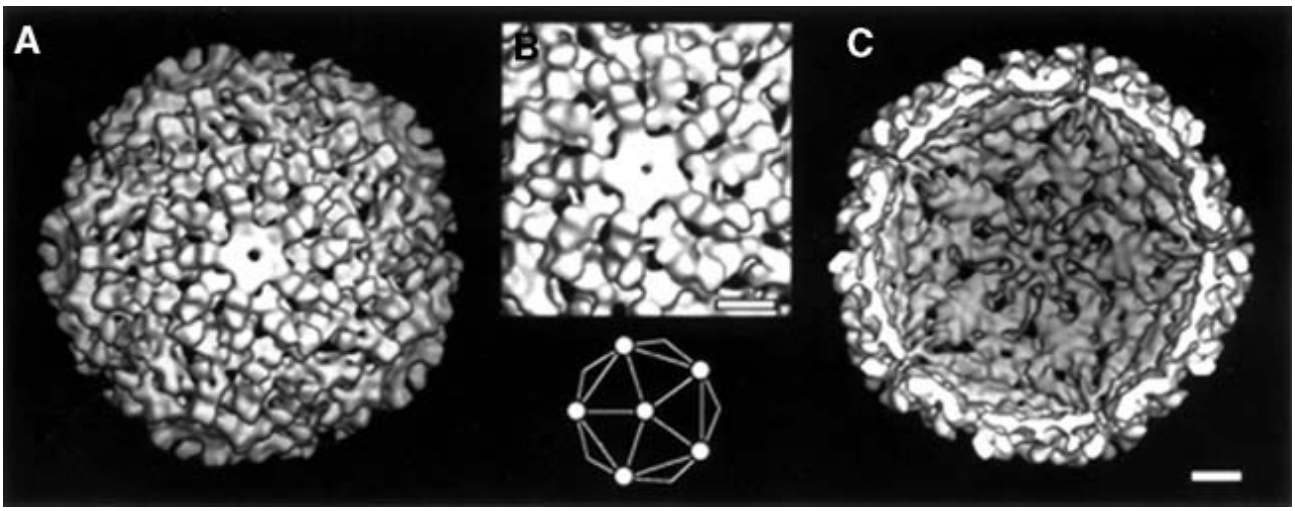


Figure 1 Cryo-electron microscopic reconstruction of L-A viral particles shows them to have icosahedral symmetry with a T=1 triangulation number. Each particle is composed of 120 Gag molecules, 60 pairs of Gag molecules with the members of a pair in two different environments. The outer (**A**, **B**) and inner (**C**) surfaces are shown at 20 Å resolution. Scale bar, 50 Å. (Reproduced with permission from Steven AC, Trus BL, Booy FP *et al* (1997) The making and breaking of symmetry in virus capsid assembly: glimpses of capsid biology from cryoelectron microscopy. *FASEB J.* 11: 733.)

widening of the scope of application of yeast studies to virology. Yeast RNA replicons are summarized in **Table 1**.

L-A Replication Cycle

L-A is a dsRNA virus that has a single 4.6 kb segment, and viral particles contain a single molecule of L-A dsRNA. The viral particles have a 76 kDa major coat protein (Gag) and a minor 170 kDa protein (Gag-Pol) whose N-terminal portion is a Gag monomer and whose C-terminal portion binds ssRNA and is the RNA polymerase. A conservative transcriptase activity of the particles produces viral plus-strands and extrudes them from the particles. The plus ssRNA is translated to form viral particle proteins (see below) and is also the species packaged to form new viral particles. These particles then make the minus-strand on the plus-strand template to form L-A dsRNA-containing particles and complete the cycle (**Fig. 2**).

Satellite viruses of L-A, such as M_1, use the L-A-encoded coat proteins. Again, a single viral plus-strand is packaged to form new particles, but after replication and transcription, the new transcripts often remain inside the viral particle where a second (or more) dsRNA molecule is formed by a second replication event. This is called 'headful replication' to distinguish it from the 'headful packaging' phenomena seen in many bacteriophages. In the case of L-A and its satellites, a single viral plus-strand is packaged and then it replicates inside the particle until the head is full. Then all new transcripts are extruded from the particle.

The *cis* signals responsible for packaging and replication have been defined on L-A (**Fig. 3**) and M_1. The packaging proteins recognize a stem–loop structure located about 400 nucleotides from the 3′ end of either L-A or M_1. The sequence of the stem is not important, only that it is a stem, but an A residue that protrudes on the 5′ side of the stem is critical, as is the loop sequence. The replication reaction requires that the template has both an internal site that largely overlaps with the packaging site (and may be identical to it) and a specific 3′-terminal sequence and structure.

The packaging of viral (+) strands involves recognition of the packaging site by residues 67–213 of the Pol domain of the Gag-Pol fusion protein. There are two copies of the fusion protein per particle.

L-A Expression: Ribosomal Frameshifting in a dsRNA Virus

L-A plus-strands encode the 76 kDa viral major coat protein (called Gag in analogy with retroviruses) and a 170 kDa Gag-Pol fusion protein, formed, as in the case of retroviruses, by a −1 ribosomal frameshift (**Fig. 3**). The structure responsible for the frameshifting is a slippery site, GGGUUUA, followed by an RNA pseudoknot, both located in the region of overlap of the *gag* and *pol* open reading frames. The efficiency of frameshifting appears to be critical for viral propagation, suggesting that the balance of Gag and Gag-Pol fusion proteins may be important in the assembly process. Since no eukaryotic cellular gene is known to use −1 ribosomal frameshifting as part of its expression strategy, these results suggest that drugs affecting this process may be useful for antiviral therapy.

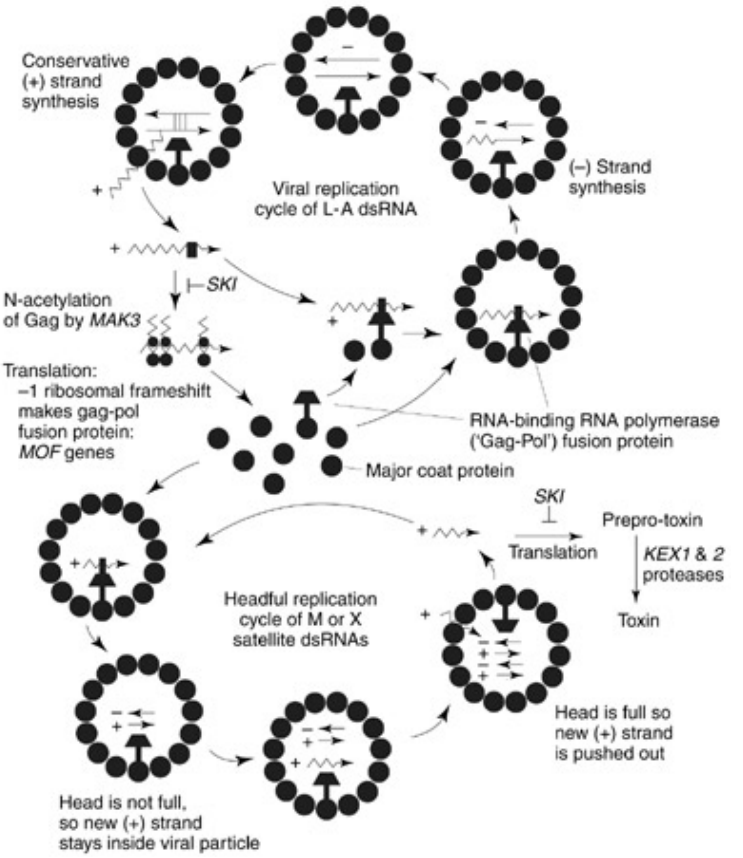


Figure 2 Replication cycles of L-A and its satellites.

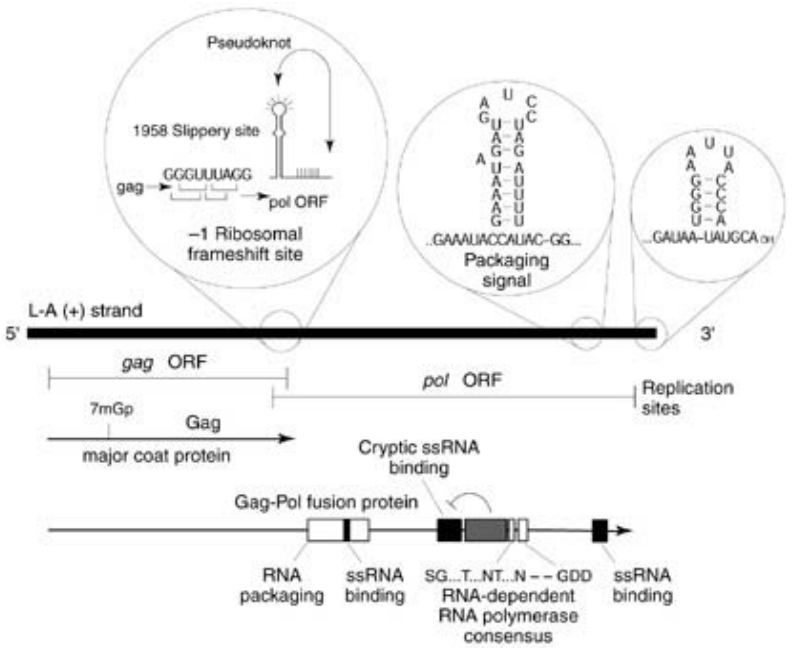


Figure 3 Expression of L-A-encoded information. The two L-A ORFs encode a chimeric RNA polymerase-RNA binding protein with a major coat protein domain. ORF 1 (Gag) is the major coat protein, whereas ORF 2 (Pol) encodes a region with the consensus sequence patterns for the RNA-dependent RNA polymerases of plus ssRNA viruses and dsRNA viruses. Pol also has three ssRNA binding domains, the N-terminal one of which is necessary for packaging viral (+) strands.

Table 2 Chromosomal genes affecting yeast RNA viruses

Gene(s)	*Encodes*	*RNA affected*	*Mechanism*
MAK3	*N*-Acetyltransferase	L-A, M	Viral assembly
*MAK*1, 7, 8, 11, 16, 18, 27, ...	60S ribosomal subunit proteins and assembly factors	L-A, M	Expression of viral poly(A)-mRNA
SKI1/XRN1	5′-exoribonuclease	L-A, M	Viral mRNA stability
*SKI*2, 3, 8	System blocking translation of nonpoly(A) mRNA	L-A, M, L-BC, 20S	Blocks viral translation
*SKI*6	3′-exoribonuclease required for 60S ribosomal subunit assembly	L-A, M	Blocks viral translation
porin, *NUC*1	Mitochondrial functions	L-A	?
*CLO*1	?	L-BC	?

The structure of the Gag-Pol fusion protein suggested a packaging mechanism for L-A in which the Pol part of the fusion protein captures a viral (+) strand and the Gag part primes polymerization of the coat by association with Gag molecules. This results in the natural packaging of one viral (+) strand per particle.

Why do L-A, retroviruses and a number of plus single-stranded (ss) RNA viruses use ribosomal frameshifting and readthrough of termination codons to make Gag-Pol and other fusion proteins? dsRNA viruses, plus ssRNA viruses and retroviruses all use their plus-strands as mRNA, as the form of the genome that is packaged to make new particles and as a template for replication. If they were to use splicing or RNA editing for the purpose of fusing open reading frames, they would be generating mutants unless the spliced out region contained a site essential for packaging or replicating the genome. Retroviruses often splice, but they remove the packaging site in so doing so that the spliced mRNAs will not be packaged. Insofar as is known, plus ssRNA viruses and dsRNA viruses do not splice, perhaps for this reason. Minus ssRNA viruses do splice and edit their RNAs.

Host Functions

Over 40 chromosomal genes affecting the propagation and expression of the L-A or M dsRNA genomes in yeast have been defined and a single gene is needed for L-BC (**Table 2**). Chromosomal (host) mutants that lose the M_1 genome are generally called *mak* mutants (for maintenance of killer). Among the 30 genes of this type are three, *MAK*3, *MAK*10 and *MAK*31, which are necessary for the propagation of L-A. *MAK*3 is now known to encode an *N*-acetyltransferase responsible for acetylating the N-terminus of the major coat protein (Gag) encoded by L-A. This acetylation appears to be necessary for the assembly of viral particles. This parallels the N-terminal myristylation necessary for proper assembly of poliovirus and proper localization of retrovirus Gag protein assembly.

Over 20 *MAK* genes encode proteins of the 60S ribosomal subunit or other factors necessary for 60S ribosomal subunit assembly. Deficiency of 60S subunits leads to loss or decreased copy number of L-A and M dsRNAs. The mechanism of this effect may be due to inefficient translation of the viral mRNA, which lacks 3′ poly(A). It has been suggested that the mRNA 3′ poly(A) structure facilitates the joining of the 60S subunit to the 40S subunit waiting at the initiator AUG. A deficiency of 60S subunits should therefore adversely affect translation of nonpoly(A) mRNAs (e.g. viral mRNA) more than the poly(A)$^+$ cellular mRNA.

Mutations of any of seven genes, called *SKI* genes (for superkiller, the phenotype of the mutants), result in elevated copy number of L-A, M, L-BC and 20S RNA (23S RNA has not been tested). *SKI*1 (= *XRN*1) encodes a 5′-exoribonuclease specific for uncapped RNAs (such as viral (+) strands) and known to be the major enzyme degrading cellular mRNAs. The L-A Gag protein can decap cellular mRNAs, probably as a means to distract the *SKI1* exoribonuclease from degrading viral mRNAs by providing other substrates to engage its attention.

Mutations in *SKI*2, 3, 6 or 8 result in derepressed translation of mRNAs lacking a 3′-poly(A) structure. It has been suggested that they act by affecting 60S ribosome biogenesis, but so far only *ski6* mutants have been proven to have altered 60S subunits. Ski3p is also known to be a nuclear protein, and Ski2p is highly homologous to a mammalian nucleolar protein. In strains carrying M dsRNA, *ski* mutants are cold-sensitive, temperature-sensitive or unable to grow at any temperature, depending on the presence of other nonchromosomal factors. The SKI system acts on L-A, M, L-BC and 20S RNA (see below), to lower their copy numbers in the cell.

A distinct system, affecting only L-A, is defined by mutations in the mitochondrial outer membrane

porin and the major mitochondrial nuclease (*NUC1*). Growth on nonfermentable carbon sources is also known to elevate the copy number of L-A, indicating that a complex relationship exists between these viruses and mitochondrial functions.

Killer Toxin Processing and Mammalian Prohormone Processing

The killer toxin is encoded by M_1 as a precursor protein which apparently gives immunity to cells carrying M_1. The toxin has two subunits which, in close analogy to insulin, are processed from the preproprotein by removal of a signal sequence and a peptide between the subunits (**Fig. 4**). This analogy with mammalian prohormone processing is more than superficial. The *kex*1 and *kex*2 mutations were originally isolated based on their inability to secrete K_1 killer toxin and the yeast α mating pheromone. The *KEX2* product was then found to be a protease cleaving specifically C-terminal pairs of basic residues, and *KEX1* is another protease that removes the two basic residues. This is just like the processing of insulin, pro-opiomelanocortin and other mammalian hormones. Indeed, using sequence information from *KEX2* or functional complementation of *kex*2 mutants, several mammalian protease genes have been isolated which are candidates for the physiological prohormone processing enzymes. Moreover, the yeast *KEX2* gene can substitute for the mammalian proteases in processing prohormones.

Toxin Action

The K_1 killer toxin must bind first to (1 → 6) β-D-glucan, a major structural component of yeast cell walls. The K_{28} toxin binds first to cell wall mannans. However, since spheroplasts remain sensitive to these toxins, there must be a receptor further downstream in the process. The K_1 toxin acts by creating proton pores in the membrane.

20S RNA Replicon and T and W dsRNA

20S RNA was discovered in 1971 as a species whose synthesis is induced when cells are transferred to the media that are used to induce meiosis and sporulation in yeast (potassium acetate medium). Subsequent studies showed that some strains were unable to produce 20S RNA under these conditions, and that there was no role for 20S RNA in sporulation. Thus, some strains that could sporulate made no 20S RNA and some that could not sporulate could make 20S RNA. It was also shown that the ability to induce 20S RNA synthesis was inherited as a non-mendelian factor.

Recently, most of 20S RNA has been cloned and

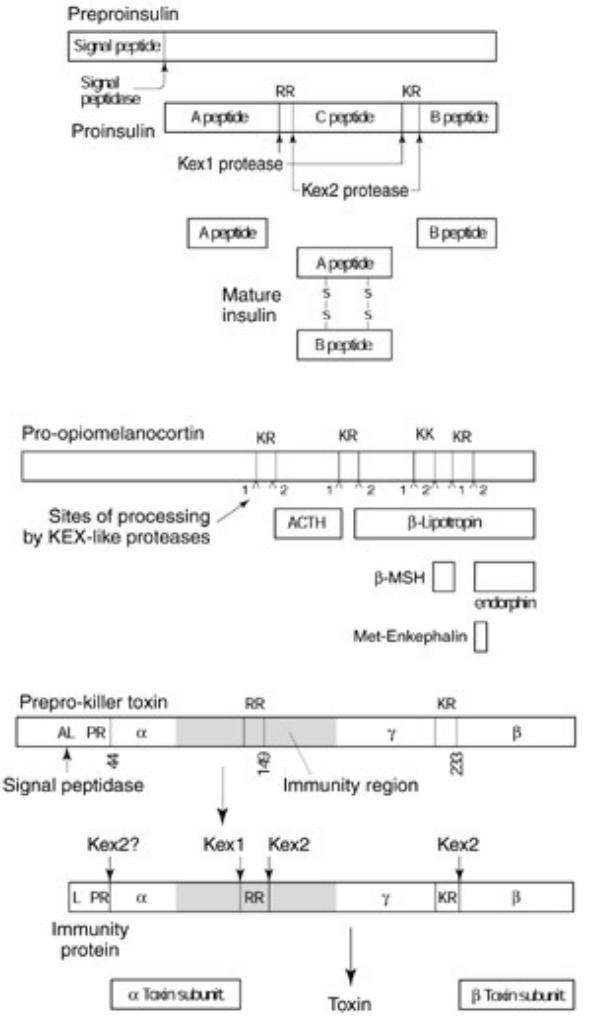


Figure 4 Preprotoxin processing resembles mammalian hormone processing. The two subunits of both insulin and killer toxin are cleaved out of their precursor hormones by Kex-like proteases. These enzymes were first discovered in the killer system and this led to their discovery in mammalian cells.

sequenced. 20S RNA is not encoded by any cellular DNA (chromosomal or otherwise). It replicates via an RNA to RNA mechanism. Electron microscopy of 20S RNA coated with T4 gene 32 protein and cross-linked with glutaraldehyde showed mostly circles, and dimer length molecules of both plus and minus polarity have also been detected *in vivo*, consistent with a rolling circle mode of replication. However, the inability to clone all the way around the 20S RNA circle suggests that either there is unusual structure at the uncloneable site, or a fundamentally linear molecule whose ends are held together by some structure. A linear structure is also indicated by biochemical experiments. At this time the structure of 20S RNA remains unclear.

Two low copy number dsRNAs, called T and W,

were described in 1984. Neither was homologous to cellular DNAs and the copy number of both was induced by growth at elevated temperatures. W was recently cloned and sequenced and was found to be identical to 20S RNA. Indeed, 20S RNA copy number is also induced at high temperature. Since the copy number of 20S RNA is always 10-fold or more that of W, W may be viewed as a replicative form of 20S RNA. Their sequence shows a single long open reading frame encoding a protein of about 90 kDa with some homology to the RNA-dependent RNA polymerases of plus-strand RNA viruses. T dsRNA encodes a similar putative RNA-dependent RNA polymerase. These proteins are found in extracts specifically associated with their respective templates, and the 20S RNA polymerase activity has actually been demonstrated *in vitro*.

Applications

Since killer strains kill nonkiller strains when cocultivated, brewing strains have been modified to include a killer virus so that strains present in the grapes (barley, hops, etc.) will not spoil the brewing process. This application is in use today. Vectors based on the toxin secretion signals have also been developed.

Future Perspectives

The ease with which yeast is manipulated genetically and the high yields of L-A virus that can be easily obtained from engineered strains have made possible extensive characterization of the genetics of this virus and its satellites and their relationship with their host. The *in vitro* replication, packaging and transcription systems were the first developed among the dsRNA viruses of eukaryotes, and few such systems have been developed for any RNA viruses. It is likely that the mechanisms found for L-A will not be unique to this system. Among the important problems to be solved are (1) development of an RNA transfection system so that the L-A virus may be used as a vector, (2) further pursuit of the mechanisms of transcription, replication and packaging and application of this information to viruses of higher systems, (3) further elucidation of the mechanisms by which the *SKI* genes control virus propagation, (4) further understanding of the role of host genes that the virus uses for its propagation, and (5) study of the role of various host components in ribosomal frameshifting and search for pharmacologic agents affecting this process – a new approach to antivirals.

The 20S RNA system (and the related T dsRNA system) have only begun to be studied. The question of the nature of the circularity of 20S RNA and the mechanism by which its propagation is controlled, including the massive 10 000-fold amplification on acetate medium, are of great interest. If it is a true circle of RNA, it is likely to self-cleave and self-ligate as do related viroid systems.

See also: **Partitiviruses – fungal (*Partitiviridae*); Prions: Yeast and Fungi; Retroviruses – type D (*Retroviridae*); Totiviruses (*Totiviridae*): General features, *Ustilago maydis* viruses; Vectors: Animal viruses, Plant viruses; Viroids.**

Further Reading

Bussey H (1991) K1 killer toxin, a pore-forming protein from yeast. *Mol. Microbiol.* 5: 2339.

Esteban R, Rodriguez-Cousino N and Esteban LM (1993) Genomic organization of T and W, a new family of double-stranded RNAs from *Saccharomyces cerevisiae*. *Prog. Nucl. Acid Res.* 46: 155.

Steiner DF, Smeekens SP, Ohagi S and Chan SJ (1992) The new enzymology of precursor processing endoproteases. *J. Biol. Chem.* 267: 23435.

Wickner RB (1996) Double-stranded RNA viruses of yeast. *Microbiol Rev* 60: 250.

Wickner RB (1996) Viruses of yeasts, fungi and parasitic microorganisms. In: Fields BN, Knipe DM, Howley PM, *et al* (eds) *Fields Virology*, 3rd edn. Philadelphia: Lippincott–Raven.

YELLOW FEVER VIRUS (*FLAVIVIRIDAE*)

Thomas P Monath, OraVax Inc., Cambridge, Massachusetts, USA

History

Yellow fever was first described as a disease entity in 1648 in Mexico. The origins of the disease are in doubt, but the susceptibility of New World – but not African – monkey species to lethal infection (suggesting contact with the virus in relatively recent times) indicates an African origin of the virus. Whether or

were described in 1984. Neither was homologous to cellular DNAs and the copy number of both was induced by growth at elevated temperatures. W was recently cloned and sequenced and was found to be identical to 20S RNA. Indeed, 20S RNA copy number is also induced at high temperature. Since the copy number of 20S RNA is always 10-fold or more that of W, W may be viewed as a replicative form of 20S RNA. Their sequence shows a single long open reading frame encoding a protein of about 90 kDa with some homology to the RNA-dependent RNA polymerases of plus-strand RNA viruses. T dsRNA encodes a similar putative RNA-dependent RNA polymerase. These proteins are found in extracts specifically associated with their respective templates, and the 20S RNA polymerase activity has actually been demonstrated *in vitro*.

Applications

Since killer strains kill nonkiller strains when cocultivated, brewing strains have been modified to include a killer virus so that strains present in the grapes (barley, hops, etc.) will not spoil the brewing process. This application is in use today. Vectors based on the toxin secretion signals have also been developed.

Future Perspectives

The ease with which yeast is manipulated genetically and the high yields of L-A virus that can be easily obtained from engineered strains have made possible extensive characterization of the genetics of this virus and its satellites and their relationship with their host. The *in vitro* replication, packaging and transcription systems were the first developed among the dsRNA viruses of eukaryotes, and few such systems have been developed for any RNA viruses. It is likely that the mechanisms found for L-A will not be unique to this system. Among the important problems to be solved are (1) development of an RNA transfection system so that the L-A virus may be used as a vector, (2) further pursuit of the mechanisms of transcription, replication and packaging and application of this information to viruses of higher systems, (3) further elucidation of the mechanisms by which the *SKI* genes control virus propagation, (4) further understanding of the role of host genes that the virus uses for its propagation, and (5) study of the role of various host components in ribosomal frameshifting and search for pharmacologic agents affecting this process – a new approach to antivirals.

The 20S RNA system (and the related T dsRNA system) have only begun to be studied. The question of the nature of the circularity of 20S RNA and the mechanism by which its propagation is controlled, including the massive 10 000-fold amplification on acetate medium, are of great interest. If it is a true circle of RNA, it is likely to self-cleave and self-ligate as do related viroid systems.

See also: **Partitiviruses – fungal (*Partitiviridae*); Prions: Yeast and Fungi; Retroviruses – type D (*Retroviridae*); Totiviruses (*Totiviridae*): General features, *Ustilago maydis* viruses; Vectors: Animal viruses, Plant viruses; Viroids.**

Further Reading

Bussey H (1991) K1 killer toxin, a pore-forming protein from yeast. *Mol. Microbiol.* 5: 2339.

Esteban R, Rodriguez-Cousino N and Esteban LM (1993) Genomic organization of T and W, a new family of double-stranded RNAs from *Saccharomyces cerevisiae*. *Prog. Nucl. Acid Res.* 46: 155.

Steiner DF, Smeekens SP, Ohagi S and Chan SJ (1992) The new enzymology of precursor processing endoproteases. *J. Biol. Chem.* 267: 23435.

Wickner RB (1996) Double-stranded RNA viruses of yeast. *Microbiol Rev* 60: 250.

Wickner RB (1996) Viruses of yeasts, fungi and parasitic microorganisms. In: Fields BN, Knipe DM, Howley PM, *et al* (eds) *Fields Virology*, 3rd edn. Philadelphia: Lippincott–Raven.

YELLOW FEVER VIRUS (*FLAVIVIRIDAE*)

Thomas P Monath, OraVax Inc., Cambridge, Massachusetts, USA

History

Yellow fever was first described as a disease entity in 1648 in Mexico. The origins of the disease are in doubt, but the susceptibility of New World – but not African – monkey species to lethal infection (suggesting contact with the virus in relatively recent times) indicates an African origin of the virus. Whether or

not yellow fever in its enzootic form (transmitted between monkeys and sylvan mosquito species) predated the Spanish Conquest in tropical America, it was the slave trade that led to the introduction from Africa of the domestic mosquito vector, *Aedes aegypti* and the emergence of epidemic (urban) yellow fever. Yellow fever was one of the great scourges of mankind during the eighteenth and nineteenth centuries, with epidemics affecting coastal cities in the Americas, Europe and West Africa. The mode of spread of the disease was the subject of great debate. Mosquito transmission was suggested as early as 1848, was emphasized by the Cuban physician, Carlos Finlay in 1881, and finally was proven by Walter Reed and his colleagues in 1900. Reed *et al* also demonstrated that yellow fever was a filterable 'virus', although the etiology of the disease remained in dispute until isolation of the virus in 1927. In the decade that followed, quantitative virological and serological methods were established, allowing precise diagnostic, epidemiological and pathogenesis studies as well as the development and evaluation of live, attenuated vaccines.

Taxonomy and Classification

Yellow fever virus is the prototype member of the family *Flaviviridae*, genus *Flavivirus*, and the virus after which the family and genus were named (*flavus*, Lat. yellow). The genus consists of at least 68 viruses grouped on the basis of shared antigenic determinants and physicochemical properties (see below). The relatively specific neutralization test using polyclonal antisera has been used to distinguish at least eight antigenic complexes to which closely-related flaviviruses are assigned. Neutralization epitopes of yellow fever virus are sufficiently different from other flaviviruses to preclude assignment to a complex.

Properties of the Virion

Virions are spherical, 40–50 nm in diameter, and have short surface projections. The nucleocapsid has icosahedral symmetry, contains the RNA genome and a single core protein, and is surrounded by a lipid bilayer envelope. Approximately 66% of the virion is composed of protein, 12% carbohydrate and 17% lipid. Viral infectivity is rapidly inactivated by heat (56°C for 30 min), ultraviolet radiation and lipid detergents.

Morphogenesis of yellow fever virus occurs at intracellular (endoplasmic reticular, ER) membranes. Mature virions accumulate in these membranes, are transported to the cell surface, and released by exocytosis. Budding of virions into the lumen of the ER is rarely observed and may be an extremely rapid event. In secretory cells (such as the mosquito salivary gland and a variety of exocrine and endocrine glands infected in mammals), virion assembly occurs in concert with host cell secretory components and virions are released in secretory granules. Flavivirus infection often proceeds without markedly disturbing host cell function or macromolecular synthesis.

Properties of the Genome

The viral genome is composed of a single linear strand of infectious (positive polarity) RNA, 10 862 nucleotides in length, with a mass of 3750 kDa. The 5′ terminus has a type 1 cap, and the 3′ terminus is not polyadenylated. The GC content is 49.7%. The viral genome contains a single long open reading frame 10 233 nucleotides in length, encoding 11 viral proteins (**Fig. 1**). The remainder of the genome comprises short 5′ (118 nucleotides) and 3′ (511 nucleotides) noncoding regions. Nucleotide conservation is seen between the 5′ terminus of the plus strand and the 3′ terminus of the minus strand, serving as common recognition sequences for the viral polymerase. The 3′ terminus forms a stable hairpin loop involved in binding to the capsid protein.

Viral Proteins

The three virion structural proteins are encoded by the 5′ one-fourth of the genome, in the sequence C (capsid), prM (precursor to the mature membrane (M) protein) and E (envelope) (**Fig. 1**). The remainder of the open reading frame encodes eight nonstructural proteins, in the order illustrated. Translation begins at the 5′ terminus of the viral genome, and the individual proteins are produced after translation by a series of enzymatic cleavages. The C protein (12–14 kDa) interacts with RNA in the virion nucleocapsid; its hydrophobic C terminus anchors nucleocapsids to ER membranes, and provides signal sequence for prM. The prM glycoprotein (18–19 kDa), present intracellularly, is cleaved by a furin-like protease at the time of virus maturation to form M in the extracellular virion. The M protein spans the viral membrane, and has exposed antigenic domains that may play a minor role in the induction of protective immunity. However, the E glycoprotein (53–54 kDa) is the major surface structure and subserves many biological functions, including cell attachment, hemagglutination and neutralization. Important to these functions is the three-dimensional configuration of E protein, determined by disulfide bonding. Epitopes with strain-specific, type-specific and flavivirus group-reactive specificities are present in the E glycoprotein. As is the case for similar proteins, the C

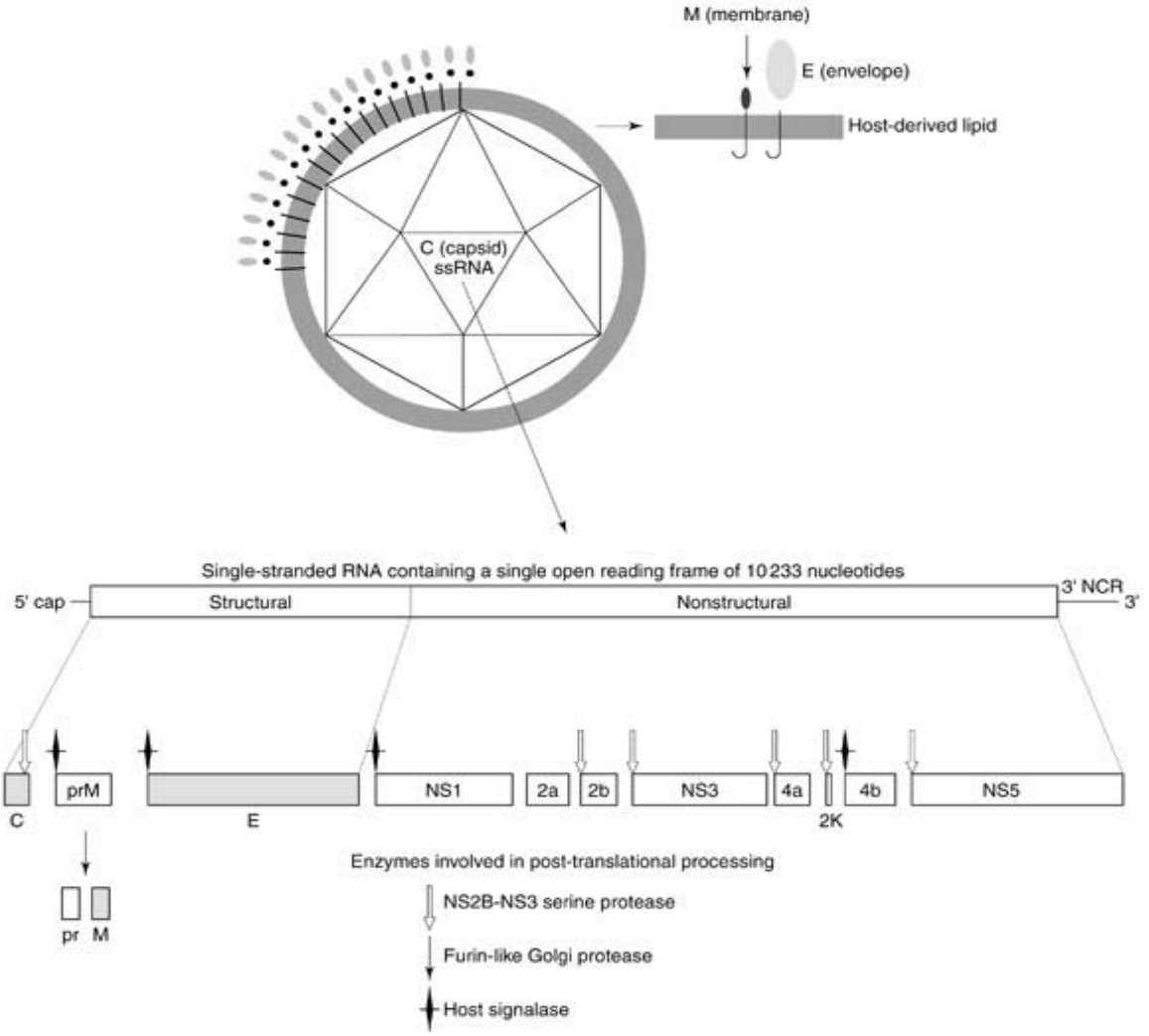


Figure 1 Yellow fever (flavivirus) virion and genome, showing the structural and nonstructural protein coding regions. The genome is characterized by type 1 5′ cap structure, followed by a short noncoding region and a long open reading frame of over 10 000 nucleotides encoding the structural (C-prM-E) and nonstructural (NS1–NS5) proteins. The genome lacks a 3′ poly(A) tail.

terminus contains hydrophobic sequences forming the protein anchor in the lipid membrane. The crystallographic structure of the E glycoprotein reveals a head-to-tail dimer composed of a 170 Å long rod anchored to the membrane at its basal end, with its long axis parallel to the virion surface. The C-terminus resembles an immunoglobulin constant domain and is connected by a flexible region to the central part of the molecule (domain I) with up-and-down topology having eight antiparallel β strands and containing the N-terminus. Two long loops (domain II) extending laterally are responsible for dimerization. A conserved stretch of 14 amino acids at the tip of one of the domain II loops constitutes the fusion domain responsible for internalization of nucleocapsids from endosomes into the cytoplasm of the infected cell. Domain III contains ligands involved in binding cell receptors. Neutralization determinants are conformational, and are scattered on the outer surface of all three flavivirus structural domains. The nonstructural NS1 glycoprotein is found both within infected cells and extracellularly (as circulating antigen with complement-fixing properties). Expressed on the cell surface, NS1 is a target for antibodies involved in clearance of virus infection. The highly conserved NS3 protein (68–70 kDa) has helicase, protease and RNA triphosphatase functions. NS5 (103–105 kDa) is the RNA polymerase. The functions of NS2A, NS2B, NS4A and NS4B are not clear.

Replication

After gaining entry to the cell, genomic, plus-strand RNA is translated and RNA replication proceeds by

Table 1 Amino acid differences between Asibi virus and attenuated 17D vaccines

Nucleotide	*Gene*	*Amino acid*	*Asibi*	*17D-204 and 17DD vaccines*
854	M	36	Leu	Phe
1127	E	52	Gly	Arg
1482		170	Ala	Val
1491		173	Thr	Ile
1572		200	Lys	Thr
1870		299	Met	Ile
1887		305	Ser	Phe
2112		380	Thr	Arg
2193		407	Ala	Val
3371	NS1	307	Ile	Val
3860	NS2a	118	Met	Val
4007		167	Thr	Ala
4022		172	Thr	Ala
4056		183	Ser	Phe
4505	NS2b	109	Ile	Leu
6023	NS3	485	Asp	Asn
6876	NS4a	146	Val	Ala
7171	NS4b	95	Ile	Met
10142	NS5	836	Glu	Lys
10338		900	Pro	Leu
10367	(3'NCR)		U	C
10418			U	C
10800			G	A
10847			A	C

synthesis of complementary minus strands. New plus strands are transcribed from genome-length minus-strand templates. Duplex RNA molecules (replicative forms) are thus formed in the infected cell. New genome-length plus strands serve as mRNAs for translation of structural and nonstructural proteins, as templates for transcription of new minus strands for replication, and as genomes for inclusion into nucleocapsids for production of mature virus particles.

Variation and Evolution

Nucleotide sequence analysis of the E gene revealed that yellow fever virus evolved early in the lineage of mosquito-borne flaviviruses, approximately 3000 years ago.

Yellow fever virus is distantly related to other flaviviruses by serologic tests and in terms of homology in RNA sequences. Strains of yellow fever virus from different geographic regions can be distinguished by RNA fingerprinting and are presently classified into three genotypes, one from West Africa, one from Central and East Africa, and one from South America. A high degree of homology has been noted between strains belonging to one topotype.

Molecular Basis of Virulence

The genomes of the attenuated 17D and the French neurotropic vaccines and their wild-type parents, Asibi and the French viscerotropic virus have been compared. The 17D and Asibi viruses differ at 20 amino acids, and four nucleotides in the 3′ noncoding region (**Table 1**). Because of the functional importance of the E protein in attachment and entry to cells, one or more of the seven amino acid differences that separate Asibi and the vaccine strains are likely to play a role in attenuation. The role of the E glycoprotein in neurovirulence of flaviviruses has been established by studies in which the E gene of a nonneurovirulent virus has been replaced by the corresponding gene of a virulent virus, resulting in a conversion to neurovirulent phenotype. Four of the seven amino acid differences in the E gene are nonconservative ($52^{Gly \rightarrow Arg}$; $200^{Lys \rightarrow Thr}$; $305^{Ser \rightarrow Phe}$; and $380^{Thr \rightarrow Arg}$). At least three wild yellow fever virus strains with different passage histories or geographic origins are identical at these codons, suggesting that the

mutations in 17D vaccine are in part responsible for attenuation. Residues 52 and 200 are located at the base of domain II, where mutations might affect acid-dependent conformational change in the endosome required for entry. The conservative change at position $173^{Thr \rightarrow Ile}$ corresponds to a site in tick-borne encephalitis virus at which a neutralization escape mutant had reduced neuroinvasiveness in mice. Residue 173 encodes an epitope recognized by wild-type specific monoclonal antibody and reversion at this site is correlated with the neurovirulence phenotype of a plaque variant recovered from a 17D vaccine. The non-conservative changes at E-305 and E-380 are located in domain III, which contains the determinants involved in tropism and cell attachment. Residue 305 is located on the outer surface of domain III and residue 380 is located in a highly conserved region in mosquito-borne flaviviruses implicated in cell receptor interactions. Sequence analysis of virus recovered from the brain of a 3-year-old child in the USA, who died of encephalitis following 17D immunization, revealed a mutation near the E-305 residue (at $303^{Glu \rightarrow Lys}$); the mutant had increased neurovirulence for mice and monkeys. Other studies emphasize the multigenic nature of virulence and suggest that one or more of the 10 amino acid changes in the nonstructural proteins or the changes in the 3′ noncoding region of the virus may contribute to the attenuation of 17D vaccine.

Little is known about the molecular basis of viscerotropism (the ability of wild-type yellow fever virus to replicate and damage nonneural tissue, particularly the liver), or the mutations responsible for loss of this trait in 17D vaccine. Sequence comparison of the French viscerotropic strain with that of the French neurotropic vaccine (FNV) revealed 77 (0.7%) nucleotide and 35 (1%) amino acid changes scattered throughout the genome, with the highest frequency of mutations in the C, M, E, NS2a, 2K and NS4b proteins. The large number of differences and lack of biological data on the role of these mutations preclude speculations on the genetic basis of viscerotropism. Sequence comparison of FNV with 17D vaccines (which also have attenuated viscerotropism) revealed only two shared differences from the parental and other wild-type yellow fever viruses. These common differences, which evolved during the development of vaccine strains by completely distinct processes, were at positions in the M protein ($35^{Leu \rightarrow Phe}$) and NS4b ($95^{Ile \rightarrow Met}$). It is unclear whether these mutations are involved in loss of viscerotropism.

Host Range and Virus Propagation

A highly conserved region in domain III of the E glycoprotein incorporates an RGD motif and is the probable site of virus–cell attachment. Cell membrane receptors also remain to be elucidated. Because of the broad host range of the flaviviruses, cell receptors may be molecules with conserved structure across the chordate and arthropod phyla. Yellow fever and other flaviviruses enter cells by typical receptor-mediated endocytosis.

Yellow fever virus replicates and produces cytopathic effects and plaque formation in a wide variety of cell cultures, including: primary chick and duck embryo cells; continuous porcine, hamster, rabbit and monkey kidney cell lines; and cells of human origin (e.g. HeLa, KB, Chang liver, SW-13 cells). The virus replicates in Fc-receptor bearing macrophages and macrophage cell lines, and replication is enhanced by antibody. Mosquito cell lines, especially *Ae. pseudoscutellaris* (AP-61) cells, are highly susceptible and are often used for primary isolation or efficient laboratory propagation of virus.

The most sensitive method for virus isolation and assay is the intrathoracic inoculation of mosquitoes, such as *Toxorhynchites* spp. or *Ae. aegypti*. Infected mosquitoes show no signs of illness, and the presence of virus must be demonstrated by immunofluorescence or subpassage to a susceptible host. Infant mice succumb within 6–8 days to encephalitis; at about 8 days of age, mice become resistant to lethal infection by the peripheral route, while remaining susceptible to intracerebral challenge. After parenteral virus infection, a number of subhuman primate species develop fatal hepatitis resembling the human disease. The only nonprimate species that develops lethal hepatitis in response to yellow fever infection is the European hedgehog, *Erinaceus europaeus*. Antibodies have been found in a wide variety of wild vertebrates collected in the field. Wild animals have also been experimentally infected with yellow fever virus, including rodents, bats and marsupials. With the possible exception of opossums in South America, the data do not support a role for nonprimate species in transmission cycles.

Geographic and Seasonal Distribution

Yellow fever virus presently occurs in tropical areas of South America and Africa. *Ae. aegypti*-infested regions of North and Central America, the Caribbean and southern Europe were intermittently invaded by the disease until the early part of this century and are still considered at risk, should the virus be introduced. Despite the prevalence of *Ae. aegypti* in tropical Asia, yellow fever has never reached that continent, possibly because transmission in Africa and South America occurs in relatively inaccessible areas and because crossprotection is afforded by immunity to dengue

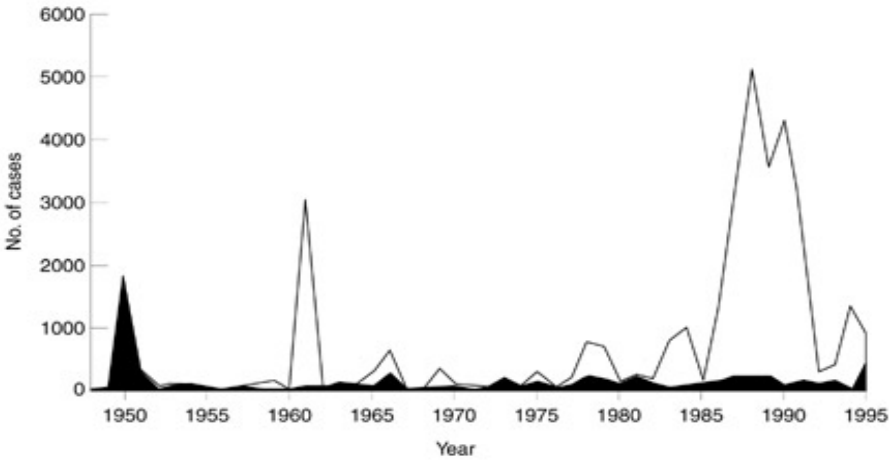


Figure 2 Officially reported incidence of yellow fever cases in South America (■) and Africa (□). The recent upsurge in incidence is due to a series of severe epidemics in Nigeria. Official reports greatly underestimate the true morbidity due to this disease.

viruses; dengue immunity is nearly universal in many parts of Asia. Asian populations of *Ae. aegypti* are relatively inefficient vectors; thus, vector competence may also provide a partial barrier to the spread of yellow fever in Asia.

The breeding of many mosquito species engaged in yellow fever transmission occurs in tree-holes and is highly dependent on rainfall. Transmission waxes during the tropical rainy season and wanes or ceases during the dry season. Breeding of the domestic mosquito, *Ae. aegypti*, occurs in manmade containers used for water storage and is thus less influenced by seasonal rainfall; where this species becomes involved in virus transmission, outbreaks may occur during the dry season.

Disease Incidence

Official notifications underestimate the true incidence of the disease by 10- to 250-fold. The number of cases officially reported annually from South America and Africa is shown in **Fig. 2**, and their geographic distribution in **Fig. 3**. The incidence in Africa has increased dramatically in recent years, due principally to a series of epidemics in Nigeria. Some epidemics in Nigeria and elsewhere in Africa have been very large, involving 100 000 or more cases, with case-fatality rates of over 20%.

Epidemiology and Transmission

Yellow fever virus is present in the blood of infected, susceptible hosts (humans and subhuman primates) for several days, during which mosquito vectors taking a blood-meal may become infected. The virus undergoes sequential replication in the midgut epithelium, body and salivary glands of the mosquito, a temperature-dependent process that takes a week or more to complete – the so-called extrinsic incubation period – before the vector is capable of transmitting virus by refeeding on a second host.

Two cycles of transmission are distinguished by the hosts and vectors involved. The urban cycle involves humans as the viremic host and *Ae. aegypti* mosquitoes, breeding in manmade containers. The forest cycle involves virus transmission between subhuman primates and tree-hole breeding mosquito vectors. The rate of transmission of yellow fever virus in the forest cycle can vary greatly, depending on the density of vectors and the available population of immunologically susceptible monkeys. In South America, some primate species succumb to fatal disease, and die-offs of these animals may provide a clue to yellow fever activity; in Africa, monkeys generally develop subclinical infections. Humans exposed in the forest to infected tree-hole mosquitoes may acquire yellow fever. In South America, this form (so-called jungle yellow fever) accounts for all cases in the last 50 years. Jungle yellow fever strikes mainly young adult males engaged in timbering and agricultural pursuits in the Amazon and Orinoco river basins. The principal mosquito vectors are species of the genus *Haemagogus*. The disappearance in the early 1940s of urban yellow fever, once a major endemic and epidemic disease in South American towns and cities, was attributed to successful programs to control the domestic vector, *Ae. aegypti*. However, in the last 10–15 years, *Ae. aegypti* has reinvaded much of the territory in South America from which it had formerly been eliminated, raising the spectre of urban epidemics in the future. Although

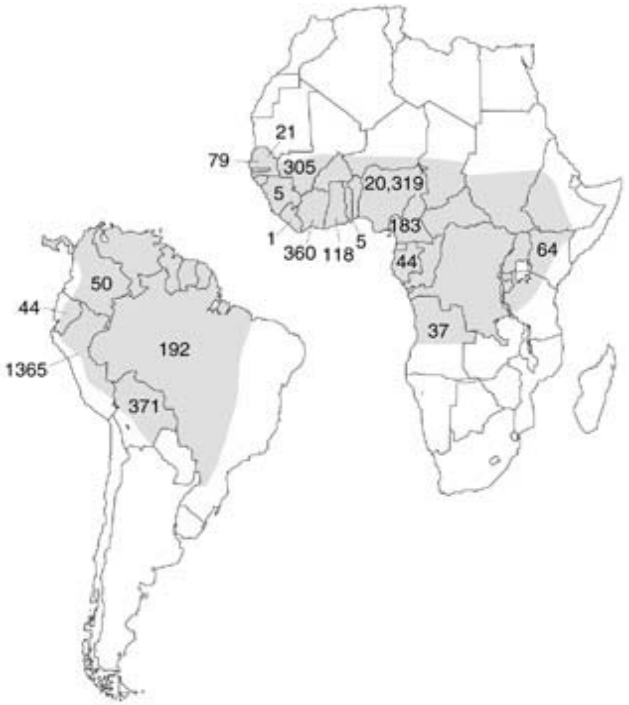


Figure 3 Geographic distribution of yellow fever cases, 1985–1996.

vaccine-induced immunity provides a degree of protection against such outbreaks, the level of coverage is presently incomplete.

The epidemiology of yellow fever in Africa is more complex than in the New World. The virus is present in a vast area of tropical Africa. Sporadic cases of jungle yellow fever occur in the rainforest zone, where the virus is maintained in a cycle involving monkeys and *Ae. africanus* mosquitoes, but such cases are rarely recognized because of inadequate surveillance. Unlike South America, tree-hole breeding *Aedes* spp. are also involved in epidemic transmission. Amplification of the virus transmission cycle occurs in moist savanna and forest-savanna transition zones, where the density of tree-hole *Aedes* reaches high levels during the rainy season. In these circumstances, humans are frequently infected. In urban areas or areas of low rainfall, where water storage practices favor breeding of domestic *Ae. aegypti*, this vector may be involved in epidemic spread, with humans serving as the viremic host.

Clinical Features

Subclinical or abortive infections occur more frequently than full-blown yellow fever. In its classical form, the disease begins suddenly, 3–6 days after the bite of an infected mosquito, with fever, headache, low back pain, muscle aches, loss of appetite and nausea. This period of infection is clinically nonspecific, and corresponds to the viremic phase when the patient is infectious for feeding mosquitoes. It may be followed by a brief period of remission, during which signs and symptoms abate. The patient then becomes increasingly ill, entering the period of intoxication, with reappearance of fever, vomiting, dehydration, abdominal pain, and the appearance of jaundice, protein in the urine, signs of renal failure, and hemorrhages, most notably the vomiting of blood. Virus is no longer present in the blood. Approximately 20 to 50% of patients who progress to this stage die between the 7th and 10th day after onset, with deepening renal and hepatic failure, shock, delirium and convulsions.

Pathology and Pathogenesis

In humans, pathologic changes in the liver include swelling and necrosis of hepatocytes in the midzone of the liver lobule, with sparing of cells in the portal area and surrounding the central veins. Viral antigen and RNA are demonstrable by immunocytochemistry and nucleic acid hybridization in cells undergoing these pathologic changes, and cytopathology is mediated by direct viral injury. Hepatocytes may undergo apoptosis. Inflammatory changes are absent or minimal, and patients with hepatitis who recover do not develop residual scarring or cirrhosis. The kidneys show acute tubular necrosis, probably the result of reduced perfusion of blood rather than direct viral

injury. Focal degeneration of muscle cells may be present in the heart. Spleen and lymph nodes show necrosis of B cell areas. The brain shows edema and petechial hemorrhages, but viral invasion and encephalitis are very rare events. Hemorrhage results principally from decreased synthesis of clotting factors by the liver. The mediators of hypotension and shock remain to be elucidated.

The pathogenesis of yellow fever has been studied in subhuman primates and mice, but our level of understanding is at the descriptive rather than the mechanistic level. Susceptible monkeys develop an illness similar to humans. Initial sites of virus replication have not been clearly defined, but probably include lymphatic tissue draining the site of virus inoculation. Viremia follows, with infection of the liver. Early virus replication occurs in fixed macrophages (Kupffer cells) in the liver, which undergo necrosis. The virus then invades hepatocytes, which develop accelerated cytopathologic changes during the 24 hours before death. Yellow fever virus demonstrates both viscerotropism (infection of liver, lymphoid and other visceral tissues) and neurotropism (infection and inflammation of the brain). In primates, viscerotropic infection is the rule; animals inoculated intracerebrally replicate virus in brain tissue and develop histopathologic evidence of encephalitis, but die of hepatitis. In contrast, mice develop brain infection without evidence of hepatitis.

Immune Response

Antibodies of both IgM and IgG subclass measurable by binding assays (immunofluorescence, ELISA), hemagglutination inhibition and neutralization appear 5–7 days after onset of illness (8–14 days after infection). IgM antibodies tend to wane to low or undetectable levels between 6 and 12 months after infection, although, in one study IgM neutralizing antibodies were still detectable several years after yellow fever immunization. Complement-fixing antibodies appear in the second week after infection, wane between 3–6 months later, provide a reliable indicator of recent wild-type yellow fever infection, and are rarely demonstrable after yellow fever vaccination. IgG neutralizing antibodies persist for at least 35 years, probably for life, and provide solid protection against reinfection. Antibodies to heterologous flaviviruses, such as Zika, dengue and Wesselsbron viruses, provide partial crossprotection against yellow fever. Complement-dependent antibody-mediated cytolysis of cells with exposed NS1 protein sequences may play a role in virus clearance and recovery, as well as in protection in the previously-immunized host. Little is known about cell-mediated responses in yellow fever. Limited studies in mice suggest that nonspecific resistance is mediated by interferon, NK cells and macrophages, and that cytotoxic T cells are important in clearance of yellow fever infection.

Prevention and Control

The control of domestic *Ae. aegypti* mosquitoes is an important measure, but has proven difficult to sustain. The most effective approach to prevention is immunization of the human population in endemic areas. Yellow fever 17D is a live, attenuated vaccine produced in embryonated eggs. Over 300 million people have been immunized with this inexpensive product, which has proven safe and highly effective. Recent efforts have focused on the production of a new vaccine derived from a full-length cDNA clone that generates infectious transcripts. By this approach it may ultimately be possible to produce chimeric vaccines with heterologous cassette genes incorporated into the 17D yellow fever vaccine backbone.

See also: **Dengue viruses (*Flaviviridae*); Pathogenesis: Animal viruses; Replication of viruses; Zoonoses.**

Further Reading

Barrett ADT (1997) Yellow fever vaccine. *Biologicals* 25: 17.

Chambers TJ, Hahn CS, Galler R and Rice CM (1990) Flavivirus genome organization, expression, and replication. *Annu. Rec. Microbiol.* 44: 649.

Chang GJ, Cropp CB, Kinney RM, Trent DW and Gubler DJ (1995) Nucleotide sequence variation of the envelope protein gene identifies two distinct genotypes of yellow fever virus. *J. Virol.* 69: 5773.

Monath TP (1986) Pathobiology of the flaviviruses. In: Schlesinger S and Schlesinger MJ (eds) *The Togaviridae and Flaviviridae*, p. 375. New York: Plenum Press.

Monath TP (1987) Yellow fever: a medically neglected disease. *Rec. Infect. Dis.* 9: 165.

Monath TP (ed.) (1989) Yellow fever. In: *The Arboviruses: Epidemiology and Ecology*, vol. V, p. 139. Boca Raton: CRC Press.

Monath TP (1991) Yellow fever: Victor, Victoria? *Am. J. Trop. Med. Hyg.* 45: 1.

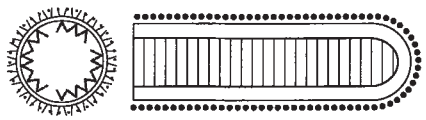
Rice CM, Lenches EM, Eddy SR *et al* (1985) Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evolution. *Science* 229: 726.

Robertson SE, Hull BP, Tomori O *et al* (1996) Yellow fever: a decade of reemergence (reprinted). *JAMA* 276: 1157.

Zanotto PM, Gould EA, Gao GF *et al* (1996) Population dynamics of flaviviruses revealed by molecular phylogenies. *Proc. Natl Acad. Sci. USA* 93: 548.

Z

ZOONOSES



Thomas M Yuill, Institute for Environmental Studies, University of Wisconsin, Madison, Wisconsin, USA

Introduction

Zoonoses are diseases transmissible from vertebrate animals, other than humans, to people. Hundreds of viruses in a wide range of families are zoonotic. Mammals, birds, reptiles and probably amphibians are reservoir or amplifier hosts for these viral zoonoses. Frequently, these viruses cause little or no overt disease in their nonhuman vertebrate hosts. Some zoonotic viruses have very limited host ranges, others may infect a wide range of vertebrates. Human infection may vary from unapparent to fatal disease. Some viral zoonoses have been recognized since ancient times, others have become public health problems recently. Both new and old viral zoonoses are especially important in emerging and re-emerging virus diseases.

Transmission

Transmission of zoonotic viruses may occur by a variety of routes. Rabies is transmitted by *direct contact* through the bite of an infected animal. *Indirect contact* transmission of other zoonoses occurs by ingestion of contaminated food and water, as is the case with the hantaviral and arenaviral hemorrhagic fevers. *Nosocomial* arenavirus and filovirus infections have been problems in hospitals caring for patients where isolation has not been adequate. *Aerosol transmission* of Venezuelan equine encephalitis virus has occasionally been a problem in the laboratory, where virus-bearing microdroplets have been widely circulated, with many resulting human cases. *Vertical (in utero)* transmission of arenaviruses results in persistent virus infection in the offspring. A large number of viral zoonoses throughout the world are *vector-* or *arthropod-borne*.

Some Common Zoonotic Viruses Around the World

Viral zoonotic diseases occur on every continent except, perhaps, Antarctica. Some are found around the world, in a variety of ecological settings. Others are found only in very limited ecologic and geographic foci. The panorama of viral zoonotic diseases is constantly changing. Although hundreds of viruses have been shown to infect both humans and animals, the importance of many of these viruses, as agents of either human or animal disease, has not yet been established. Some of the more important viral zoonoses will be discussed briefly.

Rabies virus

Rabies is one of the oldest reported zoonoses. Rabies virus infection causes nervous system disease that ends in death. Animals can become infected without nervous system involvement or disease, develop antibodies, and survive, but play no role in transmission. Classical rabies is found all around the world except in Antarctica, Britain, the Hawaiian Islands, Australia and New Zealand. Transmission occurs by the bite of an infected mammal that has virus in its saliva. Aerosol (droplet) transmission to humans has been reported, although rarely. Dogs and cats are the main reservoirs, especially in the tropical developing countries where more than 99% of all human deaths due to rabies occur. In the industrialized countries, wild mammals are the main reservoirs and the species involved vary from region to region. Translocation of rabid wild animals by hunting groups has been a cause of rabies spread in the USA. The principal species are: in North America, skunks, raccoons and foxes; in Europe, foxes; and in the Caribbean, mongooses. Bats in all enzootic regions harbor rabies, with vampire bats especially important in the Neotropics. Rabies virus is classified in the *Lyssavirus* genus of the family *Rhabdoviridae*. Genetic relationships between rabies isolates from different species and geographic areas have been established by genomic sequence analysis. Rabies-related viruses have been found in bats in Australia (Australian bat lyssavirus) and Britain and other European countries

(European bat lyssaviruses). Still more distantly-related lyssaviruses, some zoonotic, are found in Africa. Diagnosis is based on characteristic altered behavior of infected mammals, confirmed by isolation of virus or demonstration of intracellular antigen by immunofluorescence or of virus genomic sequences by nested reverse transcription–polymerase chain reaction (RT-PCR) confirmed by Southern blot hybridization. Postexposure treatment is accomplished by thorough washing of the bite wound, administration of hyperimmune serum or globulin, and administration of antirabies vaccine (prepared in human diploid cells, purified chick embryos or purified Vero cells). Dogs and cats in enzootic areas should be vaccinated. Other domestic animals and humans at high risk should be also vaccinated. Vaccination campaigns of free-ranging red fox populations in Europe and raccoons and coyotes in the USA have been carried out by oral administration of recombinant vaccinia-vectored vaccines in bait.

Hantavirus hemorrhagic fevers and pulmonary syndrome viruses

Hantaviruses are a newly-recognized complex of public health importance. These viruses belong to the *Hantavirus* genus of the *Bunyaviridae* family. Hantaan virus causes Korean hemorrhagic fever, and occurs in east Asia, especially in Korea, China and eastern Russia. Closely related rodent-associated hantaviruses cause milder hemorrhagic fever with renal syndrome in humans and have been reported from Scandinavia, Europe and Russia (Puumala virus), The Balkans (Dobrava-Belgrade virus), Russia and Asia as well as port areas around the world (Seoul virus). The similarity of many *Rattus* spp.-associated viruses globally suggests that movement of rats through commercial shipping has spread the virus as well. Seoul virus has been found in laboratory rats, with transmission to personnel there. Direct transmission of these viruses among rodents or from rodents to humans may occur by bite. A group of hantaviruses have been newly recognized with the appearance of highly fatal hantavirus pulmonary syndrome (HPS) in humans. As of June, 1998, 183 cases of HPS from 29 states and 24 cases from three Canadian provinces have been reported. Western hemisphere rodent-maintained hantaviruses that cause HPS include Sin Nombre (Alaska, across Canada, the USA except the southeast, south to central Mexico), New York (eastern USA, Canada and Mexico), Black Creek Canal (eastern Canada, USA; northern South America), Bayou (south central and southeast USA), Andes (southern South America) and Laguna Negra from a vesper mouse (southern South America). Lechiguanas and Oran viruses have also been associated with HPS cases in Argentina. There is evidence of direct human-to-human transmission of Andes virus. Several other rodent hantaviruses newly discovered in Europe, Asia and the Americas have not been associated with human disease. Hantaviruses are harbored by wild rodents which often live in close association with humans. Virus is shed in urine and other excreta. Outbreaks of HPS have been associated with ecological changes and invasion of human habitations by expanding rodent populations. Diagnosis has been complicated by the lack of efficient and sensitive isolation and serological methods, which explains why these 'new' viruses have only recently been isolated and characterized. Cell cultures, immunofluorescence or RT-PCR are used to isolate or detect virus, or to test for antibody. Because some of these agents are highly pathogenic to humans, work with them must be carried out in facilities that provide a high degree of biocontainment. Rodent control and avoidance of exposure to rodent excreta, especially in dust, are the only methods available currently for prevention of transmission to humans.

Arenavirus hemorrhagic fever viruses

Arenavirus hemorrhagic fever (HF) viruses, like the hantaviruses, are rodent associated, and can also cause severe disease in humans. Lymphocytic choriomeningitis (LCM) virus, the first arenavirus isolated, came from a person with encephalitis residing in the USA. Junin virus occurs in Argentina, Machupo virus in Bolivia, Guanarito virus in Venezuela, Sabia virus in Brazil and Lassa virus in West Africa. A complex of related arenaviruses that do not cause human disease occur in the Americas and Africa. These pathogenic arenaviruses establish persistent infection in their rodent hosts, and virus is shed in urine, infecting humans who live in close contact with these contaminated environments. Transmission may occur when people are in contact with blood or fresh carcasses of infected rodents. Lassa fever is also transmitted in rural hospitals to other people in contact with blood from viremic patients. Diagnosis is accomplished by virus isolation in cell culture, but detection of virus may require use of specific antibody and immunofluorescent or enzyme labeling. These techniques, as well as cDNA hybridization probes, are also used to detect viral products in tissues of infected humans or rodents. Diagnosis may also be established serologically by a diagnostic rise of specific IgG, or detection of IgM antibodies. Except for LCM virus, isolation of HF viruses pathogenic for humans requires the use of biosafety level 4 facilities. Serological tests such as ELISA can be done reliably

with inactivated antigen at a lower level of biosafety. Control of these diseases is attempted mainly by reduction of rodent populations, as was done successfully with Bolivian HF, and by avoidance of dust containing rodent excreta. Prevention of human disease by vaccination is possible. A live attenuated vaccine has been developed for Argentine HF, and a vaccinia-vectored vaccine has been developed for Lassa fever. Ribivirin is effective for treating arenavirus infection if administered early in the course of infection.

Yellow fever virus

Yellow fever (YF), first recognized clinically in the Neotropics in the sixteenth century, periodically ravaged human populations in the Americas, Europe and Africa until as recently as the beginning of the twentieth century. Infection causes hemorrhagic disease with severe liver damage and death in up to half of the most acute cases. Presently, the virus is maintained enzootically in Africa and the tropical Americas in primates and arboreal mosquitoes. Humans or primates transport the virus from its sylvan cycle in forested areas to rural or urban areas, where other vector mosquitoes transmit it. YF may be a public health time bomb: there has been an alarming increase in human cases in West Africa in recent years, and, for the first time in history, in Kenya in 1992–1993. Although the number of 'jungle' YF cases in the American tropics has not increased as dramatically, re-establishment of the major urban vector, *Aedes aegypti*, and the recent appearance and spread of a potential YF mosquito vector, the Asian tiger mosquito (*Ae. albopictus*), in the hemisphere is a cause for serious concern. The risk of introduction of YF into cities is real. There have been recent instances of individuals infected acutely with sylvan yellow fever traveling to four South American urban centers infested with *Ae. aegypti*, but, fortunately, without subsequent transmission. YF virus is a flavivirus in the *Togaviridae* family. Diagnosis is by virus isolation in cell culture or in inoculated mosquitoes, detection of circulating antigens, demonstration of a significant raise in specific YF virus antibodies, or by observing viral inclusion bodies or antigen in tissues taken at postmortem examination. Insecticide spraying and elimination of breeding sites in homes can be used for vector control in epidemic situations. Disease can be prevented in humans by vaccination. Unfortunately, many countries have not been able to maintain an adequate level of immunity in populations in areas of risk and have to resort to emergency immunization programs when cases are detected. Because cases often occur in remote areas, it is difficult for many national health services to provide adequate vaccination coverage quickly in the face of YF outbreaks.

West Nile virus

West Nile (WN) virus, a close relative of Japanese encephalitis virus, occurs from India and Pakistan westward through the Middle East and into Africa, and northward into Europe and the republics of the former USSR. Mild infection to acute febrile disease with rash, and occasional encephalitis (mainly in the elderly), is produced in humans. *Culex* spp. are the main vectors, and birds are the vertebrate hosts. Diagnosis is by virus isolation and serological means. Unlike the related flavivirus encephalitides, WN virus can be isolated from the blood of human patients relatively commonly.

Chikungunya virus

Chikungunya (CHIK) virus has been responsible for acute febrile disease with rash and severe arthralgia in people in Africa and Asia. The epidemiology in Africa is similar to yellow fever; CHIK virus is maintained in sylvan or savanna cycles involving wild primates and arboreal *Aedes* mosquitoes. In both Africa and Asia, the virus also has an urban cycle involving humans and *Ae. aegypti* mosquitoes. Two outbreaks in Thailand in 1995 also involved *Ae. albopictus* mosquitoes. Virus can be recovered frequently from the blood of acute phase patients. The virus is an alphavirus of the *Togaviridae* family. The virus can be isolated in mammalian or mosquito cell cultures, although suckling mouse and mosquito inoculation are also sensitive systems. The virus has been transmitted to laboratory workers in the laboratory via aerosols created during virus isolation and passage. Serologic tests (neutralizing antibody or IgM capture ELISA test) are useful for diagnosis. Control of the sylvan and savanna enzootic cycles is not feasible. The urban cycle can be controlled by reduction of *Ae. aegypti* breeding sites and adult populations. No vaccine is available.

Sindbis virus

Sindbis (SIN) virus is one of the most widely distributed arthropod-borne viruses in the world, being found in Africa, Europe, Asia and Australia. Disease in humans is usually mild, and is characterized by acute fever, with arthralgia and rash. SIN virus is maintained in wild bird populations, with transmission by *Culex* spp. mosquitoes. In Africa and the Middle East, WN is often found in the same ecosystems where WN virus is being transmitted. The virus is an alphavirus of the *Togaviridae*. Phylogenetic analysis indicates that there is one major genetic

cluster of western SIN virus strains in Africa and another in Australia and Asia. There is evidence of some geographic mixing western strains of SIN virus that suggests long-distance transport via migrating birds. Diagnosis is accomplished by virus isolation, demonstration of virus genomic sequences by RT-PCR or by serological means. There is no vaccine available. Since many of the mosquito vectors breed in extensive rice fields, large-scale control would be expensive.

Crimean–Congo hemorrhagic fever virus

Crimean–Congo hemorrhagic fever (CCHF) virus is very widely distributed, and is found from eastern Europe and the Crimean, eastward through the Middle East to western China, and southward through Africa to South Africa. CCHF is characterized by severe hemorrhagic fever with hepatitis, with case mortality of 10–50%. Asymptomatic infections also occur. Maintenance of CCHF virus involves horizontal transmission from *Hyalomma* ticks to mammals, and vertical transmission in ticks through the eggs. Immature ticks feed on small mammals, and the adult ticks feed on cattle and other large mammals. *Hyalomma* ticks have also been found on birds migrating between Europe and Africa – a mechanism for long-distance dispersal of the virus. Recent human CCHF cases in workers handling livestock and their products in Saudi Arabia and the United Arab Emirates have been attributed to importation of infected cattle and their ticks from Somalia and the Sudan. Diagnosis is by virus isolation, demonstration of viral nucleic acid sequences by RT-PCR, *in situ* hybridization or immunohistochemistry or by serological techniques. There are no vaccine or tick control measures available.

Sandfly fever viruses

Sandfly fever (Sicilian, Naples and Toscana) viruses are endemic in the Mediterranean area (southern Europe, North Africa and Southwest Asia). They cause acute febrile disease in humans, with occasional aseptic meningitis. In central Italy, Toscana virus caused one-third of previously undiagnosed cases of aseptic meningitis. The viruses are members of the *Phlebovirus* genus of the *Bunyaviridae*. They are transovarially and horizontally transmitted by phlebotomine sandflies. Wild mammals are presumed reservoirs.

Viruses Occurring in the Americas

Encephalitis viruses

Venezuelan equine encephalitis (VEE) has been a major public and animal health problem in tropical and subtropical areas of the Americas. VEE virus is made up of a closely related complex of subtypes with several varieties, which have differing epidemiologies, geographic distributions and disease importance. The epizootic/epidemic (VEE IAB and IC) virus variants are of greatest concern. In equine animals, the virus causes acute encephalitis, and case fatality may approach 80%. Survivors may have serious neurological deficits. Although the case fatality rate in humans is low (less than 1%), the large numbers of acutely infected people that occur during an epidemic may completely overwhelm the local health care system. VEE IAB and IC viruses are maintained in northern South America, where they have periodically swept through Venezuela and Colombia in epidemic waves, with occasional extension into Ecuador and massively through Central America into Mexico and South Texas. Epidemic spread depends on the availability of susceptible equine populations (the amplifying host) and abundant mosquito vectors of several species. Although the interepidemic maintenance systems remain undefined, there is some recent evidence that the epidemic form may be enzootic in Central America, causing sporadic cases in equine animals there. There is other evidence that the epizootic strains may arise by mutation of subtype ID enzootic virus. The enzootic strains are maintained in limited foci involving rodents and *Culex* (*Melanconion*) spp. mosquitoes from Florida to Argentina. With the exception of subtype IE, which caused epizootics in horses in 1993 in Chiapas and in 1996 in Oaxaca State, Mexico, these virus strains do not cause disease in equine animals, but can cause acute febrile illness in humans. The VEE complex viruses are in the *Alphavirus* genus of the *Togaviridae*. Diagnosis is usually done by antibody detection (IgG or IgM ELISA test) because virus isolation from clinical cases is difficult (except from the blood of early febrile cases in infected herds of equine animals), although RT-PCR may detect viral genomic sequences. Viruses are subtyped with monoclonal antibodies in plaque-reduction neutralization or ELISA tests. There is an effective live, attenuated vaccine for both human and equine use. Because the maintenance of equine herd immunity is costly, most animal health agencies do not carry out ongoing, intensive vaccination campaigns. Thus, the risk of reoccurrence of explosive outbreaks remains.

Eastern (EEE) and western (WEE) equine encephalitis viruses occur in epidemic form in North America, but have also been found in Central and South America. Generally, EEE is maintained in eastern North America but has caused scattered epizootics and cases in the Caribbean, and in Central and South America. EEE virus can be divided into a North

American–Caribbean clade, an Amazon Basin clade, and a Trinidad, Venezuela, Guyana, Ecuador and Argentina clade. WEE occurs in western and prairie states and provinces and along the west coast. WEE has caused sporadic cases of encephalitis in equine animals, but not humans, in Argentina and Uruguay. Both involve wild birds and mosquito vectors, with spillover into equine populations and humans, causing clinical encephalitis and death. Central nervous sequelae may occur among survivors. Diagnosis is by isolation of the virus in mice or cell cultures, by demonstration of viral RNA by RT-PCR or of viral antigen by capture ELISA. Isolation from brain tissue of clinical encephalitis cases is difficult. Demonstration of IgG antibody by a variety of tests, or IgM antibody by capture ELISA, is of diagnostic value. Effective vaccines are available commercially for equine animals, and experimental vaccines are used for laboratory personnel. Effective mosquito abatement to control vector populations has been carried out in the West for many years. Insecticide application is used for vector control in epidemic situations.

St Louis encephalitis (SLE) virus occurs from Canada to Argentina and causes sporadic but extensive epidemics in the USA, with most epidemics occurring in the west, down the Ohio and Mississippi valleys into Texas, and in Florida. Wild passerine birds are amplifying hosts in North America, but in the Southeastern USA and the Neotropics mammals may play an epidemiologic role in virus maintenance and transmission. SLE virus is transmitted by *Culex* spp. mosquitoes in the USA. SLE virus is a flavivirus of the *Togaviridae*, and is closely related to Japanese encephalitis virus. RNA oligonucleotide fingerprint patterns indicate that the virus varies by geographic region. In humans, SLE is characterized by febrile disease, with subsequent encephalitis or aseptic meningitis, and strikes older people more often than the young. Diagnosis is accomplished by virus isolation, demonstration of viral genomic sequences by RT-PCR or antigens by ELISA capture. Serological diagnosis is made by demonstration of rising titer of IgG by standard techniques, or presence of specific IgM by ELISA tests. Since no vaccine is available, SLE prevention and control relies on surveillance, vector control and screening of dwelling windows and doors.

Powassan (POW) virus is a North American member of the flavivirus tick-borne encephalitis complex. Although POW virus is widely distributed across the USA and Canada, and westward into far eastern Russia, disease (febrile, with encephalitis) has only been detected in the eastern states and provinces of North America. The transmission cycle involves small mammals and *Ixodes* ticks.

La Crosse (LAC) and other California serogroup encephalitides are human pathogens in North America. LAC encephalitis is the most common arthropod-borne virus disease in this region, affecting mainly preschool-aged children. It is endemic in the Upper Midwest, but occasional cases occur elsewhere. Although fatality is uncommon, the disease is severe enough to cause prolonged hospitalization. LAC virus is maintained transovarially in treehole breeding *Ae. triseriatus* mosquitoes with horizontal transmission to small forest mammal reservoirs and to humans. The other California group viruses affecting people have similar epidemiologies, but do not cause disease as commonly. California encephalitis virus was isolated in California, and has occasionally caused human disease there. Snowshoe hare (SSH) virus occurs in the Northern USA and across Canada, and has caused human encephalitis in the eastern provinces. Jamestown Canyon (JC) virus is widely distributed across the USA, and has been shown to cause human disease, mainly in adults in the Midwest, and to infect deer. Like LAC virus, these other viruses have the same close epidemiological relationship with their *Aedes* vectors. These viruses are members of the California serogroup of the *Bunyavirus* genus of the *Bunyaviridae* family. SSH virus is an antigenic variant of LAC virus. Oligonucleotide analysis has shown differences in LAC viruses from the eastern and the western parts of its geographic range in the Midwest. Diagnosis is nearly always by serological means, with demonstration of an antibody rise during illness. There are no vaccines for the LAC group viruses. LAC has been controlled in a few places by eliminating vector breeding sites through filling treeholes and removing discarded rubber tires.

Colorado tick fever virus

Colorado tick fever (CTF) is endemic in sagebrush–pine–juniper habitats of the higher elevations (over 1200 meters) in the mountains of the western states and provinces of North America. Although seldom fatal, CTF can cause serious disease in humans, with prolonged convalescence. CTF may present as hemorrhagic or central nervous system disease, and is most severe in preadolescent children. Males are infected over twice as frequently as are females. The virus is transmitted by and overwinters in *Dermacentor andersoni*. Wild rodents are the vertebrate hosts, and develop a prolonged viremia. CTF virus is classified in the *Coltivirus* genus of the family *Reoviridae* and is serologically related to Eyach virus from Germany. Diagnosis is by virus isolation from erythrocytes, or demonstration of CTF viral antigen by immunofluorescence, during the long viremia.

Serologic diagnosis may be problematic because antibodies develop late in the course of infection. Avoidance of tick bites is the main preventive measure available, but control of rodents and the ticks that inhabit their burrows can be applied in foci of virus maintenance in the field.

Vesicular stomatitis virus

Vesicular stomatitis (VS) virus is of major economic concern as a cause of acute, febrile vesicular disease in cattle, mainly in Central and northern South America and in the USA. Both of the major serotypes, VS-Indiana and VS-New Jersey, cause influenza-like illness in humans and are an occupational hazard to people handling cattle. The VS viruses comprise a complex of related serotypes and subtypes in the Americas, with related vesiculoviruses (*Rhabdoviridae* family) viruses in Africa and Asia. Many of these viruses are transmitted horizontally and transovarially by phlebotomine sandflies, with evidence for infection of wild rodents and other small mammals. However, the role of these mammals in the epidemiology of VS viruses is unclear because they do not develop viremia. Thus, the host and vector transmission cycles of VS viruses in the Americas are not well understood. Diagnosis is established by isolation of the virus in cell cultures, demonstration of antigen in vesicular fluid or tissues of infected animals, or demonstration of specific RNA sequences by RT-PCR. Serological diagnosis may be complicated by lack of specificity of tests and by normal fluctuations of antibody titers. The experimental vaccines developed for use in domestic animals have not yet been commercialized.

Other Neotropical viruses

Oropouche virus, a Simbu serogroup bunyavirus, causes epidemics, occasionally severe, of acute febrile disease with arthralgia and occasional aseptic meningitis in humans in the Brazilian and Peruvian Amazon as well as Panama. During rainy season epidemics, the virus is transmitted by *Culicoides paraensis* biting midges. Enzootic maintenance cycles are believed to involve forest mammals and arboreal mosquitoes.

Mayaro (MAY) virus occurs epidemically in the Brazilian and Bolivian Amazon Basin, and has also been associated with human disease in Surinam and Trinidad. In humans, the acute, nonfatal, febrile disease with rash is clinically similar to CHIK, an alphavirus to which it is antigenically and taxonomically related. MAY virus appears to be maintained in nature in a cycle similar to that of yellow fever, with arboreal mosquito vectors and primate hosts, but also involving other mammals and birds.

Rocio virus was first isolated from fatal human encephalitis cases during an explosive outbreak of acute febrile disease in coastal Sao Paulo State, Brazil in 1975, after which time sporadic outbreaks have continued. This virus is an ungrouped flavivirus in the *Togaviridae*, and is serologically related to Murray Valley encephalitis virus from Australia. The epidemiology is unclear but probably involves wild birds, and several mosquito species are suspected vectors.

Viruses Occurring in Europe

Tahyna (TAH) virus is widely distributed in Europe, and has been reported in Africa. TAH virus produces an influenza-like febrile disease, with occasional central nervous system involvement. The virus is a bunyavirus of the California serogroup, in the *Bunyaviridae*. Like La Crosse virus, small forest mammals are TAH virus reservoirs, and the virus is horizontally and transovarially transmitted by *Aedes* mosquitoes. There are no effective control measures.

Omsk hemorrhagic fever occurs in a localized area of western Siberia. Disease can be severe, with up to 3% case fatality, and sequelae are common. This virus is a member of the tick-borne encephalitis (TBE) complex of the flaviviruses. The virus is epizootic in wild muskrats, which had been introduced into the area, and is associated with ixodid ticks. Muskrat handlers are at highest risk of infection. Water voles and other rodents are also vertebrate hosts of the virus. TBE virus vaccine is used in high-risk individuals to provide protection.

Central European tick-borne encephalitis (CETBE) virus is also a member of the TBE complex of flaviviruses. Because recreation in wooded areas has increased in recent years, CETBE has become the most frequent arthropod-borne disease in Europe. The virus occurs in deciduous forests in western Europe from the Mediterranean countries, westward to France, and northward to the Scandinavian countries. It is maintained in a transmission cycle involving small mammals and *Ixodes* spp. ticks. Human infection also occurs through the consumption of unpasteurized milk from infected cows. Infection can be prevented by an inactivated vaccine and avoidance of tick bites.

Cowpox virus is an orthopoxvirus in the *Poxviridae*. It has a wide host range. Domestic cats are the most important source of human infection, transmitting the virus from wild rodent reservoirs to people. In addition to cattle, this virus has produced severe, generalized infections in a variety of incidental animal hosts in zoos and circuses, including elephants and large cats, which may die. Humans develop typical poxvirus lesions (vesicle and pustule formation),

usually on the hands. Laboratory diagnosis (characterization of isolated virus) is required to differentiate cowpox from other nodule-forming zoonotic poxviruses such as orf virus, bovine papular stomatitis virus and pseudopoxvirus, which are worldwide in distribution.

Viruses Occurring in Africa

Rift Valley fever virus

Rift Valley fever (RVF) is among the most serious arbovirus infections in Africa today. Repeated RVF epidemics in sub-Saharan Africa cause serious disease in small ruminant animals and humans. RVF disease has expanded its historical geographic range in the livestock-raising areas of eastern and southern Africa over the past 25 years, causing a massive epidemic in Egypt in 1977–1978, appearing in epidemic form along the Mauritania–Senegal border 10 years later and in Madagascar in 1990–1991. Cattle, sheep and humans are affected. Abortion storms with febrile disease and bloody diarrhea occur in ruminant animals, and mortality may be heavy in young stock. Most infected humans develop febrile disease, with prolonged convalescence. A few individuals develop more severe disease, with liver necrosis, hemorrhagic pneumonia, meningoencephalitis and retinitis with vision loss. The human case fatality rate is less than 1%. RVF virus is in the *Phlebovirus* genus of the *Bunyaviridae*. In sub-Saharan Africa, RVF virus is closely tied to its *Aedes* mosquito vectors. RVF vectors transmit the virus transovarially and horizontally. The virus persists in mosquito eggs laid around seasonally flooded pools and depressions called 'dambos'. When the rains come and dambos flood, the eggs hatch and infected mosquitoes emerge and begin transmission. The vertebrate reservoir hosts of RVF virus are unknown. Agricultural development projects in Africa must take into account that creation of larval habitats (artificial dambos) may lead to epidemics of RVF, as happened in West Africa. Diagnosis depends on virus isolation and serologic testing. Field and laboratory workers need to exercise caution to avoid becoming infected by exposure to the virus during postmortem examination of animals or processing materials in the laboratory. A high level of biosecurity is required. Both live attenuated and inactivated vaccines are available for animals, but the unpredictability of scattered, sporadic RVF outbreaks across sub-Saharan Africa is a major obstacle for implementation of extensive, cost-effective vaccination programs. An inactivated vaccine is available for laboratory and field workers at high risk of infection.

Marburg and Ebola viruses

The reappearance of epidemic Ebola disease in Kikwit, Democratic Republic of the Congo (formerly Zaire) in 1995 and Makokou, Gabon in 1996 again focused international attention on this hemorrhagic disease. Marburg and Ebola viruses have sporadically caused severe hemorrhagic fever in humans. Marburg virus, although of African origin, first appeared in laboratory workers in Germany who had handled cell cultures originating from African primates. Later, epidemics of severe hemorrhagic fever occurred in the Sudan and in Zaire, and Ebola virus was isolated. These viruses produce hemorrhagic shock syndrome and visceral organ necrosis, and have the highest case fatality rate (30–90%) of the hemorrhagic fevers. These viruses, with their bizarre filamentous, pleomorphic morphology, belong to the *Filoviridae* family. They are presumed to be zoonotic, but their hosts in nature and mechanisms of transmission in the field have not been determined. Most of the Makokou, Gabon patients had very recently butchered chimpanzees. A variant of Ebola virus has been isolated from chimpanzees from Côte D'Ivoire, but since wild primates suffer severe disease, they are unlikely to be maintenance reservoirs. Nosocomial transmission of Marburg and Ebola viruses has occurred frequently; a high level of patient isolation and biosafety containment are essential to avoid hospital- and laboratory-acquired infection. An outbreak of simian hemorrhagic disease, caused by a Marburg/Ebola-like filovirus, occurred in a primate-holding facility, but without evidence of related human disease among animal care personnel. The virus can be detected by electron microscopic examination of tissue or isolation of the virus in cell culture. Serologic diagnosis is accomplished by means of indirect immunofluorescence or ELISA test, with antigen specificity confirmed by western blot. No vaccines or control measures are available.

Monkeypox virus

The largest epidemic of human monkeypox ever documented occurred in the Katako-Kombe area of the Democratic Republic of the Congo (formerly Zaire) in 1996–1997, with over 500 people becoming ill and five deaths. Primary monkey-to-human transmission occurred, as did subsequent secondary human-to-human spread. Human monkeypox is a severe, smallpox-like illness. Monkeypox belongs to the *Orthopoxvirus* genus of the *Poxviridae*. The virus is enzootic in wild primates and squirrels in the rainforests of West and Central Africa. Vaccinia is protective against infection, but its use has been discontinued with the eradication of smallpox. A new

drug, cidofovir, has been reported to be effective in preventing overt disease and death in monkeys infected experimentally.

O'nyong-nyong virus

O'nyong-nyong virus disease initially occurred in 1959 in Uganda and spread to Kenya, Tanzania, Malawi and Senegal, infecting 2 million people. O'nyong-nyong virus is considered a subtype of Chikungunya virus, and is widespread in eastern sub-Saharan Africa. The disease it produces and its epidemiology are similar to CHIK, with *Anopheles* spp. as the main mosquito vector. Igbo Ora virus is also antigenically related to CHIK virus, and has been isolated from humans in West Africa.

Semliki Forest virus

Semliki Forest (SF) virus caused an extensive epidemic of human disease in Bangui, Central African Republic, in 1987. SF virus is an alphavirus in the *Togaviridae*. It occurs across East, Central and West Africa, and has been isolated from various mosquitoes and from wild birds. Antibodies have been also found in wild mammals. The SF virus maintenance cycle probably involves *Ae. africanus* mosquitoes and vervet monkeys.

Orungo virus

Orungo (ORU) virus caused mild epidemic disease (fever, nausea, headache and rash) in Nigeria. The virus occurs in a band across Africa from Uganda to Sierra Leone. It is an orbivirus in the *Reoviridae*. ORU is probably mosquito transmitted, but the species that transmit it in nature are not known. Although the vertebrate reservoir hosts are unknown, wild primates have antibody and are suspected to be involved in virus maintenance.

Viruses Occurring in Asia

Kyasanur Forest disease (KFD) was first recognized in India in 1957, when an acute hemorrhagic disease appeared in wild monkeys and people frequenting forested areas. KFD has been slowly spreading in India. KFD virus is a member of the tick-borne encephalitis complex of flaviviruses. The basic virus maintenance cycle involves forest mammals (primates, rodents, bats and insectivores) and ixodid ticks, mainly *Haemaphysalis spinigera*. The virus can be isolated in mice and cell cultures, including tick cells. An inactivated vaccine provides some protection to people at risk of infection.

Japanese encephalitis virus

Japanese encephalitis (JE) is found in a broad area from far eastern Russia, northeastern Asia through China and Southeast Asia to Papua New Guinea and the Torres Strait Islands of Australia and westward into India. One of the world's arthropod-borne encephalitides, JE causes the greatest number of clinical human cases – thousands annually – predominantly in children. It produces encephalitis in humans and horses, and acute febrile disease with abortion in swine, an amplifying host. Herons and egrets are wildlife amplifying hosts. The virus is transmitted by *Culex* spp. mosquitoes. The overwintering mechanism in temperate Asia is unknown. JE virus is a member of a complex of four related flaviviruses in the *Togaviridae* family. RNA oligonucleotide analysis indicates that there are differences in JE virus isolates from different vertebrate species, and between strains from different geographic areas. Diagnosis is by means of virus isolation or demonstration of JE virus genomic sequences by RT-PCR, IgG antibody rise, or of specific IgM in acute sera. Prevention of disease is mainly through vaccination of humans, horses and swine. A bivalent vaccine is used that incorporates the two recognized antigenic variants. Insecticides and integrated pest control measures that include natural compounds (*Bacillus thurengiensis* toxins), larvicidal fish, and larval habitat modification have been successfully used in China. Use of pyrethroid-impregnated bed netting can also prevent transmission.

Viruses Occurring in Australia

Murray Valley encephalitis virus

Murray Valley encephalitis (MVE) occurs primarily in southeastern Australia, with cases appearing occasionally in other parts of the country and in Papua New Guinea. Febrile disease leads to encephalitis and, in severe cases, death. Neurologic sequelae are common in survivors. Children are predominantly affected. Large water birds are the main vertebrate amplifying hosts, but mammals are also reservoirs. The virus is transmitted mainly by *Culex annulirostris* mosquitoes. It is maintained in northern Australia and New Guinea and is believed to be introduced into southern Australia in years of high rainfall. MVE virus is a flavivirus of the *Togaviridae* and is closely related to Japanese encephalitis. RNA sequencing indicates that the Australian strains of MVE virus are similar to, but different from Papua New Guinea isolates. Like JE, MVE diagnosis depends on virus isolation and serological testing. No vaccine is

available. Control is achieved through application of larvicides.

Ross River virus

Ross River (RR) virus has caused annual epidemics of febrile disease with polyarthritis and rash in tropical and temperate eastern Australia. Within the past two decades, RR virus has spread through several Pacific islands in epidemic form and appears to have become endemic in New Caledonia. Convalescence can be long. RR virus is an alphavirus of the *Togaviridae* family. The enzootic maintenance cycles of RR virus in Australia are not well defined, but wild and domestic mammals appear to be the reservoir hosts, and the principal mosquito vectors are salt marsh *Aedes* spp. and freshwater *Culex* spp. In the Pacific Islands, the virus was probably transmitted from person to person by *Aedes* mosquitoes.

Kunjin virus

Kunjin virus has caused scattered cases of fever, myalgia and polyarthralgia across Australia and in clusters in central Queensland and northern Western Australia. Kunjin virus is a flavivirus of the *Togaviridae*, and is related to WN and MVE viruses. The virus is believed to be maintained in wild bird–*Cx annulirostris* transmission cycles similar to MVE virus.

Burmah Forest virus

Burmah Forest virus causes subclinical and clinical infections in humans, including fever, myalgia, polyarthalgia and rash. It is an alphavirus in the *Togaviridae*. The virus appears to be endemic in eastern Australia, and its mosquito vectors and vertebrate hosts have not been established.

Control

Control of zoonotic virus diseases is accomplished by breaking the cycle of transmission. This is usually achieved by eliminating or immunizing vertebrate hosts, and reducing vector populations. Reduction of reservoir host populations is usually not accomplished because it is too expensive, not environmentally safe, and not technically or logistically feasible. However, there have been some notable exceptions. Bolivian hemorrhagic fever, caused by Machupo virus, was controlled by reduction of its rodent hosts through intensive rodenticiding. The principal vampire bat reservoir of rabies, *Desmodus rotundus*, is being controlled by the application of warfarin-type anticoagulants, either directly to the bats themselves or to the cattle upon which they feed (with no harm to the cattle). Control programs like these have to be continuous to be effective. Their reduction or discontinuation results in host population recovery through reproduction and immigration, which may result in re-emergence of disease sweeping through the increasing, susceptible cohort.

Immunization of hosts is both promising and discouraging. After decades of research, safe and effective rabies vaccines are being used for immunization of humans, domesticated animals and some wildlife species. The human diploid cell vaccine is extremely effective, free of adverse effects and widely available but at a cost too high for use in many developing countries. Safe, effective animal vaccines of cell culture origin are on the market. After some initial public resistance, raccoon populations in the eastern USA and wild foxes in Europe are being successfully immunized by means of an oral, vaccinia-vectored recombinant vaccine. This experience illustrates the need for public understanding, in order to counteract fear of the unknown – in this case, field use of a genetically engineered virus. Other vectored rabies vaccines are under development for control of mongoose-transmitted rabies in the Caribbean. Vaccines will not be developed for many zoonotic viral diseases that affect relatively few people and are of very limited concern geographically. With limited markets for new vaccines, there is no economic incentive to justify the several millions of dollars for the research and testing required for licensure and commercialization of these products.

Vector control is another promising but difficult area of zoonoses reduction or elimination. Insecticide application has become more problematic because both vectors themselves, as well as public opinion, have become more resistant to their use. Integrated pest management techniques, well developed for the control of many crop insects, along with the use of natural pesticides such as *Bacillus thurengiensis* toxin, offers promise for the effective, environmentally safe control of dipterous vectors. Control of tick vectors is likely to remain a problem for some time to come.

Emerging and Reemerging Zoonoses

Ecological change

Human disturbance has become a feature of nearly every part of the planet. All too often these disturbances create habitats that favor increases in populations of key hosts and vectors, with subsequent increased transmission of viral zoonoses. Nowhere are ecological changes happening more rapidly and

profoundly than in the world's tropics. Conversion of tropical forests to agricultural ecosystems simplifies diverse ecosystems and provides either native or introduced host or vector species the conditions necessary to become more abundant, and sustain intensified virus transmission in areas where people live and work. In Africa, recent yellow fever epidemics have been increasing dramatically in agricultural areas. Some agricultural irrigation development projects have created extensive vector breeding habitats, with an increase in mosquito-transmitted disease. The extensive dams constructed in Senegal were followed by epidemics of Rift Valley fever, with numerous cases of disease in humans and small ruminant animals. The public health consequences of development projects must never be overlooked.

Global climate change will also bring ecological changes and shifts of human populations that will affect the occurrence of viral zoonoses. Although experts debate the geography, severity and rapidity of the oncoming greenhouse effect, there is general consensus that changes in the global climate will happen with unprecedented speed. With those changes will come alterations in the geography of natural and agricultural ecosystems, with corresponding changes in the distribution of zoonotic diseases and the intensity of their transmission. It is clear that El Niño–southern oscillation phenomena have increased rainfall, with resulting increases in rodent populations and occurrence of hantavirus pulmonary syndrome in the Southwestern USA, and increased breeding sites for mosquito vectors of Rift Valley fever virus in Africa. While it is not possible to predict accurately what the world will be like in 100 years, nor what zoonotic diseases are likely to be most troublesome, it is certain that things will be different, and constant surveillance will be essential to avoid serious problems or deal promptly and effectively with the ones that arise.

Movement of zoonotic viruses can result from the displacement of infected animals, contaminated animal products and virus-carrying arthropod vectors. It is clear that illegal translocation of wild raccoons for hunting purposes, with inadvertent movement of rabies-infected animals, was responsible for initiating the recent rabies epizootic in the Middle Atlantic states. Pets, sport, laboratory and agricultural animals are moving around the world as never before. Although international and national regulations have been established to prevent movement of infected individuals, it is not possible to test for all possible zoonotic viruses, and prevent them from crossing international boundaries. Moreover, significant numbers of animals of high commercial value move illegally. Psittacine and other exotic birds worth hundreds to thousands of dollars each cross illegally into the USA, despite intensive government efforts to halt this smuggling. The importation of highly virulent Newcastle disease (ND) virus has been occasionally linked to smuggled birds. The costly ND outbreak (more than $50 million to control) in southern California in the early 1970s was linked to the importation of an infected pet bird from Asia. Subsequently, highly lethal ND virus has been found in smuggled parrots. Although not a serious infection in humans, ND is highly transmissible and can be extremely lethal in poultry and costly to that industry.

Zoonotic viruses may be transported by movement of arthropod vectors, too. Just as the yellow fever mosquito, *Ae. aegypti*, moved around the world in water casks aboard sailing vessels, mosquitoes are transported around the world in international commerce. Ships still transport mosquito vectors. The Asian tiger mosquito, *Ae. albopictus*, has become established in the Western Hemisphere after multiple introductions in eggs deposited in used tires. This mosquito is capable of transmitting yellow fever, Venezuelan equine encephalitis, Jamestown Canyon (JC) and La Crosse (LAC) encephalitis viruses. It remains to be seen if this highly competitive exotic vector, with its peridomestic habits, will carry yellow fever virus from its jungle cycle to the urban, human amplified cycle in the American tropics, increase the numbers of La Crosse encephalitis cases in south temperate or subtropical areas of the USA, or facilitate emergence of LACV × JCV recombinants. Perhaps of greater concern, modern transport aircraft have been shown to move vector mosquitoes internationally. Nature can move vectors as well. For example, windblown biting insects, such as culicoid midges, have been shown to account for the spread of some arthropod-borne virus diseases of animals, in some cases over long distances. There is no reason to believe that vectors of human diseases cannot move similarly.

Human activity alters animal populations, contact between wild and domestic animals, and human–animal interactions, changing the occurrence of zoonotic diseases and the risk of infection to humans. For example, emergence of new influenza strains is related to the interaction of populations of people, pigs and aquatic birds. It is hypothesized that the emergence of the new H5N1 influenza strain in Hong Kong in 1997 was the result of a chain of events resulting in transmission from migrating shorebirds to ducks, to chickens, and finally to humans. Fortunately, this pathogenic strain was not easily transmitted from human to human, or a serious pandemic could have resulted.

Social change

Increasing human populations place great demands on the public health and other government services, especially in developing countries where needs for zoonosis diagnosis, control and prevention are greatest and resources are most limited. Some preventive measures could be implemented by the people who live in the affected areas themselves, and at minimal cost, if they knew why and how they needed to do it. Public education and information is essential for control and prevention of zoonotic diseases; however, it takes more than civic action to deal with them. Delivery of public education, disease surveillance and diagnosis and the technical materials and logistical support for control or preventive programs depend on national or international scientific and financial support. Because serious zoonotic viral diseases, such as rabies, yellow fever, Venezuelan equine encephalitis and the hemorrhagic fevers, know nothing of international boundaries, international technical cooperation and financial support are imperative.

See also: ***Bunyaviridae*: General features; Replication; Diagnostic techniques: Detection of viral antigens, nucleic acids and specific antibodies; Isolation and identification by culture and microscopy; Encephalitis viruses (*Flaviviridae*): Encephalitis viruses and related viruses causing hemorrhagic disease; Tick-borne encephalitis and Wesselsbron viruses; Hantaviruses (*Bunyaviridae*); Lassa, Junin, Machupo and Guanarito viruses (*Arenaviridae*); Rhabdoviruses (*Rhabdoviridae*): Plant rhabdoviruses; Ungrouped mammalian, bird and fish rhabdoviruses; Emerging and re-emerging virus diseases; Marburg and Ebola viruses (*Filoviridae*).**

Further Reading

Beran GW and Steele JH (eds) (1994) *Handbook of Zoonoses,* Section B, Viral, 2nd edn. Boca Raton: CRC Press.

Fenner FJ, Gibbs EPJ, Murphy FA *et al* (eds) (1987) *Veterinary Virology.* San Diego: Academic Press.

Fields BN, Knipe DM and Howley PM (eds) (1990) *Virology,* 2nd edn. New York: Raven Press.

Hubbert WT, McCulloch WF and Schnurrenberg PR (eds) (1975) *Diseases Transmitted from Animals to Man* 6th edn. Springfield IL: CC Thomas.

Hugh-Jones M, Hubbert WT and Hagstad HV (1995) *Zoonoses: Recognition, Control and Prevention.* Ames: Iowa State University Press.

Monath TP (ed.) (1988–1989). *The Arboviruses: Epidemiology and Ecology,* vols I–V. Boca Raton: CRC Press.

APPENDIX

This appendix lists the updated ICTV virus name index published in the 1999 Seventh ICTV Report.[1] It lists all virus names, with nomenclature based on the ICTV code of 1999. Because of differences in publication schedules, some virus names in *Encyclopedia* entries may differ form those listed in the Appendix. While not all viruses listed are addressed by the *Encyclopedia*, the list provides a ready reference source for all viruses, from the most recognized to the most obscure.

Species names are in italics; names not in italics indicate synonym, strain, serotype, genotype, and clade or isolate names. Abbreviation virus names are enclosed in parenthesis. The family name is also indicated for each virus name.

10924 virus, *Arenaviridae*
12056 virus, *Arenaviridae*
1324Cg/79 virus, *Bunyaviridae*
3076 virus, *Arenaviridae*
3099 virus, *Arenaviridae*
3739 virus, *Arenaviridae*
63U-11 virus, (63UV), *Bunyaviridae*
75V 2374 virus, (V2374V), *Bunyaviridae*
75V 2621 virus, (V2621V), *Bunyaviridae*
78V 2441 virus, (V2441V), *Bunyaviridae*

AA288-77 virus, *Arenaviridae*
Abadina virus, (ABAV), *Reoviridae*
Abelson murine leukemia virus, (AbMLV), *Retroviridae*
Above Maiden virus, *Reoviridae*
Abras virus, (ABRV), *Bunyaviridae*
Abraxas grossulariata cypovirus 8, (AgCPV-8), *Reoviridae*
Abu Hammad virus, (AHV), *Bunyaviridae*
Abu Mina virus, (ABMV), *Bunyaviridae*
Abutilon mosaic virus, (AbMV), *Geminiviridae*
Abutilon yellows virus, (AbYV), *Closteroviridae*
Acado virus, (ACDV), *Reoviridae*
Acalypha yellow mosaic virus, (AYMV), *Geminiviridae*
Acara virus, (ACAV), *Bunyaviridae*
Acciptrid herpesvirus 1, (AcHV-1), *Herpesviridae*
Acetobacter phage pKG-2, (pKG-2), *Myoviridae*
Acetobacter phage pKG-3, (pKG-3), *Myoviridae*
Acherontia atropas virus, (AaV), *Tetraviridae*
Acheta domestica densovirus, (AdDNV), *Parvoviridae*
Acholeplasma phage 0c1r, (0c1r), *Inoviridae*
Acholeplasma phage 10tur, (10tur), *Inoviridae*
Acholeplasma phage L2, (L2), *Plasmaviridae*
Acholeplasma phage M1, (M1), *Plasmaviridae*
Acholeplasma phage MV-G51, (G51), *Inoviridae*
Acholeplasma phage MV-L1, (L1), *Inoviridae*
Acholeplasma phage MV-L51, (L51), *Inoviridae*
Acholeplasma phage O1, (O1), *Plasmaviridae*
Acholeplasma phage v1, (v1), *Plasmaviridae*
Acholeplasma phage v2, (v2), *Plasmaviridae*
Acholeplasma phage v4, (v4), *Plasmaviridae*
Acholeplasma phage v5, (v5), *Plasmaviridae*
Acholeplasma phage v7, (v7), *Plasmaviridae*
Acid-stable equine picornaviruses, (EqPV), *Picornaviridae*
Acinetobacter phage 133, (133), *Myoviridae*
Acinetobacter phage 205, (205), *Leviviridae*
Acinetobacter phage 531, (531), *Siphoviridae*
Acinetobacter phage A10/45, (A10/45), *Myoviridae*
Acinetobacter phage A3/2, (A3/2), *Myoviridae*
Acinetobacter phage A36, (A36), *Podoviridae*
Acinetobacter phage BS46, (BS46), *Myoviridae*
Acinetobacter phage E13, (E13), *Siphoviridae*
Acinetobacter phage E14, (E14), *Myoviridae*
Acinetobacter phage E4, (E4), *Myoviridae*
Acinetobacter phage E5, (E5), *Myoviridae*

[1] van Regenmortel *et al.* (1999) *Virus Taxonomy Seventh ICTV Report*. Academic Press, London, UK.

Acipenserid herpesvirus 1, (AciHV-1), *Herpesviridae*
Acipenserid herpesvirus 2, (AciHV-2), *Herpesviridae*
Acrobasis zelleri entomopoxvirus 'L', (AZEV), *Poxviridae*
Actias selene cypovirus 4, (AsCPV-4), *Reoviridae*
Actinomycetes phage 108/016, (108/016), *Myoviridae*
Actinomycetes phage 119, (119), *Siphoviridae*
Actinomycetes phage A1-Dat, (A1-Dat), *Siphoviridae*
Actinomycetes phage Bir, (Bir), *Siphoviridae*
Actinomycetes phage M_1, (M_1), *Siphoviridae*
Actinomycetes phage MSP8, (MSP8), *Siphoviridae*
Actinomycetes phage P-a-1, (P-a-1), *Siphoviridae*
Actinomycetes phage R_1, (R_1), *Siphoviridae*
Actinomycetes phage R_2, (R_2), *Siphoviridae*
Actinomycetes phage SK1, (SK1), *Myoviridae*
Actinomycetes phage SV2, (SV2), *Siphoviridae*
Actinomycetes phage VP5, (VP5), *Siphoviridae*
Actinomycetes phage ΦC, (ΦC), *Siphoviridae*
Actinomycetes phage ϕ115-A, (ϕ115-A), *Siphoviridae*
Actinomycetes phage ϕ150A, (ϕ150A), *Siphoviridae*
Actinomycetes phage ϕ31C, (ϕ31C), *Siphoviridae*
Actinomycetes phage ϕUW21, (ϕUW21), *Siphoviridae*
Acute bee paralysis virus, (ABPV), Unassigned
Acyrthosiphon pisum virus, (APV), Unassigned
Adelaide River virus, (ARV), *Rhabdoviridae*
Adeno-associated virus 1, (AAV-1), *Parvoviridae*
Adeno-associated virus 2, (AAV-2), *Parvoviridae*
Adeno-associated virus 3, (AAV-3), *Parvoviridae*
Adeno-associated virus 4, (AAV-4), *Parvoviridae*
Adeno-associated virus 5, (AAV-5), *Parvoviridae*
Adeno-associated virus 6, (AAV-6), *Parvoviridae*
Aedes aegypti densovirus, (AaDNV), *Parvoviridae*
Aedes aegypti entomopoxvirus, (AAEV), *Poxviridae*
Aedes albopictus densovirus, (AlDNV), *Parvoviridae*
Aedes pseudoscutellaris densovirus, (ApDNV), *Parvoviridae*
Aedes sollicitans NPV, (AesoNPV), *Baculoviridae*
Aedes taeniorhynchus iridescent virus, *Iridoviridae*
Aeromonas phage 1, (1), *Myoviridae*
Aeromonas phage 25, (25), *Myoviridae*
Aeromonas phage 29, (29), *Myoviridae*
Aeromonas phage 31, (31), *Myoviridae*
Aeromonas phage 37, (37), *Myoviridae*
Aeromonas phage 40R, (40R), *Myoviridae*
Aeromonas phage 40RR2.8t, (40RR2.8t), *Myoviridae*
Aeromonas phage 43, (43), *Myoviridae*
Aeromonas phage 51, (51), *Myoviridae*
Aeromonas phage 59.1, (59.1), *Myoviridae*
Aeromonas phage 65, (65), *Myoviridae*
Aeromonas phage Aa-1, (Aa-1), *Podoviridae*
Aeromonas phage Aeh1, (Aeh1), *Myoviridae*
Aeromonas phage Aeh2, (Aeh2), *Myoviridae*
African cassava mosaic virus, (ACMV), *Geminiviridae*
African green monkey cytomegalovirus, *Herpesviridae*
African green monkey EBV-like virus, *Herpesviridae*
African green monkey polyomavirus, (AGMPyV), *Polyomaviridae*
African horse sickness virus 1 to 9, (AHSV-1 to 9), *Reoviridae*
African horse sickness virus, (AHSV), *Reoviridae*
African swine fever virus, (ASFV), *Asfarviridae*
AG80-663 virus, (AG80V), *Togaviridae*
AG83-1746 virus, (AG1746V), *Bunyaviridae*
AG83-497 virus, (AG497V), *Bunyaviridae*
Agaricus bisporus virus 1, (ABV-1), Unassigned
Agaricus bisporus virus 4, (AbV-4), *Partitiviridae*
Ageratum yellow vein virus, (AYVV), *Geminiviridae*
Aglais urticae cypovirus 2, (AuCPV-2), *Reoviridae*
Aglais urticae cypovirus 6, (AuCPV-6), *Reoviridae*
Aglaonema bacilliform virus, (ABV), *Caulimoviridae*
Agraulis vanillae cypovirus 2, (AvaCPV-2), *Reoviridae*
Agraulis vanillae densovirus, (AvDNV), *Parvoviridae*
Agraulis vanillae virus, (AvV), *Tetraviridae*
Agrobacterium phage PIIBNV6, (PIIBNV6), *Myoviridae*
Agrobacterium phage PS8, (PS8), *Siphoviridae*
Agrobacterium phage PT11, (PT11), *Siphoviridae*
Agrobacterium phage Ψ, (Ψ), *Siphoviridae*
Agrochola helvolva cypovirus 6, (AhCPV-6), *Reoviridae*
Agrochola lychnidis cypovirus 6, (AlCPV-6), *Reoviridae*
Agropyron mosaic virus, (AgMV), *Potyviridae*
Agrotis segetum cypovirus 9, (AsCPV-9), *Reoviridae*
Aguacate virus, (AGUV), *Bunyaviridae*
Ahlum waterborne virus, (AWBV), *Tombusviridae*
Aichi virus, (AiV), *Picornaviridae*
Aino virus, (AINOV), *Bunyaviridae*
Akabane virus, (AKAV), *Bunyaviridae*
AKR (endogenous), murine leukemia virus, (AKRMLV), *Retroviridae*
Alajuela virus, (ALJV), *Bunyaviridae*
Alcaligenes phage 8764, (8764), *Siphoviridae*
Alcaligenes phage A5/A6, (A5/A6), *Siphoviridae*
Alcaligenes phage A6, (A6), *Myoviridae*
Alcelaphine herpesvirus 1, (AlHV-1), *Herpesviridae*
Alcelaphine herpesvirus 2, (AlHV-2), *Herpesviridae*
Alenquer virus, (ALEV), *Bunyaviridae*
Aleutian disease virus, *Parvoviridae*
Aleutian mink disease virus, (AMDV), *Parvoviridae*
Alfalfa cryptic virus 1, (ACV-1), *Partitiviridae*
Alfalfa cryptic virus 2, (ACV-2), *Partitiviridae*
Alfalfa latent virus, *Carlavirus*
Alfalfa mosaic virus, (AMV), *Bromoviridae*
Alfuy virus, (ALFV), *Flaviviridae*

Alligatorweed stunting virus, (AWSV), *Closteroviridae*
Allomyces arbuscula virus, (AAV), Unassigned
Almeirim virus, (ALMV), *Reoviridae*
Almpiwar virus, (ALMV), *Rhabdoviridae*
Alpinia mosaic virus, (AlpMV), *Potyviridae*
Alstroemeria carlavirus, *Carlavirus*
Alstroemeria mosaic virus, (AlMV), *Potyviridae*
Alstroemeria streak virus, (AlStV), *Potyviridae*
Altamira virus, (ALTV), *Reoviridae*
Alternanthera mosaic virus, (AltMV), *Potexvirus*
Alteromonas phage PM2, (PM2), *Corticoviridae*
Althea rosea enation virus, (AREV), *Geminiviridae*
Amapari virus, (AMAV), *Arenaviridae*
Amaranthus leaf mottle virus, (AmLMV), *Potyviridae*
Amazon lily mosaic virus, (ALiMV), *Potyviridae*
Ambystoma tigrinum stebbensi virus, (ATV), *Iridoviridae*
American ground squirrel cytomegalovirus, *Herpesviridae*
American hop latent virus, (AHLV), *Carlavirus*
American oyster reovirus 13p2V, (13p2V), Reoviridae
American plum line pattern virus, (APLPV), *Bromoviridae*
Amsacta moorei entomopoxvirus 'L', (AMEV), *Poxviridae*
Amyelosis chronic stunt virus, (ACSV), *Caliciviridae*
AN 20410 virus, *Arenaviridae*
AN 21366 virus, *Arenaviridae*
Anagrapha falcifera NPV, (AnfaMNPV), *Baculoviridae*
Anagyris vein yellowing virus, *Tymovirus*
Anaitis plagiata cypovirus 3, (ApCPV-3), *Reoviridae*
Anaitis plagiata cypovirus 6, (ApCPV-6), *Reoviridae*
Ananindeua virus, (ANUV), *Bunyaviridae*
Anatid herpesvirus 1, (AnHV-1), *Herpesviridae*
Andasibe virus, (ANDV), *Reoviridae*
Andean potato latent virus, (APLV), *Tymovirus*
Andean potato mottle virus, (APMoV), *Comoviridae*
Andes virus, (ANDV), *Bunyaviridae*
Aneilema virus, (AneV), *Potyviridae*
Angel fish reovirus, (AFRV), *Reoviridae*
Anguillid herpesvirus 1, (AngHV-1), *Herpesviridae*
Anhanga virus, (ANHV), *Bunyaviridae*
Anhembi virus, (AMBV), *Bunyaviridae*
Anomala cuprea entomopoxvirus, (ACEV), *Poxviridae*
Anopheles A virus, (ANAV), *Bunyaviridae*
Anopheles B virus, (ANBV), *Bunyaviridae*
Antequera virus, (ANTV), *Bunyaviridae*
Antheraea eucalypti virus (AeV) *Tetraviridae*
Antheraea mylitta cypovirus 4, (AmCPV-4), *Reoviridae*
Antheraea pernyi cypovirus 4, (ApCPV-4), *Reoviridae*
Anthoxanthum latent blanching virus, (ALBV), *Hordeivirus*
Anthoxanthum mosaic virus, (AntMV), *Potyviridae*
Anthriscus latent virus, (AntLV), *Carlavirus*
Anthriscus yellows virus, (AYV), *Sequiviridae*
Anti xanthomista cypovirus 6, (AxCPV-6), *Reoviridae*
Anticarsia gemmatalis iridescent virus, (AGIV), *Iridoviridae*
Anticarsia gemmatalis MNPV, (AgMNPV), *Baculoviridae*
Aotine herpesvirus 1, (AoHV-1), *Herpesviridae*
Aotine herpesvirus 3, (AoHV-3), *Herpesviridae*
Apanteles crassicornis bracovirus, (AcBV), *Polydnaviridae*
Apanteles fumiferanae bracovirus, (AfBV), *Polydnaviridae*
Apeu virus, (APEUV), *Bunyaviridae*
Aphid lethal paralysis virus, (ALPV), Unassigned
Aphodius tasmaniae entomopoxvirus, (ATEV), *Poxviridae*
Apis iridescent virus, *Iridoviridae*
Apoi virus, (APOIV), *Flaviviridae*
Aporophyla lutulenta cypovirus 10, (AlCPV-10), *Reoviridae*
Apple chlorotic leaf spot virus, (ACLSV), *Trichovirus*
Apple dimple fruit viroid, (ADFVd), *Pospiviroidae*
Apple fruit crinkle viroid, (AFCVd), *Avsunviroidae*
Apple mosaic virus, (ApMV), *Bromoviridae*
Apple scar skin viroid, (ASSVd), *Pospiviroidae*
Apple stem grooving virus, (ASGV), *Capillovirus*
Apple stem pitting virus, (ASPV), *Foveavirus*
Aquareovirus A, (ARV-A), *Reoviridae*
Aquareovirus B, (ARV-B), *Reoviridae*
Aquareovirus C, (ARV-C), *Reoviridae*
Aquareovirus D, (ARV-D), *Reoviridae*
Aquareovirus E, (ARV-E), *Reoviridae*
Aquareovirus F, (ARV-F), *Reoviridae*
Aquilegia necrotic mosaic virus, (ANMV), *Caulimoviridae*
Aquilegia virus, (AqV), *Potyviridae*
Arabidopsis thaliana Ta1 virus, (AthTa1V), *Pseudoviridae*
Arabis mosaic virus large satellite RNA, Satellite
Arabis mosaic virus small satellite RNA, Satellite
Arabis mosaic virus, (ArMV), *Comoviridae*
Araguari virus, (ARAV), Unassigned
Aransas Bay virus, (ABV), *Bunyaviridae*
Araujia mosaic virus, (ArjMV), *Potyviridae*
Arbia virus, (ARBV), *Bunyaviridae*
Arboledas virus, (ADSV), *Bunyaviridae*
Arbroath virus, (ABRV), *Reoviridae*
Arctia caja cypovirus 2, (AcCPV-2), *Reoviridae*
Arctia caja cypovirus 3, (AcCPV-3), *Reoviridae*
Arctia villica cypovirus 2, (AviCPV-2), *Reoviridae*

Arctic squirrel hepatitis virus, (ASHV), *Hepadnaviridae*
Argentine turtle herpesvirus, *Herpesviridae*
Arkansas bee virus, (ABV), Unassigned
Arkonam virus, *Reoviridae*
Armadillidium vulgare iridescent virus, *Iridoviridae*
Armstrong virus, *Arenaviridae*
Aroa virus, (AROAV), *Flaviviridae*
Arphia conspersa entomopoxvirus 'O', (ACOEV), *Poxviridae*
Arracacha latent virus, (ALV), *Carlavirus*
Arracacha virus A, (AVA), *Comoviridae*
Arracacha virus B, (AVB), *Comoviridae*
Arracacha virus Y, (AVY), *Potyviridae*
Artichoke Aegean ringspot virus, (AARSV), *Comoviridae*
Artichoke curly dwarf virus, (ACDV), *Potexvirus*
Artichoke Italian latent virus, (AILV), *Comoviridae*
Artichoke latent virus M, (ArLVM), *Carlavirus*
Artichoke latent virus S, (ArLVS), *Carlavirus*
Artichoke latent virus, (ArLV), *Potyviridae*
Artichoke mottled crinkle virus, (AMCV), *Tombusviridae*
Artichoke vein banding virus, (AVBV), *Comoviridae*
Artichoke yellow ringspot virus, (AYRSV), *Comoviridae*
Artogeia rapae granulovirus, (ArGV), *Baculoviridae*
Aruac virus, (ARUV), *Rhabdoviridae*
Arumowot virus, (AMTV), *Bunyaviridae*
Ascogaster argentifrons bracovirus, (AaBV), *Polydnaviridae*
Ascogaster quadridentata bracovirus, (AqBV), *Polydnaviridae*
Asinine herpesvirus 1, *Herpesviridae*
Asinine herpesvirus 2, *Herpesviridae*
Asinine herpesvirus 3, *Herpesviridae*
Asparagus virus 1, (AV-1), *Potyviridae*
Asparagus virus 2, (AV-2), *Bromoviridae*
Asparagus virus 3, (AV-3), *Potexvirus*
Aspergillus foetidus virus F, (AFV-F), Unassigned
Aspergillus foetidus virus S, (AFV-S), *Totiviridae*
Aspergillus niger virus S, (AnV-S), *Totiviridae*
Aspergillus ochraceous virus, (AoV), *Partitiviridae*
Asystasia gangetica mottle virus, (AGMoV), *Potyviridae*
Asystasia golden mosaic virus, (AGMV), *Geminiviridae*
Ateline herpesvirus 1, (AtHV-1), *Herpesviridae*
Ateline herpesvirus 2, (AtHV-2), *Herpesviridae*
Ateline herpesvirus 3, (AtHV-3), *Herpesviridae*
Atkinsonella hypxylon virus, (AhV), *Partitiviridae*
Atlantic salmon reovirus ASV, (ASRV), *Reoviridae*
Atlantic salmon reovirus HBR, (HBRV), *Reoviridae*
Atlantic salmon reovirus TSV, (TSRV), *Reoviridae*
Atropa belladonna virus, (AtBV), *Rhabdoviridae*
Aucuba bacilliform virus, (AuBV), *Caulimoviridae*
Aura virus, (AURAV), *Togaviridae*
Australian bat lyssavirus, (ABLV), *Rhabdoviridae*
Australian grapevine viroid, (AGVd), *Pospiviroidae*
Autographa californica MNPV, (AcMNPV), *Baculoviridae*
Autographa gamma cypovirus 12, (AgCPV-12), *Reoviridae*
Auzduk disease virus, *Poxviridae*
AV 9310135 virus, *Arenaviridae*
Avalon virus, (AVAV), *Bunyaviridae*
Avian adeno-associated virus, (AAAV), *Parvoviridae*
Avian carcinoma Mill Hill virus 2, (ACMHV-2), *Retroviridae*
Avian encephalomyelitis-like virus, (AEV), *Picornaviridae*
Avian entero-like virus 2 to 4, (AELV-2 to 4), *Picornaviridae*
Avian leukosis virus - HPRS103, (ALV-J), *Retroviridae*
Avian leukosis virus - RSA, (ALV-A), *Retroviridae*
Avian leukosis virus, (ALV), *Retroviridae*
Avian myeloblastosis virus, (AMV), *Retroviridae*
Avian myelocytomatosis virus 29, (AMCV-29), *Retroviridae*
Avian nephritis virus 1 to 3, (ANV-1 to 3), *Picornaviridae*
Avian orthoreovirus 176, (ARV-176), *Reoviridae*
Avian orthoreovirus S1133, (ARV-S1133), *Reoviridae*
Avian orthoreovirus SK138a, (ARV-138), *Reoviridae*
Avian orthoreovirus, (ARV), *Reoviridae*
Avian parainfluenza virus 1, (APMV-1), *Paramyxoviridae*
Avian paramyxovirus 2 (Yucaipa), (APMV-2), *Paramyxoviridae*
Avian paramyxovirus 3, (APMV-3), *Paramyxoviridae*
Avian paramyxovirus 4, (APMV-4), *Paramyxoviridae*
Avian paramyxovirus 5 (Kunitachi), (APMV-5), *Paramyxoviridae*
Avian paramyxovirus 6, (APMV-6), *Paramyxoviridae*
Avian paramyxovirus 7, (APMV-7), *Paramyxoviridae*
Avian paramyxovirus 8, (APMV-8), *Paramyxoviridae*
Avian paramyxovirus 9, (APMV-9), *Paramyxoviridae*
Avian pneumovirus, *Paramyxoviridae*
Avian sarcoma virus CT10, (ASV-CT10), *Retroviridae*
Avocado sunblotch viroid, (ASBVd), *Avsunviroidae*
Azotobacter phage A12, (A12), *Podoviridae*
Azuki bean mosaic virus, *Potyviridae*

B19 virus, (B19V), *Parvoviridae*
Babahoya virus, (BABV), *Bunyaviridae*
Babanki virus, *Togaviridae*
Baboon herpesvirus, *Herpesviridae*

Baboon orthoreovirus, (BRV), *Reoviridae*
Baboon polyomavirus 2, (PPyV), *Polyomaviridae*
Bacillus phage 1A, (1A), *Siphoviridae*
Bacillus phage 2C, (2C), *Myoviridae*
Bacillus phage AP50, (AP50), *Tectiviridae*
Bacillus phage AR1, (AR1), *Myoviridae*
Bacillus phage AR13, (AR13), *Podoviridae*
Bacillus phage B103, (B103), *Podoviridae*
Bacillus phage B1715V1, (B1715V1), *Siphoviridae*
Bacillus phage Bace-11, (Bace-11), *Myoviridae*
Bacillus phage BLE, (BLE), *Siphoviridae*
Bacillus phage CP-54, (CP-54), *Myoviridae*
Bacillus phage G, (G), *Myoviridae*
Bacillus phage GA-1, (GA-1), *Podoviridae*
Bacillus phage GS1, (GS1), *Myoviridae*
Bacillus phage I9, (I9), *Myoviridae*
Bacillus phage II, (II), *Siphoviridae*
Bacillus phage IPy-1, (IPy-1), *Siphoviridae*
Bacillus phage M2, (M2), *Podoviridae*
Bacillus phage mor1, (mor1), *Siphoviridae*
Bacillus phage MP13, (MP13), *Myoviridae*
Bacillus phage MP15, (MP15), *Siphoviridae*
Bacillus phage MY2, (MY2), *Podoviridae*
Bacillus phage Nf, (Nf), *Podoviridae*
Bacillus phage NLP-1, (NLP-1), *Myoviridae*
Bacillus phage PBP1, (PBP1), *Siphoviridae*
Bacillus phage PBS1, (PBS1), *Myoviridae*
Bacillus phage PZA, (PZA), *Podoviridae*
Bacillus phage PZE, (PZE), *Podoviridae*
Bacillus phage SF5, (SF5), *Podoviridae*
Bacillus phage SN45, (SN45), *Siphoviridae*
Bacillus phage SP01, (SP01), *Myoviridae*
Bacillus phage SP10, (SP10), *Myoviridae*
Bacillus phage SP15, (SP15), *Myoviridae*
Bacillus phage SP3, (SP3), *Myoviridae*
Bacillus phage SP5, (SP5), *Myoviridae*
Bacillus phage SP50, (SP50), *Myoviridae*
Bacillus phage SP8, (SP8), *Myoviridae*
Bacillus phage SP82, (SP82), *Myoviridae*
Bacillus phage SPP1, (SPP1), *Siphoviridae*
Bacillus phage Spy-2, (Spy-2), *Myoviridae*
Bacillus phage Spy-3, (Spy-3), *Myoviridae*
Bacillus phage SPβ, (SPβ), *Siphoviridae*
Bacillus phage SST, (SST), *Myoviridae*
Bacillus phage SW, (SW), *Myoviridae*
Bacillus phage Tb10, (Tb10), *Siphoviridae*
Bacillus phage TP-15, (TP15), *Siphoviridae*
Bacillus phage type F, (type F), *Siphoviridae*
Bacillus phage α, (α), *Siphoviridae*
Bacillus phage ϕ105, (ϕ105), *Siphoviridae*
Bacillus phage ϕ15, (ϕ15), *Podoviridae*
Bacillus phage ϕ25, (ϕ25), *Myoviridae*
Bacillus phage ϕ29, (ϕ29), *Podoviridae*
Bacillus phage ϕBa1, (ϕBa1), *Podoviridae*
Bacillus phage ϕe, (ϕe), *Myoviridae*
Bacillus phage ϕNS11, (ϕNS11), *Tectiviridae*
Bagaza virus, (BAGV), *Flaviviridae*
Bahia Grande virus, (BGV), *Rhabdoviridae*
Bahig virus, (BAHV), *Bunyaviridae*
Bajra streak virus, (BaSV), *Geminiviridae*
Bakau virus, (BAKV), *Bunyaviridae*
Bakel virus, (BAKV), *Bunyaviridae*
Baku virus, (BAKUV), *Reoviridae*
Bald eagle herpesvirus, *Herpesviridae*
Bamboo mosaic virus satellite RNA, Satellite
Bamboo mosaic virus, (BaMV), *Potexvirus*
Banana bract mosaic virus, (BBrMV), *Potyviridae*
Banana bunchy top virus, (BBTV), *Nanovirus*
Banana streak virus, (BSV), *Caulimoviridae*
Banded krait herpesvirus, *Herpesviridae*
Bandia virus, (BDAV), *Bunyaviridae*
Bangoran virus, (BGNV), *Rhabdoviridae*
Bangui virus, (BGIV), *Bunyaviridae*
Banna virus (China), (BAV-Ch), *Reoviridae*
Banna virus (China-HN131), (BAV-HN131V), *Reoviridae*
Banna virus (China-HN191), (BAV-HN191V), *Reoviridae*
Banna virus (China-HN295), (BAV-HN295V), *Reoviridae*
Banna virus (China-HN59), (BAV-HN59V), *Reoviridae*
Banna virus (Indonesia-6423), (BAV-In6423), *Reoviridae*
Banna virus (Indonesia-6969), (BAV-In6969), *Reoviridae*
Banna virus (Indonesia-7043), (BAV-In7043), *Reoviridae*
Banna virus, (BAV), *Reoviridae*
Banzi virus, (BANV), *Flaviviridae*
Barfin flounder nervous necrosis virus, (BFNNV), *Nodaviridae*
Barley mild mosaic virus, (BaMMV), *Potyviridae*
Barley stripe mosaic virus, (BSMV), *Hordeivirus*
Barley virus B1, (BarV-B1), *Potexvirus*
Barley yellow dwarf virus – GPV, (BYDV-GPV), *Luteoviridae*
Barley yellow dwarf virus – MAV, (BYDV-MAV), *Luteoviridae*
Barley yellow dwarf virus – PAV, (BYDV-PAV), *Luteoviridae*
Barley yellow dwarf virus – RGV, *Luteoviridae*
Barley yellow dwarf virus – RMV, (BYDV-RMV), *Luteoviridae*
Barley yellow dwarf virus – SGV, (BYDV-SGV), *Luteoviridae*
Barley yellow dwarf virus satellite RNA, Satellite
Barley yellow mosaic virus, (BaYMV), *Potyviridae*
Barley yellow striate mosaic virus, (BYSMV), *Rhabdoviridae*

Barmah Forest virus, (BFV), *Togaviridae*
Barramundi virus-1, (BaV), *Picornaviridae*
Barranqueras virus, (BQSV), *Bunyaviridae*
Barur virus, (BARV), *Rhabdoviridae*
Bashkiria Cg18-20 virus, *Bunyaviridae*
Batai virus, (BATV), *Bunyaviridae*
Batama virus, (BMAV), *Bunyaviridae*
Batken virus, *Orthomyxoviridae*
Batu Cave virus, (BCV), *Flaviviridae*
Bauline virus, (BAUV), *Reoviridae*
Bayou virus, (BAYV), *Bunyaviridae*
Bdellovibrio phage MAC 1, (MAC-1), *Microviridae*
Bdellovibrio phage MAC 1′, (MAC-1′), *Microviridae*
Bdellovibrio phage MAC 2, (MAC-2), *Microviridae*
Bdellovibrio phage MAC 4, (MAC-4), *Microviridae*
Bdellovibrio phage MAC 4′, (MAC-4′), *Microviridae*
Bdellovibrio phage MAC 5, (MAC-5), *Microviridae*
Bdellovibrio phage MAC 7, (MAC-7), *Microviridae*
Beak and feather disease virus, (BFDV), *Circoviridae*
BeAn 157575 virus, (BeAnV-157575), *Rhabdoviridae*
BeAn 277 virus, (GMAV), *Bunyaviridae*
BeAn 293022 virus, *Arenaviridae*
BeAn 47693 virus, (BUJV), *Bunyaviridae*
BeAn 70563 virus, *Arenaviridae*
BeAn 8582 virus, (CAPV), *Bunyaviridae*
Bean angular mosaic virus, *Carlavirus*
Bean calico mosaic virus, (BCaMV), *Geminiviridae*
Bean common mosaic necrosis virus, (BCMNV), *Potyviridae*
Bean common mosaic virus, (BCMV), *Potyviridae*
Bean dwarf mosaic virus, (BDMV), *Geminiviridae*
Bean golden mosaic virus – Brazil, (BGMV-Br), *Geminiviridae*
Bean golden mosaic virus – Dom. Rep., (BGMV-DR), *Geminiviridae*
Bean golden mosaic virus – Guatemala, (BGMV-Gua), *Geminiviridae*
Bean golden mosaic virus – Puerto Rico, (BGMV-PR), *Geminiviridae*
Bean leafroll virus, (BLRV), *Luteoviridae*
Bean mild mosaic virus, (BMMV), *Tombusviridae*
Bean pod mottle virus, (BPMV), *Comoviridae*
Bean rugose mosaic virus, (BRMV), *Comoviridae*
Bean yellow dwarf virus, (BeYDV), *Geminiviridae*
Bean yellow mosaic virus, (BYMV), *Potyviridae*
Bean yellow vein-banding virus, (BYVBV), *Umbravirus*
BeAr 328208 virus, (BAV), *Bunyaviridae*
Bearded iris mosaic virus, *Potyviridae*
Bebaru virus, (BEBV), *Togaviridae*
Bee iridescent virus, *Iridoviridae*
Bee virus X, (BXV), Unassigned
Bee virus Y, (BYV), Unassigned
Beet cryptic virus 1, (BCV-1), *Partitiviridae*
Beet cryptic virus 2, (BCV-2), *Partitiviridae*
Beet cryptic virus 3, (BCV-3), *Partitiviridae*
Beet curly top virus Iran/CFH, (BCTV-CFH), *Geminiviridae*
Beet curly top virus Worland, (BCTV-Wor), *Geminiviridae*
Beet curly top virus, (BCTV), *Geminiviridae*
Beet leaf curl virus, (BLCV), *Rhabdoviridae*
Beet mild yellowing virus, (BMYV), *Luteoviridae*
Beet mosaic virus, (BtMV), *Potyviridae*
Beet necrotic yellow vein virus satellite - like RNA5, Satellite
Beet necrotic yellow vein virus, (BNYVV), *Benyvirus*
Beet pseudoyellows virus, (BPYV), *Closteroviridae*
Beet ringspot virus satellite RNA, Satellite
Beet ringspot virus, (BRSV), *Comoviridae*
Beet soil-borne mosaic virus, (BSBMV), *Benyvirus*
Beet soil-borne virus, (BSBV), *Pomovirus*
Beet virus Q, (BVQ), *Pomovirus*
Beet western yellows virus, (BWYV), *Luteoviridae*
Beet yellow stunt virus, (BYSV), *Closteroviridae*
Beet yellows virus, (BYV), *Closteroviridae*
BeH 2251 virus, (CDUV), *Bunyaviridae*
Belem virus, (BLMV), *Bunyaviridae*
Belladonna mottle virus, (BeMV), *Tymovirus*
Belmont virus, (BELV), *Bunyaviridae*
Beltera virus, (BELTV), *Bunyaviridae*
Benevides virus, (BENV), *Bunyaviridae*
Benfica virus, (BENV), *Bunyaviridae*
Berkeley bee virus, (BBV), Unassigned
Bermejo virus, *Bunyaviridae*
Bermuda grass etched-line virus, (BELV), *Marafivirus*
Berrimah virus, (BRMV), *Rhabdoviridae*
Bertioga virus, (BERV), *Bunyaviridae*
Bhanja virus, (BHAV), *Bunyaviridae*
Bhendi yellow vein mosaic virus, (BYVMV), *Geminiviridae*
Bidens mosaic virus, (BiMV), *Potyviridae*
Bidens mottle virus, (BiMoV), *Potyviridae*
Biken-1 virus, *Bunyaviridae*
Bimbo virus, (BBOV), *Rhabdoviridae*
Bimiti virus, (BIMV), *Bunyaviridae*
Birao virus, (BIRV), *Bunyaviridae*
Biston betularia cypovirus 6, (BbCPV-6), *Reoviridae*
Bivens Arm virus, (BAV), *Rhabdoviridae*
BK polyomavirus, (BKPyV), *Polyomaviridae*
Black beetle iridescent virus, *Iridoviridae*
Black beetle virus, (BBV), *Nodaviridae*
Black Creek Canal virus, (BCCV), *Bunyaviridae*
Black footed penguin herpesvirus, *Herpesviridae*
Black queen cell virus, (BQCV), Unassigned
Black raspberry necrosis virus, (BRNV), Unassigned
Black stork herpesvirus, *Herpesviridae*
Blackcurrant reversion associated virus, (BRAV), *Comoviridae*
Blackeye cowpea mosaic virus, *Potyviridae*

Blackgram mottle virus, (BMoV), *Tombusviridae*
Bloodland Lake virus, (BLLV), *Bunyaviridae*
Blue crab virus, (BCV), *Rhabdoviridae*
Blue River virus, *Bunyaviridae*
Blueberry leaf mottle virus, (BLMoV), *Comoviridae*
Blueberry mosaic viroid-like RNA, (BluMVd-RNA), *Avsunviroidae*
Blueberry red ringspot virus, (BRRV), *Caulimoviridae*
Blueberry scorch virus, (BlScV), *Carlavirus*
Blueberry shock virus, (BlShV), *Bromoviridae*
Blueberry shoestring virus, (BSSV), *Sobemovirus*
Bluetongue virus 1 to 24, (BTV-1 to 24), *Reoviridae*
Bluetongue virus, (BTV), *Reoviridae*
B-lymphotropic polyomavirus, (LPyV), *Polyomaviridae*
Boa herpesvirus, *Herpesviridae*
Bobaya virus, (BOBV), *Bunyaviridae*
Bobia virus, (BIAV), *Bunyaviridae*
Bobwhite quail herpesvirus, *Herpesviridae*
Bohle iridovirus, (BIV), *Iridoviridae*
Boid herpesvirus 1, (BoiHV-1), *Herpesviridae*
Boletus virus X, (BolVX), *Potexvirus*
Boloria dia cypovirus 2, (BdCPV-2), *Reoviridae*
Bombyx mori cypovirus 1, (BmCPV-1), *Reoviridae*
Bombyx mori densovirus, (BmDNV), *Parvoviridae*
Bombyx mori mag virus, (BmoMagV), *Metaviridae*
Bombyx mori NPV, (BmNPV), *Baculoviridae*
Boolarra virus, (BoV), *Nodaviridae*
Boraceia virus, (BORV), *Bunyaviridae*
Border disease virus BD31, *Flaviviridae*
Border disease virus X818, *Flaviviridae*
Border disease virus, (BDV), *Flaviviridae*
Borna disease virus, (BDV), *Bornaviridae*
Botambi virus, (BOTV), *Bunyaviridae*
Boteke virus, (BTKV), *Rhabdoviridae*
Bouboui virus, (BOUV), *Flaviviridae*
Bovine adeno-associated virus, (BAAV), *Parvoviridae*
Bovine adenovirus 1, (BAdV-1), *Adenoviridae*
Bovine adenovirus 10, (BAdV-10), *Adenoviridae*
Bovine adenovirus 2, (BAdV-2), *Adenoviridae*
Bovine adenovirus 3, (BAdV-3), *Adenoviridae*
Bovine adenovirus 4 to 8, (BAdV-4 to 8), *Adenoviridae*
Bovine adenovirus 9, (BAdV-9), *Adenoviridae*
Bovine adenovirus A, (BAdV-A), *Adenoviridae*
Bovine adenovirus B, (BAdV-B), *Adenoviridae*
Bovine adenovirus C, (BAdV-C), *Adenoviridae*
Bovine astrovirus 1, (BAstV-1), *Astroviridae*
Bovine astrovirus 2, (BAstV-2), *Astroviridae*
Bovine astrovirus, (BAstV), *Astroviridae*
Bovine calicivirus, (VESV/Bos-1), *Caliciviridae*
Bovine encephalitis virus, *Herpesviridae*
Bovine enteric calicivirus, (BoCV), *Caliciviridae*
Bovine enterovirus 1, (BEV-1), *Picornaviridae*
Bovine enterovirus 2, (BEV-2), *Picornaviridae*
Bovine enterovirus, (BEV), *Picornaviridae*
Bovine ephemeral fever virus, (BEFV), *Rhabdoviridae*
Bovine foamy virus, (BFV), *Retroviridae*
Bovine herpesvirus 1, (BoHV-1), *Herpesviridae*
Bovine herpesvirus 2, (BoHV-2), *Herpesviridae*
Bovine herpesvirus 4, (BoHV-4), *Herpesviridae*
Bovine herpesvirus 5, (BoHV-5), *Herpesviridae*
Bovine immunodeficiency virus, (BIV), *Retroviridae*
Bovine leukemia virus, (BLV), *Retroviridae*
Bovine mamillitis virus, *Herpesviridae*
Bovine papillomavirus 1 to 4, (BPV-1 to 4), *Papillomaviridae*
Bovine papillomavirus, (BPV), *Papillomaviridae*
Bovine papular stomatitis virus, (BPSV), *Poxviridae*
Bovine parainfluenza virus 3, (BPIV-3), *Paramyxoviridae*
Bovine parvovirus, (BPV), *Parvoviridae*
Bovine polyomavirus, (BPyV), *Polyomaviridae*
Bovine respiratory syncytial virus, (BRSV), *Paramyxoviridae*
Bovine rhinovirus 1, (BRV-1), *Picornaviridae*
Bovine rhinovirus 2, (BRV-2), *Picornaviridae*
Bovine rhinovirus 3, (BRV-3), *Picornaviridae*
Bovine viral diarrhea virus 1 CP7, *Flaviviridae*
Bovine viral diarrhea virus 1 NADL, *Flaviviridae*
Bovine viral diarrhea virus 1 Osloss, *Flaviviridae*
Bovine viral diarrhea virus 1 SD-1, *Flaviviridae*
Bovine viral diarrhea virus 1, (BVDV-1), *Flaviviridae*
Bovine viral diarrhea virus 2 C413, *Flaviviridae*
Bovine viral diarrhea virus 2 strain 890, *Flaviviridae*
Bovine viral diarrhea virus 2, (BVDV-2), *Flaviviridae*
Box turtle virus 3, (TV3), *Iridoviridae*
Bozo virus, (BOZOV), *Bunyaviridae*
Brachypodium yellow streak virus, (BYSV), Unassigned
Bramble yellow mosaic virus, (BrmYMV), *Potyviridae*
Brazilian wheat spike virus, (BWSpV), *Tenuivirus*
Broad bean mottle virus, (BBMV), *Bromoviridae*
Broad bean necrosis virus, (BBNV), *Pomovirus*
Broad bean stain virus, (BBSV), *Comoviridae*
Broad bean true mosaic virus, (BBTMV), *Comoviridae*
Broad bean wilt virus 1, (BBWV-1), *Comoviridae*
Broad bean wilt virus 2, (BBWV-2), *Comoviridae*
Broadhaven virus, (BRDV), *Reoviridae*
Broccoli necrotic yellows virus, (BNYV), *Rhabdoviridae*
Brome mosaic virus, (BMV), *Bromoviridae*
Brome streak virus, (BStV), *Potyviridae*
Bromus striate mosaic virus, (BrSMV), *Geminiviridae*
Brucella phage Tb, (Tb), *Podoviridae*
Bruconha virus, (BRUV), *Bunyaviridae*
Bryonia mottle virus, (BryMoV), *Potyviridae*

BT 4971 virus, (PATV), *Bunyaviridae*
Bubaline herpesvirus 1, (BuHV-1), *Herpesviridae*
Budgerigar fledgling polyomavirus, (BFPyV), *Polyomaviridae*
Buenaventura virus, (BUEV), *Bunyaviridae*
Buffalopox virus, (BPXV), *Poxviridae*
Buggy Creek virus, *Togaviridae*
Bujaru virus, (BUJV), *Bunyaviridae*
Bukalasa bat virus, (BBV), *Flaviviridae*
Bunyamwera virus, (BUNV), *Bunyaviridae*
Bunyip creek virus, (BCV), *Reoviridae*
Burdock stunt viroid, (BuSVd), *Avsunviroidae*
Burdock yellows virus, (BuYV), *Closteroviridae*
Bushbush virus, (BSBV), *Bunyaviridae*
Bussuquara virus, (BSQV), *Flaviviridae*
Buthus occitanus reovirus, (BoRV), *Reoviridae*
Butterbur mosaic virus, (ButMV), *Carlavirus*
Buttonwillow virus, (BUTV), *Bunyaviridae*
B-virus; Herpesvirus simiae, *Herpesviridae*
Bwamba virus, (BWAV), *Bunyaviridae*

Cabassou virus, (CABV), *Togaviridae*
Cacao swollen shoot virus, (CSSV), *Caulimoviridae*
Cacao virus, (CACV), *Bunyaviridae*
Cacao yellow mosaic virus, (CYMV), *Tymovirus*
Cache Valley virus, (CVV), *Bunyaviridae*
Cacipacore virus, (CPCV), *Flaviviridae*
Cactus virus 2, (CV-2), *Carlavirus*
Cactus virus X, (CVX), *Potexvirus*
Caddo Canyon virus, (CDCV), *Bunyaviridae*
Caenorhabditis elegans Cer1 virus, (CelCer1V), *Metaviridae*
Caimito virus, (CAIV), *Bunyaviridae*
Calanthe mild mosaic virus, (CalMMV), *Potyviridae*
Calchaqui virus, (CQIV), *Rhabdoviridae*
California encephalitis virus, (CEV), *Bunyaviridae*
California harbor seal poxvirus, (SPV), *Poxviridae*
California hare coltivirus, (CTFV-Ca), *Reoviridae*
Callimorpha quadripuntata virus, (CqV), *Tetraviridae*
Callistephus chinensis chlorosis virus, (CCCV), *Rhabdoviridae*
Calliteara pudibunda virus, (CpV), *Tetraviridae*
Callitrichine herpesvirus 1, (CalHV-1), *Herpesviridae*
Callitrichine herpesvirus 2, (CalHV-2), *Herpesviridae*
Calopogonium yellow vein virus, (CalYVV), *Tymovirus*
Camel contagious ecthyma virus, *Poxviridae*
Camellia yellow mottle virus, (CYMoV), *Varicosavirus*
Camelpox virus, (CMLV), *Poxviridae*
Campoletis aprilis ichnovirus, (CaIV), *Polydnaviridae*
Campoletis flavicincta ichnovirus, (CfIV), *Polydnaviridae*
Campoletis sonorensis ichnovirus, (CsIV), *Polydnaviridae*
Campoletis sp. *ichnovirus*, (CspIV), *Polydnaviridae*
Camptochironomus tentans entomopoxvirus, (CTEV), *Poxviridae*
Cananeia virus, (CNAV), *Bunyaviridae*
Canary reed mosaic virus, (CRMV), *Potyviridae*
Canarypox virus, (CNPV), *Poxviridae*
Canavalia maritima mosaic virus, (CnMMV), *Potyviridae*
Canid herpesvirus 1, (CaHV-1), *Herpesviridae*
Caninde virus, (CANV), *Reoviridae*
Canine adeno-associated virus, (CAAV), *Parvoviridae*
Canine adenovirus 1, (CAdV-1), *Adenoviridae*
Canine adenovirus 2, (CAdV-2), *Adenoviridae*
Canine adenovirus, (CAdV), *Adenoviridae*
Canine calicivirus, (CaCV), *Caliciviridae*
Canine distemper virus, (CDV), *Paramyxoviridae*
Canine herpesvirus, *Herpesviridae*
Canine minute virus, (CMV), *Parvoviridae*
Canine oral papillomavirus, (COPV), *Papillomaviridae*
Canine parvovirus, (CPV), *Parvoviridae*
Canna yellow mottle virus, (CaYMV), *Caulimoviridae*
Cano Delgadito virus, (CADV), *Bunyaviridae*
Cape Wrath virus, (CWV), *Reoviridae*
Caper latent virus, (CapLV), *Carlavirus*
Capim virus, (CAPV), *Bunyaviridae*
Caprine adenovirus, (GAdV), *Adenoviridae*
Caprine arthritis encephalitis virus, (CAEV), *Retroviridae*
Caprine herpesvirus 1, (CpHV-1), *Herpesviridae*
Capuchin herpesvirus AL-5, *Herpesviridae*
Capuchin herpesvirus AP-18, *Herpesviridae*
Carajas virus, (CJSV), *Rhabdoviridae*
Caraparu virus, (CARV), *Bunyaviridae*
Caraway latent virus, (CawLV), *Carlavirus*
Carcinus mediterraneus W2 virus, (CcRV-W2), *Reoviridae*
Cardamine chlorotic fleck virus, (CCFV), *Tombusviridae*
Cardamine latent virus, (CaLV), *Carlavirus*
Cardamom mosaic virus, (CdMV), *Potyviridae*
Cardiochiles nigriceps bracovirus, (CnBV), *Polydnaviridae*
Carey Island virus, (CIV), *Flaviviridae*
Carnation bacilliform virus, (CBV), *Rhabdoviridae*
Carnation cryptic virus 1, (CCV-1), *Partitiviridae*
Carnation cryptic virus 2, (CCV-2), *Partitiviridae*
Carnation etched ring virus, (CERV), *Caulimoviridae*
Carnation Italian ringspot virus, (CIRV), *Tombusviridae*
Carnation latent virus, (CLV), *Carlavirus*

Carnation mottle virus, (CarMV), *Tombusviridae*
Carnation necrotic fleck virus, (CNFV), *Closteroviridae*
Carnation ringspot virus, (CRSV), *Tombusviridae*
Carnation vein mottle virus, (CVMoV), *Potyviridae*
Carnation yellow stripe virus, (CYSV), *Tombusviridae*
Carp pox herpesvirus, *Herpesviridae*
Carrot latent virus, (CtLV), *Rhabdoviridae*
Carrot mosaic virus, (CtMV), *Potyviridae*
Carrot mottle mimic virus, (CMoMV), *Umbravirus*
Carrot mottle virus, (CMoV), *Umbravirus*
Carrot red leaf virus, (CtRLV), *Luteoviridae*
Carrot temperate virus 1, (CTeV-1), *Partitiviridae*
Carrot temperate virus 2, (CTeV-2), *Partitiviridae*
Carrot temperate virus 3, (CTeV-3), *Partitiviridae*
Carrot temperate virus 4, (CTeV-4), *Partitiviridae*
Carrot thin leaf virus, (CTLV), *Potyviridae*
Carrot yellow leaf virus, (CYLV), *Closteroviridae*
Casinaria arjuna ichnovirus, (CarIV), *Polydnaviridae*
Casinaria forcipata ichnovirus, (CfoIV), *Polydnaviridae*
Casinaria infesta ichnovirus, (CiIV), *Polydnaviridae*
Casinaria sp. *ichnovirus*, (CaspIV), *Polydnaviridae*
Casphalia extranea densovirus, (CeDNV), *Parvoviridae*
Cassava American latent virus, (CsALV), *Comoviridae*
Cassava common mosaic virus, (CsCMV), *Potexvirus*
Cassava green mottle virus, (CsGMV), *Comoviridae*
Cassava Ivorian bacilliform virus, (CsIBV), Unassigned
Cassava symptomless virus, (CsSLV), *Rhabdoviridae*
Cassava vein mosaic virus, (CsVMV), *Caulimoviridae*
Cassava virus C, (CsVC), *Ourmiavirus*
Cassava virus X, (CsVX), *Potexvirus*
Cassia mild mosaic virus, (CasMMV), *Carlavirus*
Cassia yellow blotch virus, (CYBV), *Bromoviridae*
Cassia yellow spot virus, (CasYSV), *Potyviridae*
Catu virus, (CATUV), *Bunyaviridae*
Cauliflower mosaic virus, (CaMV), *Caulimoviridae*
Caulobacter phage ϕCb12r, (ϕCB12r), *Leviviridae*
Caulobacter phage ϕCb2, (ϕCb2), *Leviviridae*
Caulobacter phage ϕCb23r, (ϕCb23r), *Leviviridae*
Caulobacter phage ϕCb4, (ϕCb4), *Leviviridae*
Caulobacter phage ϕCb5, (ϕCb5), *Leviviridae*
Caulobacter phage ϕCb8r, (ϕCb8r), *Leviviridae*
Caulobacter phage ϕCb9, (ϕCb9), *Leviviridae*
Caulobacter phage ϕCP18, (ϕCP18), *Leviviridae*
Caulobacter phage ϕCP2, (ϕCP2), *Leviviridae*
Caulobacter phage ϕCr14, (ϕCr14), *Leviviridae*
Caulobacter phage ϕCr28, (ϕCr28), *Leviviridae*
Caulobacter phage ϕCr24, (ϕCr24), *Myoviridae*
Berne virus, (BEV), *Coronviridae*
Bovine coronavirus, (BCoV), *Coronviridae*
Bovine torovirus, (BoTV), *Coronviridae*
Breda virus, (BRV), *Coronviridae*
Canine coronavirus, (CCoV), *Coronviridae*
Caulobacter phage ϕCd1, (ϕCd1), *Podoviridae*
Caviid herpesvirus 1, (CavHV-1), *Herpesviridae*
Caviid herpesvirus 2, (CavHV-2), *Herpesviridae*
Caviid herpesvirus 3, (CavHV-3), *Herpesviridae*
CbaAr 426 virus, (CAV), *Bunyaviridae*
Cebine herpesvirus 1, (CbHV-1), *Herpesviridae*
Cebine herpesvirus 2, (CbHV-2), *Herpesviridae*
Celery mosaic virus, (CeMV), *Potyviridae*
Celery yellow mosaic virus, (CeYMV), *Potyviridae*
Cell fusing agent virus, (CFAV), *Flaviviridae*
Centrosema mosaic virus, (CenMV), *Potexvirus*
Ceratitis capitata reovirus, *Reoviridae*
Ceratitis I virus, (CIV), *Reoviridae*
Ceratitis V virus, (CVV), Unassigned
Ceratobium mosaic virus, (CerMV), *Potyviridae*
Cercopithecine herpesvirus 1, (CeHV-1), *Herpesviridae*
Cercopithecine herpesvirus 10, (CeHV-10), *Herpesviridae*
Cercopithecine herpesvirus 12, (CeHV-12), *Herpesviridae*
Cercopithecine herpesvirus 13, (CeHV-13), *Herpesviridae*
Cercopithecine herpesvirus 14, (CeHV-14), *Herpesviridae*
Cercopithecine herpesvirus 15, (CeHV-15), *Herpesviridae*
Cercopithecine herpesvirus 16, (CeHV-16), *Herpesviridae*
Cercopithecine herpesvirus 17, (CeHV-17), *Herpesviridae*
Cercopithecine herpesvirus 2, (CeHV-2), *Herpesviridae*
Cercopithecine herpesvirus 3, (CeHV-3), *Herpesviridae*
Cercopithecine herpesvirus 4, (CeHV-4), *Herpesviridae*
Cercopithecine herpesvirus 5, (CeHV-5), *Herpesviridae*
Cercopithecine herpesvirus 8, (CeHV-8), *Herpesviridae*
Cercopithecine herpesvirus 9, (CeHV-9), *Herpesviridae*
Cercopithecine herpesvirus SA8, *Herpesviridae*
Cereal chlorotic mottle virus, (CCMoV), *Rhabdoviridae*
Cereal yellow dwarf – RPV satellite virus, Satellite
Cereal yellow dwarf virus – rpv, (CYDV-RPV), *Luteoviridae*
Cervid herpesvirus 1, (CvHV-1), *Herpesviridae*
Cervid herpesvirus 2, (CvHV-2), *Herpesviridae*

Cestrum yellow leaf curling virus, (CmYLCV), *Caulimoviridae*
Cetacean calicivirus, (VESV/Tur-1), *Caliciviridae*
Cetacean morbillivirus virus, (CeMV), *Paramyxoviridae*
CEV BFS-283, (CEV), *Bunyaviridae*
Chaco virus, (CHOV), *Rhabdoviridae*
Chagres virus, *Bunyaviridae*
Chamois contagious ecthyma virus, *Poxviridae*
Chandipura virus, (CHPV), *Rhabdoviridae*
Chandiru virus, (CDUV), *Bunyaviridae*
Changuinola virus, (CGLV), *Reoviridae*
Changuinola virus, (CGLV), *Reoviridae*
Channel catfish reovirus, (CRV), *Reoviridae*
Channel catfish virus, *Herpesviridae*
Chara australis virus, (CAV), Unassigned
Chara corallina virus, Unassigned
Charleville virus, (CHVV), *Rhabdoviridae*
Chayote mosaic virus, (ChMV), *Tymovirus*
Chelonid herpesvirus 1, (ChHV-1), *Herpesviridae*
Chelonid herpesvirus 2, (ChHV-2), *Herpesviridae*
Chelonid herpesvirus 3, (ChHV-3), *Herpesviridae*
Chelonid herpesvirus 4, (ChHV-4), *Herpesviridae*
Chelonus altitudinis bracovirus, (CalBV), *Polydnaviridae*
Chelonus blackburni bracovirus, (CbBV), *Polydnaviridae*
Chelonus inanitus bracovirus, (CinaBV), *Polydnaviridae*
Chelonus insularis bracovirus, (CinsBV), *Polydnaviridae*
Chelonus nr. curvimaculatus bracovirus, (CcBV), *Polydnaviridae*
Chelonus texanus bracovirus, (CtBV), *Polydnaviridae*
Chenopodium necrosis virus, (ChNV), *Tombusviridae*
Chenuda virus, (CNUV), *Reoviridae*
Chenuda virus, (CNUV), *Reoviridae*
Cherry green ring mottle virus, (CGRMV), *Foveavirus*
Cherry leaf roll virus, (CLRV), *Comoviridae*
Cherry rasp leaf virus, (CRLV), *Comoviridae*
Cherry rosette virus, (CRV), *Comoviridae*
Cherry virus A, (CVA), *Capillovirus*
Chick syncytial virus, (CSV), *Retroviridae*
Chicken anemia virus, (CAV), *Circoviridae*
Chicken parvovirus, (ChPV), *Parvoviridae*
Chicken rotavirus 555, (AvRV-G/555), *Reoviridae*
Chicken rotavirus A4, (AvRV-F/A4), *Reoviridae*
Chicken rotavirus 132, (AvRV-D/132), *Reoviridae*
Chickpea bushy dwarf virus, (CpBDV), *Potyviridae*
Chickpea chlorotic dwarf virus, (CpCDV), *Geminiviridae*
Chickpea filiform virus, (CpFV), *Potyviridae*
Chickpea stunt disease associated virus, (CpSDaV), *Luteoviridae*
Chicory yellow blotch virus, (ChYBV), *Carlavirus*
Chicory yellow mottle virus large satellite RNA, Satellite
Chicory yellow mottle virus satellite RNA, Satellite
Chicory yellow mottle virus, (ChYMV), *Comoviridae*
Chikungunya virus, (CHIKV), *Togaviridae*
Chilibre virus VP-118D, (CHIV), *Bunyaviridae*
Chilibre virus, (CHIV), *Bunyaviridae*
Chilli veinal mottle virus, (ChiVMV), *Potyviridae*
Chilo iridescent virus, *Iridoviridae*
Chim virus, (CHIMV), *Bunyaviridae*
Chimpanzee adenovirus C2, (ChAdV-C2), *Adenoviridae*
Chimpanzee adenovirus strain Y34, (ChAdV-Y34), *Adenoviridae*
Chimpanzee foamy virus human isolate, (CFV/Hu), *Retroviridae*
Chimpanzee foamy virus, (CFV), *Retroviridae*
Chinese rape mosaic virus, *Tobamovirus*
Chinese yam necrotic mosaic virus, (ChYNMV), *Carlavirus*
Chino del tomate virus, (CdTV), *Geminiviridae*
Chinook salmon reovirus B, (GRCV), *Reoviridae*
Chinook salmon reovirus DRC, (DRCRV), *Reoviridae*
Chinook salmon reovirus ICR, (ICRV), *Reoviridae*
Chinook salmon reovirus LBS, (LBSV), *Reoviridae*
Chinook salmon reovirus YRC, (YRCV), *Reoviridae*
Chironomus attenuatus entomopoxvirus, (CAEV), *Poxviridae*
Chironomus luridus entomopoxvirus, (CLEV), *Poxviridae*
Chironomus plumosus entomopoxvirus, (CPEV), *Poxviridae*
Chlamydia phage 1, (Ch-1), *Microviridae*
Chloris striate mosaic virus, (CSMV), *Geminiviridae*
Chobar Gorge virus, (CGV), *Reoviridae*
Chobar Gorge virus, (CGV), *Reoviridae*
Choristoneura biennis entomopoxvirus 'L', (CBEV), *Poxviridae*
Choristoneura conflicta entomopoxvirus 'L', (CCEV), *Poxviridae*
Choristoneura diversuma entomopoxvirus 'L', (CDEV), *Poxviridae*
Choristoneura fumiferana cypovirus 7, (CfCPV-7), *Reoviridae*
Choristoneura fumiferana entomopoxvirus 'L', (CFEV), *Poxviridae*
Choristoneura fumiferana MNPV, (CfMNPV), *Baculoviridae*
Chorizagrotis auxiliars entomopoxvirus 'L', (CXEV), *Poxviridae*
Chronic bee paralysis virus, (CBPV), Unassigned

Chronic bee-paralysis satellite virus, Satellite
Chrysanthemum aspermy virus, *Bromoviridae*
Chrysanthemum chlorotic mottle viroid, (CChMVd), *Avsunviroidae*
Chrysanthemum frutescens virus, (CFV), *Rhabdoviridae*
Chrysanthemum stem necrosis virus, (CSNV), *Bunyaviridae*
Chrysanthemum stunt viroid, (CSVd), *Pospiviroidae*
Chrysanthemum vein chlorosis virus, (CVCV), *Rhabdoviridae*
Chrysanthemum virus B, (CVB), *Carlavirus*
Chub reovirus, (CHRV), *Reoviridae*
Chum salmon reovirus CSV, (CSRV), *Reoviridae*
Chum salmon reovirus F, (PSRV), *Reoviridae*
Chysochromulina brevifilum virus PW1, (CbV-PW1), *Phycodnaviridae*
Chysochromulina brevifilum virus PW3, (CbV-PW3), *Phycodnaviridae*
Ciconiid herpesvirus 1, (CiHV-1), *Herpesviridae*
Cimex lactularius reovirus, (ClRV), *Reoviridae*
Citrus bent leaf viroid, (CBLVd), *Pospiviroidae*
Citrus cachexia viroid, *Pospiviroidae*
Citrus exocortis viroid, (CEVd), *Pospiviroidae*
Citrus III viroid, (CVd-III), *Pospiviroidae*
Citrus IV viroid, (CVd-IV), *Pospiviroidae*
Citrus leaf rugose virus, (CiLRV), *Bromoviridae*
Citrus leprosis virus, (CiLV), *Rhabdoviridae*
Citrus mosaic virus, (CMBV), *Caulimoviridae*
Citrus psorosis virus, (CPsV), *Ophiovirus*
Citrus tatter leaf virus, *Capillovirus*
Citrus tristeza virus, (CTV), *Closteroviridae*
Citrus variegation virus, (CVV), *Bromoviridae*
Cladosporium fulvum T-1 virus, (CfuT1V), *Metaviridae*
Classical swine fever virus Alfort/187, *Flaviviridae*
Classical swine fever virus Alfort-Tübingen, *Flaviviridae*
Classical swine fever virus Brescia, *Flaviviridae*
Classical swine fever virus C strain, *Flaviviridae*
Classical swine fever virus, (CSFV), *Flaviviridae*
Clitoria yellow mosaic virus, (CtYMV), *Potyviridae*
Clitoria yellow vein virus, (CYVV), *Tymovirus*
Clo Mor virus, (CMV), *Bunyaviridae*
Clostridium phage CEβ, (CEβ), *Myoviridae*
Clostridium phage F1, (F1), *Siphoviridae*
Clostridium phage HM2, (HM2), *Podoviridae*
Clostridium phage HM3, (HM3), *Myoviridae*
Clostridium phage HM7, (HM7), *Siphoviridae*
Cloudy wing virus, (CWV), Unassigned
Clover enation virus, (ClEV), *Rhabdoviridae*
Clover yellow mosaic virus, (ClYMV), *Potexvirus*
Clover yellow vein virus, (ClYVV), *Potyviridae*
Clover yellows virus, (CYV), *Closteroviridae*
CoAr 1071 virus, (CA1071V), *Bunyaviridae*
CoAr 3624 virus, (CA3624V), *Bunyaviridae*
CoAr 3627 virus, (CA3627V), *Bunyaviridae*
Coastal Plains virus, (CPV), *Rhabdoviridae*
Cocal virus, (COCV), *Rhabdoviridae*
Cockatoo entero-like virus, (CELV), *Picornaviridae*
Cocksfoot mild mosaic virus, (CMMV), *Sobemovirus*
Cocksfoot mottle virus, (CoMV), *Sobemovirus*
Cocksfoot streak virus, (CSV), *Potyviridae*
Cocoa necrosis virus, (CoNV), *Comoviridae*
Coconut cadang-cadang viroid, (CCCVd), *Pospiviroidae*
Coconut foliar decay virus, (CFDV), *Nanovirus*
Coconut tinangaja viroid, (CTiVd), *Pospiviroidae*
Coffee ringspot virus, (CoRSV), *Rhabdoviridae*
Coho salmon reovirus CSR, (CSRV), *Reoviridae*
Coho salmon reovirus ELC, (ELCV), *Reoviridae*
Coho salmon reovirus SCS, (SCSV), *Reoviridae*
ColAn 57389 virus, (CA57389V), *Bunyaviridae*
Cole latent virus, (CoLV), *Carlavirus*
Coleus blumei viroid 1, (CbVd-1), *Pospiviroidae*
Coleus blumei viroid 2, (CbVd-2), *Pospiviroidae*
Coleus blumei viroid 3, (CbVd-3), *Pospiviroidae*
Colletotrichum lindemuthianum virus, (CLV), Unassigned
Colocasia bobone disease virus, (CBDV), *Rhabdoviridae*
Colombian datura virus, (CDV), *Potyviridae*
Colony B North virus, *Reoviridae*
Colony virus, (COYV), *Reoviridae*
Colorado tick fever virus, (CTFV), *Reoviridae*
Columbia SK virus, *Picornaviridae*
Columbid herpesvirus 1, (CoHV-1), *Herpesviridae*
Columnea latent viroid, (CLVd), *Pospiviroidae*
Commelina mosaic virus, (ComMV), *Potyviridae*
Commelina virus X, (ComVX), *Potexvirus*
Commelina yellow mottle virus, (ComYMV), *Caulimoviridae*
Connecticut virus, (CNTV), *Rhabdoviridae*
Convict Creek 107 virus, *Bunyaviridae*
Convict Creek 74 virus, *Bunyaviridae*
Corfou virus, (CFUV), *Bunyaviridae*
Coriander feathery red vein virus, (CFRVV), *Rhabdoviridae*
Cormorant herpesvirus, *Herpesviridae*
Corriparta virus, (CORV), *Reoviridae*
Corriparta virus, (CORV), *Reoviridae*
Coryneform phage A19, (A19), *Myoviridae*
Coryneforms phage 7/26, (7/26), *Podoviridae*
Coryneforms phage AN25S-1, (AN25S-1), *Podoviridae*
Coryneforms phage Arp, (Arp), *Siphoviridae*
Coryneforms phage BL3, (BL3), *Siphoviridae*
Coryneforms phage CONX, (CONX), *Siphoviridae*
Coryneforms phage MT, (MT), *Siphoviridae*
Coryneforms phage α, (α), *Siphoviridae*

Coryneforms phage β, (β), *Siphoviridae*
Coryneforms phage ϕA8010, (ϕA8010), *Siphoviridae*
Costelytra zealandica iridescent virus, *Iridoviridae*
Cote d'Ivoire Ebola virus, (CIEBOV), *Filoviridae*
Cotesia congregata bracovirus, (CcBV), *Polydnaviridae*
Cotesia flavipes bracovirus, (CfBV), *Polydnaviridae*
Cotesia glomerata bracovirus, (CgBV), *Polydnaviridae*
Cotesia hyphantriae bracovirus, (ChBV), *Polydnaviridae*
Cotesia kariyai bracovirus, (CkBV), *Polydnaviridae*
Cotesia marginiventris bracovirus, (CmaBV), *Polydnaviridae*
Cotesia melanoscela bracovirus, (CmeBV), *Polydnaviridae*
Cotesia rubecula bracovirus, (CrBV), *Polydnaviridae*
Cotesia schaeferi bracovirus, (CsBV), *Polydnaviridae*
Cotia virus, (CPV), *Poxviridae*
Cotton leaf crumple virus, (CLCrV), *Geminiviridae*
Cotton leaf curl virus - Pakistan 1, (CLCuV-Pk1), *Geminiviridae*
Cotton leaf curl virus - Pakistan 2, (CLCuV-Pk2), *Geminiviridae*
Cottontail rabbit herpesvirus, *Herpesviridae*
Cottontail rabbit papillomavirus, (CRPV), *Papillomaviridae*
Cow parsnip mosaic virus, (CPaMV), *Rhabdoviridae*
Cowbone Ridge virus, (CRV), *Flaviviridae*
Cowpea aphid-borne mosaic virus, (CABMV), *Potyviridae*
Cowpea chlorotic mottle virus, (CCMV), *Bromoviridae*
Cowpea golden mosaic virus, (CPGMV), *Geminiviridae*
Cowpea green vein banding virus, (CGVBV), *Potyviridae*
Cowpea mild mottle virus, (CPMMV), *Carlavirus*
Cowpea mosaic virus, (CPMV), *Comoviridae*
Cowpea mottle virus, (CPMoV), *Tombusviridae*
Cowpea rugose mosaic virus, (CPRMV), *Potyviridae*
Cowpea severe mosaic virus, (CPSMV), *Comoviridae*
Cowpox virus, (CPXV), *Poxviridae*
Crane herpesvirus, *Herpesviridae*
Cricetid herpesvirus, (CrHV-1), *Herpesviridae*
Cricket paralysis virus, (CrPV), "CrPV-like viruses"
Crimean-Congo hemorrhagic fever virus, *Bunyaviridae*
Crimson clover latent virus, (CCLV), *Comoviridae*
Crinum mosaic virus, (CriMV), *Potyviridae*
Croatian clover virus, (CroCV), *Potyviridae*
Crocus tomasinianus virus, *Potyviridae*
Croton yellow vein mosaic virus, (CYVMV), *Geminiviridae*
Crowpox virus, (CRPV), *Poxviridae*
Cryphonectria hypovirus 1-EP713, (CHV1-EP713), *Hypoviridae*
Cryphonectria hypovirus 1-EP747, (CHV1-EP747), *Hypoviridae*
Cryphonectria hypovirus 2-NB58, (CHV2-NB58), *Hypoviridae*
Cryphonectria hypovirus 3-GH2, (CHV3-GH2), *Hypoviridae*
Cryphonectria hypovirus 4-SR2, (CHV4-SR2), *Hypoviridae*
Cryphonectria parasitica mitovirus 1-NB631, (CpMV1-NB631), *Narnaviridae*
CSIRO village virus, (CVGV), *Reoviridae*
Cucumber chlorotic spot virus, (CCSV), *Closteroviridae*
Cucumber cryptic virus, (CuCV), *Partitiviridae*
Cucumber green mottle mosaic virus, (CGMMV), *Tobamovirus*
Cucumber leaf spot virus, (CLSV), *Tombusviridae*
Cucumber mosaic virus satellite RNA, Satellite
Cucumber mosaic virus, (CMV), *Bromoviridae*
Cucumber necrosis virus, (CuNV), *Tombusviridae*
Cucumber pale fruit viroid, *Pospiviroidae*
Cucumber soil-borne virus, (CuSBV), *Tombusviridae*
Cucurbit aphid-borne yellows virus, (CABYV), *Luteoviridae*
Cucurbit yellow stunting disorder virus, (CYSDV), *Closteroviridae*
CUMC-B11 virus, *Bunyaviridae*
Cyanobacteria phage A-4(L), (A-4(L)), *Podoviridae*
Cyanobacteria phage AC-1, (AC-1), *Podoviridae*
Cyanobacteria phage AS-1, (AS-1), *Myoviridae*
Cyanobacteria phage LPP-1, (LPP-1), *Podoviridae*
Cyanobacteria phage N1, (N1), *Myoviridae*
Cyanobacteria phage S-2L, (S-2L), *Siphoviridae*
Cyanobacteria phage S-4L, (S-4L), *Siphoviridae*
Cyanobacteria phage S-6(L), (S-6(L)), *Myoviridae*
Cyanobacteria phage SM-1, (SM-1), *Podoviridae*
Cycas necrotic stunt virus, (CNSV), *Comoviridae*
Cydia pomonella granulovirus, (CpGV), *Baculoviridae*
Cymbidium mosaic virus, (CymMV), *Potexvirus*
Cymbidium ringspot virus satellite RNA, Satellite
Cymbidium ringspot virus, (CymRSV), *Tombusviridae*
Cynara virus, (CraV), *Rhabdoviridae*
Cynodon mosaic virus, (CynMV), *Carlavirus*
Cynosurus mottle virus, (CnMoV), *Sobemovirus*
Cypovirus 1, (CPV-1), *Reoviridae*
Cypovirus 10, (CPV-10), *Reoviridae*
Cypovirus 11, (CPV-11), *Reoviridae*
Cypovirus 12, (CPV-12), *Reoviridae*
Cypovirus 13, (CPV-13), *Reoviridae*
Cypovirus 14, (CPV-14), *Reoviridae*
Cypovirus 2, (CPV-2), *Reoviridae*

Cypovirus 3, (CPV-3), *Reoviridae*
Cypovirus 4, (CPV-4), *Reoviridae*
Cypovirus 5, (CPV-5), *Reoviridae*
Cypovirus 6, (CPV-6), *Reoviridae*
Cypovirus 7, (CPV-7), *Reoviridae*
Cypovirus 8, (CPV-8), *Reoviridae*
Cypovirus 9, (CPV-9), *Reoviridae*
Cyprinid herpesvirus 1, (CyHV-1), *Herpesviridae*
Cyprinid herpesvirus 2, (CyHV-2), *Herpesviridae*
Cypripedium calceolus virus, (CypCV), *Potyviridae*

Dab lymphocystis disease virus, *Iridoviridae*
Dabakala virus, (DABV), *Bunyaviridae*
Dacus oleae reovirus, (DoRV), *Reoviridae*
D'Aguilar virus, (DAGV), *Reoviridae*
Dahlia mosaic virus, (DMV), *Caulimoviridae*
Dak AN B 188d virus, *Arenaviridae*
Dakar bat virus, (DBV), *Flaviviridae*
DakArK 7292 virus, (DAKV-7292), *Rhabdoviridae*
Danaus plexippus cypovirus 3, (DpCPV-3), *Reoviridae*
Dandelion latent virus, (DaLV), *Carlavirus*
Dandelion yellow mosaic virus, (DaYMV), *Sequiviridae*
Daphne virus S, (DVS), *Carlavirus*
Daphne virus X, (DVX), *Potexvirus*
Daphne virus Y, (DVY), *Potyviridae*
Dapple apple viroid, *Pospiviroidae*
Darna trima virus, (DtV), *Tetraviridae*
Dasheen mosaic virus, (DsMV), *Potyviridae*
Dasychira pudibunda cypovirus 2, (DpCPV-2), *Reoviridae*
Dasychira pudibunda virus, (DpV), *Tetraviridae*
Datura distortion mosaic virus, (DDMV), *Potyviridae*
Datura mosaic virus, (DTMV), *Potyviridae*
Datura necrosis virus, (DNV), *Potyviridae*
Datura shoestring virus, (DSSV), *Potyviridae*
Datura virus 437, (DV-437), *Potyviridae*
Datura yellow vein virus, (DYVV), *Rhabdoviridae*
Deer fibroma virus, *Papillomaviridae*
Deer papillomavirus, (DPV), *Papillomaviridae*
Deformed wing virus, (DWV), Unassigned
Demodema boranensis entomopoxvirus, (DBEV), *Poxviridae*
Dendrobium leaf streak virus, (DLSV), *Rhabdoviridae*
Dendrobium mosaic virus, *Potyviridae*
Dendrobium vein necrosis virus, (DVNV), *Closteroviridae*
Dendrolimus spectabilis cypovirus 1, (DsCPV-1), *Reoviridae*
Dengue virus type 1, (DENV-1), *Flaviviridae*
Dengue virus type 2, (DENV-2), *Flaviviridae*
Dengue virus type 3, (DENV-3), *Flaviviridae*
Dengue virus type 4, (DENV-4), *Flaviviridae*
Dengue virus, (DENV), *Flaviviridae*
Dera Ghazi Khan virus, *(DGKV)*, *Bunyaviridae*
Dermolepida albohirtum entomopoxvirus, (DAEV), *Poxviridae*
Desert Shield virus, (DSV-395), *Caliciviridae*
Desmodium mosaic virus, (DesMV), *Potyviridae*
Desmodium yellow mottle virus, (DYMoV), *Tymovirus*
Desulfurolobus virus DAFV, (DAFV), *Lipothrixiviridae*
Dhori virus, (DHOV), *Orthomyxoviridae*
Diadegma acronyctae ichnovirus, (DaIV), *Polydnaviridae*
Diadegma interruptum ichnovirus, (DiIV), *Polydnaviridae*
Diadegma terebrans ichnovirus, (DtIV), *Polydnaviridae*
Diadromus pulchellus ascovirus 1a, (DpAV-1a), *Ascoviridae*
Diadromus pulchellus reovirus, (DpRV), *Reoviridae*
Diatraea saccharalis densovirus, (DsDNV), *Parvoviridae*
Dicentrarchus labrax encephalitis virus, (DlEV), *Nodaviridae*
Digitaria streak virus, (DSV), *Geminiviridae*
Digitaria striate mosaic virus, (DiSMV), *Geminiviridae*
Digitaria striate virus, (DiSV), *Rhabdoviridae*
Diodea vein chlorosis virus, (DVCV), *Closteroviridae*
Diolcogaster facetosa bracovirus, (DfBV), *Polydnaviridae*
Dioscorea alata ring mottle virus, *Potyviridae*
Dioscorea bacilliform virus, (DBV), *Caulimoviridae*
Dioscorea green banding virus, *Potyviridae*
Dioscorea latent virus, (DLV), *Potexvirus*
Dioscorea trifida virus, (DTV), *Potyviridae*
Dipladenia mosaic virus, (DipMV), *Potyviridae*
Diplocarpon rosae virus, (DrV), *Partitiviridae*
Dobrava-Belgrade virus, (DOBV), *Bunyaviridae*
Dock mottling mosaic virus, (DMMV), *Potyviridae*
Doctor fish virus, (DFV), *Iridoviridae*
Dolichos yellow mosaic virus, (DoYMV), *Geminiviridae*
Dolphin poxvirus, (DOV), *Poxviridae*
Dorcopsis wallaby herpesvirus, *Herpesviridae*
Douglas virus, (DOUV), *Bunyaviridae*
Drosophila ananassae Tom virus, (DanTomV), *Metaviridae*
Drosophila C virus, (DCV), "CrPV-like viruses"
Drosophila F virus, (DFV), *Reoviridae*
Drosophila line virus, (DLV), *Nodaviridae*
Drosophila melanogaster 176 virus, (Dme176V), *Metaviridae*

Drosophila melanogaster 1731 virus, (Dme1731V), *Pseudoviridae*
Drosophila melanogaster 297 virus, (Dme297V), *Metaviridae*
Drosophila melanogaster 412 virus, (Dme412V), *Metaviridae*
Drosophila melanogaster copia virus, (DmeCopV), *Pseudoviridae*
Drosophila melanogaster gypsy virus, (DmeGypV), *Metaviridae*
Drosophila melanogaster mdg1 virus, (DmeMdg1V), *Metaviridae*
Drosophila melanogaster mdg4 virus, (DmeMdg4V), *Metaviridae*
Drosophila melanogaster micropia virus, (DmeMicV), *Metaviridae*
Drosophila P virus, (DPV), Unassigned
Drosophila S virus, (DSV), *Reoviridae*
Drosophila virilis Ulysses virus, (DviUllV), *Metaviridae*
Drosophila X virus, (DXV), *Birnaviridae*
Drosophilia A virus, (DAV), Unassigned
Duck adenovirus 2, (DAdV-2), *Adenoviridae*
Duck adenovirus, (DAdV), *Adenoviridae*
Duck astrovirus 1, (DAstV-1), *Astroviridae*
Duck astrovirus, (DAstV), *Astroviridae*
Duck hepatitis B virus, (DHBV), *Hepadnaviridae*
Duck hepatitis virus 1, (DHV-1), *Picornaviridae*
Duck hepatitis virus 3, (DHV-3), *Picornaviridae*
Duck plague herpesvirus, *Herpesviridae*
Dugbe virus, (DUGV), *Bunyaviridae*
Dulcamara mottle virus, (DuMV), *Tymovirus*
Dulcamara virus A, (DuVA), *Carlavirus*
Dulcamara virus B, (DuVB), *Carlavirus*
Dusona sp. *ichnovirus*, (DspIV), *Polydnaviridae*
Duvenhage virus, (DUVV), *Rhabdoviridae*

East African cassava mosaic virus, (EACMV), *Geminiviridae*
Eastern equine encephalitis virus, (EEEV), *Togaviridae*
Echinochloa hoja blanca virus, (EHBV), *Tenuivirus*
Echinochloa ragged stunt virus, (ERSV), *Reoviridae*
Eclipta yellow vein virus, (EYVV), *Geminiviridae*
Ectocarpus fasciculatus virus a, (EfV-a), *Phycodnaviridae*
Ectocarpus siliculosus virus 1, (EsV-1), *Phycodnaviridae*
Ectocarpus siliculosus virus a, (EsV-a), *Phycodnaviridae*
Ectromelia virus, (ECTV), *Poxviridae*
Edge Hill virus, (EHV), *Flaviviridae*
Edmonston virus, *Paramyxoviridae*
EDS virus, *Adenoviridae*
Eel virus American, (EVA), *Rhabdoviridae*
Eel virus B12, (EEV-B12), *Rhabdoviridae*
Eel virus C26, (EEV-C26), *Rhabdoviridae*
EgAN 1825-61 virus, (EGAV), *Bunyaviridae*
Egg drop syndrome virus, (DAdV-1), *Adenoviridae*
Eggplant green mosaic virus, (EGMV), *Potyviridae*
Eggplant latent viroid, (ELVd), *Avsunviroidae*
Eggplant mild mottle virus, (EMMV), *Carlavirus*
Eggplant mosaic virus, (EMV), *Tymovirus*
Eggplant mottled crinkle virus, (EMCV), *Tombusviridae*
Eggplant mottled dwarf virus, (EMDV), *Rhabdoviridae*
Eggplant severe mottle virus, (ESMoV), *Potyviridae*
Eggplant virus, *Carlavirus*
Eggplant yellow mosaic virus, (EYMV), *Geminiviridae*
Egtved virus, *Rhabdoviridae*
Egypt bee virus, (EBV), Unassigned
El Moro Canyon virus, (ELMCV), *Bunyaviridae*
Elapid herpesvirus 1, (EpHV-1), *Herpesviridae*
Elderberry latent virus, (ElLV), *Tombusviridae*
Elderberry symptomless virus, (ESLV), *Carlavirus*
Elderberry virus A, *Carlavirus*
Elephant loxodontol herpesvirus, *Herpesviridae*
Elephantid herpesvirus 1, (ElHV-1), *Herpesviridae*
Ellidaey virus, (ELLV), *Reoviridae*
Elm mottle virus, (EMoV), *Bromoviridae*
Embu virus, (ERV), *Poxviridae*
Encephalomyocarditis virus, (EMCV), *Picornaviridae*
Endive necrotic mosaic virus, (ENMV), *Potyviridae*
Enseada virus, (ENSV), *Bunyaviridae*
Entamoeba virus, (ENTV), *Rhabdoviridae*
Entebbe bat virus, (ENTV), *Flaviviridae*
Enterobacteria phage ϕK, (ϕK), *Microviridae*
Enterobacteria phage μ2, (μ2), *Leviviridae*
Enterobacteria phage 01, (01), *Myoviridae*
Enterobacteria phage 1, (1), *Myoviridae*
Enterobacteria phage 102, (102), *Siphoviridae*
Enterobacteria phage 103, (103), *Siphoviridae*
Enterobacteria phage 11F, (11F), *Myoviridae*
Enterobacteria phage 121, (121), *Myoviridae*
Enterobacteria phage 150, (150), *Siphoviridae*
Enterobacteria phage 16-19, (16-19), *Myoviridae*
Enterobacteria phage 168, (168), *Siphoviridae*
Enterobacteria phage 174, (174), *Siphoviridae*
Enterobacteria phage 186, (186), *Myoviridae*
Enterobacteria phage 1ϕ1, (1ϕ1), *Microviridae*
Enterobacteria phage 1ϕ3, (1ϕ3), *Microviridae*
Enterobacteria phage 1ϕ7, (1ϕ7), *Microviridae*
Enterobacteria phage 1ϕ9, (1ϕ9), *Microviridae*
Enterobacteria phage 299, (299), *Myoviridae*
Enterobacteria phage 2D/13, (2D/13), *Microviridae*
Enterobacteria phage 3, (3), *Myoviridae*
Enterobacteria phage 3T+, (3T+), *Myoviridae*

Enterobacteria phage 50, (50), *Myoviridae*
Enterobacteria phage 5845, (5845), *Myoviridae*
Enterobacteria phage 66F, (66F), *Myoviridae*
Enterobacteria phage 7-11, (7-11), *Podoviridae*
Enterobacteria phage 7480b, (7480b), *Podoviridae*
Enterobacteria phage 8893, (8893), *Myoviridae*
Enterobacteria phage 9/0, (9/0), *Myoviridae*
Enterobacteria phage 9266, (9266), *Myoviridae*
Enterobacteria phage AE2, (AE2), *Inoviridae*
Enterobacteria phage B6, (B6), *Leviviridae*
Enterobacteria phage B7, (B7), *Leviviridae*
Enterobacteria phage BA14, (Ba14), *Podoviridae*
Enterobacteria phage BE/1, (BE/1), *Microviridae*
Enterobacteria phage Beccles, (Beccles), *Myoviridae*
Enterobacteria phage BF23, (BF23), *Siphoviridae*
Enterobacteria phage BZ13, (BZ13), *Leviviridae*
Enterobacteria phage C-1, (C-1), *Leviviridae*
Enterobacteria phage C16, (C16), *Myoviridae*
Enterobacteria phage C-2, (C-2), *Inoviridae*
Enterobacteria phage C2, (C2), *Leviviridae*
Enterobacteria phage D108, (D108), *Myoviridae*
Enterobacteria phage D20, (D20), *Siphoviridae*
Enterobacteria phage *D2A*, (D2A), *Myoviridae*
Enterobacteria phage D6, (D6), *Myoviridae*
Enterobacteria phage *D8*, (D8), *Myoviridae*
Enterobacteria phage DdVI, (DdVI), *Myoviridae*
Enterobacteria phage dϕ3, (dϕ3), *Microviridae*
Enterobacteria phage dϕ4, (dϕ4), *Microviridae*
Enterobacteria phage dϕ5, (dϕ5), *Microviridae*
Enterobacteria phage Ec9, (Ec9), *Inoviridae*
Enterobacteria phage Esc-7-11, (Esc-7-11), *Podoviridae*
Enterobacteria phage f1, (f1), *Inoviridae*
Enterobacteria phage F10, (F10), *Myoviridae*
Enterobacteria phage f2, (f2), *Leviviridae*
Enterobacteria phage F7, (F7), *Myoviridae*
Enterobacteria phage FC3-9, (FC3-9), *Myoviridae*
Enterobacteria phage fcan, (fcan), *Leviviridae*
Enterobacteria phage fd, (fd), *Inoviridae*
Enterobacteria phage FI, (FI), *Leviviridae*
Enterobacteria phage Folac, (Folac), *Leviviridae*
Enterobacteria phage fr, (fr), *Leviviridae*
Enterobacteria phage Fsα, (Fsα), *Myoviridae*
Enterobacteria phage G13, (G13), *Microviridae*
Enterobacteria phage G14, (G14), *Microviridae*
Enterobacteria phage G4, (G4), *Microviridae*
Enterobacteria phage G6, (G6), *Microviridae*
Enterobacteria phage GA, (GA), *Leviviridae*
Enterobacteria phage H, (H), *Podoviridae*
Enterobacteria phage H-19J, (H-19J), *Siphoviridae*
Enterobacteria phage Hi, (Hi), *Siphoviridae*
Enterobacteria phage HK022, (HK022), *Siphoviridae*
Enterobacteria phage HK97, (HK97), *Siphoviridae*
Enterobacteria phage HR, (HR), *Inoviridae*
Enterobacteria phage I_2-2, (I_2-2), *Inoviridae*
Enterobacteria phage ID2, (ID2), *Leviviridae*
Enterobacteria phage If1, (If1), *Inoviridae*
Enterobacteria phage IKe, (IKe), *Inoviridae*
Enterobacteria phage IV, (IV), *Podoviridae*
Enterobacteria phage Iα, (Iα), *Leviviridae*
Enterobacteria phage j2, (j2), *Myoviridae*
Enterobacteria phage Jersey, (Jersey), *Siphoviridae*
Enterobacteria phage JP34, (JP34), *Leviviridae*
Enterobacteria phage JP501, (JP501), *Leviviridae*
Enterobacteria phage K11, (K11), *Podoviridae*
Enterobacteria phage Kl3, (Kl3), *Myoviridae*
Enterobacteria phage Kl9, (Kl9), *Myoviridae*
Enterobacteria phage KU1, (KU1), *Leviviridae*
Enterobacteria phage L, (L), *Podoviridae*
Enterobacteria phage L17, (L17), *Tectiviridae*
Enterobacteria phage LP7, (LP7), *Podoviridae*
Enterobacteria phage M, (M), *Leviviridae*
Enterobacteria phage M11, (M11), *Leviviridae*
Enterobacteria phage M12, (M12), *Leviviridae*
Enterobacteria phage M13, (M13, Ff), *Inoviridae*
Enterobacteria phage M20, (M20), *Microviridae*
Enterobacteria phage Mg40, (Mg40), *Podoviridae*
Enterobacteria phage MS2, (MS2), *Leviviridae*
Enterobacteria phage Mu, (Mu), *Myoviridae*
Enterobacteria phage Mu-1, (Mu-1), *Myoviridae*
Enterobacteria phage MX1, (MX1), *Leviviridae*
Enterobacteria phage N4, (N4), *Podoviridae*
Enterobacteria phage NL95, (NL95), *Leviviridae*
Enterobacteria phage P1, (P1), *Myoviridae*
Enterobacteria phage P1D, (P1D), *Myoviridae*
Enterobacteria phage P2, (P2), *Myoviridae*
Enterobacteria phage P22, (P22), *Podoviridae*
Enterobacteria phage P7, (P7), *Myoviridae*
Enterobacteria phage PA-2, (PA-2), *Siphoviridae*
Enterobacteria phage PB, (PB), *Siphoviridae*
Enterobacteria phage pilHα, (pilHα), *Leviviridae*
Enterobacteria phage Pk2, (Pk2), *Myoviridae*
Enterobacteria phage PR3, (PR3), *Tectiviridae*
Enterobacteria phage PR4, (PR4), *Tectiviridae*
Enterobacteria phage PR5, (PR5), *Tectiviridae*
Enterobacteria phage PR64FS, (PR64FS), *Inoviridae*
Enterobacteria phage PR772, (PR772), *Tectiviridae*
Enterobacteria phage PRD1, (PRD1), *Tectiviridae*
Enterobacteria phage PSA78, (PSA78), *Podoviridae*
Enterobacteria phage PST, (PST), *Myoviridae*
Enterobacteria phage PTB, (PTB), *Podoviridae*
Enterobacteria phage Qβ, (Qβ), *Leviviridae*
Enterobacteria phage R, (R), *Podoviridae*
Enterobacteria phage R17, (R17), *Leviviridae*
Enterobacteria phage R23, (R23), *Leviviridae*
Enterobacteria phage R34, (R34), *Leviviridae*
Enterobacteria phage RB42, (RB42), *Myoviridae*
Enterobacteria phage RB43, (RB43), *Myoviridae*
Enterobacteria phage RB49, (RB49), *Myoviridae*
Enterobacteria phage RB69, (RB69), *Myoviridae*

Enterobacteria phage S13, (S13), *Microviridae*
Enterobacteria phage San 2, (San2), *Siphoviridae*
Enterobacteria phage sd, (sd), *Podoviridae*
Enterobacteria phage SF, (SF), *Inoviridae*
Enterobacteria phage Sf6, (Sf6), *Podoviridae*
Enterobacteria phage SKII, (SKII), *Myoviridae*
Enterobacteria phage SKV, (SKV), *Myoviridae*
Enterobacteria phage SKX, (SKX), *Myoviridae*
Enterobacteria phage SMB, (SMB), *Myoviridae*
Enterobacteria phage SMP2, (SMP2), *Myoviridae*
Enterobacteria phage SP, (SP), *Leviviridae*
Enterobacteria phage SP6, (SP6), *Podoviridae*
Enterobacteria phage ST, (ST), *Leviviridae*
Enterobacteria phage St-1, (St-1), *Microviridae*
Enterobacteria phage SV14, (SV14), *Myoviridae*
Enterobacteria phage SV3, (SV3), *Myoviridae*
Enterobacteria phage T1, (T1), *Siphoviridae*
Enterobacteria phage T2, (T2), *Myoviridae*
Enterobacteria phage T3, (T3), *Podoviridae*
Enterobacteria phage T4, (T4), *Myoviridae*
Enterobacteria phage T5, (T5), *Siphoviridae*
Enterobacteria phage T6, (T6), *Myoviridae*
Enterobacteria phage T7, (T7), *Podoviridae*
Enterobacteria phage tf-1, (tf-1), *Inoviridae*
Enterobacteria phage TH1, (TH1), *Leviviridae*
Enterobacteria phage TW18, (TW18), *Leviviridae*
Enterobacteria phage TW28, (TW28), *Leviviridae*
Enterobacteria phage U3, (U3), *Microviridae*
Enterobacteria phage UC-1, (UC-1), *Siphoviridae*
Enterobacteria phage ViI, (ViI), *Myoviridae*
Enterobacteria phage ViII, (ViII), *Siphoviridae*
Enterobacteria phage VK, (VK), *Leviviridae*
Enterobacteria phage W31, (W31), *Podoviridae*
Enterobacteria phage WA/1, (WA/1), *Microviridae*
Enterobacteria phage WF/1, (WF/1), *Microviridae*
Enterobacteria phage WPK, (WPK), *Podoviridae*
Enterobacteria phage WW/1, (WW/1), *Microviridae*
Enterobacteria phage Wϕ, (Wϕ), *Myoviridae*
Enterobacteria phage X, (X), *Inoviridae*
Enterobacteria phage X-2, (X-2), *Inoviridae*
Enterobacteria phage Y, (Y), *Podoviridae*
Enterobacteria phage ZG/1, (ZG/1), *Leviviridae*
Enterobacteria phage ZG/3A, (ZG/3A), *Siphoviridae*
Enterobacteria phage ZIK/1, (ZIK/1), *Leviviridae*
Enterobacteria phage ZJ/1, (ZJ/1), *Leviviridae*
Enterobacteria phage ZJ/2, (ZJ/2), *Inoviridae*
Enterobacteria phage ZL/3, (ZL/3), *Leviviridae*
Enterobacteria phage ZS/3, (ZS/3), *Leviviridae*
Enterobacteria phage ΦD328, (ΦD328), *Siphoviridae*
Enterobacteria phage Ω8, (Ω8), *Podoviridae*
Enterobacteria phage α1, (α1), *Myoviridae*
Enterobacteria phage α10, (α10), *Microviridae*
Enterobacteria phage α15, (α15), *Leviviridae*
Enterobacteria phage α3, (α3), *Microviridae*
Enterobacteria phage β, (β), *Leviviridae*
Enterobacteria phage β4, (β4), *Siphoviridae*
Enterobacteria phage χ, (χ), *Siphoviridae*
Enterobacteria phage δ1, (δ1), *Microviridae*
Enterobacteria phage δ6, (δ6), *Microviridae*
Enterobacteria phage δA, (δA), *Inoviridae*
Enterobacteria phage ϕ80, (ϕ80), *Siphoviridae*
Enterobacteria phage ϕ92, (ϕ92), *Myoviridae*
Enterobacteria phage ϕA, (ϕA), *Microviridae*
Enterobacteria phage ϕR, (ϕR), *Microviridae*
Enterobacteria phage ϕW39, (ϕW39), *Myoviridae*
Enterobacteria phage ϕX174, (ϕX174), *Microviridae*
Enterobacteria phage ϕ1.2, (ϕ1.2), *Podoviridae*
Enterobacteria phage $\phi\gamma$, ($\phi\gamma$), *Siphoviridae*
Enterobacteria phage η8, (η8), *Microviridae*
Enterobacteria phage; λ, (λ), Siphoviridae
Enterobacteria phage μ, (μ), *Myoviridae*
Enterobacteria phage τ, (τ), *Leviviridae*
Enterobacteria phage ζ3, (ζ3), *Microviridae*
Enterobacteria phage ϕI, (ϕI), *Podoviridae*
Enterobacteria phage ϕII, (ϕII), *Podoviridae*
Enterobacteria phage ϕye03, (ϕye03), *Podoviridae*
Enterobacterial phage ViIII, (ViIII), *Podoviridae*
Enytus montanus ichnovirus, (EmIV), *Polydnaviridae*
Epirus cherry virus, (EpCV), *Ourmiavirus*
Epizootic haematopoietic necrosis virus, (EHNV), *Iridoviridae*
Epizootic hemorrhagic disease virus 1 to 8, (EHDV-1 to 8), *Reoviridae*
Epizootic hemorrhagic disease virus, (EHDV), *Reoviridae*
Epstein-Barr virus, *Herpesviridae*
Equid herpesvirus 1, (EHV-1), *Herpesviridae*
Equid herpesvirus 2, (EHV-2), *Herpesviridae*
Equid herpesvirus 3, (EHV-3), *Herpesviridae*
Equid herpesvirus 4, (EHV-4), *Herpesviridae*
Equid herpesvirus 5, (EHV-5), *Herpesviridae*
Equid herpesvirus 6, (EHV-6), *Herpesviridae*
Equid herpesvirus 7, (EHV-7), *Herpesviridae*
Equid herpesvirus 8, (EHV-8), *Herpesviridae*
Equid herpesvirus 9, (EHV-9), *Herpesviridae*
Equine abortion virus, *Herpesviridae*
Equine adeno-associated virus, (EAAV), *Parvoviridae*
Equine adenovirus 1, (EAdV-1), *Adenoviridae*
Equine adenovirus 2, (EAdV-2), *Adenoviridae*
Equine adenovirus A, (EAdV-A), *Adenoviridae*
Equine adenovirus B, (EAdV-B), *Adenoviridae*
Equine arteritis virus, (EAV), *Arteriviridae*
Equine coital exanthema virus, *Herpesviridae*
Equine encephalosis virus 1 to 7, (EEV-1 to 7), *Reoviridae*
Equine encephalosis virus, (EEV), *Reoviridae*
Equine infectious anemia virus, (EIAV), *Retroviridae*
Equine rhinitis A virus, (ERAV), *Picornaviridae*
Equine rhinitis B virus, (ERBV), *Picornaviridae*
Equine rhinopneumonitis virus, *Herpesviridae*

Equine rhinovirus 1, *Picornaviridae*
Equine rhinovirus 2, *Picornaviridae*
Equine rhinovirus 3, (ERV-3), *Picornaviridae*
Equine torovirus, (EqTV), *Coronaviridae*
Eret-147 virus, (E147V), *Bunyaviridae*
Eriborus terebrans ichnovirus, (EtIV), *Polydnaviridae*
Erinaceid herpesvirus 1, (ErHV-1), *Herpesviridae*
Eriogaster lanestris cypovirus 2, (ElCPV-2), *Reoviridae*
Eriogaster lanestris cypovirus 6, (E1CPV-6), *Reoviridae*
Erve virus, (ERVEV), *Bunyaviridae*
Erysimum latent virus, (ErLV), *Tymovirus*
Esocid herpesvirus 1, (EsHV-1), *Herpesviridae*
Essaouira virus, (ESSV), *Reoviridae*
Estero Real virus, (ERV), *Bunyaviridae*
Eubenangee virus, (EUBV), *Reoviridae*
Eubenangee virus, (EUBV), *Reoviridae*
Eucocytis meeki virus, (EmV), *Tetraviridae*
Euonymus fasciation virus, (EFV), *Rhabdoviridae*
Euonymus mosaic virus, (EuoMV), *Carlavirus*
Eupatorium yellow vein virus, (EpYVV), *Geminiviridae*
Euphorbia mosaic virus, (EuMV), *Geminiviridae*
Euphorbia ringspot virus, (EuRSV), *Potyviridae*
Euploea corea virus, (EcV), *Tetraviridae*
European bat lyssavirus 1, (EBLV-1), *Rhabdoviridae*
European bat lyssavirus 2, (EBLV-2), *Rhabdoviridae*
European brown hare syndrome virus, (EBHSV), *Caliciviridae*
European brown hare syndrome virus-BS89, (EBHSV-BS89), *Caliciviridae*
European brown hare syndrome virus-FRG, (EBHSV-FRG), *Caliciviridae*
European brown hare syndrome virus-GD, (EBHSV-GD), *Caliciviridae*
European brown hare syndrome virus-UK91, (EBHSV-UK91), *Caliciviridae*
European elk papillomavirus, (EEPV), *Papillomaviridae*
European ground squirrel cytomegalovirus, *Herpesviridae*
European hedgehog herpesvirus, *Herpesviridae*
European wheat striate mosaic virus, (EWSMV), *Tenuivirus*
Euxoa auxiliaris densovirus, (EaDNV), *Parvoviridae*
Euxoa scandens cypovirus 5, (EsCPV-5), *Reoviridae*
Everglades virus, (EVEV), *Togaviridae*
Eyach virus (France-577), (EYAV-Fr577), *Reoviridae*
Eyach virus (France-578), (EYAV-Fr578), *Reoviridae*
Eyach virus (Germany), (EYAV-Gr), *Reoviridae*
Eyach virus, (EYAV), *Reoviridae*
Faba bean necrotic yellows virus, (FBNYV), *Nanovirus*
Facey's Paddock virus, (FPV), *Bunyaviridae*
Falcon inclusion body diseases, *Herpesviridae*
Falconid herpesvirus 1, (FaHV-1), *Herpesviridae*
Farallon virus, (FARV), *Bunyaviridae*
Feldmannia irregularis virus a, (FiV-a), *Phycodnaviridae*
Feldmannia species virus a, (FsV-a), *Phycodnaviridae*
Feldmannia species virus, (FsV), *Phycodnaviridae*
Felid herpesvirus 1, (FeHV-1), *Herpesviridae*
Feline astrovirus 1, (FAstV-1), *Astroviridae*
Feline astrovirus, (FAstV), *Astroviridae*
Feline calicivirus CFI/68, (FCV-CFI/68), *Caliciviridae*
Feline calicivirus F9, (FCV-F9), *Caliciviridae*
Feline calicivirus, (FCV), *Caliciviridae*
Feline coronavirus, (FCoV), *Coronaviridae*
Feline foamy virus, (FFV), *Retroviridae*
Feline immunodeficiency virus (Oma), (FIV-O), *Retroviridae*
Feline immunodeficiency virus, (FIV), *Retroviridae*
Feline infectious peritonitis virus, (FIPV), *Coronaviridae*
Feline leukemia virus, (FeLV), *Retroviridae*
Feline panleukopenia virus, (FPLV), *Parvoviridae*
Feline parvovirus, (FPV), *Parvoviridae*
Feline rhinotracheitis virus, *Herpesviridae*
Fescue cryptic virus, (FCV), *Partitiviridae*
Festuca leaf streak virus, (FLSV), *Rhabdoviridae*
Festuca necrosis virus, (FNV), *Closteroviridae*
Fetal rhesus kidney virus, *Polyomaviridae*
Ficus carica virus, (FicCV), *Potyviridae*
Field mouse herpesvirus, *Herpesviridae*
Fig virus S, (FVS), *Carlavirus*
Figulus subleavis entomopoxvirus, (FSEV), *Poxviridae*
Figwort mosaic virus, (FMV), *Caulimoviridae*
Fiji disease virus, (FDV), *Reoviridae*
Fin V 707 virus, (FINV), *Bunyaviridae*
Finger millet mosaic virus, (FMMV), *Rhabdoviridae*
Finkel-Biskis-Jinkins murine sarcoma virus, (FBJMSV), *Retroviridae*
Flame chlorosis virus, (FlCV), Unassigned
Flanders virus, (FLAV), *Rhabdoviridae*
Flexal virus, (FLEV), *Arenaviridae*
Flock house virus, (FHV), *Nodaviridae*
Flounder lymphocystis disease virus, (FLDV), *Iridoviridae*
Flounder virus, *Iridoviridae*
Fomede virus, (FV), *Reoviridae*
Foot-and-mouth disease virus type A, (FMDV-A), *Picornaviridae*
Foot-and-mouth disease virus type Asia 1, (FMDV-Asia1), *Picornaviridae*

Foot-and-mouth disease virus type C, (FMDV-C), *Picornaviridae*
Foot-and-mouth disease virus type O, (FMDV-O), *Picornaviridae*
Foot-and-mouth disease virus type SAT 1, (FMDV-SAT1), *Picornaviridae*
Foot-and-mouth disease virus type SAT 2, (FMDV-SAT2), *Picornaviridae*
Foot-and-mouth disease virus type SAT 3, (FMDV-SAT3), *Picornaviridae*
Foot-and-mouth disease virus, (FMDV), *Picornaviridae*
Forecariah virus, (FORV), *Bunyaviridae*
Fort Morgan virus, (FMV), *Togaviridae*
Fort Sherman virus, (FSV), *Bunyaviridae*
Foula virus, (FOUV), *Reoviridae*
Fowl adenovirus 1 (CELO, 112, Phelps), (FAdV-1), *Adenoviridae*
Fowl adenovirus 10 (C-2B, M11, CFA20), (FAdV-10), *Adenoviridae*
Fowl adenovirus 11 (380), (FAdV-11), *Adenoviridae*
Fowl adenovirus 2 (GAL-1, 685, SR48), (FAdV-2), *Adenoviridae*
Fowl adenovirus 3 (SR49, 75), (FAdV-3), *Adenoviridae*
Fowl adenovirus 4 (KR-5, J-2), (FAdV-4), *Adenoviridae*
Fowl adenovirus 5 (340, TR22), (FAdV-5), *Adenoviridae*
Fowl adenovirus 6 (CR119, 168), (FAdV-6), *Adenoviridae*
Fowl adenovirus 7 (YR36, X-11), (FAdV-7), *Adenoviridae*
Fowl adenovirus 8a (TR59, T-8, CFA40), (FAdV-8a), *Adenoviridae*
Fowl adenovirus 8b (764, B3), (FAdV-8b), *Adenoviridae*
Fowl adenovirus 9 (A2, 90), (FAdV-9), *Adenoviridae*
Fowl adenovirus A, (FAdV-A), *Adenoviridae*
Fowl adenovirus B, (FAdV-B), *Adenoviridae*
Fowl adenovirus C, (FAdV-C), *Adenoviridae*
Fowl adenovirus D, (FAdV-D), *Adenoviridae*
Fowl adenovirus E, (FAdV-E), *Adenoviridae*
Fowl calicivirus, (FCV), *Caliciviridae*
Fowlpox virus, (FWPV), *Poxviridae*
Foxtail mosaic virus, (FoMV), *Potexvirus*
Fragaria chiloensis virus, (FClV), *Bromoviridae*
Frangipani mosaic virus, (FrMV), *Tobamovirus*
Fraser Point virus, (FPV), *Bunyaviridae*
Freesia leaf necrosis virus, (FLNV), *Varicosavirus*
Freesia mosaic virus, (FreMV), *Potyviridae*
Friend murine leukemia virus, (FrMLV), *Retroviridae*
Frijoles virus VP-161A, (FRIV), *Bunyaviridae*
Frijoles virus, (FRIV), *Bunyaviridae*
Frog adenovirus, (FrAdV-1), *Adenoviridae*
Frog herpesvirus 4, *Herpesviridae*
Frog virus 3, (FV-3), *Iridoviridae*
Fuchsia latent virus, (FLV), *Carlavirus*
Fujinami sarcoma virus, (FuSV), *Retroviridae*
Fukuoka virus, (FUKAV), *Rhabdoviridae*
Furcraea necrotic streak virus, (FNSV), *Tombusviridae*
Fusarium oxysporum Skippy virus, (FoxSkiV), *Metaviridae*

GA391 virus, *Arenaviridae*
Gabek Forest virus, (GFV), *Bunyaviridae*
Gadgets Gully virus, (GGYV), *Flaviviridae*
Gaeumannomyces graminis virus 019/6-A, (GgV-019/6-A), *Partitiviridae*
Gaeumannomyces graminis virus 45/101-C, (GGV-45/101C), Unassigned
Gaeumannomyces graminis virus 87-1-H, (GgV-87-1-H), *Totiviridae*
Gaeumannomyces graminis virus T1-A, (GgV-T1-A), *Partitiviridae*
Galinsoga mosaic virus, (GaMV), *Tombusviridae*
Galleria cell line virus, (GmCLV), Unassigned
Galleria mellonella densovirus, (GmDNV), *Parvoviridae*
Galleria mellonella MNPV, (GmMNPV), *Baculoviridae*
Gallid herpesvirus 1, (GaHV-1), *Herpesviridae*
Gallid herpesvirus 2, (GaHV-2), *Herpesviridae*
Gallid herpesvirus 3, (GaHV-3), *Herpesviridae*
Gamboa virus, (GAMV), *Bunyaviridae*
Gan Gan virus, (GGV), *Bunyaviridae*
Ganjam virus, *Bunyaviridae*
Garba virus, (GARV), *Rhabdoviridae*
Gardner-Arnstein feline sarcoma virus, (GAFeSV), *Retroviridae*
Garland chrysanthemum temperate virus, (GCTV), *Partitiviridae*
Garlic common latent virus, (GarCLV), *Carlavirus*
Garlic dwarf virus, (GDV), *Reoviridae*
Garlic latent virus, (GarLV), *Carlavirus*
Garlic mite-borne filamentous virus, (GarMbFV), *Allexivirus*
Garlic mite-borne latent virus, (GarMbLV), *Allexivirus*
Garlic mosaic virus, (GarMV), *Carlavirus*
Garlic virus 2, *Potyviridae*
Garlic virus A, (GarV-A), *Allexivirus*
Garlic virus B, (GarV-B), *Allexivirus*
Garlic virus C, (GarV-C), *Allexivirus*
Garlic virus D, (GarV-D), *Allexivirus*
Garlic virus X, (GarV-X), *Allexivirus*
Garlic virus, *Potyviridae*
Gazelle herpesvirus, *Herpesviridae*

GB virus A, (GBV-A), *Flaviviridae*
GB virus B, (GBV-B), *Flaviviridae*
GB virus C, (GBV-C), *Flaviviridae*
GBV-A-like agents, (GBV-A-like), *Flaviviridae*
Gentiana latent virus, (GenLV), *Carlavirus*
Geotrupes sylvaticus entomopoxvirus, (GSEV), *Poxviridae*
Gerbera symptomless virus, (GeSLV), *Rhabdoviridae*
Germiston virus, (GERV), *Bunyaviridae*
Getah virus, (GETV), *Togaviridae*
Giardia lamblia virus, (GLV), *Totiviridae*
Gibbon ape leukemia virus, (GALV), *Retroviridae*
Ginger chlorotic fleck virus, (GCFV), *Sobemovirus*
Gloriosa stripe mosaic virus, (GSMV), *Potyviridae*
Glycine mosaic virus, (GMV), *Comoviridae*
Glycine mottle virus, (GMoV), *Tombusviridae*
Glypta fumiferanae ichnovirus, (GfIV), *Polydnaviridae*
Glypta sp. *ichnovirus*, (GspIV), *Polydnaviridae*
Glyptapanteles flavicoxis bracovirus, (GflBV), *Polydnaviridae*
Glyptapanteles indiensis bracovirus, (GiBV), *Polydnaviridae*
Glyptapanteles liparidis bracovirus, (GlBV), *Polydnaviridae*
Goat adenovirus 1, 2, (GAdV-1, 2), *Adenoviridae*
Goat herpesvirus, *Herpesviridae*
Goatpox virus, (GTPV), *Poxviridae*
Goeldichironomus haloprasimus entomopoxvirus, (GHEV), *Poxviridae*
Golden shiner reovirus, (GSV), *Reoviridae*
Goldfish herpesvirus, *Herpesviridae*
Goldfish virus 1, (GFV-1), *Iridoviridae*
Gomoka virus, *Reoviridae*
Gomphrena virus, (GoV), *Rhabdoviridae*
Gonometa podocarpi virus, (GpV), Unassigned
Gonometa rufibrunnea cypovirus 3, (GrCPV-3), *Reoviridae*
Goose adenovirus 1 to 3, (GoAdV-1 to 3), *Adenoviridae*
Goose adenovirus, (GoAdV), *Adenoviridae*
Goose parvovirus, (GPV), *Parvoviridae*
Gordil virus, (GORV), *Bunyaviridae*
Gorilla herpesvirus, *Herpesviridae*
Gossas virus, (GOSV), *Rhabdoviridae*
Grand Arbaud virus, (GAV), *Bunyaviridae*
Grapevine Algerian latent virus, (GALV), *Tombusviridae*
Grapevine berry inner necrosis virus, (GINV), *Trichovirus*
Grapevine Bulgarian latent virus satellite RNA, Satellite
Grapevine Bulgarian latent virus, (GBLV), *Comoviridae*
Grapevine chrome mosaic virus, (GCMV), *Comoviridae*
Grapevine fanleaf virus satellite RNA, Satellite
Grapevine fanleaf virus, (GFLV), *Comoviridae*
Grapevine fleck virus, (GFkV), Unassigned
Grapevine leafroll-associated virus 1, (GLRaV-1), *Closteroviridae*
Grapevine leafroll-associated virus 2, (GLRaV-2), *Closteroviridae*
Grapevine leafroll-associated virus 3, (GLRaV-3), *Closteroviridae*
Grapevine leafroll-associated virus 4, (GLRaV-4), *Closteroviridae*
Grapevine leafroll-associated virus 5, (GLRaV-5), *Closteroviridae*
Grapevine leafroll-associated virus 6, (GLRaV-6), *Closteroviridae*
Grapevine leafroll-associated virus 7, (GLRaV-7), *Closteroviridae*
Grapevine Tunisian ringspot virus, (GTRSV), *Comoviridae*
Grapevine virus A, (GVA), *Vitivirus*
Grapevine virus B, (GVB), *Vitivirus*
Grapevine virus C, (GVC), *Vitivirus*
Grapevine virus D, (GVD), *Vitivirus*
Grapevine yellow speckle viroid 1, (GYSVd-1), *Pospiviroidae*
Grapevine yellow speckle viroid 2, (GYSVd-2), *Pospiviroidae*
Grass carp reovirus, (GCRV), *Reoviridae*
Grass carp rhabdovirus, *Rhabdoviridae*
Gray Lodge virus, (GLOV), *Rhabdoviridae*
Great Island virus, (GIV), *Reoviridae*
Great Island virus, (GIV), *Reoviridae*
Great Saltee Island virus, (GSIV), *Reoviridae*
Great Saltee virus, (GRSV), *Bunyaviridae*
Green lizard herpesvirus, *Herpesviridae*
Grey kangaroo poxvirus, (KXV), *Poxviridae*
Grey patch disease of turtles, *Herpesviridae*
Grimsey virus, (GSYV), *Reoviridae*
Ground squirrel hepatitis virus, (GSHV), *Hepadnaviridae*
Groundnut bud necrosis virus, (GBNV), *Bunyaviridae*
Groundnut chlorotic fan-spot virus, (GCFSV), *Bunyaviridae*
Groundnut crinkle virus, *Carlavirus*
Groundnut eyespot virus, (GEV), *Potyviridae*
Groundnut ringspot virus, (GRSV), *Bunyaviridae*
Groundnut rosette assistor virus, (GRAV), *Luteoviridae*
Groundnut rosette virus satellite RNA, Satellite
Groundnut rosette virus, (GRV), *Umbravirus*
Groundnut yellow spot virus, (GYSV), *Bunyaviridae*
Gruid herpesvirus 1, (GrHV-1), *Herpesviridae*

GU71U 344 virus, (GU344V), *Bunyaviridae*
GU71U 350 virus, (GU350V), *Bunyaviridae*
Guajara virus, (GJAV), *Bunyaviridae*
Guama virus, (GMAV), *Bunyaviridae*
Guanarito virus, (GTOV), *Arenaviridae*
Guar green sterile virus, *Potyviridae*
Guar symptomless virus, (GSLV), *Potyviridae*
Guaratuba virus, (GTBV), *Bunyaviridae*
Guaroa virus, (GROV), *Bunyaviridae*
Guinea grass mosaic virus, (GGMV), *Potyviridae*
Guinea pig adenovirus 1, (GPAdV-1), *Adenoviridae*
Guinea pig adenovirus, (GPAdV), *Adenoviridae*
Guinea pig Chlamydia phage, (GPCh), *Microviridae*
Guinea pig cytomegalovirus, *Herpesviridae*
Guinea pig herpesvirus 3, *Herpesviridae*
Guinea pig herpesvirus, *Herpesviridae*
Guinea pig type-C oncovirus, (GPCOV), *Retroviridae*
Guineafowl transmissible enteritis virus, (GTEV), *Picornaviridae*
Gumbo Limbo virus, (GLV), *Bunyaviridae*
Guppy virus 6, (GV6), *Iridoviridae*
Gurupi virus, (GURV), *Reoviridae*
Gynura latent virus, (GyLV), *Carlavirus*
Gypsy moth virus, (GMV), *Nodaviridae*

H-1 virus, (H-1PV), *Parvoviridae*
H-32580 virus, (H32580V), *Bunyaviridae*
Habenaria mosaic virus, (HaMV), *Potyviridae*
Haematopoietic necrosis herpesvirus of goldfish, *Herpesviridae*
Haemophilus phage HP1, (HP1), *Myoviridae*
Haemophilus phage S2, (S2), *Myoviridae*
Halobacterium phage Hs1, (Hs1), *Myoviridae*
Halobacterium phage ϕH, (ϕH), *Myoviridae*
Hamster herpesvirus, *Herpesviridae*
Hamster polyomavirus, (HaPyV), *Polyomaviridae*
Hantaan 76-118 virus, *Bunyaviridae*
Hantaan virus, (HTNV), *Bunyaviridae*
Harbour seal herpesvirus, *Herpesviridae*
Harbour seals picorna-like virus, (SPLV), *Picornaviridae*
Hard clam reovirus, (HCRV), *Reoviridae*
Hardy-Zuckerman feline sarcoma virus, (HZFeSV), *Retroviridae*
Hare fibroma virus, (FIBV), *Poxviridae*
Hart Park virus, (HPV), *Rhabdoviridae*
Hartebeest malignant catarrhal fever virus, *Herpesviridae*
Hart's tongue fern mottle virus, (HTFMoV), Unassigned
Harvey murine sarcoma virus, (HaMSV), *Retroviridae*
Hawaii virus, (HV), *Caliciviridae*
Hazara virus, (HAZV), *Bunyaviridae*
HB virus, (HBPV), *Parvoviridae*
HB55 virus, *Bunyaviridae*
HCV cluster 1, (HCV-1), *Flaviviridae*
HCV cluster 2, (HCV-J6), *Flaviviridae*
HCV cluster 3, (HCV-NZL1), *Flaviviridae*
HCV cluster 4, (HCV-ED43), *Flaviviridae*
HCV cluster 5, (HCV-EVH1480), *Flaviviridae*
HCV cluster 6, (HCV-EUHK2), *Flaviviridae*
Helenium virus S, (HVS), *Carlavirus*
Helenium virus Y, (HVY), *Potyviridae*
Helicoverpa armigera stunt virus, (HaSV) *Tetraviridae*
Helicoverpa zea SNPV, (HzSNPV), *Baculoviridae*
Heliothis armigera cypovirus ('B' strain), *Reoviridae*
Heliothis armigera cypovirus 11, (HaCPV-11), *Reoviridae*
Heliothis armigera cypovirus 14 ('A' strain), (HaCPV-14), *Reoviridae*
Heliothis armigera cypovirus 5, (HaCPV-5), *Reoviridae*
Heliothis armigera cypovirus 8, (HaCPV-8), *Reoviridae*
Heliothis armigera entomopoxvirus 'L', (HAVE), *Poxviridae*
Heliothis armigera iridescent virus, *Iridoviridae*
Heliothis virescens ascovirus 1a, (HvAV-1a), *Ascoviridae*
Heliothis zea cypovirus 11, (HzCPV-11), *Reoviridae*
Heliothis zea virus 1, (HzV-1), Unassigned
Heliothis/Helicoverpa zea iridescent virus, *Iridoviridae*
Helleborus mosaic virus, (HeMV), *Carlavirus*
Helminthosporium maydis virus, (HMV), Unassigned
Helminthosporium victoriae virus 145S, (HvV-145S), *Partitiviridae*
Helminthosporium victoriae virus 190S, (HvV-190S), *Totiviridae*
Henbane mosaic virus, (HMV), *Potyviridae*
Hepatitis A virus, (HAV), *Picornaviridae*
Hepatitis B virus, (HBV), *Hepadnaviridae*
Hepatitis C virus, (HCV), *Flaviviridae*
Hepatitis delta virus, (HDV), *Deltavirus*
Hepatitis E virus, (HEV), "HEV-like viruses"
Hepatitis G virus, (HGV-1), *Flaviviridae*
Hepatopancreatic parvo-like virus of shrimps, *Parvoviridae*
Heracleum latent virus, (HLV), *Vitivirus*
Heracleum virus 6, (HV-6), *Closteroviridae*
Heron hepatitis B virus, (HHBV), *Hepadnaviridae*
Herpes simplex virus 1, *Herpesviridae*
Herpes simplex virus 2, *Herpesviridae*
Herpesvirus aotus 1, *Herpesviridae*
Herpesvirus aotus 3, *Herpesviridae*
Herpesvirus ateles strain 73, *Herpesviridae*

Herpesvirus ateles, *Herpesviridae*
Herpesvirus cuniculi, *Herpesviridae*
Herpesvirus cyclopsis, *Herpesviridae*
Herpesvirus marmota, *Herpesviridae*
Herpesvirus pan, *Herpesviridae*
Herpesvirus papio 2, *Herpesviridae*
Herpesvirus papio, *Herpesviridae*
Herpesvirus pottos, *Herpesviridae*
Herpesvirus saimiri, *Herpesviridae*
Herpesvirus salmonis, *Herpesviridae*
Herpesvirus sanguinus, *Herpesviridae*
Herpesvirus scophthalmus, *Herpesviridae*
Herpesvirus simiae, *Herpesviridae*
Herpesvirus sylvilagus, *Herpesviridae*
Herpesvirus tamarinus, *Herpesviridae*
Heteronychus arator iridescent virus, *Iridoviridae*
Hibiscus chlorotic ringspot virus, (HCRSV), *Tombusviridae*
Hibiscus latent ringspot virus, (HLRSV), *Comoviridae*
Highlands J virus, (HJV), *Togaviridae*
Himetobi P virus, (HiPV), "CrPV-like viruses"
Hincksia hinckiae virus a, (HhV-a), *Phycodnaviridae*
Hippeastrum latent virus, *Carlavirus*
Hippeastrum mosaic virus, (HiMV), *Potyviridae*
Hippotragine herpesvirus 18, (HiHV-1), *Herpesviridae*
Hirame rhabdovirus, (HIRRV), *Rhabdoviridae*
Hog cholera virus, *Flaviviridae*
HoJo virus, *Bunyaviridae*
Holcus lanatus yellowing virus, (HLYV), *Rhabdoviridae*
Holcus streak virus, (HSV), *Potyviridae*
Hollyhock leaf curl virus, (HLCV), *Geminiviridae*
Honeysuckle latent virus, (HnLV), *Carlavirus*
Honeysuckle yellow vein mosaic virus, (HYVMV), *Geminiviridae*
Hop latent viroid, (HpLVd), *Pospiviroidae*
Hop latent virus, (HpLV), *Carlavirus*
Hop mosaic virus, (HpMV), *Carlavirus*
Hop stunt viroid, (HSVd), *Pospiviroidae*
Hop trefoil cryptic virus 1, (HTCV-1), *Partitiviridae*
Hop trefoil cryptic virus 2, (HTCV-2), *Partitiviridae*
Hop trefoil cryptic virus 3, (HTCV-3), *Partitiviridae*
Hordeum mosaic virus, (HoMV), *Potyviridae*
Hordeum vulgare BARE-1 virus, (HvuBar1V), *Pseudoviridae*
Horsegram yellow mosaic virus, (HgYMV), *Geminiviridae*
Horseradish curly top virus, (HrCTV), *Geminiviridae*
Horseradish latent virus, (HRLV), *Caulimoviridae*
Hosta virus X, (HVX), *Potexvirus*
HR80-39, *Bunyaviridae*
Hsiung kaplow herpesvirus, *Herpesviridae*
Hughes virus, (HUGV), *Bunyaviridae*
Human adenovirus 1, 2, 5, 6, (HAdV-1, 2, 5, 6), *Adenoviridae*
Human adenovirus 12, 18, 31, (HAdV-12, 18, 31), *Adenoviridae*
Human adenovirus 16, 21, 34, 35, 50, (HAdV-16, 21, 34, 35, 50), *Adenoviridae*
Human adenovirus 20, 22–30, 32, 33, (HAdV-20, 22–30, 32, 33), *Adenoviridae*
Human adenovirus 3, 7, 11, 14, (HAdV-3, 7, 11, 14), *Adenoviridae*
Human adenovirus 36-39, 42–49, 51, (HAdV-36–39, 42-49, 51), *Adenoviridae*
Human adenovirus 4, (HAdV-4), *Adenoviridae*
Human adenovirus 40, 41, (HAdV-40, 41), *Adenoviridae*
Human adenovirus 8–10, 13, 15, 17, 19, (HAdV-8–10, 13, 15, 17, 19), *Adenoviridae*
Human adenovirus A, (HAdV-A), *Adenoviridae*
Human adenovirus B, (HAdV-B), *Adenoviridae*
Human adenovirus C, (HAdV-C), *Adenoviridae*
Human adenovirus D, (HAdV-D), *Adenoviridae*
Human adenovirus E, (HAdV-E), *Adenoviridae*
Human adenovirus F, (HAdV-F), *Adenoviridae*
Human astrovirus 1, (HAstV-1), *Astroviridae*
Human astrovirus 2, (HAstV-2), *Astroviridae*
Human astrovirus 3, (HAstV-3), *Astroviridae*
Human astrovirus 4, (HAstV-4), *Astroviride*
Human astrovirus 5, (HAstV-5), *Astroviridae*
Human astrovirus 6, (HAstV-6), *Astroviridae*
Human astrovirus 7, (HAstV-7), *Astroviridae*
Human astrovirus 8, (HAstV-8), *Astroviridae*
Human astrovirus, (HAstV), *Astroviridae*
Human coronavirus 229E, (HCoV-229E), *Coronaviridae*
Human coronavirus OC43, (HCoV-OC43), *Coronaviridae*
Human coxsackievirus A1, (CV-A1), *Picornaviridae*
Human coxsackievirus A10, (CV-A10), *Picornaviridae*
Human coxsackievirus A11, (CV-A11), *Picornaviridae*
Human coxsackievirus A12, (CV-A12), *Picornaviridae*
Human coxsackievirus A13, (CV-A13), *Picornaviridae*
Human coxsackievirus A14, (CV-A14), *Picornaviridae*
Human coxsackievirus A15, (CV-A15), *Picornaviridae*
Human coxsackievirus A16, (CV-A16), *Picornaviridae*
Human coxsackievirus A17, (CV-A17), *Picornaviridae*
Human coxsackievirus A18, (CV-A18), *Picornaviridae*

Human coxsackievirus A19, (CV-A19), *Picornaviridae*
Human coxsackievirus A2, (CV-A2), *Picornaviridae*
Human coxsackievirus A20, (CV-A20), *Picornaviridae*
Human coxsackievirus A21, (CV-A21), *Picornaviridae*
Human coxsackievirus A22, (CV-A22), *Picornaviridae*
Human coxsackievirus A24, (CV-A24), *Picornaviridae*
Human coxsackievirus A3, (CV-A3), *Picornaviridae*
Human coxsackievirus A4, (CV-A4), *Picornaviridae*
Human coxsackievirus A5, (CV-A5), *Picornaviridae*
Human coxsackievirus A6, (CV-A6), *Picornaviridae*
Human coxsackievirus A7, (CV-A7), *Picornaviridae*
Human coxsackievirus A8, (CV-A8), *Picornaviridae*
Human coxsackievirus A9, (CV-A9), *Picornaviridae*
Human coxsackievirus B1, (CV-B1), *Picornaviridae*
Human coxsackievirus B2, (CV-B2), *Picornaviridae*
Human coxsackievirus B3, (CV-B3), *Picornaviridae*
Human coxsackievirus B4, (CV-B4), *Picornaviridae*
Human coxsackievirus B5, (CV-B5), *Picornaviridae*
Human coxsackievirus B6, (CV-B6), *Picornaviridae*
Human cytomegalovirus, *Herpesviridae*
Human echovirus 1, (E-1), *Picornaviridae*
Human echovirus 11, (E-11), *Picornaviridae*
Human echovirus 12, (E-12), *Picornaviridae*
Human echovirus 13, (E-13), *Picornaviridae*
Human echovirus 14, (E-14), *Picornaviridae*
Human echovirus 15, (E-15), *Picornaviridae*
Human echovirus 16, (E-16), *Picornaviridae*
Human echovirus 17, (E-17), *Picornaviridae*
Human echovirus 18, (E-18), *Picornaviridae*
Human echovirus 19, (E-19), *Picornaviridae*
Human echovirus 2, (E-2), *Picornaviridae*
Human echovirus 20, (E-20), *Picornaviridae*
Human echovirus 21, (E-21), *Picornaviridae*
Human echovirus 22, (E-22), *Picornaviridae*
Human echovirus 23, (E-23), *Picornaviridae*
Human echovirus 24, (E-24), *Picornaviridae*
Human echovirus 25, (E-25), *Picornaviridae*
Human echovirus 26, (E-26), *Picornaviridae*
Human echovirus 27, (E-27), *Picornaviridae*
Human echovirus 29, (E-29), *Picornaviridae*
Human echovirus 3, (E-3), *Picornaviridae*
Human echovirus 30, (E-30), *Picornaviridae*
Human echovirus 31, (E-31), *Picornaviridae*
Human echovirus 32, (E-32), *Picornaviridae*
Human echovirus 33, (E-33), *Picornaviridae*
Human echovirus 4, (E-4), *Picornaviridae*
Human echovirus 5, (E-5), *Picornaviridae*
Human echovirus 6, (E-6), *Picornaviridae*
Human echovirus 7, (E-7), *Picornaviridae*
Human echovirus 9, (E-9), *Picornaviridae*
Human enterovirus 68, (EV-68), *Picornaviridae*
Human enterovirus 69, (EV-69), *Picornaviridae*
Human enterovirus 70, (EV-70), *Picornaviridae*
Human enterovirus 71, (EV-71), *Picornaviridae*
Human enterovirus A, (HEV-A), *Picornaviridae*
Human enterovirus B, (HEV-B), *Picornaviridae*
Human enterovirus C, (HEV-C), *Picornaviridae*
Human enterovirus D, (HEV-D), *Picornaviridae*
Human foamy virus, HFV, *Retroviridae*
Human hepatitis A virus, (HHAV), *Picornaviridae*
Human herpesvirus 1, (HHV-1), *Herpesviridae*
Human herpesvirus 2, (HHV-2), *Herpesviridae*
Human herpesvirus 3, (HHV-3), *Herpesviridae*
Human herpesvirus 4, (HHV-4), *Herpesviridae*
Human herpesvirus 5, (HHV-5), *Herpesviridae*
Human herpesvirus 6, (HHV-6), *Herpesviridae*
Human herpesvirus 7, (HHV-7), *Herpesviridae*
Human herpesvirus 8, (HHV-8), *Herpesviridae*
Human immunodeficiency virus 1 90CR056, (HIV-1.90CR056), *Retroviridae*
Human immunodeficiency virus 1 93BR020, (HIV-1.93BR020), *Retroviridae*
Human immunodeficiency virus 1 ANT70, (HIV-1.ANT70), *Retroviridae*
Human immunodeficiency virus 1 ARV-2/SF-2, (HIV-1.ARV-2/SF-2), *Retroviridae*
Human immunodeficiency virus 1 BRU (LAI), (HIV-1.BRU(LAI)), *Retroviridae*
Human immunodeficiency virus 1 ELI, (HIV-1.ELI), *Retroviridae*
Human immunodeficiency virus 1 ETH2220, (HIV-1.ETH2220), *Retroviridae*
Human immunodeficiency virus 1 HXB2, (HIV-1.HXB2), *Retroviridae*
Human immunodeficiency virus 1 MN, (HIV-1.MN), *Retroviridae*
Human immunodeficiency virus 1 NDK, (HIV-1.NDK), *Retroviridae*
Human immunodeficiency virus 1 RF, (HIV-1.RF), *Retroviridae*
Human immunodeficiency virus 1 U455, (HIV-1.U455), *Retroviridae*
Human immunodeficiency virus 1, (HIV-1), *Retroviridae*
Human immunodeficiency virus 2 BEN, (HIV-2.BEN), *Retroviridae*
Human immunodeficiency virus 2 D205, (HIV-2.D205), *Retroviridae*
Human immunodeficiency virus 2 EHOA, (HIV-2.EHOA), *Retroviridae*
Human immunodeficiency virus 2 ISY, (HIV-2.ISY), *Retroviridae*
Human immunodeficiency virus 2 ROD, (HIV-2.ROD), *Retroviridae*

Human immunodeficiency virus 2 ST, (HIV-2.ST), *Retroviridae*
Human immunodeficiency virus 2 UC1, (HIV-2.UC1), *Retroviridae*
Human immunodeficiency virus 2, (HIV-2), *Retroviridae*
Human papillomavirus 1 to 82, (HPV-1 to 82), *Papillomaviridae*
Human papillomavirus, (HPV), *Papillomaviridae*
Human parainfluenza virus 1, (HPIV-1), *Paramyxoviridae*
Human parainfluenza virus 2, (HPIV-2), *Paramyxoviridae*
Human parainfluenza virus 3, (HPIV-3), *Paramyxoviridae*
Human parainfluenza virus 4, (HPIV-4), *Paramyxoviridae*
Human parainfluenza virus 4a, *Paramyxoviridae*
Human parainfluenza virus 4b, *Paramyxoviridae*
Human parechovirus 1, (HPeV-1), *Picornaviridae*
Human parechovirus 2, (HPeV-2), *Picornaviridae*
Human parechovirus, (HPeV), *Picornaviridae*
Human poliovirus 1, (PV-1), *Picornaviridae*
Human poliovirus 2, (PV-2), *Picornaviridae*
Human poliovirus 3, (PV-3), *Picornaviridae*
Human respiratory syncytial virus A2, *Paramyxoviridae*
Human respiratory syncytial virus B1, *Paramyxoviridae*
Human respiratory syncytial virus S2, *Paramyxoviridae*
Human respiratory syncytial virus, (HRSV), *Paramyxoviridae*
Human rhinovirus 1, (HRV-1), *Picornaviridae*
Human rhinovirus 10 to 13, (HRV-10 to 13), *Picornaviridae*
Human rhinovirus 11, (HRV-11), *Picornaviridae*
Human rhinovirus 14, (HRV-14), *Picornaviridae*
Human rhinovirus 15, (HRV-15), *Picornaviridae*
Human rhinovirus 16, (HRV-16), *Picornaviridae*
Human rhinovirus 17 to 20, (HRV-17 to 20), *Picornaviridae*
Human rhinovirus 2, (HRV-2), *Picornaviridae*
Human rhinovirus 21, (HRV-21), *Picornaviridae*
Human rhinovirus 22 to 28, (HRV-22 to 28), *Picornaviridae*
Human rhinovirus 29, (HRV-29), *Picornaviridae*
Human rhinovirus 3, (HRV-3), *Picornaviridae*
Human rhinovirus 30 to 38, (HRV-30 to 38), *Picornaviridae*
Human rhinovirus 36, (HRV-36), *Picornaviridae*
Human rhinovirus 39, (HRV-39), *Picornaviridae*
Human rhinovirus 4 to 6, (HRV-4 to 6), *Picornaviridae*
Human rhinovirus 40 to 48, (HRV-40 to 48), *Picornaviridae*
Human rhinovirus 49, (HRV-49), *Picornaviridae*
Human rhinovirus 50, (HRV-50), *Picornaviridae*
Human rhinovirus 51 to 57, (HRV-51 to 57), *Picornaviridae*
Human rhinovirus 58, (HRV-58), *Picornaviridae*
Human rhinovirus 59 to 61, (HRV-59 to 61), *Picornaviridae*
Human rhinovirus 62, (HRV-62), *Picornaviridae*
Human rhinovirus 63 to 64, (HRV-63 to 64), *Picornaviridae*
Human rhinovirus 65, (HRV-65), *Picornaviridae*
Human rhinovirus 66 to 71, (HRV-66 to 71), *Picornaviridae*
Human rhinovirus 7, (HRV-7), *Picornaviridae*
Human rhinovirus 72, (HRV-72), *Picornaviridae*
Human rhinovirus 73 to 84, (HRV-73 to 84), *Picornaviridae*
Human rhinovirus 8, (HRV-8), *Picornaviridae*
Human rhinovirus 85, (HRV-85), *Picornaviridae*
Human rhinovirus 86 to 88, (HRV-86 to 88), *Picornaviridae*
Human rhinovirus 89, (HRV-89), *Picornaviridae*
Human rhinovirus 9, (HRV-9), *Picornaviridae*
Human rhinovirus 90 to 100, (HRV-90 to 100), *Picornaviridae*
Human rhinovirus A, (HRV-A), *Picornaviridae*
Human rhinovirus B, (HRV-B), *Picornaviridae*
Human T-lymphotropic virus 1, (HTLV-1), *Retroviridae*
Human T-lymphotropic virus 2, (HTLV-2), *Retroviridae*
Human torovirus, (HuTV), *Coronaviridae*
Humpty Doo virus, (HDOOV), *Rhabdoviridae*
Humulus japonicus virus, (HJV), *Bromoviridae*
Huncho virus, (HUAV), *Reoviridae*
Hungarian datura innoxia virus, (HDIV), *Potyviridae*
HV114 virus, *Bunyaviridae*
Hyacinth mosaic virus, (HyaMV), *Potyviridae*
Hyalophora cecropia virus, (HcV), *Tetraviridae*
Hydra viridis Chlorella virus 1, (HVCV-1), *Phycodnaviridae*
Hydra viridis Chlorella virus 2, (HVCV-2), *Phycodnaviridae*
Hydra viridis Chlorella virus 3, (HVCV-3), *Phycodnaviridae*
Hydrangea latent virus, (HdLV), *Carlavirus*
Hydrangea mosaic virus, (HdMV), *Bromoviridae*
Hydrangea ringspot virus, (HdRSV), *Potexvirus*
Hyloicus pinastri cypovirus 2, (HpCPV-2), *Reoviridae*
Hyphomicrobium phage Hyϕ30, (Hyϕ30), *Podoviridae*
Hypochoeris mosaic virus, (HyMV), *Furovirus*

Hypocritae jacobeae virus, (HjV), *Tetraviridae*
Hypomicrogaster canadensis bracovirus, (HcBV), *Polydnaviridae*
Hypomicrogaster ectdytolophae bracovirus, (HecBV), *Polydnaviridae*
Hyposoter annulipes ichnovirus, (HaIV), *Polydnaviridae*
Hyposoter exiguae ichnovirus, (HeIV), *Polydnaviridae*
Hyposoter exiguae reovirus, (HeRV), *Reoviridae*
Hyposoter fugitivus ichnovirus, (HfIV), *Polydnaviridae*
Hyposoter lymantriae ichnovirus, (HlIV), *Polydnaviridae*
Hyposoter pilosulus ichnovirus, (HpIV), *Polydnaviridae*
Hyposoter rivalis ichnovirus, (HrIV), *Polydnaviridae*
Hypovirulence-associated virus, *Hypoviridae*

Iaco virus, (IACOV), *Bunyaviridae*
Ibaraki virus isolate 318, (IBAV), *Reoviridae*
Icoaraci virus, *Bunyaviridae*
Ictalurid herpesvirus 1, (IcHV-1), *Herpesviridae*
Ieri virus, (IERIV), *Reoviridae*
Ieri virus, (IERIV), *Reoviridae*
Ife virus, (IFEV), *Reoviridae*
Iguape virus, (IGUV), *Flaviviridae*
Ilesha virus, (ILEV), *Bunyaviridae*
Ilheus virus, (ILHV), *Flaviviridae*
Impatiens latent virus, (ILV), *Carlavirus*
Impatiens necrotic spot virus, (INSV), *Bunyaviridae*
Inachis io cypovirus 2, (IiCPV-2), *Reoviridae*
Indian cassava mosaic virus, (ICMV), *Geminiviridae*
Indian cobra herpesvirus, *Herpesviridae*
Indian peanut clump virus, (IPCV), *Pecluvirus*
Indian pepper mottle virus, (IPMoV), *Potyviridae*
Indian tomato bunchy top viroid, *Pospiviroidae*
Indian tomato leaf curl virus, (IToLCV), *Geminiviridae*
Indonesian soybean dwarf virus, (ISDV), *Luteoviridae*
Infectious bovine rhinotracheitis virus, *Herpesviridae*
Infectious bronchitis virus, (IBV), *Coronaviridae*
Infectious bursal disease virus 002-73, *Birnaviridae*
Infectious bursal disease virus 23/82, *Birnaviridae*
Infectious bursal disease virus 52/70, *Birnaviridae*
Infectious bursal disease virus Australian 002-73, *Birnaviridae*
Infectious bursal disease virus Cu-1, *Birnaviridae*
Infectious bursal disease virus Edgar, *Birnaviridae*
Infectious bursal disease virus Farragher, *Birnaviridae*
Infectious bursal disease virus GPF-1E, *Birnaviridae*
Infectious bursal disease virus KS, *Birnaviridae*
Infectious bursal disease virus OH, *Birnaviridae*
Infectious bursal disease virus OKYM attenuated, *Birnaviridae*
Infectious bursal disease virus OKYM, *Birnaviridae*
Infectious bursal disease virus P2, *Birnaviridae*
Infectious bursal disease virus PBG-98, *Birnaviridae*
Infectious bursal disease virus QC-2, *Birnaviridae*
Infectious bursal disease virus STC, *Birnaviridae*
Infectious bursal disease virus UK661, *Birnaviridae*
Infectious bursal disease virus, (IBDV), *Birnaviridae*
Infectious flacherie virus, (IFV), Unassigned
Infectious hematopoietic necrosis virus, (IHNV), *Rhabdoviridae*
Infectious laryngotracheitis virus, *Herpesviridae*
Infectious pancreatic necrosis virus DRT, *Birnaviridae*
Infectious pancreatic necrosis virus Jasper, *Birnaviridae*
Infectious pancreatic necrosis virus N1, *Birnaviridae*
Infectious pancreatic necrosis virus Sp, *Birnaviridae*
Infectious pancreatic necrosis virus, (IPNV), *Birnaviridae*
Influenza A virus, (FLUAV), *Orthomyxoviridae*
Influenza B virus, (FLUBV), *Orthomyxoviridae*
Influenza C virus, (FLUCV), *Orthomyxoviridae*
Ingwavuma virus, (INGV), *Bunyaviridae*
INH-95551 virus, *Arenaviridae*
Inini virus, (INIV), *Bunyaviridae*
Inkoo virus, (INKV), *Bunyaviridae*
Inner Farne virus, (INFV), *Reoviridae*
Insect iridescent virus 28, *Iridoviridae*
Invertebrate iridescent virus 1, (IIV-1), *Iridoviridae*
Invertebrate iridescent virus 10, *Iridoviridae*
Invertebrate iridescent virus 16, (IIV-16), *Iridoviridae*
Invertebrate iridescent virus 18, *Iridoviridae*
Invertebrate iridescent virus 2, (IIV-2), *Iridoviridae*
Invertebrate iridescent virus 21, (IIV-21), *Iridoviridae*
Invertebrate iridescent virus 22, (IIV-22), *Iridoviridae*
Invertebrate iridescent virus 23, (IIV-23), *Iridoviridae*
Invertebrate iridescent virus 24, (IIV-24), *Iridoviridae*
Invertebrate iridescent virus 29, (IIV-29), *Iridoviridae*
Invertebrate iridescent virus 3, (IIV-3), *Iridoviridae*
Invertebrate iridescent virus 30, (IIV-30), *Iridoviridae*
Invertebrate iridescent virus 31, (IIV-31), *Iridoviridae*
Invertebrate iridescent virus 32, *Iridoviridae*
Invertebrate iridescent virus 6, (IIV-6), *Iridoviridae*
Invertebrate iridescent virus 9, (IIV-9), *Iridoviridae*
Ippy virus, (IPPYV), *Arenaviridae*
Iranian wheat stripe virus, (IWSV), *Tenuivirus*
Iresine viroid 1, (IrVd-1), *Pospiviroidae*
Iris fulva mosaic virus, (IFMV), *Potyviridae*
Iris germanica leaf stripe virus, (IGLSV), *Rhabdoviridae*
Iris mild mosaic virus, (IMMV), *Potyviridae*
Iris severe mosaic virus, (ISMV), *Potyviridae*
Iris yellow spot virus, (IYSV), *Bunyaviridae*

Irituia virus, (IRIV), *Reoviridae*
Isachne mosaic virus*, (IsaMV), *Potyviridae*
Isfahan virus, (ISFV), *Rhabdoviridae*
Isla Vista virus, (ISLAV), *Bunyaviridae*
Isopod iridescent virus, *Iridoviridae*
Israel turkey meningoencephalomyelitis virus, (ITV), *Flaviviridae*
Issyk-Kul virus, (ISKV), *Bunyaviridae*
Itaituba virus, (ITAV), *Bunyaviridae*
Itaporanga virus, (ITPV), *Bunyaviridae*
Itaqui virus, (ITQV), *Bunyaviridae*
Itimirim virus, (ITIV), *Bunyaviridae*
Itupiranga virus, (ITUV), *Reoviridae*
Ivy vein clearing virus, (IVCV), *Rhabdoviridae*

Jaagsiekte sheep retrovirus, (JSRV), *Retroviridae*
Jacareacanga virus, (JACV), *Reoviridae*
Jamanxi virus, (JAMV), *Reoviridae*
Jamestown Canyon virus, (JCV), *Bunyaviridae*
Japanaut virus, (JAPV), *Reoviridae*
Japanese eel herpesvirus, *Herpesviridae*
Japanese encephalitis virus, (JEV), *Flaviviridae*
Japanese flounder nervous necrosis virus, (JFNNV), *Nodaviridae*
Japanese pear fruit dimple viroid, *Pospiviroidae*
Jari virus, (JARIV), *Reoviridae*
Jatropha mosaic virus, (JMV), *Geminiviridae*
JC polyomavirus, (JCPyV), *Polyomaviridae*
JD254 virus, (DGKV), *Bunyaviridae*
Joa virus, (JOAV), *Bunyaviridae*
Johnsongrass mosaic virus, (JGMV), *Potyviridae*
Johnston Atoll virus, (JAV), Unassigned
Joinjakaka virus, (JOIV), *Rhabdoviridae*
Jonquil mild mosaic virus, *Potyviridae*
Josiah virus, *Arenaviridae*
Juan Diaz virus, (JDV), *Bunyaviridae*
Jugra virus, (JUGV), *Flaviviridae*
Juncopox virus, (JNPV), *Poxviridae*
Junøn virus, (JUNV), *Arenaviridae*
Junonia coenia densovirus, (JcDNV), *Parvoviridae*
Jurona virus, (JURV), *Rhabdoviridae*
Jutiapa virus, (JUTV), *Flaviviridae*

K27 virus, *Bunyaviridae*
Kachemak Bay virus, (KBV), *Bunyaviridae*
Kadam virus, (KADV), *Flaviviridae*
Kadipiro virus (Java-7075), (KDV-Ja7075), *Reoviridae*
Kadipiro virus, (KDV), *Reoviridae*
Kaeng Khoi virus, (KKV), *Bunyaviridae*
Kaikalur virus, (KAIV), *Bunyaviridae*
Kairi virus, (KRIV), *Bunyaviridae*
Kaisodi virus, (KSOV), *Bunyaviridae*
Kala Iris virus, (KIRV), *Reoviridae*
Kalanchoe latent virus, (KLV), *Carlavirus*
Kalanchoë mosaic virus, (KMV), *Potyviridae*
Kalanchoe top-spotting virus, (KTSV), *Caulimoviridae*
Kamese virus, (KAMV), *Rhabdoviridae*
Kamiiso-8Cr-95 virus, *Bunyaviridae*
Kammavanpettai virus, (KMPV), *Reoviridae*
Kannamangalam virus, (KANV), *Rhabdoviridae*
Kao Shuan virus, (KSV), *Bunyaviridae*
Kaposi's sarcoma-associated herpesvirus, *Herpesviridae*
Karimabad virus, (KARV), *Bunyaviridae*
Karshi virus, (KSIV), *Flaviviridae*
Kasba virus (Chuzan virus), (KASV), *Reoviridae*
Kashmir bee virus, (KBV), Unassigned
Kasokero virus, (KASV), *Bunyaviridae*
Kawino virus, (KaV), Unassigned
Kazan virus, *Bunyaviridae*
Kedougou virus, (KEDV), *Flaviviridae*
Kelp fly virus, (KFV), Unassigned
Kemerovo virus, (KEMV), *Reoviridae*
Kenai virus, (KENV), *Reoviridae*
Kennedya virus Y, (KVY), *Potyviridae*
Kennedya yellow mosaic virus, (KYMV), *Tymovirus*
Kern Canyon virus, (KCV), *Rhabdoviridae*
Ketapang virus, (KETV), *Bunyaviridae*
Keterah virus, (KTRV), *Bunyaviridae*
Keuraliba virus, (KEUV), *Rhabdoviridae*
Keystone virus, (KEYV), *Bunyaviridae*
Khabarovsk virus, (KHAV), *Bunyaviridae*
Kharagysh virus, (KHAV), *Reoviridae*
Khasan virus, (KHAV), *Bunyaviridae*
KI-83-262 virus, *Bunyaviridae*
KI-85-1 virus, *Bunyaviridae*
KI-88-15 virus, *Bunyaviridae*
Kilham polyomavirus, (KPyV), *Polyomaviridae*
Kilham rat virus, (KRV), *Parvoviridae*
Kimberley virus, (KIMV), *Rhabdoviridae*
Kindia virus, (KINV), *Reoviridae*
Kinkajou herpesvirus, *Herpesviridae*
Kirsten murine sarcoma virus, (KiMSV), *Retroviridae*
Kismayo virus, (KISV), *Bunyaviridae*
Klamath virus, (KLAV), *Rhabdoviridae*
Kluyvera phage Kvp1, (Kvp1), *Podoviridae*
Kodzha virus AP92, *Bunyaviridae*
Kodzha virus C68031, *Bunyaviridae*
Kodzha virus, (CCHFV), *Bunyaviridae*
Kokobera virus, (KOKV), *Flaviviridae*
Kolongo virus, (KOLV), *Rhabdoviridae*
Konjac mosaic virus, (KoMV), *Potyviridae*
Koolpinyah virus, (KOOLV), *Rhabdoviridae*
Koongol virus, (KOOV), *Bunyaviridae*
Kotonkon virus, (KOTV), *Rhabdoviridae*
Koutango virus, (KOUV), *Flaviviridae*

Kowanyama virus, (KOWV), *Bunyaviridae*
Kunjin virus, (KUNV), *Flaviviridae*
Kurthia phage 6, (6), *Podoviridae*
Kurthia phage 7, (7), *Podoviridae*
Kwatta virus, (KWAV), *Rhabdoviridae*
Kyasanur Forest disease virus, (KFDV), *Flaviviridae*
Kyuri green mottle mosaic virus, (KGMMV), *Tobamovirus*
Kyzylagach virus, *Togaviridae*

L99 virus, *Bunyaviridae*
La Crosse virus, (LACV), *Bunyaviridae*
La Joya virus, (LJV), *Rhabdoviridae*
Lacanobia oleracea cypovirus 2, (LoCPV-2), Reoviridae
Lacertid herpesvirus, (LaHV-1), *Herpesviridae*
Lactate dehydrogenase-elevating virus, (LDV), *Arteriviridae*
Lactobacillus phage 222a, (222a), *Myoviridae*
Lactobacillus phage 223, (223), *Siphoviridae*
Lactobacillus phage fri, (fri), *Myoviridae*
Lactobacillus phage hv, (hv), *Myoviridae*
Lactobacillus phage hw, (hw), *Myoviridae*
Lactobacillus phage lb6, (lb6), *Siphoviridae*
Lactobacillus phage PL-1, (PL-1), *Siphoviridae*
Lactobacillus phage y5, (y5), *Siphoviridae*
Lactobacillus phage ϕFSW, (ϕFSW), *Siphoviridae*
Lactococcus phage 1358, (1358), *Siphoviridae*
Lactococcus phage 1483, (1483), *Siphoviridae*
Lactococcus phage 3ML, (3ML), *Siphoviridae*
Lactococcus phage 936, (936), *Siphoviridae*
Lactococcus phage 949, (949), *Siphoviridae*
Lactococcus phage bIL67, (bIL67), *Siphoviridae*
Lactococcus phage BK5-T, (BK5-T), *Siphoviridae*
Lactococcus phage c2, (c2), *Siphoviridae*
Lactococcus phage c6A, (PBc6A), *Siphoviridae*
Lactococcus phage KSY1, (KSY1), *Podoviridae*
Lactococcus phage ML3, (ML3), *Siphoviridae*
Lactococcus phage ml3, (ml3), *Siphoviridae*
Lactococcus phage P001, (P001), *Siphoviridae*
Lactococcus phage P107, (P107), *Siphoviridae*
Lactococcus phage P335, (P335), *Siphoviridae*
Lactococcus phage PO34, (PO34), *Podoviridae*
Lactococcus phage PO87, (PO87), *Siphoviridae*
Lactococcus phage ϕvML3, (ϕvML3), *Siphoviridae*
Laelia red leafspot virus, (LRLV), *Rhabdoviridae*
LaFrance isometric virus, (LFIV), Unassigned
Lagos bat virus, (LBV), *Rhabdoviridae*
Laguna Negra virus, (LANV), *Bunyaviridae*
Lake Clarendon virus, (LCV), *Reoviridae*
Lake victoria cormorant herpesvirus, *Herpesviridae*
Lamium mild mosaic virus, (LMMV), *Comoviridae*
Landjia virus, (LJAV), *Rhabdoviridae*
Landlocked salmon reovirus, (LSRV), *Reoviridae*
Langat virus, (LGTV), *Flaviviridae*
Langur virus, (LNGV), *Retroviridae*
Lanjan virus, (LJNV), *Bunyaviridae*
La-Piedad-Michoacan-Mexico virus, *Paramyxoviridae*
Lapine parvovirus, (LPV), *Parvoviridae*
Largemouth bass virus, (LMBV), *Iridoviridae*
Las Maloyas virus, (LMV), *Bunyaviridae*
Lasiocampa quercus cypovirus 6, (LqCPV-6), Reoviridae
Lassa virus, (LASV), *Arenaviridae*
Lates calcarifer encephalitis virus, (LcEV), *Nodaviridae*
Latino virus, (LATV), *Arenaviridae*
Lato river virus, (LRV), *Tombusviridae*
Latoia viridissima virus, (LvV), Unassigned
Launea arborescens stunt virus, (LArSV), *Rhabdoviridae*
Le Dantec virus, (LDV), *Rhabdoviridae*
Leanyer virus, (LEAV), *Bunyaviridae*
Lebombo virus 1, (LEBV-1), *Reoviridae*
Lebombo virus, (LEBV), *Reoviridae*
Lechiguanas virus, *Bunyaviridae*
Lednice virus, (LEDV), *Bunyaviridae*
Lee virus, *Bunyaviridae*
Leek white stripe virus, (LWSV), *Tombusviridae*
Leek yellow stripe virus, (LYSV), *Potyviridae*
Legume yellows virus, *Luteoviridae*
Leishmania RNA virus 1-1, (LRV1-1), *Totiviridae*
Leishmania RNA virus 1-10, (LRV1-10), *Totiviridae*
Leishmania RNA virus 1-11, (LRV1-11), *Totiviridae*
Leishmania RNA virus 1-12, (LRV1-12), *Totiviridae*
Leishmania RNA virus 1-2, (LRV1-2), *Totiviridae*
Leishmania RNA virus 1-3, (LRV1-3), *Totiviridae*
Leishmania RNA virus 1-4, (LRV1-4), *Totiviridae*
Leishmania RNA virus 1-5, (LRV1-5), *Totiviridae*
Leishmania RNA virus 1-6, (LRV1-6), *Totiviridae*
Leishmania RNA virus 1-7, (LRV1-7), *Totiviridae*
Leishmania RNA virus 1-8, (LRV1-8), *Totiviridae*
Leishmania RNA virus 1-9, (LRV1-9), *Totiviridae*
Leishmania RNA virus 2-1, (LRV2-1), *Totiviridae*
Lemon scented thyme leaf chlorosis virus, (LSTCV), *Rhabdoviridae*
Lentinus edodes virus, (LEV), Unassigned
Leonurus mosaic virus, (LeMV), *Geminiviridae*
Leporid herpesvirus 1, (LeHV-1), *Herpesviridae*
Leporid herpesvirus 2, (LeHV-2), *Herpesviridae*
Leporid herpesvirus 3, (LeHV-3), *Herpesviridae*
Lethocerus columbinae iridescent virus, *Iridoviridae*
Lettuce big-vein virus, (LBVV), *Varicosavirus*
Lettuce chlorosis virus, (LCV), *Closteroviridae*
Lettuce infectious yellows virus, (LIYV), *Closteroviridae*
Lettuce mosaic virus, (LMV), *Potyviridae*

Lettuce necrotic yellows virus, (LNYV), *Rhabdoviridae*
Lettuce speckles mottle virus, (LSMV), *Umbravirus*
Leuconostoc phage pro2, (pro2), *Siphoviridae*
Leucorrhinia dubia densovirus, (LdDNV), *Parvoviridae*
Lilac chlorotic leafspot virus, (LiCLV), *Capillovirus*
Lilac mottle virus, (LiMoV), *Carlavirus*
Lilac ring mottle virus, (LiRMoV), *Bromoviridae*
Lilac ringspot virus, (LiRSV), *Carlavirus*
Lilium henryi del1 virus, (LheDel1V), *Metaviridae*
Lily mild mottle virus, (LiMMoV), *Potyviridae*
Lily mottle virus, (LMoV), *Potyviridae*
Lily symptomless virus, (LSV), *Carlavirus*
Lily virus X, (LVX), *Potexvirus*
Limabean golden mosaic virus, (LGMV), *Geminiviridae*
Lipovnik virus, (LIPV), *Reoviridae*
Lisianthus necrosis virus, (LNV), *Tombusviridae*
Lissonota sp. ichnovirus, (LspIV), *Polydnaviridae*
Listeria phage 2389, (2389), *Siphoviridae*
Listeria phage 2671, (2671), *Siphoviridae*
Listeria phage 2685, (2685), *Siphoviridae*
Listeria phage 4211, (4211), *Myoviridae*
Listeria phage A511, (A511), *Myoviridae*
Listeria phage H387, (H387), *Siphoviridae*
Little cherry virus, (LChV), *Closteroviridae*
Liverpool vervet herpesvirus, *Herpesviridae*
Ljungan virus, (LV), *Picornaviridae*
Llano Seco virus, (LLSV), *Reoviridae*
Locusta migratoria entomopoxvirus 'O', (LMEV), *Poxviridae*
Lokern virus, (LOKV), *Bunyaviridae*
Lolium ryegrass virus, (LoRV), *Rhabdoviridae*
Lone Star virus, (LSV), *Bunyaviridae*
Lordsdale virus, (LDV), *Caliciviridae*
Lorisine herpesvirus 1, (LoHV-1), *Herpesviridae*
Lotus stem necrosis, (LoSNV), *Rhabdoviridae*
Louping ill virus British subtype, *Flaviviridae*
Louping ill virus Irish subtype, *Flaviviridae*
Louping ill virus Spanish subtype, *Flaviviridae*
Louping ill virus Turkish subtype, *Flaviviridae*
Louping ill virus, (LIV), *Flaviviridae*
LP virus, *Arenaviridae*
Lucerne Australian latent virus, (LALV), *Comoviridae*
Lucerne Australian symptomless virus, (LASV), *Comoviridae*
Lucerne enation virus, (LEV), *Rhabdoviridae*
Lucerne transient streak virus satellite RNA, Satellite
Lucerne transient streak virus, (LTSV), *Sobemovirus*
Lucké frog herpesvirus, *Herpesviridae*
Lucké triturus virus 1; CP4-4398B (276), *Iridoviridae*
LUIII virus, (LUIIIV), *Parvoviridae*
Lukuni virus, (LUKV), *Bunyaviridae*
Lumbo virus, (LUMV), *Bunyaviridae*
Lumpy skin disease virus, (LSDV), *Poxviridae*
Lundy virus, (LUNV), *Reoviridae*
Lupin leaf curl virus, (LLCV), *Geminiviridae*
Lupin yellow vein virus, (LYVV), *Rhabdoviridae*
Lychnis ringspot virus, (LRSV), *Hordeivirus*
Lychnis symptomless virus, (LycSLV), *Potexvirus*
Lymantria dispar cypovirus 1, (LdCPV-1), *Reoviridae*
Lymantria dispar cypovirus 11, (LdCPV-11), *Reoviridae*
Lymantria dispar MNPV, (LdMNPV), *Baculoviridae*
Lymantria dubia densovirus, (LdDNV), *Parvoviridae*
Lymantria ninayi virus (Greenwood), (LNV), *Nodaviridae*
Lymantria ninayi virus, (LnV), *Tetraviridae*
Lymantria ninayi virus, (LnV), Unassigned
Lymphocystis disease virus 1, (LCDV-1), *Iridoviridae*
Lymphocystis disease virus 2, (LCDV-2), *Iridoviridae*
Lymphocytic choriomeningitis virus, (LCMV), *Arenaviridae*

M'Poko virus, (MPOV), *Bunyaviridae*
M459 virus, (BWAV), *Bunyaviridae*
M67U5 virus, (MNTV), *Bunyaviridae*
Macaua virus, (MCAV), *Bunyaviridae*
Machupo virus, (MACV), *Arenaviridae*
Maciel virus, *Bunyaviridae*
Maclura mosaic virus, (MacMV), *Potyviridae*
Macropipus depurator P virus, (MdRV-P), *Reoviridae*
Macropodid herpesvirus 1, (MaHV-1), *Herpesviridae*
Macropodid herpesvirus 2, (MaHV-2), *Herpesviridae*
Macroptilium golden mosaic virus, (MGMV), *Geminiviridae*
Macrotyloma mosaic virus, (MaMV), *Geminiviridae*
Madrid virus, (MADV), *Bunyaviridae*
Maguari virus, (MAGV), *Bunyaviridae*
Mahogany Hammock virus, (MHV), *Bunyaviridae*
Maiden virus, (MDNV), *Reoviridae*
Main Drain virus, (MDV), *Bunyaviridae*
Maize chlorotic dwarf virus, (MCDV), *Sequiviridae*
Maize chlorotic mottle virus, (MCMV), *Tombusviridae*
Maize dwarf mosaic virus, (MDMV), *Potyviridae*
Maize mosaic virus, (MMV), *Rhabdoviridae*
Maize rayado fino virus, (MRFV), *Marafivirus*
Maize rough dwarf virus, (MRDV), *Reoviridae*
Maize stem borer virus, (MSBV), Unassigned
Maize sterile stunt virus, (MSSV), *Rhabdoviridae*
Maize streak virus, (MSV), *Geminiviridae*
Maize stripe virus, (MSpV), *Tenuivirus*
Maize white line mosaic satellite virus, Satellite
Maize white line mosaic virus, (MWLMV), Unassigned

Mal de Rio Cuarto virus, (MRCV), *Reoviridae*
Malacky/Ma32/94 virus, *Bunyaviridae*
Malacosoma disstria cypovirus 8, (MdCPV-8), *Reoviridae*
Malacosoma neustria cypovirus 2, (MnCPV-2), *Reoviridae*
Malacosoma neustria cypovirus 3, (MnCPV-3), *Reoviridae*
Malakal virus, (MALV), *Rhabdoviridae*
Malignant catarrhal fever virus, *Herpesviridae*
Malpais Spring virus, (MSPV), *Rhabdoviridae*
Malva silvestris virus, (MaSV), *Rhabdoviridae*
Malva vein clearing virus, (MVCV), *Potyviridae*
Malva veinal necrosis virus, (MVNV), *Potexvirus*
Malva yellows virus, *Luteoviridae*
Malvaceous chlorosis virus, (MCV), *Geminiviridae*
Mamestra brassicae cypovirus 11, (MbCPV-11), *Reoviridae*
Mamestra brassicae cypovirus 12, (MbCPV-12), *Reoviridae*
Mamestra brassicae cypovirus 2, (MbCPV-2), *Reoviridae*
Mamestra brassicae cypovirus 7, (MbCPV-7), *Reoviridae*
Mamestra brassicae MNPV, (MbMNPV), *Baculoviridae*
Mammalian orthoreovirus 1-Lang, (MRV-1), *Reoviridae*
Mammalian orthoreovirus 2-D5/Jones, (MRV-2), *Reoviridae*
Mammalian orthoreovirus 3-Dearing, (MRV-3), *Reoviridae*
Mammalian orthoreovirus, (MRV), *Reoviridae*
Manawa virus, (MWAV), *Bunyaviridae*
Manawatu virus, (MwV), *Nodaviridae*
Manitoba virus, (MNTBV), *Rhabdoviridae*
Manzanilla virus, (MANV), *Bunyaviridae*
Mapputta virus, (MAPV), *Bunyaviridae*
Maprik virus, (MPKV), *Bunyaviridae*
Mapuera virus, (MPRV), *Paramyxoviridae*
Maraba virus, (MARAV), *Rhabdoviridae*
Maracuja mosaic virus, (MarMV), *Tobamovirus*
Marble spleen disease virus, *Adenoviridae*
Marburg virus Marburg Ravn, *Filoviridae*
Marburg virus Musoke, *Filoviridae*
Marburg virus Ozolin, *Filoviridae*
Marburg virus Popp, *Filoviridae*
Marburg virus Ratayczak, *Filoviridae*
Marburg virus Voege, *Filoviridae*
Marburg virus, (MARV), *Filoviridae*
Marco virus, (MCOV), *Rhabdoviridae*
Marek's disease virus type 1, *Herpesviridae*
Marek's disease virus type 2, *Herpesviridae*
Marigold mottle virus, (MaMoV), *Potyviridae*
Marituba virus, (MTBV), *Bunyaviridae*
Marmomid herpesvirus 1, (MarHV-1), *Herpesviridae*
Marmoset cytomegalovirus, *Herpesviridae*
Marmoset herpesvirus, *Herpesviridae*
Marmosetpox virus, (MPV), *Poxviridae*
Marrakai virus, (MARV), *Reoviridae*
MARU 10962 virus, (GAMV), *Bunyaviridae*
Mason-Pfizer monkey virus, (MPMV), *Retroviridae*
Masou salmon reovirus MSV, (MSRV), *Reoviridae*
Matruh virus, (MTRV), *Bunyaviridae*
Matucare virus, (MATV), *Reoviridae*
Maus Elberfeld virus, *Picornaviridae*
Mayaro virus, (MAYV), *Togaviridae*
Mboke virus, (MBOV), *Bunyaviridae*
MC2 virus, *Arenaviridae*
Meaban virus, (MEAV), *Flaviviridae*
Measles virus, (MeV), *Paramyxoviridae*
Medical lake macaque herpesvirus, *Herpesviridae*
Megakepasma mosaic virus, (MegMV), *Closteroviridae*
Melandrium yellow fleck virus, (MYFV), *Bromoviridae*
Melanoplus sanguinipes entomopoxvirus 'O', (MSEV), *Poxviridae*
Melao virus, (MELV), *Bunyaviridae*
Meleagrid herpesvirus 1, (MeHV-1), *Herpesviridae*
Melilotus latent virus, (MeLV), *Rhabdoviridae*
Melilotus mosaic virus, (MeMV), *Potyviridae*
Melolontha melolontha entomopoxvirus, (MMEV), *Poxviridae*
Melon leaf curl virus, (MLCV), *Geminiviridae*
Melon necrotic spot virus, (MNSV), *Tombusviridae*
Melon rugose mosaic virus, (MRMV), *Tymovirus*
Melon variegation virus, (MVV), *Rhabdoviridae*
Melon vein-banding mosaic virus, (MVBMV), *Potyviridae*
Mengovirus, *Picornaviridae*
Mermet virus, (MERV), *Bunyaviridae*
Methanobacterium phage ΦF3, (ΦF3), *Siphoviridae*
Methanobacterium phage ΨM1, (ΨM1), *Siphoviridae*
Methanobrevibacter phage PG, (PG), *Siphoviridae*
Mexican papita viroid, (MPVd), *Pospiviroidae*
Mexico virus, (MXV), *Caliciviridae*
Mibuna temperate virus, (MTV), *Partitiviridae*
Mice minute virus, (MMV), *Parvoviridae*
Michigan alfalfa virus, *Luteoviridae*
Micrococcus phage N1, (N1), *Siphoviridae*
Micrococcus phage N5, (N5), *Siphoviridae*
Micromonas pusilla virus GM1, (MpV-GM1), *Phycodnaviridae*
Micromonas pusilla virus PB6, (MpV-PB6), *Phycodnaviridae*
Micromonas pusilla virus PB7, (MpV-PB7), *Phycodnaviridae*
Micromonas pusilla virus PB8, (MpV-PB8), *Phycodnaviridae*

Micromonas pusilla virus PL1, (MpV-PL1), *Phycodnaviridae*
Micromonas pusilla virus SG1, (MpV-SG1), *Phycodnaviridae*
Micromonas pusilla virus SP1, (MpV-SP1), *Phycodnaviridae*
Micromonas pusilla virus SP2, (MpV-SP2), *Phycodnaviridae*
Microplitis croceipes bracovirus, (McBV), *Polydnaviridae*
Microplitis demolitor bracovirus, (MdBV), *Polydnaviridae*
Microtus pennyslvanicus herpesvirus, *Herpesviridae*
Middelburg virus, (MIDV), *Togaviridae*
Milk vetch dwarf virus, (MDV), *Nanovirus*
Milker's nodule virus, *Poxviridae*
Mill Door virus, (MDRV), *Reoviridae*
Mimosa bacilliform virus, (MBV), *Caulimoviridae*
Minatitlan virus, (MNTV), *Bunyaviridae*
Mink calicivirus, (MCV), *Caliciviridae*
Mink enteritis virus, (MEV), *Parvoviridae*
Minnal virus, (MINV), *Reoviridae*
Mirabilis mosaic virus, (MiMV), *Caulimoviridae*
Mirim virus, (MIRV), *Bunyaviridae*
Miscanthus streak virus, (MiSV), *Geminiviridae*
Mitchell river virus, (MRV), *Reoviridae*
MM-2325 virus, (BAKV), *Bunyaviridae*
Mobala virus, (MOBV), *Arenaviridae*
Modoc virus, (MODV), *Flaviviridae*
Moju virus, (MOJUV), *Bunyaviridae*
Mojui Dos Campos virus, (MDCV), *Bunyaviridae*
Mokola virus, (MOKV), *Rhabdoviridae*
Molinia streak virus, (MoSV), *Tombusviridae*
Mollicutes phage Br1, (Br1), *Myoviridae*
Mollicutes phage C3, (C3), *Podoviridae*
Mollicutes phage L3, (L3), *Podoviridae*
Molluscum contagiosum virus, (MOCV), *Poxviridae*
Molluscum-like poxvirus, (MOV), *Poxviridae*
Moloney murine leukemia virus, (MoMLV), *Retroviridae*
Moloney murine sarcoma virus, (MoMSV), *Retroviridae*
Monkeypox virus, (MPXV), *Poxviridae*
Mono Lake virus, (MLV), *Reoviridae*
Monongahela virus, *Bunyaviridae*
Montana myotis leukoencephalitis virus, (MMLV), *Flaviviridae*
Monte Dourado virus, (MDOV), *Reoviridae*
Mopeia virus, (MOPV), *Arenaviridae*
Moravia/Ma5302V virus, *Bunyaviridae*
Moriche virus, (MORV), *Bunyaviridae*
Moroccan pepper virus, (MPV), *Tombusviridae*
Moroccan watermelon mosaic virus, (MWMV), *Potyviridae*
Mosqueiro virus, (MQOV), *Rhabdoviridae*
Mosquito iridescent virus, *Iridoviridae*
Mossuril virus, (MOSV), *Rhabdoviridae*
Mount Elgon bat virus, (MEBV), *Rhabdoviridae*
Mouse cytomegalovirus, *Herpesviridae*
Mouse herpesvirus strain 68, *Herpesviridae*
Mouse mammary tumor virus, (MMTV), *Retroviridae*
Mouse parvovirus 1, (MPV), *Parvoviridae*
Mouse thymic herpesvirus, *Herpesviridae*
Movar virus, *Herpesviridae*
MP 401 virus, (NDV), *Bunyaviridae*
MRM31 virus, (KOOV), *Bunyaviridae*
Mucambo virus, (MUCV), *Togaviridae*
Mudjinbarry virus, (MUDV), *Reoviridae*
Muir Springs virus, (MSV), *Rhabdoviridae*
Mulberry latent virus, (MLV), *Carlavirus*
Mulberry ringspot virus, (MRSV), *Comoviridae*
Mule deer poxvirus, (DPV), *Poxviridae*
Muleshoe virus, (MULV), *Bunyaviridae*
Mumps virus, (MuV), *Paramyxoviridae*
Mungbean mosaic virus, (MbMV), *Potyviridae*
Mungbean mottle virus, (MMoV), *Potyviridae*
Mungbean yellow mosaic virus, (MYMV), *Geminiviridae*
Munguba virus, (MUNV), *Bunyaviridae*
Murid herpesvirus 1, (MuHV-1), *Herpesviridae*
Murid herpesvirus 2, (MuHV-2), *Herpesviridae*
Murid herpesvirus 3, (MuHV-3), *Herpesviridae*
Murid herpesvirus 4, (MuHV-4), *Herpesviridae*
Murid herpesvirus 5, (MuHV-5), *Herpesviridae*
Murid herpesvirus 6, (MuHV-6), *Herpesviridae*
Murine adenovirus 1, (MAdV-1), *Adenoviridae*
Murine adenovirus 2, (MAdV-2), *Adenoviridae*
Murine adenovirus A, (MAdV-A), *Adenoviridae*
Murine adenovirus B, (MAdV-B), *Adenoviridae*
Murine hepatitis virus, (MHV), *Coronaviridae*
Murine leukemia virus, (MLV), *Retroviridae*
Murine parainfluenza virus 1, *Paramyxoviridae*
Murine pneumonia virus, (MPV), *Paramyxoviridae*
Murine pneumotropic virus, (MPtV), *Polyomaviridae*
Murine polyomavirus, (MPyV), *Polyomaviridae*
Murray Valley encephalitis virus, (MVEV), *Flaviviridae*
Murre virus, (MURV), *Bunyaviridae*
Murutucu virus, (MURV), *Bunyaviridae*
Musca domestica reovirus, (MdRV), *Reoviridae*
Muscovy duck parvovirus, (MDPV), *Parvoviridae*
Mushroom bacilliform virus, (MBV), *Barnaviridae*
Mushroom virus 4, *Partitiviridae*
Muskmelon vein necrosis virus, (MuVNV), *Carlavirus*
Mycobacterium phage I3, (I3), *Myoviridae*
Mycobacterium phage L5, (L5), *Siphoviridae*
Mycobacterium phage lacticola, (lacticola), *Siphoviridae*

Mycobacterium phage Leo, (Leo), *Siphoviridae*
Mycobacterium phage minetti, (minetti), *Siphoviridae*
Mycobacterium phage phlei, (GS4E), *Siphoviridae*
Mycobacterium phage R1-Myb, (R1-Myb), *Siphoviridae*
Mycobacterium phage ϕ17, (ϕ17), *Podoviridae*
Mycogone perniciosa virus, (MpV), *Totiviridae*
Mykines virus, (MYKV), *Reoviridae*
Mynahpox virus, (MYPV), *Poxviridae*
Myriotrichia clavaeformis virus a, (McV-a), *Phycodnaviridae*
Myrobalan latent ringspot virus (160), (MLRSV), *Comoviridae*
Myrobalan latent ringspot virus satellite RNA, Satellite
Myxoma virus, (MYXV), *Poxviridae*

Nairobi sheep disease virus, (NSDV), *Bunyaviridae*
Nandina mosaic virus, (NaMV), *Potexvirus*
Nandina stem pitting virus, (NSPV), *Capillovirus*
Naranjal virus, (NJLV), *Flaviviridae*
Narcissus degeneration virus, (NDV), *Potyviridae*
Narcissus late season yellows virus, (NLSYV), *Potyviridae*
Narcissus latent virus, (NLV), *Potyviridae*
Narcissus mosaic virus, (NMV), *Potexvirus*
Narcissus tip necrosis virus, (NTNV), *Tombusviridae*
Narcissus yellow stripe virus, (NYSV), *Potyviridae*
Nasoule virus, (NASV), *Rhabdoviridae*
Nasturtium mosaic virus, *Potyviridae*
Navarro virus, (NAVV), *Rhabdoviridae*
Ndelle virus, (NDEV), *Reoviridae*
Ndumu virus, (NDUV), *Togaviridae*
Neckar river virus, (NRV), *Tombusviridae*
Negro coffee mosaic virus, (NeCMV), *Potexvirus*
Nelson Bay orthoreovirus, (NBV), *Reoviridae*
Neodiprion sertifer NPV, (NeseNPV), *Baculoviridae*
Nepuyo virus, (NEPV), *Bunyaviridae*
Nerine latent virus, (NeLV), *Carlavirus*
Nerine virus X, (NVX), *Potexvirus*
Nerine virus Y, (NVY), *Potyviridae*
Nerine virus, (NV), *Potyviridae*
Nerine yellow stripe virus, (NeYSV), *Potyviridae*
Netivot virus, (NETV), *Reoviridae*
New Minto virus, (NMV), *Rhabdoviridae*
New York virus, (NYV), *Bunyaviridae*
New Zealand virus, (NZV), *Nodaviridae*
Newcastle disease virus, (NDV), *Paramyxoviridae*
Nezara viridula virus-1, (NVV-1), Unassigned
Ngaingan virus, (NGAV), *Rhabdoviridae*
Ngari virus, (NRIV), *Bunyaviridae*
Ngoupe virus, (NGOV), *Reoviridae*
Nicotiana glutinosa stunt viroid, (NGSVd), *Avsunviroidae*
Nicotiana tabacum Tnt1 virus, (NtaTnt1V), *Pseudoviridae*
Nicotiana tabacum Tto1 virus, (NtaTto1V), *Pseudoviridae*
Nicotiana velutina mosaic virus, (NVMV), Unassigned
Nilaparvata lugens reovirus, (NLRV), *Reoviridae*
Nile crocodile poxvirus, (CRV), *Poxviridae*
Nique virus, (NIQV), *Bunyaviridae*
Nkolbisson virus, (NKOV), *Rhabdoviridae*
NMH10 virus, *Bunyaviridae*
NMR-11 virus, *Bunyaviridae*
Noctua pronuba cypovirus 7, (NpCPV-7), *Reoviridae*
Nodamura virus, (NoV), *Nodaviridae*
Nola virus, (NOLAV), *Bunyaviridae*
North Clett virus, (NCLV), *Reoviridae*
North End virus, (NEDV), *Reoviridae*
Northern cereal mosaic virus, (NCMV), *Rhabdoviridae*
Northern pike herpesvirus, *Herpesviridae*
Northway virus, (NORV), *Bunyaviridae*
Norwalk virus, (NV), *Caliciviridae*
Norwalk virus, (NV), *Caliciviridae*
Nothoscordum mosaic virus, (NoMV), *Potyviridae*
Ntaya virus, (NTAV), *Flaviviridae*
Nudaurelia capensis β virus, (NβV) *Tetraviridae*
Nudaurelia capensis ε virusa, (NεV), *Tetraviridae*
Nudaurelia capensis ω virus, (NωV), *Tetraviridae*
Nudaurelia cytherea cypovirus 8, (NcCPV-8), *Reoviridae*
Nugget virus, (NUGV), *Reoviridae*
Nyabira virus, (NYAV), *Reoviridae*
Nyamanini virus, (NYMV), Unassigned
Nyando virus, (NDV), *Bunyaviridae*

O'nyong-nyong virus, (ONNV), *Togaviridae*
Oak-Vale virus, (OVRV), *Rhabdoviridae*
Oat blue dwarf virus, (OBDV), *Marafivirus*
Oat chlorotic stunt virus, (OCSV), *Tombusviridae*
Oat golden stripe virus, *Furovirus*
Oat mosaic virus, (OMV), *Potyviridae*
Oat necrotic mottle virus, (ONMV), *Potyviridae*
Oat sterile dwarf virus, (OSDV), *Reoviridae*
Oat striate mosaic virus, (OSMV), *Rhabdoviridae*
Obodhiang virus, (OBOV), *Rhabdoviridae*
Obuda pepper virus, (ObPV), *Tobamovirus*
Oceanside virus, (OCV), *Bunyaviridae*
Ockelbo virus, *Togaviridae*
Odontoglossum ringspot virus, (ORSV), *Tobamovirus*
Odrenisrou virus, (ODRV), *Bunyaviridae*

Oedaleus senigalensis entomopoxvirus 'O', (OSEV), *Poxviridae*
Oilseed rape mosaic virus, *Tobamovirus*
Oita virus, (OITAV), *Rhabdoviridae*
Okhotskiy virus, (OKHV), *Reoviridae*
Okola virus, (OKOV), *Bunyaviridae*
Okra leaf curl virus, (OLCV), *Geminiviridae*
Okra mosaic virus, (OkMV), *Tymovirus*
Olesicampe benefactor ichnovirus, (ObIV), *Polydnaviridae*
Olesicampe geniculatae ichnovirus, (OgIV), *Polydnaviridae*
Olifantsvlei virus, (OLIV), *Bunyaviridae*
Olive latent ringspot virus, (OLRSV), *Comoviridae*
Olive latent virus 1, (OLV-1), *Tombusviridae*
Olive latent virus 2, (OLV-2), *Bromoviridae*
Oliveros virus, (OLVV), *Arenaviridae*
Omo virus, (OMOV), *Bunyaviridae*
Omsk hemorrhagic fever virus, (OHFV), *Flaviviridae*
Oncorhynchus masou herpesvirus, *Herpesviridae*
Onion mite-borne latent virus, (OMbLV), *Allexivirus*
Onion yellow dwarf virus, (OYDV), *Potyviridae*
Ononis yellow mosaic virus, (OYMV), *Tymovirus*
Operophtera brumata cypovirus 2, (ObCPV-2), *Reoviridae*
Operophtera brumata cypovirus 3, (ObCPV-3), *Reoviridae*
Operophtera brumata entomopoxvirus 'L', (OBEV), *Poxviridae*
Opogonia iridescent virus, *Iridoviridae*
Oran virus, *Bunyaviridae*
Orangutan herpesvirus, *Herpesviridae*
Orchid fleck virus, (OFV), Unassigned
Orf virus, (ORFV), *Poxviridae*
Orgyia pseudosugata cypovirus 5, (OpCPV-5), *Reoviridae*
Orgyia pseudotsugata MNPV, (OpMNPV), *Baculoviridae*
Orgyia pseudotsugata SNPV, (OpSNPV), *Baculoviridae*
Oriboca virus, (ORIV), *Bunyaviridae*
Oriximina virus, (ORXV), *Bunyaviridae*
Ornithogalum mosaic virus, (OrMV), *Potyviridae*
Oropouche virus, (OROV), *Bunyaviridae*
Orungo virus 1 to 4, (ORUV-1 to 4), *Reoviridae*
Orungo virus, (ORUV), *Reoviridae*
Oryctes rhinoceros virus, (OrV), Unassigned
Ossa virus, (OSSAV), *Bunyaviridae*
Ostreid herpesvirus 1, (OsHV-1), *Herpesviridae*
Ouango virus, (OUAV), *Rhabdoviridae*
Oubi virus, (OUBIV), *Bunyaviridae*
Ourem virus, (OURV), *Reoviridae*
Ourmia melon virus, (OuMV), *Ourmiavirus*
Ovine adeno-associated virus, (OAAV), *Parvoviridae*
Ovine adenovirus 1, (OAdV-1), *Adenoviridae*
Ovine adenovirus 2-5, (OAdV-2 to 5), *Adenoviridae*
Ovine adenovirus 6, (OAdV-6), *Adenoviridae*
Ovine adenovirus A, (OAdV-A), *Adenoviridae*
Ovine adenovirus B, (OAdV-B), *Adenoviridae*
Ovine adenovirus C, (OAdV-C), *Adenoviridae*
Ovine adenovirus isolate 287, (OAdV-287), *Adenoviridae*
Ovine astrovirus 1, (OAstV-1), *Astroviridae*
Ovine astrovirus, (OAstV), *Astroviridae*
Ovine herpesvirus 1, (OvHV-1), *Herpesviridae*
Ovine herpesvirus 2, (OvHV-2), *Herpesviridae*
Ovine papillomavirus 1, (OPV-1), *Papillomaviridae*
Ovine papillomavirus 2, (OPV-2), *Papillomaviridae*
Ovine papillomavirus, (OPV), *Papillomaviridae*
Ovine pulmonary adenocarcinoma virus, *Retroviridae*
Owl hepatosplenitis herpesvirus, *Herpesviridae*

p2b-2 virus, *Arenaviridae*
p360 virus, *Bunyaviridae*
Pacific oyster herpesvirus, *Herpesviridae*
Pacific pond turtle herpesvirus, *Herpesviridae*
Pacora virus, (PCAV), *Bunyaviridae*
Pacui virus, (PACV), *Bunyaviridae*
Pahayokee virus, (PAHV), *Bunyaviridae*
Painted turtle herpesvirus, *Herpesviridae*
Pakistani cotton leaf curl virus, (PCLCuV), *Geminiviridae*
Palestina virus, (PLSV), *Bunyaviridae*
Palm mosaic virus, (PalMV), *Potyviridae*
Palyam virus, (PALV), *Reoviridae*
Palyam virus, (PALV), *Reoviridae*
Pampa virus, (PAMV), *Arenaviridae*
PAn 18400 virus, *Arenaviridae*
Pangola stunt virus, (PaSV), *Reoviridae*
Panicum mosaic satellite virus, Satellite
Panicum mosaic virus satellite RNA, Satellite
Panicum mosaic virus, (PMV), *Tombusviridae*
Panicum streak virus, (PanSV), *Geminiviridae*
Panolis flammea NPV, (PaflNPV), *Baculoviridae*
Papaya leaf curl virus, (PaLCV), *Geminiviridae*
Papaya leaf distortion mosaic virus, (PLDMV), *Potyviridae*
Papaya mosaic virus, (PapMV), *Potexvirus*
Papaya ringspot virus, (PRSV), *Potyviridae*
Papilio machaon cypovirus 2, (PmCPV-2), *Reoviridae*
Paprika mild mottle virus, (PaMMV), *Tobamovirus*
Paramecium bursaria Chlorella virus 1, (PBCV-1), *Phycodnaviridae*
Paramecium bursaria Chlorella virus A1, (PBCV-A1), *Phycodnaviridae*
Paramecium bursaria Chlorella virus AL1A, (PBCV-AL1A), *Phycodnaviridae*

Paramecium bursaria Chlorella virus AL2A, (PBCV-AL2A), *Phycodnaviridae*
Paramecium bursaria Chlorella virus AL2C, (PBCV-AL2C), *Phycodnaviridae*
Paramecium bursaria Chlorella virus B1, (PBCV-B1), *Phycodnaviridae*
Paramecium bursaria Chlorella virus BJ2C, (PBCV-BJ2C), *Phycodnaviridae*
Paramecium bursaria Chlorella virus CA1A, (PBCV-CA1A), *Phycodnaviridae*
Paramecium bursaria Chlorella virus CA1D, (PBCV-CA1D), *Phycodnaviridae*
Paramecium bursaria Chlorella virus CA2A, (PBCV-CA2A), *Phycodnaviridae*
Paramecium bursaria Chlorella virus CA4A, (PBCV-CA4A), *Phycodnaviridae*
Paramecium bursaria Chlorella virus CA4B, (PBCV-CA4B), *Phycodnaviridae*
Paramecium bursaria Chlorella virus CVBII, (PBCV-CVBII), *Phycodnaviridae*
Paramecium bursaria Chlorella virus CVK2, (PBCV-CVK2), *Phycodnaviridae*
Paramecium bursaria Chlorella virus CVU1, (PBCV-CVU1), *Phycodnaviridae*
Paramecium bursaria Chlorella virus G1, (PBCV-G1), *Phycodnaviridae*
Paramecium bursaria Chlorella virus IL2A, (PBCV-IL2A), *Phycodnaviridae*
Paramecium bursaria Chlorella virus IL2B, (PBCV-IL2B), *Phycodnaviridae*
Paramecium bursaria Chlorella virus IL3A, (PBCV-IL3A), *Phycodnaviridae*
Paramecium bursaria Chlorella virus IL3D, (PBCV-IL3D), *Phycodnaviridae*
Paramecium bursaria Chlorella virus IL5-2s1, (PBCV-IL5-2s1), *Phycodnaviridae*
Paramecium bursaria Chlorella virus M1, (PBCV-M1), *Phycodnaviridae*
Paramecium bursaria Chlorella virus MA1D, (PBCV-MA1D), *Phycodnaviridae*
Paramecium bursaria Chlorella virus MA1E, (PBCV-MA1E), *Phycodnaviridae*
Paramecium bursaria Chlorella virus NC1A, (PBCV-NC1A), *Phycodnaviridae*
Paramecium bursaria Chlorella virus NC1B, (PBCV-NC1B), *Phycodnaviridae*
Paramecium bursaria Chlorella virus NC1C, (PBCV-NC1C), *Phycodnaviridae*
Paramecium bursaria Chlorella virus NC1D, (PBCV-NC1D), *Phycodnaviridae*
Paramecium bursaria Chlorella virus NE8A, (PBCV-NE8A), *Phycodnaviridae*
Paramecium bursaria Chlorella virus NE8D, (PBCV-NE8D), *Phycodnaviridae*
Paramecium bursaria Chlorella virus NY2A, (PBCV-NY2A), *Phycodnaviridae*
Paramecium bursaria Chlorella virus NY2B, (PBCV-NY2B), *Phycodnaviridae*
Paramecium bursaria Chlorella virus NY2C, (PBCV-NY2C), *Phycodnaviridae*
Paramecium bursaria Chlorella virus NY2F, (PBCV-NY2F), *Phycodnaviridae*
Paramecium bursaria Chlorella virus NYb1, (PBCV-NYb1), *Phycodnaviridae*
Paramecium bursaria Chlorella virus NYs1, (PBCV-NYs1), *Phycodnaviridae*
Paramecium bursaria Chlorella virus R1, (PBCV-R1), *Phycodnaviridae*
Paramecium bursaria Chlorella virus SC1A, (PBCV-SC1A), *Phycodnaviridae*
Paramecium bursaria Chlorella virus SC1B, (PBCV-SC1B), *Phycodnaviridae*
Paramecium bursaria Chlorella virus SH6A, (PBCV-SH6A), *Phycodnaviridae*
Paramecium bursaria Chlorella virus XY6E, (PBCV-XY6E), *Phycodnaviridae*
Paramecium bursaria Chlorella virus XZ3A, (PBCV-XZ3A), *Phycodnaviridae*
Paramecium bursaria Chlorella virus XZ4A, (PBCV-XZ4A), *Phycodnaviridae*
Paramecium bursaria Chlorella virus XZ4C, (PBCV-XZ4C), *Phycodnaviridae*
Paramecium bursaria Chlorella virus XZ5C, (PBCV-XZ5C), *Phycodnaviridae*
Paramushir virus, *Bunyaviridae*
Paraná virus, (PARV), *Arenaviridae*
Parapoxvirus of red deer in New Zealand, (PVNZ), *Poxviridae*
Paravaccinia virus, *Poxviridae*
Pariacato virus, (PaV), *Nodaviridae*
Parietaria mottle virus, (PMoV), *Bromoviridae*
Parma wallaby herpesvirus, *Herpesviridae*
Paroo river virus, (PRV), *Reoviridae*
Parrot herpesvirus, *Herpesviridae*
Parry Creek virus, (PCRV), *Rhabdoviridae*
Parsley latent virus, (PaLV), Unassigned
Parsley virus 5, (PaV-5), *Potexvirus*
Parsley virus, (PaV), *Rhabdoviridae*
Parsnip mosaic virus, (ParMV), *Potyviridae*
Parsnip virus 3, (ParV-3), *Potexvirus*
Parsnip virus 5, (ParV-5), *Potexvirus*
Parsnip yellow fleck virus, (PYFV), *Sequiviridae*
Parvo-like virus of crabs, (PCV84), *Parvoviridae*
Paspalum striate mosaic virus, (PSMV), *Geminiviridae*
Passiflora latent virus, (PLV), *Carlavirus*
Passion fruit mottle virus, (PFMoV), *Potyviridae*
Passion fruit ringspot virus, (PFRSV), *Potyviridae*
Passion fruit woodiness virus, (PWV), *Potyviridae*

Passion fruit yellow mosaic virus, (PFYMV), *Tymovirus*
Pasteurella phage 22, (22), *Podoviridae*
Pasteurella phage 32, (32), *Siphoviridae*
Pasteurella phage AU, (AU), *Myoviridae*
Pasteurella phage AU, (AU), *Myoviridae*
Pasteurella phage C-2, (C-2), *Siphoviridae*
Pata virus, (PATAV), *Reoviridae*
Patas monkey herpesvirus deltaherpesvirus, *Herpesviridae*
Patchouli mild mosaic virus, (PatMMV), *Comoviridae*
Patchouli mottle virus, (PatMoV), *Potyviridae*
Patchouli virus X, (PatVX), *Potexvirus*
Pathum Thani virus, (PTHV), *Bunyaviridae*
Patois virus, (PATV), *Bunyaviridae*
Pea early-browning virus, (PEBV), *Tobravirus*
Pea enation mosaic virus satellite RNA, Satellite
Pea enation mosaic virus-1, (PEMV-1), *Luteoviridae*
Pea enation mosaic virus-2, (PEMV-2), *Umbravirus*
Pea green mottle virus, (PGMV), *Comoviridae*
Pea leafroll virus, *Luteoviridae*
Pea mild mosaic virus, (PMiMV), *Comoviridae*
Pea mosaic virus, *Potyviridae*
Pea necrosis virus, *Potyviridae*
Pea seed-borne mosaic virus, (PSbMV), *Potyviridae*
Pea streak virus, (PeSV), *Carlavirus*
Peach dapple viroid, *Pospiviroidae*
Peach latent mosaic viroid, (PLMVd), *Avsunviroidae*
Peach rosette mosaic virus, (PRMV), *Comoviridae*
Peacockpox virus, (PKPV), *Poxviridae*
Peanut bud necrosis virus, *Bunyaviridae*
Peanut chlorotic ring mottle virus, *Potyviridae*
Peanut chlorotic streak virus, (PCSV), *Caulimoviridae*
Peanut clump virus, (PCV), *Pecluvirus*
Peanut green mottle virus, (PeGMoV), *Potyviridae*
Peanut mild mottle virus, *Potyviridae*
Peanut mottle virus, (PeMoV), *Potyviridae*
Peanut stripe virus, *Potyviridae*
Peanut stunt virus satellite RNA, Satellite
Peanut stunt virus, (PSV), *Bromoviridae*
Peanut yellow mosaic virus, (PeYMV), *Tymovirus*
Peanut yellow spot virus, *Bunyaviridae*
Pear blister canker viroid, (PBCVd), *Pospiviroidae*
Pear rusty skin viroid, *Pospiviroidae*
Pear vein yellows virus, *Foveavirus*
Peaton virus, (PEAV), *Bunyaviridae*
Pecteilis mosaic virus, (PcMV), *Potyviridae*
Pectinophora gossypiella cypovirus 11, (PgCPV-11), *Reoviridae*
Pectinophora gossypiella virus, (PgV), Unassigned
Pelargonium flower break virus, (PFBV), *Tombusviridae*
Pelargonium leaf curl virus, (PLCV), *Tombusviridae*
Pelargonium vein clearing virus, (PVCV), *Rhabdoviridae*
Pelargonium zonate spot virus, (PZSV), Unassigned
Penaeus monodon NPV, (PemoNPV), *Baculoviridae*
Penguinpox virus, (PEPV), *Poxviridae*
Penicillium brevicompactum virus, (PbV), *Partitiviridae*
Penicillium chrysogenum virus, (PcV), *Partitiviridae*
Penicillium cyaneo-fulvum virus, (Pc-fV), *Partitiviridae*
Penicillium stoloniferum virus F, (PsV-F), *Partitiviridae*
Penicillium stoloniferum virus S, (PsV-S), *Partitiviridae*
Pepino latent virus, *Carlavirus*
Pepino mosaic virus, (PepMV), *Potexvirus*
Pepper huasteco virus, (PHV), *Geminiviridae*
Pepper leaf curl virus, (PepLCV), *Geminiviridae*
Pepper mild mosaic virus, (PMMV), *Potyviridae*
Pepper mild mottle virus, (PMMoV), *Tobamovirus*
Pepper mild tigré virus, (PepMTV), *Geminiviridae*
Pepper mottle virus, (PepMoV), *Potyviridae*
Pepper ringspot virus, (PepRSV), *Tobravirus*
Pepper severe mosaic virus, (PepSMV), *Potyviridae*
Pepper vein banding virus, (PVBV), *Potyviridae*
Pepper veinal mottle virus, (PVMV), *Potyviridae*
Perch hyperplasia virus, (PHV), *Retroviridae*
Percid herpesvirus 1, (PeHV-1), *Herpesviridae*
Perconia circinata virus, (PeCV), Unassigned
Perdicid herpesvirus 1, (PdHV-1), *Herpesviridae*
Pergamino virus, *Bunyaviridae*
Perilla mottle virus, (PerMoV, *Potyviridae*
Perinet virus, (PERV), *Rhabdoviridae*
Periplanata fuliginosa densovirus, (PfDNV), *Parvoviridae*
Peru tomato mosaic virus, (PTV), *Potyviridae*
Peste-des-petits-ruminants virus, (PPRV), *Paramyxoviridae*
Pestivirus of giraffe, *Flaviviridae*
Petevo virus, (PETV), *Reoviridae*
Petuluma, (FIV-P), *Retroviridae*
Petunia asteroid mosaic virus, (PetAMV), *Tombusviridae*
Petunia flower mottle virus, (PetFMV), *Potyviridae*
Petunia vein clearing virus, (PVCV), *Caulimoviridae*
Phalacrocoracid herpesvirus 1, (PhHV-1), *Herpesviridae*
Phalaenopsis chlorotic spot virus, (PhCSV), *Rhabdoviridae*
Phalera bucephala cypovirus 2, (PbCPV-2), *Reoviridae*
Phanerotoma flavitestacea bracovirus, (PfBV), *Polydnaviridae*
Pheasant adenovirus, (PhAdV-1), *Adenoviridae*

Phialophora radicicola virus 2-2-A, (PrV-2-2-A), *Partitiviridae*
Philosamia cynthia x ricini virus, (PxV), *Tetraviridae*
Phlogophera meticulosa cypovirus 3, (PmCPV-3), Reoviridae
Phlogophora meticulosa cypovirus 8, (PmCPV-8), *Reoviridae*
Phnom Penh bat virus, (PPBV), *Flaviviridae*
Phocid herpesvirus 1, (PhoHV-1), *Herpesviridae*
Phocine distemper virus, (PDV), *Paramyxoviridae*
Pholetesor ornigis bracovirus, (PoBV), *Polydnaviridae*
Physalis mottle virus, (PhyMV), *Tymovirus*
Physalis severe mottle virus, (PhySMV), *Bunyaviridae*
Physarum polycephalum Tp1 virus, (PpoTp1V), *Pseudoviridae*
Pichinde virus, (PICV), *Arenaviridae*
Picola virus, (PIAV), *Reoviridae*
Pieris brassicae granulovirus, (PbGV, ArGV-1), *Baculoviridae*
Pieris rapae cypovirus 12, (PrCPV-12), *Reoviridae*
Pieris rapae cypovirus 2, (PrCPV-2), *Reoviridae*
Pieris rapae cypovirus 3, (PrCPV-3), *Reoviridae*
Pieris rapae densovirus, (PrDNV), *Parvoviridae*
Pigeon adenovirus, (PiAdV), *Adenoviridae*
Pigeon circovirus, (PiCV), *Circoviridae*
Pigeon herpesvirus, *Herpesviridae*
Pigeon pea mosaic mottle viroid, (PPMMoVd), *Avsunviroidae*
Pigeon pea proliferation virus, (PPPV), *Rhabdoviridae*
Pigeonpox virus, (PGPV), *Poxviridae*
Pike fry rhabdovirus, (PFRV), *Rhabdoviridae*
Pineapple bacilliform virus, (PBV), *Caulimoviridae*
Pineapple chlorotic leaf streak virus, (PCLSV), *Rhabdoviridae*
Pineapple mealybug wilt-associated virus 1, (PMWaV-1), *Closteroviridae*
Pineapple mealybug wilt-associated virus 2, (PMWaV-2), *Closteroviridae*
Piper yellow mottle virus, (PYMoV), *Caulimoviridae*
Pirital virus, (PIRV), *Arenaviridae*
Piry virus, (PIRYV), *Rhabdoviridae*
Pisum virus, (PisV), *Rhabdoviridae*
Pittosporum vein yellowing virus, (PVYV), *Rhabdoviridae*
Pixuna virus, (PIXV), *Togaviridae*
Plantago asiatica mosaic virus, (PlAMV), *Potexvirus*
Plantago mottle virus, (PlMoV), *Tymovirus*
Plantago severe mottle virus, (PlSMoV), *Potexvirus*
Plantago virus 4, (PlV-4), *Caulimoviridae*
Plantain mottle virus, (PlMV), *Rhabdoviridae*
Plantain virus 6, (PlV-6), *Tombusviridae*
Plantain virus 7, (PlV-7), *Potyviridae*
Plantain virus 8, (PlV-8), *Carlavirus*
Plantain virus X, (PlVX), *Potexvirus*
Plautia stali intestine virus, (PSIV), "CrPV-like viruses"
Playas virus, (PLAV), *Bunyaviridae*
Pleioblastus mosaic virus, (PleMV), *Potyviridae*
Pleuronectid herpesvirus 1, (PlHV-1), *Herpesviridae*
Plodia interpunctella granulovirus, (PiGV), *Baculoviridae*
Plum dapple viroid, *Pospiviroidae*
Plum pox virus, (PPV), *Potyviridae*
Poa semilatent virus, (PSLV), *Hordeivirus*
Poinsettia cryptic virus, (PnCV), *Partitiviridae*
Poinsettia mosaic virus, (PnMV), *Tymovirus*
Pokeweed mosaic virus, (PkMV), *Potyviridae*
Poliovirus, (PV), *Picornaviridae*
Polistes hebraeus cypovirus 13, (PhCPV-13), *Reoviridae*
Pongine herpesvirus 1, (PoHV-1), *Herpesviridae*
Pongine herpesvirus 2, (PoHV-2), *Herpesviridae*
Pongine herpesvirus 3, (PoHV-3), *Herpesviridae*
Pongola virus, (PGAV), *Bunyaviridae*
Ponteves virus, (PTVV), *Bunyaviridae*
Poovoot virus, (POOV), *Reoviridae*
Poplar mosaic virus, (PopMV), *Carlavirus*
Populus virus, (PV), *Potyviridae*
Porcelio dilatatus reovirus, (PdRV), *Reoviridae*
Porcelio dilatatus iridescent virus, *Iridoviridae*
Porcine adenovirus 1-3, (PAdV-1 to 3), *Adenoviridae*
Porcine adenovirus 4, (PAdV-4), *Adenoviridae*
Porcine adenovirus 5, (PAdV-5), *Adenoviridae*
Porcine adenovirus A, (PAdV-B), *Adenoviridae*
Porcine adenovirus B, (PAdV-B), *Adenoviridae*
Porcine adenovirus C, (PAdV-C), *Adenoviridae*
Porcine astrovirus 1, (PAstV-1), *Astroviridae*
Porcine astrovirus, (PAstV), *Astroviridae*
Porcine circovirus, (PCV), *Circoviridae*
Porcine enteric calicivirus, (PECV), *Caliciviridae*
Porcine enterovirus 1, *Picornaviridae*
Porcine enterovirus 10, (PEV-10), *Picornaviridae*
Porcine enterovirus 11-13, (PEV-11 to 13), *Picornaviridae*
Porcine enterovirus 2-7, (PEV-2 to 7), *Picornaviridae*
Porcine enterovirus 8, (PEV-8), *Picornaviridae*
Porcine enterovirus 9, (PEV-9), *Picornaviridae*
Porcine enterovirus A, (PEV-A), *Picornaviridae*
Porcine enterovirus B, (PEV-B), *Picornaviridae*
Porcine epidemic diarrhea virus, (PEDV), *Coronaviridae*
Porcine hemagglutinating encephalomyelitis virus, (HEV), *Coronaviridae*
Porcine parvovirus, (PPV), *Parvoviridae*
Porcine respiratory and reproductive syndrome virus, *Arteriviridae*
Porcine respiratory coronavirus, (PRCoV), *Coronaviridae*

Porcine rotavirus Cowden, (PoRV-C/Cowden), *Reoviridae*
Porcine rotavirus DC-9, (PoRV-E/DC9), *Reoviridae*
Porcine rubulavirus, (PoRV), *Paramyxoviridae*
Porcine teschovirus 1, (PTV-1), *Picornaviridae*
Porcine teschovirus, (PTV), *Picornaviridae*
Porcine torovirus, (PoTV), *Coronaviridae*
Porcine type-C oncovirus, (PCOV), *Retroviridae*
Porton virus, (PORV), *Rhabdoviridae*
Potato aucuba mosaic virus, (PAMV), *Potexvirus*
Potato black ringspot virus, (PBRSV), *Comoviridae*
Potato leafroll virus, (PLRV), *Luteoviridae*
Potato mop-top virus, (PMTV), *Pomovirus*
Potato spindle tuber viroid, (PSTVd), *Pospiviroidae*
Potato virus A, (PVA), *Potyviridae*
Potato virus M, (PVM), *Carlavirus*
Potato virus S, (PVS), *Carlavirus*
Potato virus T, (PVT), *Trichovirus*
Potato virus U, (PVU), *Comoviridae*
Potato virus V, (PVV), *Potyviridae*
Potato virus X, (PVX), *Potexvirus*
Potato virus Y, (PVY), *Potyviridae*
Potato yellow dwarf virus, (PYDV), *Rhabdoviridae*
Potato yellow mosaic virus, (PYMV), *Geminiviridae*
Pothos latent virus, (PoLV) *Tombusviridae*
Potiskum virus, (POTV), *Flaviviridae*
Potosi virus, (POTV), *Bunyaviridae*
Powassan virus, (POWV), *Flaviviridae*
Precarious Point virus, (PPV), *Bunyaviridae*
Pretoria virus, (PREV), *Bunyaviridae*
Primate calicivirus, (VESV/Pan-1), *Caliciviridae*
Primate T-lymphotropic virus 1, (PTLV-1), *Retroviridae*
Primate T-lymphotropic virus 2, (PTLV-2), *Retroviridae*
Primate T-lymphotropic virus 3, (PTLV-3), *Retroviridae*
Primula mosaic virus, (PrMV), *Potyviridae*
Primula mottle virus, (PrMoV), *Potyviridae*
Prospect Hill virus, (PHV), *Bunyaviridae*
Protapanteles paleacritae bracovirus, (PpBV), *Polydnaviridae*
Prune dwarf virus, (PDV), *Bromoviridae*
Prunus necrotic ringspot virus, (PNRSV), *Bromoviridae*
Prunus virus S, (PruVS), *Carlavirus*
Pseudaletia includens densovirus, (PiDNV), *Parvoviridae*
Pseudaletia unipuncta cypovirus 11, (PuCPV-11), *Reoviridae*
Pseuderanthemum yellow vein virus, (PYVV), *Geminiviridae*
Pseudocowpox virus, (PCPV), *Poxviridae*
Pseudomonas phage 12S, (12S), *Myoviridae*
Pseudomonas phage 42, (42), *Myoviridae*
Pseudomonas phage 525, (525), *Podoviridae*
Pseudomonas phage 7s, (7s), *Leviviridae*
Pseudomonas phage D3, (D3), *Siphoviridae*
Pseudomonas phage F116, (F116), *Podoviridae*
Pseudomonas phage gh-1, (gh-1), *Podoviridae*
Pseudomonas phage Kf1, (Kf1), *Siphoviridae*
Pseudomonas phage M6, (M6), *Siphoviridae*
Pseudomonas phage PB-1, (PB-1), *Myoviridae*
Pseudomonas phage Pf1, (Pf1), *Inoviridae*
Pseudomonas phage Pf2, (Pf2), *Inoviridae*
Pseudomonas phage Pf3, (Pf3), *Inoviridae*
Pseudomonas phage PP7, (PP7), *Leviviridae*
Pseudomonas phage PP8, (PP8), *Myoviridae*
Pseudomonas phage PRR1, (PRR1), *Leviviridae*
Pseudomonas phage PS17, (PS17), *Myoviridae*
Pseudomonas phage PS4, (PS4), *Siphoviridae*
Pseudomonas phage PsP3, (PsP3), *Myoviridae*
Pseudomonas phage Pssy9220, (Psy9220), *Podoviridae*
Pseudomonas phage SD1, (SD1), *Siphoviridae*
Pseudomonas phage ϕ6, (ϕ6), *Cystoviridae*
Pseudomonas phage ϕCTX, (ϕCTX), *Myoviridae*
Pseudomonas phage ϕKZ, (ϕKZ), *Myoviridae*
Pseudomonas phage ϕPLS27, (ϕPLS27), *Podoviridae*
Pseudomonas phage ϕPLS743, (ϕPLS743), *Podoviridae*
Pseudomonas phage ϕW-14, (ϕW-14), *Myoviridae*
Pseudoplusia includens virus, (PiV), *Tetraviridae*
Pseudorabies virus, *Herpesviridae*
Psittacid herpesvirus 1, (PsHV-1), *Herpesviridae*
Psittacinepox virus, (PSPV), *Poxviridae*
Psophocarpus necrotic mosaic virus, *Carlavirus*
Pteroteinon laufella virus-1, (PlV-1), Unassigned
Puchong virus, (PUCV), *Rhabdoviridae*
Pueblo Viejo virus, (PVV), *Bunyaviridae*
Puffin Island virus, (PIV), *Bunyaviridae*
Puma lentivirus (PLV-14), (PLV), *Retroviridae*
Punta Salinas virus, (PSV), *Bunyaviridae*
Punta Toro virus D-4021A, (PTV), *Bunyaviridae*
Punta Toro virus, (PTV), *Bunyaviridae*
Purus virus, (PURV), *Reoviridae*
Puumala virus, (PUUV), *Bunyaviridae*
Python orthoreovirus, (PRV), *Reoviridae*

Qalyub virus, (QYBV), *Bunyaviridae*
Quail pea mosaic virus, (QPMV), *Comoviridae*
Quailpox virus, (QUPV), *Poxviridae*
Quaranfil virus, (QRFV), Unassigned
Queensland fruit fly virus, (QFFV), Unassigned
Quokka poxvirus, (QPV), *Poxviridae*

R22 virus, *Bunyaviridae*
Rabbit calicivirus, (RCV), *Caliciviridae*
Rabbit coronavirus, (RbCoV), *Coronaviridae*

Rabbit fibroma virus, (SFV), *Poxviridae*
Rabbit hemorrhagic disease virus, (RHDV), *Caliciviridae*
Rabbit hemorrhagic disease virus-AST89, (RHDV-AST89), *Caliciviridae*
Rabbit hemorrhagic disease virus-BS89, (RHDV-BS89), *Caliciviridae*
Rabbit hemorrhagic disease virus-FRG, (RHDV-FRG), *Caliciviridae*
Rabbit hemorrhagic disease virus-SD, (RHDV-SD), *Caliciviridae*
Rabbit hemorrhagic disease virus-V351, (RHDV-V351), *Caliciviridae*
Rabbit kidney vacuolating virus, (RKV), *Polyomaviridae*
Rabbitpox virus, (RPXV), *Poxviridae*
Rabies virus, (RABV), *Rhabdoviridae*
Raccoon parvovirus, (RPV), *Parvoviridae*
Raccoonpox virus, (RCNV), *Poxviridae*
Rachiplusia ou MNPV, (RoMNPV), *Baculoviridae*
Radi virus, (RADIV), *Rhabdoviridae*
Radish mosaic virus, (RaMV), *Comoviridae*
Radish vein clearing virus, (RaVCV), *Potyviridae*
Radish yellow edge virus, (RYEV), *Partitiviridae*
Rainbow trout virus, (RTV), *Iridoviridae*
Ranid herpesvirus 1, (RaHV-1), *Herpesviridae*
Ranid herpesvirus 2, (RaHV-2), *Herpesviridae*
Ranunculus mottle virus, (RanMoV), *Potyviridae*
Ranunculus repens symptomless virus, (RaRSV), *Rhabdoviridae*
Ranunculus white mottle virus, (RWMV), *Ophiovirus*
Raphanus virus, (RaV), *Rhabdoviridae*
Raspberry bushy dwarf virus, (RBDV), *Idaeovirus*
Raspberry ringspot virus, (RpRSV), *Comoviridae*
Raspberry vein chlorosis virus, (RVCV), *Rhabdoviridae*
Rat coronavirus, (RtCoV), *Coronaviridae*
Rat cytomegalovirus, *Herpesviridae*
Rat encephalomyelitis virus, (REV), *Picornaviridae*
Rat virus, *Parvoviridae*
Rattlesnake orthoreovirus, (RRV), *Reoviridae*
Raza virus, (RAZAV), *Bunyaviridae*
Razdan virus, (RAZV), *Bunyaviridae*
Red clover cryptic virus 2, (RCCV-2), *Partitiviridae*
Red clover mosaic virus, (RCIMV), *Rhabdoviridae*
Red clover mottle virus, (RCMV), *Comoviridae*
Red clover necrotic mosaic virus, (RCNMV), *Tombusviridae*
Red clover vein mosaic virus, (RCVMV), *Carlavirus*
Red deer herpesvirus, *Herpesviridae*
Red kangaroo poxvirus, (KPV), *Poxviridae*
Red pepper cryptic virus 1, (RPCV-1), *Partitiviridae*
Red pepper cryptic virus 2, (RPCV-2), *Partitiviridae*
Redfin perch virus, (RFPV), *Iridoviridae*
Redspotted grouper nervous necrosis virus, (RGNNV), *Nodaviridae*
Redwood Park virus, (RPV), *Iridoviridae*
Reed Ranch virus, (RRV), *Rhabdoviridae*
Regina ranavirus, (RRV), *Iridoviridae*
Reindeer herpesvirus, *Herpesviridae*
Rembrandt tulip breaking virus, (ReTBV), *Potyviridae*
Reptile calicivirus, (VESV/Cro-1), *Caliciviridae*
Resistencia virus, (RTAV), *Bunyaviridae*
Restan virus, (RESV), *Bunyaviridae*
Reston Ebola virus Philippines, *Filoviridae*
Reston Ebola virus Reston, *Filoviridae*
Reston Ebola virus Siena, *Filoviridae*
Reston Ebola virus Texas, *Filoviridae*
Reston Ebola virus, (REBOV), *Filoviridae*
Reticuloendotheliosis virus (strain T, A), (REV), *Retroviridae*
Rhesus EBV-like herpesvirus, *Herpesviridae*
Rhesus leukocyte associated herpesvirus strain 1, *Herpesviridae*
Rhesus monkey cytomegalovirus, *Herpesviridae*
Rhesus rhadinovirus, *Herpesviridae*
Rheumatoid arthritis virus, (RAV-1), *Parvoviridae*
Rhizidiomyces virus, (RZV), *Rhizidiovirus*
Rhizobium phage 16-2-12, (16-2-12), *Siphoviridae*
Rhizobium phage 16-6-2, (16-6-2), *Siphoviridae*
Rhizobium phage 2, (2), *Podoviridae*
Rhizobium phage 317, (317), *Siphoviridae*
Rhizobium phage 5, (5), *Siphoviridae*
Rhizobium phage 7-7-7, (7-7-7), *Siphoviridae*
Rhizobium phage CM_1, (CM_1), *Myoviridae*
Rhizobium phage CT4, (CT4), *Myoviridae*
Rhizobium phage m, (m), *Myoviridae*
Rhizobium phage NM1, (NM1), *Siphoviridae*
Rhizobium phage NT2, (NT2), *Siphoviridae*
Rhizobium phage S, (S), *Podoviridae*
Rhizobium phage WT1, (WT1), *Myoviridae*
Rhizobium phage ϕ2037/1, (ϕ2037/1), *Siphoviridae*
Rhizobium phage ϕ2042, (ϕ2042), *Podoviridae*
Rhizobium phage ϕgal-1/R, (ϕgal-1/R), *Myoviridae*
Rhizoctonia solani virus, (RsV), *Partitiviridae*
Rhododendron necrotic ringspot virus, (RoNRSV), *Potexvirus*
Rhopalosiphum padi virus, (RhPV), "CrPV-like viruses"
Rhubarb temperate virus, (RTV), *Partitiviridae*
Rhubarb virus 1, (RV-1), *Potexvirus*
Rhynchosia mosaic virus, (RhMV), *Geminiviridae*
RI-1 virus, *Bunyaviridae*
Ribgrass mosaic virus, (RMV), *Tobamovirus*
Rice black streaked dwarf virus, (RBSDV), *Reoviridae*
Rice dwarf virus, (RDV), *Reoviridae*
Rice gall dwarf virus, (RGDV), *Reoviridae*

rice giallume, *Luteoviridae*
Rice grassy stunt virus, (RGSV), *Tenuivirus*
Rice hoja blanca virus, (RHBV), *Tenuivirus*
Rice necrosis mosaic virus, (RNMV), *Potyviridae*
Rice ragged stunt virus, (RRSV), *Reoviridae*
Rice stripe necrosis virus, (RSNV), *Furovirus*
Rice stripe virus, (RSV), *Tenuivirus*
Rice transitory yellowing virus, (RTYV), *Rhabdoviridae*
Rice tungro bacilliform virus, (RTBV), *Caulimoviridae*
Rice tungro spherical virus, (RTSV), *Sequiviridae*
Rice wilted stunt virus, (RWSV), *Tenuivirus*
Rice yellow mottle virus satellite, Satellite
Rice yellow mottle virus, (RYMV), *Sobemovirus*
Rice yellow stunt virus, (RYSV), *Rhabdoviridae*
Rift Valley fever virus, (RVFV), *Bunyaviridae*
RIID 3229 virus, *Arenaviridae*
Rinderpest virus, (RPV), *Paramyxoviridae*
Rio Bravo virus, (RBV), *Flaviviridae*
Rio Grande cichlid virus, (RGRCV), *Rhabdoviridae*
Rio Grande virus, (RGV), *Bunyaviridae*
Rio Mamore virus, (RIOMV), *Bunyaviridae*
Rio Segundo virus, (RIOSV), *Bunyaviridae*
RM-97 virus, *Bunyaviridae*
RML 105355 virus, (RMLV), *Bunyaviridae*
Roan antelope herpesvirus, *Herpesviridae*
Robinia mosaic virus satellite RNA, Satellite
Robinia mosaic virus, *Bromoviridae*
Rochambeau virus, (RBUV), *Rhabdoviridae*
Rocio virus, (ROCV), *Flaviviridae*
Ross' goose hepatitis B virus, (RGHBV), *Hepadnaviridae*
Ross River virus, (RRV), *Togaviridae*
Rost Island virus, (RSTV), *Reoviridae*
Rotavirus A, (RV-A), *Reoviridae*
Rotavirus B, (RV-B), *Reoviridae*
Rotavirus C, (RV-C), *Reoviridae*
Rotavirus D, (RV-D), *Reoviridae*
Rotavirus E, (RV-E), *Reoviridae*
Rotavirus F, (RV-F), *Reoviridae*
Rotavirus G, (RV-G), *Reoviridae*
Rotifer birnavirus, (RBV), *Birnaviridae*
Rous sarcoma virus (Prague C), (RSV-Pr-C), *Retroviridae*
Rous sarcoma virus (Schmidt-Ruppin B), (RSV-SR-B), *Retroviridae*
Rous sarcoma virus (Schmidt-Ruppin D), (RSV-SR-D), *Retroviridae*
Rous sarcoma virus, (RSV), *Retroviridae*
Royal Farm virus, (RFV), *Flaviviridae*
RT parvovirus, (RTPV), *Parvoviridae*
Rubella virus, (RUBV), *Togaviridae*
Rubus Chinese seed-borne virus, (RCSV), *Comoviridae*
Rudbeckia mosaic virus, (RuMV), *Potyviridae*
Rupestris stem pitting-associated virus, (RSPaV), *Foveavirus*
Ryegrass cryptic virus, (RGCV), *Partitiviridae*
Ryegrass mosaic virus, (RGMV), *Potyviridae*

S 1954-847-32 virus, (TURV), *Bunyaviridae*
SA15 virus, *Herpesviridae*
SA6 virus, *Herpesviridae*
SAAAr 5133 virus, (OLIV), *Bunyaviridae*
SAAn 3518 virus, (TETEV), *Bunyaviridae*
SAAr 53 virus, (SIMV), *Bunyaviridae*
Sabiá virus, (SABV), *Arenaviridae*
Sabin virus, (SFNV), *Bunyaviridae*
Sabo virus, (SABOV), *Bunyaviridae*
Saboya virus, (SABV), *Flaviviridae*
Sacbrood virus, (SBV), Unassigned
Saccharomyces cerevisiae M virus, Satellite
Saccharomyces cerevisiae narnavirus 20S RNA, (ScNV-20S), *Narnaviridae*
Saccharomyces cerevisiae narnavirus 23S RNA, (ScNV-23S), *Narnaviridae*
Saccharomyces cerevisiae Ty1 virus, (SceTy1V), *Pseudoviridae*
Saccharomyces cerevisiae Ty2 virus, (SceTy2V), *Pseudoviridae*
Saccharomyces cerevisiae Ty3 virus, (SceTy3V), *Metaviridae*
Saccharomyces cerevisiae Ty4 virus, (SceTy4V), *Pseudoviridae*
Saccharomyces cerevisiae Ty5 virus, (SceTy5V), *Pseudoviridae*
Saccharomyces cerevisiae virus L-A (L1), (ScV-L-A), *Totiviridae*
Saccharomyces cerevisiae virus L-BC (La), (ScV-L-BC), *Totiviridae*
Sagiyama virus, *Togaviridae*
Saguaro cactus virus, (SgCV), *Tombusviridae*
Saimiriine herpesvirus 1, (SaHV-1), *Herpesviridae*
Saimiriine herpesvirus 2, (SaHV-2), *Herpesviridae*
Sainpaulia leaf necrosis virus, (SLNV), *Rhabdoviridae*
Saint-Floris virus, (SAFV), *Bunyaviridae*
Sakhalin virus, (SAKV), *Bunyaviridae*
Sal Vieja virus, (SVV), *Flaviviridae*
Salanga poxvirus, (SGV), *Poxviridae*
Salanga virus, (SGAV), *Bunyaviridae*
Salehebad I-81 virus, (SALV), *Bunyaviridae*
Salehebad virus, (SALV), *Bunyaviridae*
Salmon reovirus, (SSRV), *Reoviridae*
Salmonid herpesvirus 1, (SalHV-1), *Herpesviridae*
Salmonid herpesvirus 2, (SalHV-2), *Herpesviridae*
Sambucus vein clearing virus, (SVCV), *Rhabdoviridae*

Sammons's Opuntia virus, (SOV), *Tobamovirus*
San Angelo virus, (SAV), *Bunyaviridae*
San Juan virus, (SJV), *Bunyaviridae*
San Miguel sea lion virus, serotype 1, (VESV/SMSV-1), *Caliciviridae*
San Miguel sea lion virus, serotype 17, (VESV/SMSV-17), *Caliciviridae*
San Miguel sea lion virus, serotype 4, (VESV/SMSV-4), *Caliciviridae*
San Perlita virus, (SPV), *Flaviviridae*
Sand rat nuclear inclusion agents, *Herpesviridae*
Sandfly fever Naples virus, (SFNV), *Bunyaviridae*
Sandfly fever Sicilian virus, (SFSV), *Bunyaviridae*
Sandjimba virus, (SJAV), *Rhabdoviridae*
Sango virus, (SANV), *Bunyaviridae*
Santa Rosa virus, (SARV), *Bunyaviridae*
Santarem virus, (STMV), *Bunyaviridae*
Santee-Cooper ranavirus, (SCRV), *Iridoviridae*
Santosai temperate virus, (STV), *Partitiviridae*
Sapphire II virus, (SAPV), *Bunyaviridae*
Sapporo virus, (SV), *Caliciviridae*
Sapporo virus-Houston/86, (SV-Houston/86), *Caliciviridae*
Sapporo virus-Houston/90, (SV-Houston/90), *Caliciviridae*
Sapporo virus-London/29845, (SV-London/29845), *Caliciviridae*
Sapporo virus-Manchester, (SV-Manchester), *Caliciviridae*
Sapporo virus-Parkville, (SV-Parkville), *Caliciviridae*
Sapporo virus-Sapporo, (SV-Sapporo), *Caliciviridae*
Saraca virus, (SRAV), *Reoviridae*
Sarracenia purpurea virus, (SPV), *Rhabdoviridae*
Sathuperi virus, (SATV), *Bunyaviridae*
Satsuma dwarf virus, (SDV), *Comoviridae*
Saturnia pavonia virus, (SpV), *Tetraviridae*
Saumarez Reef virus, (SREV), *Flaviviridae*
Sawgrass virus, (SAWV), *Rhabdoviridae*
Schefflera ringspot virus, (SRV), *Caulimoviridae*
Schistocera gregaria entomopoxvirus 'O', (SGEV), *Poxviridae*
Schizosaccharomyces pombe Tf1 virus, (SpoTf1V), *Metaviridae*
Schizosaccharomyces pombe Tf2 virus, (SpoTf2V), *Metaviridae*
Sciurid herpesvirus 1, (ScHV-2), *Herpesviridae*
Sciurid herpesvirus 2, (ScHV-2), *Herpesviridae*
Scrophularia mottle virus, (ScrMV), *Tymovirus*
Sea-bass virus-1, (SBV), *Picornaviridae*
Seal distemper virus, *Paramyxoviridae*
Sealpox virus, *Poxviridae*
Seletar virus, (SELV), *Reoviridae*
Semliki Forest virus, (SFV), *Togaviridae*
Sena Madureira virus, (SMV), *Rhabdoviridae*
Sendai virus, (SeV), *Paramyxoviridae*
Seoul virus, (SEOV), *Bunyaviridae*
Sepik virus, (SEPV), *Flaviviridae*
Sericesthis iridescent virus, *Iridoviridae*
Serotype A of BCMV, *Potyviridae*
Serra do Navio virus, (SDNV), *Bunyaviridae*
Serrano golden mosaic virus, (SGMV), *Geminiviridae*
Sesame mosaic virus, *Potyviridae*
Setora nitens virus, (SnV), *Tetraviridae*
Setothosea asigna virus, (SaV), *Tetraviridae*
Shallot latent virus, (SLV), *Carlavirus*
Shallot mite-borne latent virus, (ShMbLV), *Allexivirus*
Shallot virus X, (ShV-X), *Allexivirus*
Shallot yellow stripe virus, (SYSV), *Potyviridae*
Shamonda virus, (SHAV), *Bunyaviridae*
Shark River virus, (SRV), *Bunyaviridae*
Sheep pulmonary adenomatosis associated herpesvirus, *Herpesviridae*
Sheep-associated malignant catarrhal fever virus, *Herpesviridae*
Sheeppox virus, (SPPV), *Poxviridae*
Shiant Islands virus, (SHIV), *Reoviridae*
Shokwe virus, (SHOV), *Bunyaviridae*
Shope fibroma virus, *Poxviridae*
Shuni virus, (SHUV), *Bunyaviridae*
Sialodacryoadenitis virus, (SDAV), *Coronaviridae*
Siamese cobra herpesvirus, *Herpesviridae*
Sibine fusca densovirus, (SfDNV), *Parvoviridae*
Sida golden mosaic virus, (SiGMV), *Geminiviridae*
Sida yellow vein virus, (SiYVV), *Geminiviridae*
Sigma virus, (SIGMAV), *Rhabdoviridae*
Sikhote-Alyn virus, (SAV), *Picornaviridae*
Silverwater virus, (SILV), *Bunyaviridae*
Simbu virus, (SIMV), *Bunyaviridae*
Simian adenovirus 1, 5, 7, 8, 12, 15, 17, 18, 20, 26?, 27?, (SAdV-1, 5, 7, 8, 12, 15, 17, 18, 20, 26?, 27?), *Adenoviridae*
Simian adenovirus 13, (SAdV-13), *Adenoviridae*
Simian adenovirus 16, 19, (SAdV-16, 19), *Adenoviridae*
Simian adenovirus 21, (SAdV-21), *Adenoviridae*
Simian adenovirus 22-25, (SAdV-22-25), *Adenoviridae*
Simian adenovirus 2-4, 6, (SAdV-2-4, 6), *Adenoviridae*
Simian adenovirus 9-11, 14, (SAdV-9-11, 14), *Adenoviridae*
Simian adenovirus, (SAdV), *Adenoviridae*
Simian enterovirus 1 to 18, (SEV-1 to 18), *Picornaviridae*
Simian enterovirus N125, (SEV-N125), *Picornaviridae*
Simian enterovirus N203, (SEV-N203), *Picornaviridae*
Simian foamy virus 1, (SFV-1), *Retroviridae*
Simian foamy virus 3, (SFV-3), *Retroviridae*

Simian hemorrhagic fever virus, (SHFV), *Arteriviridae*
Simian hepatitis A virus, (SHAV), *Picornaviridae*
Simian immunodeficiency virus African green monkey 155, (SIV-agm.155), *Retroviridae*
Simian immunodeficiency virus African green monkey 3, (SIV-agm.3), *Retroviridae*
Simian immunodeficiency virus African green monkey gr-1, (SIV-agm.gr), *Retroviridae*
Simian immunodeficiency virus African green monkey Sab-1, (SIV-agm.sab), *Retroviridae*
Simian immunodeficiency virus African green monkey Tan-1, (SIV-agm.tan), *Retroviridae*
Simian immunodeficiency virus African green monkey TYO, (SIV-agm.TYO), *Retroviridae*
Simian immunodeficiency virus African Green Monkey, (SIV-agm), *Retroviridae*
Simian immunodeficiency virus chimpanzee SIV, (SIV-cpz), *Retroviridae*
Simian immunodeficiency virus mandrill SIV, (SIV-mnd), *Retroviridae*
Simian immunodeficiency virus pig-tailed macaque, (SIV-mne), *Retroviridae*
Simian immunodeficiency virus red capped mangabey SIV, (SIV-rcm), *Retroviridae*
Simian immunodeficiency virus Rhesus (Maccaca mulatta), (SIV-mac), *Retroviridae*
Simian immunodeficiency virus sooty mangabey SIV-H4, (SIV-sm), *Retroviridae*
Simian immunodeficiency virus stump-tailed macaque (stm), (SIV-stm), *Retroviridae*
Simian immunodeficiency virus sykes monkey SIV, (SIV-syk), *Retroviridae*
Simian immunodeficiency virus, (SIV), *Retroviridae*
Simian retrovirus 1, (SRV-1), *Retroviridae*
Simian retrovirus 2, (SRV-2), *Retroviridae*
Simian rotavirus SA11, (SiRV-A/SA11), *Reoviridae*
Simian sarcoma virus, *Retroviridae*
Simian T-lymphotropic virus 1, (STLV-1), *Retroviridae*
Simian T-lymphotropic virus 2, (STLV-2), *Retroviridae*
Simian T-lymphotropic virus 3, (STLV-3), *Retroviridae*
Simian varicella virus, *Herpesviridae*
Simian virus 10, (SV-10), *Paramyxoviridae*
Simian virus 12, (SV-12), *Polyomaviridae*
Simian virus 40, (SV-40), *Polyomaviridae*
Simian virus 41, (SV-41), *Paramyxoviridae*
Simian virus 5, (SV-5), *Paramyxoviridae*
Simulium sp. iridescent virus, *Iridoviridae*
Simulium vittatum densovirus, (SvDNV), *Parvoviridae*
Sin Nombre virus, (SNV), *Bunyaviridae*
Sinaloa tomato leaf curl virus, (STLCV), *Geminiviridae*
Sindbis virus, (SINV), *Togaviridae*
Sint-Jem's onion latent virus, (SJOLV), *Carlavirus*
Sitke waterborne virus, (SWBV), *Tombusviridae*
Sitobion avenae virus, (SaV), Unassigned
Sixgun city virus, (SCV), *Reoviridae*
Skunk calicivirus, (VESV/SCV), *Caliciviridae*
Skunkpox virus, (SKPV), *Poxviridae*
Slow bee paralysis virus, (SBPV), Unassigned
Smelt reovirus, (SRV), *Reoviridae*
Smelt virus-1, (SmV-1), *Picornaviridae*
Smelt virus-2, (SmV-2), *Picornaviridae*
Smithiantha latent virus, (SmiLV), *Potexvirus*
Snake adenovirus, (SnAdV-1), *Adenoviridae*
Snakehead retrovirus, (SnRV), *Retroviridae*
Snakehead rhabdovirus, (SHRV), *Rhabdoviridae*
Snow Mountain virus, (SMV), *Caliciviridae*
Snowshoe hare virus, (SSHV), *Bunyaviridae*
Snyder-Theilen feline sarcoma virus, (STFeSV), *Retroviridae*
Soil-borne rye mosaic virus, (SBRMV), *Furovirus*
Soil-borne wheat mosaic virus, (SBWMV), *Furovirus*
Sokoluk virus, (SOKV), *Flaviviridae*
Solanum apical leaf curl virus, (SALCV), *Geminiviridae*
Solanum nodiflorum mottle virus satellite RNA, Satellite
Solanum nodiflorum mottle virus, (SNMoV), *Sobemovirus*
Solanum tomato leaf curl virus, (SToLCV), *Geminiviridae*
Solanum tuberosum Tst1 virus, (StuTst1V), *Pseudoviridae*
Solanum yellow leaf curl virus, (SYLCV), *Geminiviridae*
Solanum yellows virus, (SOLYV), *Luteoviridae*
Soldado virus, (SOLV), *Bunyaviridae*
Sonchus mottle virus, (SMoV), *Caulimoviridae*
Sonchus virus, (SonV), *Rhabdoviridae*
Sonchus yellow net virus, (SYNV), *Rhabdoviridae*
Sorghum chlorotic spot virus, (SrCSV), *Furovirus*
Sorghum mosaic virus, (SrMV), *Potyviridae*
Sorghum virus, (SrV), *Rhabdoviridae*
Sororoca virus, (SORV), *Bunyaviridae*
Sotkamo virus, *Bunyaviridae*
Soursop yellow blotch virus, (SYBV), *Rhabdoviridae*
South African cassava mosaic virus, (SACMV), *Geminiviridae*
South African passiflora virus, *Potyviridae*
South River virus, (SORV), *Bunyaviridae*
Southampton virus, (SHV), *Caliciviridae*
Southern bean mosaic virus, (SBMV), *Sobemovirus*
Southern cowpea mosaic virus, (SCPMV), *Sobemovirus*

Southern potato latent virus, (SoPLV), *Carlavirus*
Sowbane mosaic virus, (SoMV), *Sobemovirus*
Sowthistle yellow vein virus, (SYVV), *Rhabdoviridae*
Soybean chlorotic mottle virus, (SbCMV), *Caulimoviridae*
Soybean crinkle leaf virus, (SCLV), *Geminiviridae*
Soybean dwarf virus, (SbDV), *Luteoviridae*
Soybean mosaic virus, (SMV), *Potyviridae*
SPAr 2317 virus, (SPAV), *Bunyaviridae*
Sparrowpox virus, (SRPV), *Poxviridae*
Spartina mottle virus, (SpMV), *Potyviridae*
Spectacled caiman poxvirus, (SPV), *Poxviridae*
SPH114202 virus, *Arenaviridae*
Sphenicid herpesvirus 1, (SpHV-1), *Herpesviridae*
Spider monkey herpesvirus, *Herpesviridae*
Spinach latent virus, (SpLV), *Bromoviridae*
Spinach temperate virus, (SpTV), *Partitiviridae*
Spiroplasma phage 1-aa, (SpV1-aa), *Inoviridae*
Spiroplasma phage 1-C74, (SpV1-C74), *Inoviridae*
Spiroplasma phage 1-KC3, (SpV1/KC3), *Inoviridae*
Spiroplasma phage 1-R8A2B, (SpV1-R8A2B), *Inoviridae*
Spiroplasma phage 1-S102, (SpV1-S102), *Inoviridae*
Spiroplasma phage 1-T78, (SpV1-T78), *Inoviridae*
Spiroplasma phage 4, (Sp-4), *Microviridae*
Spiroplasma phage C1/TS2, (C1/TS2), *Inoviridae*
Spodoptera exempta cypovirus 11, (SexmCPV-11), *Reoviridae*
Spodoptera exempta cypovirus 12, (SexmCPV-12), *Reoviridae*
Spodoptera exempta cypovirus 3, (SexmCPV-3), *Reoviridae*
Spodoptera exempta cypovirus 5, (SexmCPV-5), *Reoviridae*
Spodoptera exempta cypovirus 8, (SexmCPV-8), *Reoviridae*
Spodoptera exempta MNPV, (SpexMNPV), *Baculoviridae*
Spodoptera exigua cypovirus 11, (SexgCPV-11), *Reoviridae*
Spodoptera exigua MNPV, (SeMNPV), *Baculoviridae*
Spodoptera frugiperda ascovirus 1a, (SfAV-1a), *Ascoviridae*
Spodoptera frugiperda MNPV, (SfMNPV), *Baculoviridae*
Spodoptera littoralis NPV, (SpliNPV), *Baculoviridae*
Spodoptera litura NPV, (SpltNPV), *Baculoviridae*
Spondweni virus, (SPOV), *Flaviviridae*
Spring beauty latent virus, (SBLV), *Bromoviridae*
Spring viremia of carp virus, (SVCV), *Rhabdoviridae*
Squash leaf curl virus - China, (SLCV-Ch), *Geminiviridae*
Squash leaf curl virus, (SLCV), *Geminiviridae*
Squash mosaic virus, (SqMV), *Comoviridae*
Squirrel fibroma virus, (SQFV), *Poxviridae*
Squirrel monkey retrovirus, (SMRV), *Retroviridae*
Squirrel parapoxvirus, (SPPV), *Poxviridae*
SR-11 virus, *Bunyaviridae*
Sri Lankan passion fruit mottle virus, (SLPMoV), *Potyviridae*
Sripur virus, (SRIV), *Rhabdoviridae*
St Abb's Head virus, (SAHV), *Reoviridae*
St. Abbs Head virus, (SAHV), *Bunyaviridae*
St. Louis encephalitis virus, (SLEV), *Flaviviridae*
Staphylococcus phage 107, (107), *Siphoviridae*
Staphylococcus phage 11, (11), *Siphoviridae*
Staphylococcus phage 1139, (1139), *Siphoviridae*
Staphylococcus phage 1154A, (1154A), *Siphoviridae*
Staphylococcus phage 187, (187), *Siphoviridae*
Staphylococcus phage 2848A, (2848A), *Siphoviridae*
Staphylococcus phage 392, (392), *Siphoviridae*
Staphylococcus phage 3A, (3A), *Siphoviridae*
Staphylococcus phage 44AHJD, (44AHJD), *Podoviridae*
Staphylococcus phage 77, (77), *Siphoviridae*
Staphylococcus phage B11-M15, (B11-M15), *Siphoviridae*
Staphylococcus phage P11, (P11), *Siphoviridae*
Staphylococcus phage ϕ11, (ϕ11), *Siphoviridae*
Starlingpox virus, (SLPV), *Poxviridae*
Statice virus Y, *Potyviridae*
Stickleback virus, (SBV), *Iridoviridae*
Stratford virus, (STRV), *Flaviviridae*
Strawberry crinkle virus, (SCV), *Rhabdoviridae*
Strawberry latent ringspot virus satellite RNA, Satellite
Strawberry latent ringspot virus, (SLRSV), *Comoviridae*
Strawberry mild yellow edge virus, (SMYEV), *Potexvirus*
Strawberry mild yellow edge associated virus, *Luteoviridae*
Strawberry pseudo mild yellow edge virus, (SPMYEV), *Carlavirus*
Strawberry vein banding virus, (SVBV), *Caulimoviridae*
Streptococcus phage 182, (182), *Podoviridae*
Streptococcus phage 24, (24), *Siphoviridae*
Streptococcus phage 2BV, (2BV), *Podoviridae*
Streptococcus phage A25, (A25), *Siphoviridae*
Streptococcus phage Cp-1, (Cp-1), *Podoviridae*
Streptococcus phage Cp-5, (Cp-5), *Podoviridae*
Streptococcus phage Cp-7, (Cp-7), *Podoviridae*
Streptococcus phage Cp-9, (Cp-9), *Podoviridae*
Streptococcus phage Cvir, (Cvir), *Podoviridae*
Streptococcus phage H39, (H39), *Podoviridae*
Streptococcus phage PE1, (PE1), *Siphoviridae*
Streptococcus phage VD13, (VD13), *Siphoviridae*
Streptococcus phage ω8, (ω8), *Siphoviridae*
Strigid herpesvirus 1, (StHV-1), *Herpesviridae*

Striped bass reovirus, (SBRV), *Reoviridae*
Striped jack nervous necrosis virus, (SJNNV), *Nodaviridae*
Stump-tailed macaque virus, *Polyomaviridae*
Subterranean clover mottle virus satellite RNA, Satellite
Subterranean clover mottle virus, (SCMoV), *Sobemovirus*
Subterranean clover red leaf virus, *Luteoviridae*
Subterranean clover stunt virus, (SCSV), *Nanovirus*
Sudan Ebola virus Boniface, *Filoviridae*
Sudan Ebola virus Maleo, *Filoviridae*
Sudan Ebola virus, (SEBOV), *Filoviridae*
Sugarcane bacilliform virus, (SCBV), *Caulimoviridae*
Sugarcane mild mosaic virus, (SMMV), *Closteroviridae*
Sugarcane mosaic virus, (SCMV), *Potyviridae*
Sugarcane streak virus, (SSV), *Geminiviridae*
Suid herpesvirus 1, (SuHV-1), *Herpesviridae*
Suid herpesvirus 2, (SuHV-2), *Herpesviridae*
Sulfolobus virus 1, (SSV-1), *Fuselloviridae*
Sulfolobus virus SIRV-1, (SIRV-1), *Rudiviridae*
Sulfolobus virus SIRV-2, (SIRV-2), *Rudiviridae*
Sulfolobus virus SNDV, (SNDV), "SNDV-like viruses"
Sunday Canyon virus, (SCAV), *Bunyaviridae*
Sunflower crinkle virus, (SuCV), *Umbravirus*
Sunflower mosaic virus, (SuMV), *Potyviridae*
Sunflower rugose mosaic virus, *Umbravirus*
Sunflower yellow blotch virus, (SuYBV), *Umbravirus*
Sunflower yellow ringspot virus, *Umbravirus*
Sunn-hemp mosaic virus, (SHMV), *Tobamovirus*
Sweet clover necrotic mosaic virus, (SCNMV), *Tombusviridae*
Sweet potato chlorotic leafspot virus, *Potyviridae*
Sweet potato chlorotic stunt virus, (SPCSV), *Closteroviridae*
Sweet potato feathery mottle virus, (SPFMV), *Potyviridae*
Sweet potato internal cork virus, *Potyviridae*
Sweet potato latent virus, (SPLV), *Potyviridae*
Sweet potato leaf speckling virus, (SPLSV), *Luteoviridae*
Sweet potato mild mottle virus, (SPMMV), *Potyviridae*
Sweet potato mild speckling virus, (SPMSV), *Potyviridae*
Sweet potato russet crack virus, *Potyviridae*
Sweet potato sunken vein virus, *Closteroviridae*
Sweet potato vein mosaic virus, (SPVMV), *Potyviridae*
Sweet potato virus A, *Potyviridae*
Sweet potato yellow dwarf virus, (SPYDV), *Potyviridae*
Sweetwater Branch virus, (SWBV), *Rhabdoviridae*
Swine calicivirus, (SwV-43), *Caliciviridae*
Swine cytomegalovirus, *Herpesviridae*
Swine vesicular disease virus, *Picornaviridae*
Swinepox virus, (SWPV), *Poxviridae*
Sword bean distortion mosaic virus, (SBDMV), *Potyviridae*
Synetaeris tenuifemur ichnovirus, (StIV), *Polydnaviridae*
Syr-Daria Valley fever virus, (SDFV), *Picornaviridae*

T.RVL.II 573 virus, *Arenaviridae*
Tacaiuma virus, (TCMV), *Bunyaviridae*
Tacaribe virus, (TCRV), *Arenaviridae*
Tadpole edema virus, *Iridoviridae*
Tadpole virus 2, *Iridoviridae*
Taggert virus, (TAGV), *Bunyaviridae*
Tahyna virus, (TAHV), *Bunyaviridae*
Tai virus, (TAIV), *Bunyaviridae*
Taiassui virus, (TAIAV), *Bunyaviridae*
Taino tomato mottle virus, (TToMoV), *Geminiviridae*
Tamana bat virus, (TABV), *Flaviviridae*
Tamarillo mosaic virus, (TamMV), *Potyviridae*
Tamdy virus, (TDYV), *Bunyaviridae*
Tamiami virus, (TAMV), *Arenaviridae*
Tamus red mosaic virus, (TRMV), *Potexvirus*
Tanapox virus, (TANV), *Poxviridae*
Tanga virus, (TANV), *Bunyaviridae*
Tanjong Rabok virus, (TRV), *Bunyaviridae*
Taro bacilliform virus, (TaBV), *Caulimoviridae*
Taro feathery mottle virus, (TFMoV), *Potyviridae*
Tataguine virus, (TATV), *Bunyaviridae*
Taterapox virus, (GBLV), *Poxviridae*
Taura syndrome virus, (TSV), *Picornaviridae*
TBRV-G serotype satellite RNA, Satellite
TBRV-S serotype satellite RNA, Satellite
Teasel mosaic virus, (TeaMV), *Potyviridae*
Tehran virus, (THEV), *Bunyaviridae*
Telfairia mosaic virus, (TeMV), *Potyviridae*
Telok Forest virus, (TFV), *Bunyaviridae*
Tembe virus, (TMEV), *Reoviridae*
Tembusu virus, (TMUV), *Flaviviridae*
Tench reovirus, (TNRV), *Reoviridae*
Tenebrio molitor iridescent virus, *Iridoviridae*
Tensaw virus, (TENV), *Bunyaviridae*
Tephrosia symptomless virus, (TeSV), *Tombusviridae*
Termeil virus, (TERV), *Bunyaviridae*
Tete virus, (TETEV), *Bunyaviridae*
Texas pepper virus, (TPV), *Geminiviridae*
Thailand virus, (THAIV), *Bunyaviridae*
Theiler's murine encephalomyelitis virus, (TMEV), *Picornaviridae*
Theilovirus, (ThV), *Picornaviridae*

Thermoproteus virus 1, (TTV1), *Lipothrixiviridae*
Thermoproteus virus 2, (TTV2), *Lipothrixiviridae*
Thermoproteus virus 3, (TTV3), *Lipothrixiviridae*
Thermoproteus virus TTV4, (TTV4), *Rudiviridae*
Thermus phage P37-14, (P37-14), *Tectiviridae*
Thiafora virus, (TFAV), *Bunyaviridae*
Thimiri virus, (THIV), *Bunyaviridae*
Thistle mottle virus, (ThMoV), *Caulimoviridae*
Thogoto virus, (THOV), *Orthomyxoviridae*
Thormodseyjarlettur virus, (THRV), *Reoviridae*
Thosea asigna virus, (TaV), *Tetraviridae*
Thottapalayam virus, (TPMV), *Bunyaviridae*
Tibrogargan virus, (TIBV), *Rhabdoviridae*
Tichoplusia ni cypovirus 5, (TnCPV-5), *Reoviridae*
Tick-borne encephalitis virus European subtype, *Flaviviridae*
Tick-borne encephalitis virus Far Eastern subtype, *Flaviviridae*
Tick-borne encephalitis virus Siberian subtype, *Flaviviridae*
Tick-borne encephalitis virus, (TBEV), *Flaviviridae*
Tiger puffer nervous necrosis virus, (TPNNV), *Nodaviridae*
Tiger salamander virus, *Iridoviridae*
Tillamook virus, (TILLV), *Bunyaviridae*
Tillamook virus, *Reoviridae*
Tilligerry virus, (TILV), *Reoviridae*
Timbo virus, (TIMV), *Rhabdoviridae*
Timboteua virus, (TBTV), *Bunyaviridae*
Tinaroo virus, (TINV), *Bunyaviridae*
Tindholmur virus, (TDMV), *Reoviridae*
Tipula iridescent virus, *Iridoviridae*
Tipula paludosa NPV, (TipaNPV), *Baculoviridae*
Tlacotalpan virus, (TLAV), *Bunyaviridae*
Tobacco bushy top virus, (TBTV), *Umbravirus*
Tobacco etch virus, (TEV), *Potyviridae*
Tobacco leaf curl virus, (TLCV), *Geminiviridae*
Tobacco leaf enation phytoreovirus, (TLEP), *Reoviridae*
Tobacco mild green mosaic virus, (TMGMV), *Tobamovirus*
Tobacco mosaic satellite virus, Satellite
Tobacco mosaic virus-U1, *Tobamovirus*
Tobacco mosaic virus-U2, *Tobamovirus*
Tobacco mosaic virus-vulgare, *Tobamovirus*
Tobacco mosaic virus, (TMV), *Tobamovirus*
Tobacco mottle virus, (TMoV), *Umbravirus*
Tobacco necrosis satellite virus, Satellite
Tobacco necrosis virus A, (TNV-A), *Tombusviridae*
Tobacco necrosis virus D, (TNV-D), *Tombusviridae*
Tobacco necrosis virus small satellite RNA, Satellite
Tobacco necrotic dwarf virus, (TNDV), *Luteoviridae*
Tobacco rattle virus, (TRV), *Tobravirus*
Tobacco ringspot virus satellite RNA, Satellite
Tobacco ringspot virus, (TRSV), *Comoviridae*
Tobacco streak virus, (TSV), *Bromoviridae*
Tobacco stunt virus, (TStV), *Varicosavirus*
Tobacco vein banding mosaic virus, (TVBMV), *Potyviridae*
Tobacco vein mottling virus, (TVMV), *Potyviridae*
Tobacco wilt virus, (TWV), *Potyviridae*
Tobacco yellow dwarf virus, (TYDV), *Geminiviridae*
Tobacco yellow vein virus, (TYVV), *Umbravirus*
Tobetsu-60Cr-93 virus, *Bunyaviridae*
Tomato apical stunt viroid, (TASVd), *Pospiviroidae*
Tomato aspermy virus, (TAV), *Bromoviridae*
Tomato black ring virus satellite RNA, Satellite
Tomato black ring virus, (TBRV), *Comoviridae*
Tomato bunchy top viroid, (ToBTVd), *Avsunviroidae*
Tomato bushy stunt virus satellite RNA, Satellite
Tomato bushy stunt virus, (TBSV), *Tombusviridae*
Tomato chlorosis virus, (ToCV), *Closteroviridae*
Tomato chlorotic spot virus, (TCSV), *Bunyaviridae*
Tomato golden mosaic virus, (TGMV), *Geminiviridae*
Tomato infectious chlorosis virus, (TICV), *Closteroviridae*
Tomato leaf crumple virus, (TLCrV), *Geminiviridae*
Tomato leaf curl virus-Australia, (ToLCV-Au), *Geminiviridae*
Tomato leaf curl virus-Bangalore I, (ToLCV-BanI), *Geminiviridae*
Tomato leaf curl virus-Bangalore II, (ToLCV-BanII), *Geminiviridae*
Tomato leaf curl virus-New Delhi, (ToLCV-NDe), *Geminiviridae*
Tomato leaf curl virus-Senegal, (ToLCV-Sn), *Geminiviridae*
Tomato leaf curl virus-Taiwan, (ToLCV-Tw), *Geminiviridae*
Tomato leaf curl virus-Tanzania, (ToLCV-Tz), *Geminiviridae*
Tomato leaf curl virus satellite DNA, Satellite
Tomato leafroll virus, (TLRV), *Geminiviridae*
Tomato mosaic virus, (ToMV), *Tobamovirus*
Tomato mottle virus, (ToMoV), *Geminiviridae*
Tomato pale chlorosis virus, *Carlavirus*
Tomato planta macho viroid, (TPMVd), *Pospiviroidae*
Tomato pseudo-curly top virus, (TPCTV), *Geminiviridae*
Tomato ringspot virus, (ToRSV), *Comoviridae*
Tomato severe leaf curl virus, (ToSLCV), *Geminiviridae*
Tomato spotted wilt virus, (TSWV), *Bunyaviridae*
Tomato top necrosis virus, (ToTNV), *Comoviridae*
Tomato vein yellowing virus, (TVYV), *Rhabdoviridae*

Tomato yellow dwarf virus, (ToYDV), *Geminiviridae*
Tomato yellow leaf curl virus-China, (TYLCV-Ch), *Geminiviridae*
Tomato yellow leaf curl virus-Israel, (TYLCV-Is), *Geminiviridae*
Tomato yellow leaf curl virus-Nigeria, (TYLCV-Ng), *Geminiviridae*
Tomato yellow leaf curl virus-Sardinia, (TYLCV-Sar), *Geminiviridae*
Tomato yellow leaf curl virus-Southern Saudi Arabia, (TYLCV-SSA), *Geminiviridae*
Tomato yellow leaf curl virus-Tanzania, (TYLCV-Tz), *Geminiviridae*
Tomato yellow leaf curl virus-Thailand, (TYLCV-Th), *Geminiviridae*
Tomato yellow leaf curl virus-Yemen, (TYLCV-Ye), *Geminiviridae*
Tomato yellow mosaic virus, (ToYMV), *Geminiviridae*
Tomato yellow mottle virus, (ToYMoV), *Geminiviridae*
Tomato yellow top virus, *Luteoviridae*
Tomato yellow vein streak virus, (ToYVSV), *Geminiviridae*
Tongan vanilla virus, (TVV), *Potyviridae*
Topografov virus, (TOPV), *Bunyaviridae*
Tortoise virus 5, (TV5), *Iridoviridae*
Toscana virus, (TOSV), *Bunyaviridae*
Tradescantia/Zebrina virus, (TZV), *Potyviridae*
Trager duck spleen necrosis virus, (TDSNV), *Retroviridae*
Tranosema rostrale bracovirus, (TrBV), *Polydnaviridae*
Tranosema rostrales ichnovirus, (TrIV), *Polydnaviridae*
Transmissible gastroenteritis virus, (TGEV), *Coronaviridae*
Tree shrew adenovirus 1, (TSAdV-1), *Adenoviridae*
Tree shrew adenovirus, (TSAdV), *Adenoviridae*
Tree shrew herpesvirus, *Herpesviridae*
Triatoma virus, (TrV), Unassigned
Tribec virus, (TRBV), *Reoviridae*
Tribolium castaneum Woot virus, (TcaWooV), *Metaviridae*
Trichomonas vaginalis T1 virus, Satellite
Trichomonas vaginalis virus, (TVV), *Totiviridae*
Trichoplusia ni ascovirus 1a, (TnAV-1a), *Ascoviridae*
Trichoplusia ni granulovirus, (TnGV), *Baculoviridae*
Trichoplusia ni MNPV, (TnMNPV), *Baculoviridae*
Trichoplusia ni SNPV, (TnSNPV), *Baculoviridae*
Trichoplusia ni TED virus, (TniTedV), *Metaviridae*
Trichoplusia ni virus, (TnV), *Tetraviridae*
Trichosanthes mottle virus, (TrMoV), *Potyviridae*
Tripneustis gratilla SURL virus, (TgrSurV), *Metaviridae*
Triticum aestivum chlorotic spot virus, (TACSV), *Rhabdoviridae*
Triticum aestivum WIS-2 virus, (TaeWis2V), *Pseudoviridae*
Trivittatus virus, (TVTV), *Bunyaviridae*
Trombetas virus, (TRMV), *Bunyaviridae*
Tropaeolum mosaic virus, (TrMV), *Potyviridae*
Tropaeolum virus 1, (TV-1), *Potyviridae*
Tropaeolum virus 2, (TV-2), *Potyviridae*
Trubanaman virus, (TRUV), *Bunyaviridae*
Tsuruse virus, (TSUV), *Bunyaviridae*
Tuberose mild mosaic virus, (TuMMV), *Potyviridae*
Tucunduba virus, (TUCV), *Bunyaviridae*
Tula virus, (TULV), *Bunyaviridae*
Tulare apple mosaic virus, (TAMV), *Bromoviridae*
Tulip band breaking virus, (TBBV), *Potyviridae*
Tulip breaking virus, (TBV), *Potyviridae*
Tulip chlorotic blotch virus, *Potyviridae*
Tulip mild mottle mosaic virus, (TMMMV), *Ophiovirus*
Tulip top breaking virus, *Potyviridae*
Tulip virus X, (TVX), *Potexvirus*
Tumor virus X, (TVX), *Parvoviridae*
Tunis virus, (TUNV), *Bunyaviridae*
Tupaia virus, (TUPV), *Rhabdoviridae*
Tupaiid herpesvirus 1, (TuHV-1), *Herpesviridae*
Turbot herpesvirus, *Herpesviridae*
Turbot reovirus, (TRV), *Reoviridae*
Turbot virus-1, (TuV-1), *Picornaviridae*
Turkey adenovirus 1, 2, (TAdV-1, 2), *Adenoviridae*
Turkey adenovirus, (TAdV), *Adenoviridae*
Turkey astrovirus 1, (TAstV-1), *Astroviridae*
Turkey astrovirus, (TAstV), *Astroviridae*
Turkey coronavirus, (TCoV), *Coronaviridae*
Turkey entero-like virus, (TELV), *Picornaviridae*
Turkey hemorrhagic enteritis virus (HEV), (TAdV-3), *Adenoviridae*
Turkey hepatitis virus, (THV), *Picornaviridae*
Turkey herpesvirus, *Herpesviridae*
Turkey pseudo enterovirus 1 to 2, (TPEV-1, 2), *Picornaviridae*
Turkey rhinotracheitis virus, (TRTV), *Paramyxoviridae*
Turkeypox virus, (TKPV), *Poxviridae*
Turlock virus, (TURV), *Bunyaviridae*
Turnaca rufisquamata virus, (TrV), Unassigned
Turnip crinkle virus satellite RNA, Satellite
Turnip crinkle virus, (TCV), *Tombusviridae*
Turnip mild yellows virus, *Luteoviridae*
Turnip mosaic virus, (TuMV), *Potyviridae*
Turnip rosette virus, (TRoV), *Sobemovirus*
Turnip vein-clearing virus, (TVCV), *Tobamovirus*
Turnip yellow mosaic virus, (TYMV), *Tymovirus*

Turuna virus, (TUAV), *Bunyaviridae*
Tyuleniy virus, (TYUV), *Flaviviridae*

Uasin Gishu disease virus, (UGDV), *Poxviridae*
Uganda S virus, (UGSV), *Flaviviridae*
Ulcerative disease rhabdovirus, (UDRV), *Rhabdoviridae*
Ullucus mild mottle virus, (UMMV), *Tobamovirus*
Ullucus mosaic virus, (UMV), *Potyviridae*
Ullucus virus C, (UVC), *Comoviridae*
Umatilla virus, (UMAV), *Reoviridae*
Umatilla virus, (UMAV), *Reoviridae*
Umbre virus, (UMBV), *Bunyaviridae*
Una virus, (UNAV), *Togaviridae*
Upolu virus, (UPOV), *Bunyaviridae*
UR2 sarcoma virus, (UR2SV), *Retroviridae*
Urmurtia/338Cg/92 virus, *Bunyaviridae*
Urochloa hoja blanca virus, (UHBV), *Tenuivirus*
Urucuri virus, (URUV), *Bunyaviridae*
Ustilago maydis killer M virus, Satellite
Ustilago maydis virus H1, (UmV-H1), *Totiviridae*
Usutu virus, (USUV), *Flaviviridae*
Utinga virus, (UTIV), *Bunyaviridae*
Utive virus, (UVV), *Bunyaviridae*
Uukuniemi virus S 23, (UUKV), *Bunyaviridae*
Uukuniemi virus, (UUKV), *Bunyaviridae*

Vaccinia virus, (VACV), *Poxviridae*
Vallota mosaic virus, (ValMV), *Potyviridae*
Vanilla mosaic virus, (VanMV), *Potyviridae*
Vanilla necrosis virus, *Potyviridae*
Varicella-zoster virus, *Herpesviridae*
Variola virus, (VARV), *Poxviridae*
VAV-488 virus, *Arenaviridae*
VAV-499 virus, *Arenaviridae*
Vearoy virus, (VAEV), *Reoviridae*
Vellore virus, (VELV), *Reoviridae*
Velvet tobacco mottle virus satellite RNA, Satellite
Velvet tobacco mottle virus, (VTMoV), *Sobemovirus*
Venezuelan equine encephalitis virus, (VEEV), *Togaviridae*
Vesicular exanthema of swine virus, (VESV), *Caliciviridae*
Vesicular exanthema of swine virus-A48, (VESV-A48), *Caliciviridae*
Vesicular stomatitis Alagoas virus, (VSAV), *Rhabdoviridae*
Vesicular stomatitis Indiana virus, (VSIV), *Rhabdoviridae*
Vesicular stomatitis New Jersey virus, (VSNJV), *Rhabdoviridae*
Vibrio phage 06N-22P, (06N-22P), *Myoviridae*
Vibrio phage 06N-58P, (06N-58P), *Corticoviridae*
Vibrio phage 06N-58P, *Tectiviridae*
Vibrio phage 493, (493), *Inoviridae*
Vibrio phage 4996, (4996), *Podoviridae*
Vibrio phage CTX, (CTX), *Inoviridae*
Vibrio phage fs1, (fs1), *Inoviridae*
Vibrio phage fs2, (fs2), *Inoviridae*
Vibrio phage I, (I), *Podoviridae*
Vibrio phage II, (II), *Myoviridae*
Vibrio phage III, (III), *Podoviridae*
Vibrio phage IV, (IV), *Siphoviridae*
Vibrio phage kappa, (kappa), *Myoviridae*
Vibrio phage KVP20, (KVP20), *Myoviridae*
Vibrio phage KVP40, (KVP40), *Myoviridae*
Vibrio phage nt-1, (nt-1), *Myoviridae*
Vibrio phage O6N-72P, (06N-72P), *Podoviridae*
Vibrio phage OXN-100P, (OXN-100P), *Podoviridae*
Vibrio phage OXN-52P, (OXN-52P), *Siphoviridae*
Vibrio phage P147, (P147), *Myoviridae*
Vibrio phage v6, (v6), *Inoviridae*
Vibrio phage VcA3, (VcA3), *Myoviridae*
Vibrio phage Vf12, (Vf12), *Inoviridae*
Vibrio phage Vf33, (Vf33), *Inoviridae*
Vibrio phage VP1, (VP1), *Myoviridae*
Vibrio phage VP11, (VP11), *Siphoviridae*
Vibrio phage VP3, (VP3), *Siphoviridae*
Vibrio phage VP5, (VP5), *Siphoviridae*
Vibrio phage VSK, (VSK), *Inoviridae*
Vibrio phage X29, (X29), *Myoviridae*
Vibrio phage $\alpha 3\alpha$, ($\alpha 3\alpha$), *Siphoviridae*
Vibrio phage ϕ149 (type IV), (ϕ149), *Siphoviridae*
Vibrio phage ϕVP253, (ϕVP253), *Myoviridae*
Vicia cryptic virus, (VCV), *Partitiviridae*
Vicia faba 447 cytoplasmic male sterility-associated virus, (VfCMSaV), Unassigned
Vigna sinensis mosaic virus, (VSMV), *Rhabdoviridae*
Vilyuisk human encephalomyelitis virus, (VHEV), *Picornaviridae*
Vinces virus, (VINV), *Bunyaviridae*
Vindeln/L20Cg/83 virus, *Bunyaviridae*
Viola mottle virus, (VMoV), *Potexvirus*
Viper retrovirus, (VRV), *Retroviridae*
Viral hemorrhagic septicemia virus, (VHSV), *Rhabdoviridae*
Virgin River virus, (VRV), *Bunyaviridae*
Visna/maedi virus (strain 1514), (VISNA), *Retroviridae*
Voandzeia mosaic virus, *Carlavirus*
Voandzeia necrotic mosaic virus, (VNMV) *Tymovirus*
Vole poxvirus, (VPV), *Poxviridae*
Volepox virus, (VPXV), *Poxviridae*
Volvox carteri Osser virus, (VcaOssV), *Pseudoviridae*
Vranica virus, *Bunyaviridae*

W10777 virus, *Arenaviridae*
Wad Medani virus, (WMV), *Reoviridae*
Wad Medani virus, (WMV), *Reoviridae*
Wallal virus, (WALV), *Reoviridae*
Wallal virus, (WALV), *Reoviridae*
Walleye dermal sarcoma virus, (WDSV), *Retroviridae*
Walleye epidermal hyperplasia virus 1, (WEHV-1), *Retroviridae*
Walleye epidermal hyperplasia virus 2, (WEHV-2), *Retroviridae*
Walleye epidermal hyperplasia, *Herpesviridae*
Walrus calicivirus, (WCV), *Caliciviridae*
Wanowrie virus, (WANV), *Bunyaviridae*
Warrego virus, (WARV), *Reoviridae*
Warrego virus, (WARV), *Reoviridae*
Water buffalo herpesvirus, *Herpesviridae*
Watercress yellow spot virus, (WYSV), Unassigned
Watermelon bud necrosis virus, (WBNV), *Bunyaviridae*
Watermelon chlorotic stunt virus, (WmCSV), *Geminiviridae*
Watermelon curly mottle virus, (WmCMV), *Geminiviridae*
Watermelon mosaic virus 1, *Potyviridae*
Watermelon mosaic virus 2, *Potyviridae*
Watermelon mosaic virus, (WMV), *Potyviridae*
Watermelon silver mottle virus, (WSMoV), *Bunyaviridae*
WE virus, *Arenaviridae*
Weddel waterborne virus, (WWBV), *Tombusviridae*
Weldona virus, (WELV), *Bunyaviridae*
Welsh onion yellow stripe virus, *Potyviridae*
Wesselsbron virus, (WESSV), *Flaviviridae*
West Nile virus, (WNV), *Flaviviridae*
Western equine encephalitis virus, (WEEV), *Togaviridae*
Wexford virus, (WEXV), *Reoviridae*
Whataroa virus, (WHAV), *Togaviridae*
Wheat American striate mosaic virus, (WASMV), *Rhabdoviridae*
Wheat chlorotic streak virus, (WCSV), *Rhabdoviridae*
Wheat dwarf virus, (WDV), *Geminiviridae*
Wheat rosette stunt virus, (WRSV), *Rhabdoviridae*
Wheat spindle streak mosaic virus, (WSSMV), *Potyviridae*
Wheat streak mosaic virus, (WSMV), *Potyviridae*
Wheat yellow leaf virus, (WYLV), *Closteroviridae*
Wheat yellow mosaic virus, (WYMV), *Potyviridae*
White bryony mosaic virus, (WBMV), *Carlavirus*
White bryony virus, (WBV), *Potyviridae*
White clover cryptic virus 1, (WCCV-1), *Partitiviridae*
White clover cryptic virus 2, (WCCV-2), *Partitiviridae*
White clover cryptic virus 3, (WCCV-3), *Partitiviridae*
White clover mosaic virus, (WClMV), *Potexvirus*
White clover virus L, (WClVL), Unassigned
White lupin mosaic virus, *Potyviridae*
White sturgeon herpesvirus 1, *Herpesviridae*
White Sturgeon herpesvirus 2, *Herpesviridae*
Whitewater Arroyo virus, (WWAV), *Arenaviridae*
Wild cucumber mosaic virus, (WCMV), *Tymovirus*
Wild potato mosaic virus, (WPMV), *Potyviridae*
Winter wheat mosaic virus, (WWMV), *Tenuivirus*
Winter wheat Russian mosaic virus, (WWMV), *Rhabdoviridae*
Wiseana iridescent virus, *Iridoviridae*
Wissadula golden mosaic virus, (WGMV), *Geminiviridae*
Wisteria vein mosaic virus, (WVMV), *Potyviridae*
Witlesia iridescent virus, *Iridoviridae*
Witwatersrand virus, (WITV), *Bunyaviridae*
Wongal virus, (WONV), *Bunyaviridae*
Wongorr virus CS131, (WGRV- CS131), *Reoviridae*
Wongorr virus MRM13443, (WGRV- MRM13443), *Reoviridae*
Wongorr virus V1447, (WGRV- V1447), *Reoviridae*
Wongorr virus V195, (WGRV- V195), *Reoviridae*
Wongorr virus V199, (WGRV- V199), *Reoviridae*
Wongorr virus V595, (WGRV- V595), *Reoviridae*
Wongorr virus, (WGRV), *Reoviridae*
Woodchuck hepatitis virus, (WHV), *Hepadnaviridae*
Woodchuck herpesvirus, *Herpesviridae*
Woolly monkey sarcoma virus, (WMSV), *Retroviridae*
Wound tumor virus, (WTV), *Reoviridae*
Wyeomyia virus, (WYOV), *Bunyaviridae*

Xanthomonas phage Cf16, (Cf16), *Inoviridae*
Xanthomonas phage Cf1c, (Cf1c), *Inoviridae*
Xanthomonas phage Cf1t, (Cf1t), *Inoviridae*
Xanthomonas phage Cf1tv, (Cf1tv), *Inoviridae*
Xanthomonas phage Lf, (Lf), *Inoviridae*
Xanthomonas phage RR66, (RR66), *Podoviridae*
Xanthomonas phage Xf, (Xf), *Inoviridae*
Xanthomonas phage Xfo, (Xfo), *Inoviridae*
Xanthomonas phage Xfv, (Xfv), *Inoviridae*
Xanthomonas phage XP5, (XP5), *Myoviridae*
Xestia c-nigrum granulovirus, (XecnGV), *Baculoviridae*
Xiburema virus, (XIBV), *Rhabdoviridae*
Xingu virus, (XINV), *Bunyaviridae*
XJ virus, *Arenaviridae*

Y73 sarcoma virus, (Y73SV), *Retroviridae*
Yaba monkey tumor virus, (YMTV), *Poxviridae*
Yaba-1 virus, (Y1V), *Bunyaviridae*
Yaba-7 virus, (Y7V), *Bunyaviridae*
Yacaaba virus, (YACV), *Bunyaviridae*
Yam mosaic virus, (YMV), *Potyviridae*
Yaounde virus, (YAOV), *Flaviviridae*
Yaquina Head virus, (YHV), *Reoviridae*
Yata virus, (YATAV), *Rhabdoviridae*
Yellow fever virus, (YFV), *Flaviviridae*
Yellowtail ascites virus, (YTAV), *Birnaviridae*
Yogue virus, (YOGV), *Bunyaviridae*
Yoka poxvirus, (YKV), *Poxviridae*
Yokose virus, (YOKV), *Flaviviridae*
Youcai mosaic virus, (YoMV), *Tobamovirus*
Yucca bacilliform virus, (YBV), *Caulimoviridae*
Yug Bogdanovac virus, (YBV), *Rhabdoviridae*

Zaire Ebola virus Eckron, *Filoviridae*
Zaire Ebola virus Gabon, *Filoviridae*
Zaire Ebola virus Kikwit, *Filoviridae*
Zaire Ebola virus Mayinga, *Filoviridae*
Zaire Ebola virus Tandala, *Filoviridae*
Zaire Ebola virus Zaire, *Filoviridae*
Zaire Ebola virus, (ZEBOV), *Filoviridae*
Zaliv Terpeniya virus, (ZTV), *Bunyaviridae*
Zea mays Hopscotch virus, (ZmaHopV), *Pseudoviridae*
Zea mays virus, (ZMV), *Rhabdoviridae*
Zegla virus, (ZEGV), *Bunyaviridae*
Zika virus, (ZIKV), *Flaviviridae*
Zinnia leaf curl virus, (ZiLCV), *Geminiviridae*
Zirqa virus, (ZIRV), *Bunyaviridae*
Zoysia mosaic virus, (ZoMV), *Potyviridae*
Zucchini lethal chlorosis virus, (ZLCV), *Bunyaviridae*
Zucchini yellow fleck virus, (ZYFV), *Potyviridae*
Zucchini yellow mosaic virus, (ZYMV), *Potyviridae*
Zygocactus symptomless virus, (ZSLV), *Potexvirus*

Plate 1

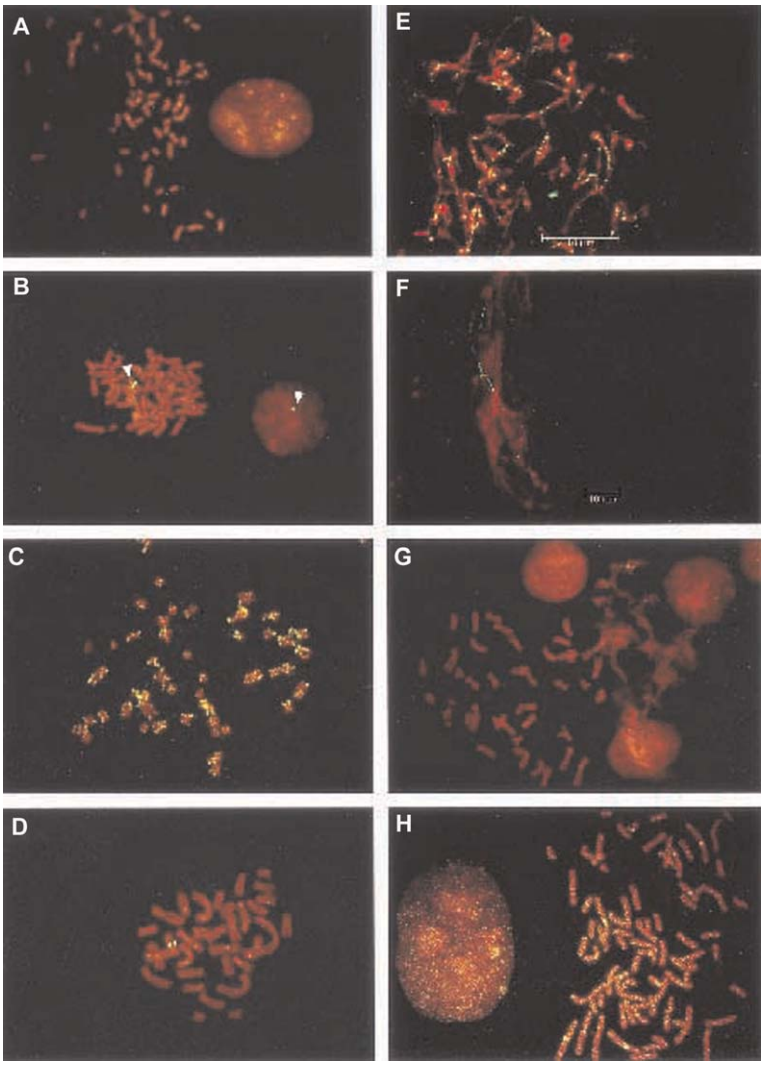


Plate 1 Association of Ad12 DNA with host cell chromosomes.(**A**) Human HeLa cells, uninfected control. (**B**) T637 cells, uninfected: arrowhead points to chromosomally integrated Ad12 genomes. (**C**) Productively Ad12-infected HeLa cells, 6 h p.i. (**D**) Abortively Ad12-superinfected T637 cells, 6 h p.i. (**E**) Productively Ad12-infected HeLa cells, 6 h p.i. (stretched chromosome preparation). (**F**) Ad12-transformed T637 cells (stretched chromosome preparation). (**G**) BHK21 cells, Ad12 DNA added to the medium. (**H**) BHK21, Ad12-DNA-TP added to the medium. Original magnification $\times$ 1250 (**A**–**D**, **G**, **H**); (**E**, **F**) see bar. (Reproduced with permission from Schröer *et al* (1997).) See article (**Adenoviruses *Adenoviridae*): Molecular Biology** for more information.

Plate 2

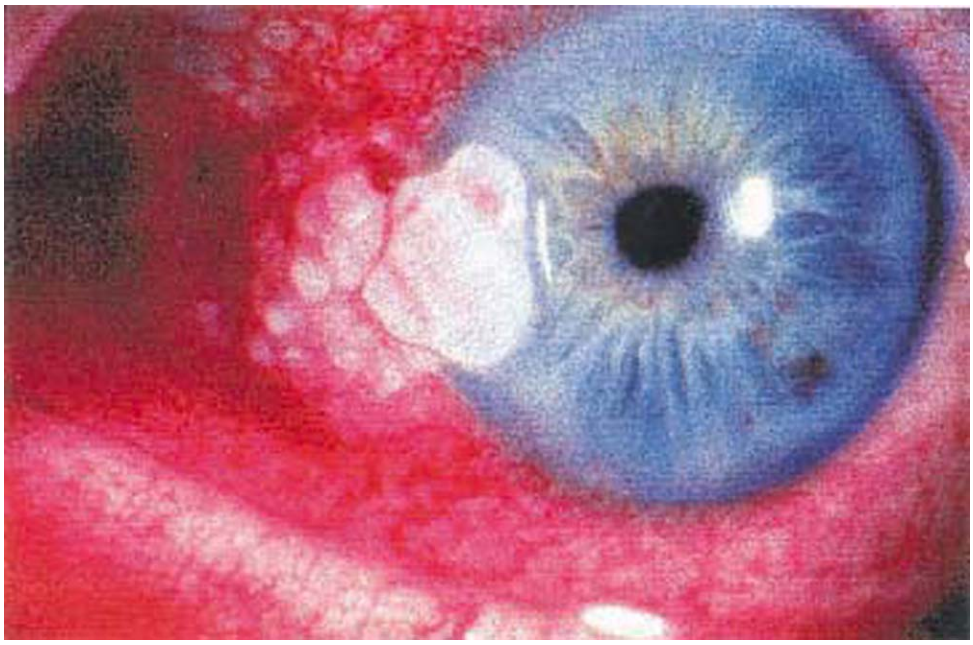


Plate 2 Squamous cell carcinoma of the corneal limbus is associated with infection by human papilloma virus types 16 and 18. See article **Eye Infections** for more information.

Plate 3

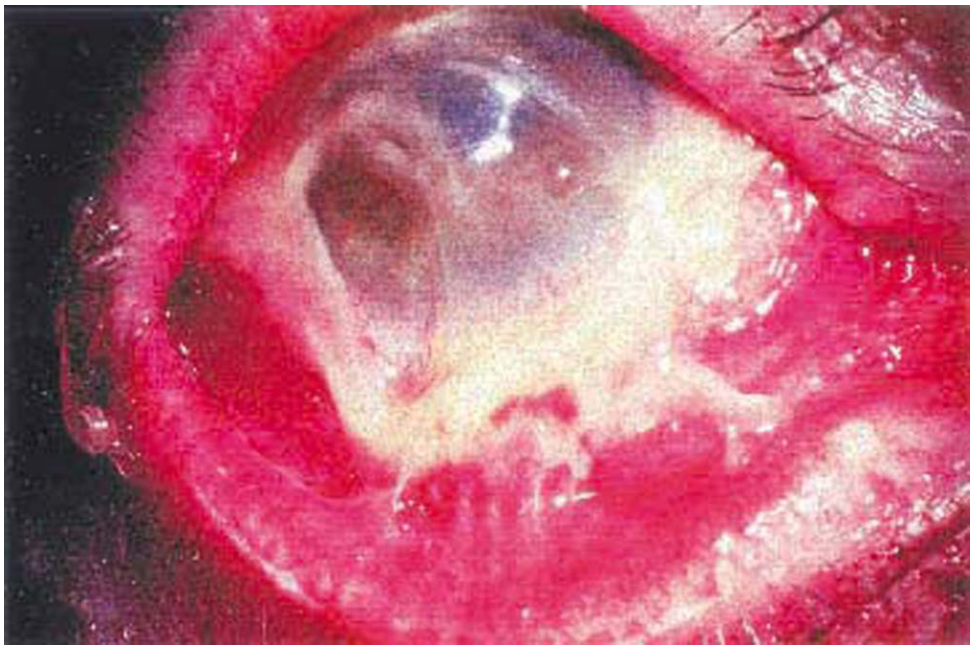


Plate 3 Epidemic keratoconjunctivitis. Infection with adenovirus serotype 19 has resulted in severe ocular surface inflammation. See article **Eye Infections** for more information.

Plate 4

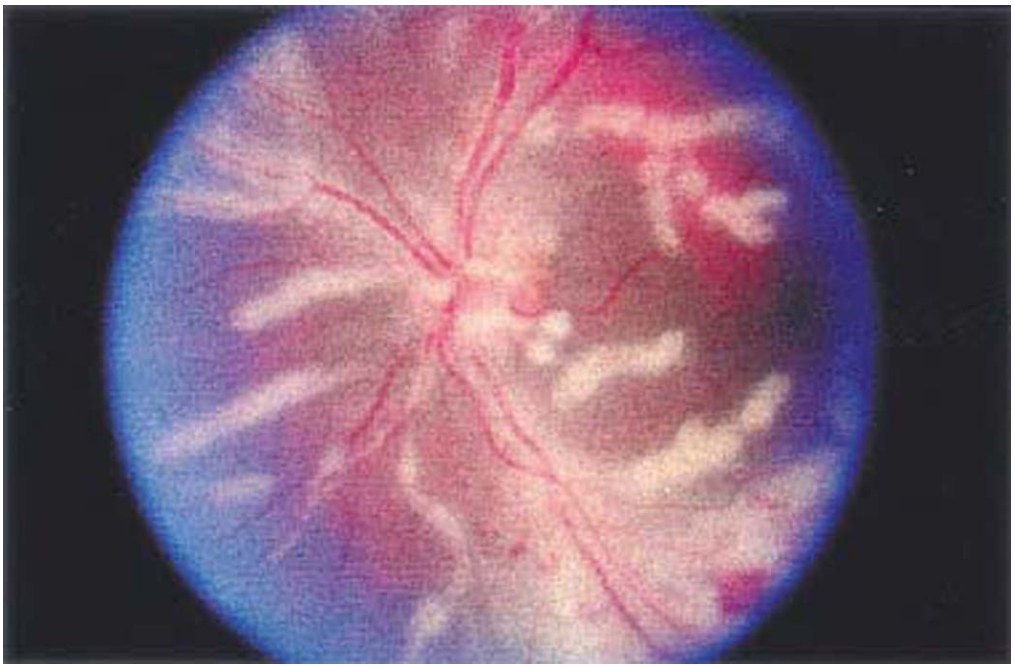


Plate 4 Cytomegalovirus retinitis. Discrete areas of perivascular necrosis and hemorrhage are typical. See article **Eye Infections** for more information.

Plate 5

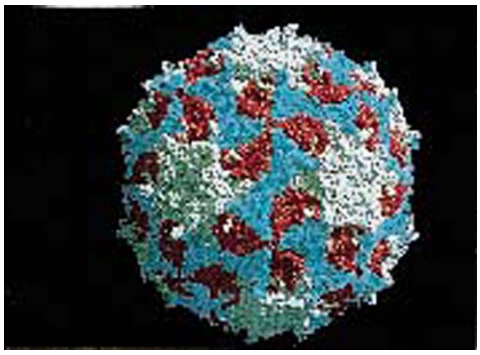


Plate 5 Computer graphics representation of the Mengo virion at a resolution of 3. ϕ 0 (Luo et al., 1987). Each small ball represents a single amino acid residue. The VP1 polypeptides are colored white, VP2 polypeptides are blue and VP3 polypeptides are red. See article **Cardioviruses** (*Picornaviridae*) for more information.

Plate 6

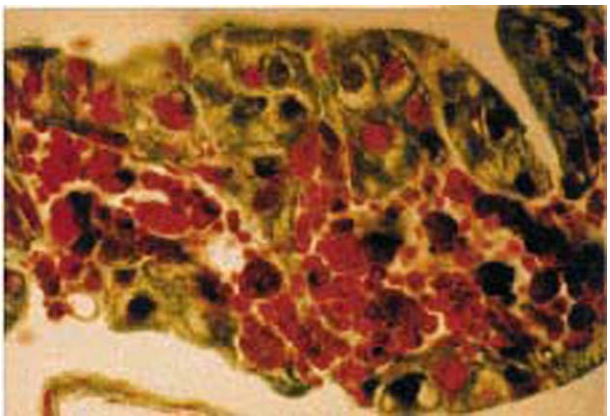


Plate 6 Section through the fat body tissue of the caterpillar, Spodoptera frugiperda, infected with an ascovirus. The green areas are uninfected cells or cells in early stages of infection. The red cells are infected cells and clusters of spherical virion-containing vesicles eventually disseminate into the hemolymph (blood) through ruptures in the basal lamina of the fat body tissue. See article **Ascoviruses (*Ascoviridae*)** for more information.

Plate 7

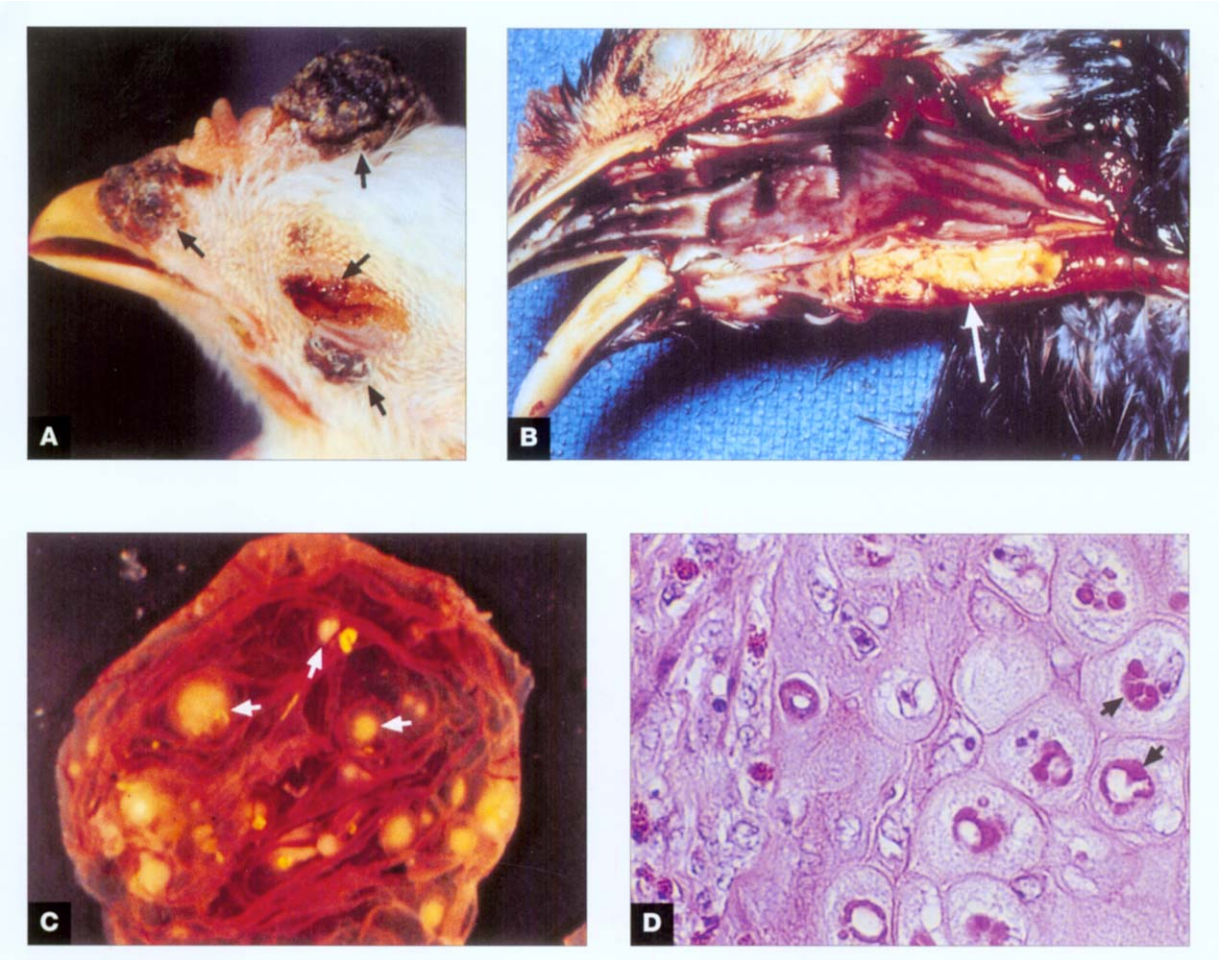


Plate 7 (**A**) Cutaneous lesions of fowlpox. (**B**) Diphtheritic fowlpox – tracheal plug. (**C**) Fowlpox virus lesions (pocks) in the chorioallantoic membrane of a developing chicken embryo. (**D**) Cells infected with fowlpox virus are enlarged and contain cytoplasmic inclusion bodies (arrows). Parts B and D are part of Fowlpox Slide Study Set prepared by Dr Tripathy for American Association of Avian Pathologists. See article **Fowlpox Viruses (*Poxviridae*)** for more information.

Plate 8

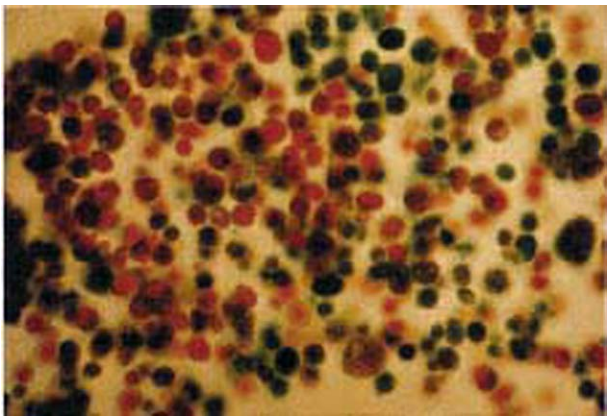


Plate 8 Hemolymph (blood) of the caterpillar, Spodoptera frugiperda, infected with an ascovirus. The green spherical vesicles are hemocytes and virion-containing vesicles in which virion assembly is at an early stage. The red vesicles contain large numbers of fully mature virions. Paraffin section stained with Hamm's strain. 900X. See article **Ascoviruses (*Ascoviridae*)** for more information.

Plate 9

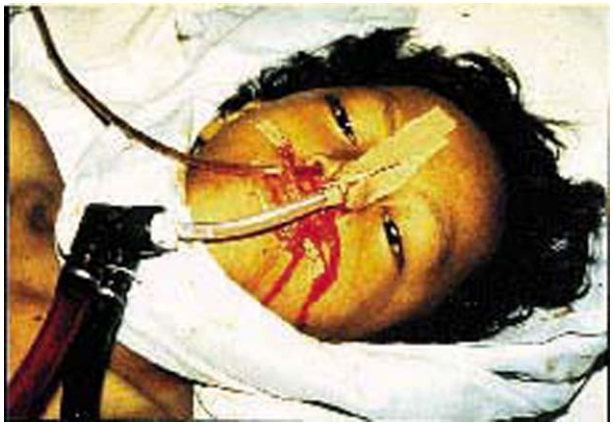


Plate 9 A child with dengue hemorrhagic fever. See article **Dengue Viruses (*Flaviviridae*)** for more information.

Plate 10

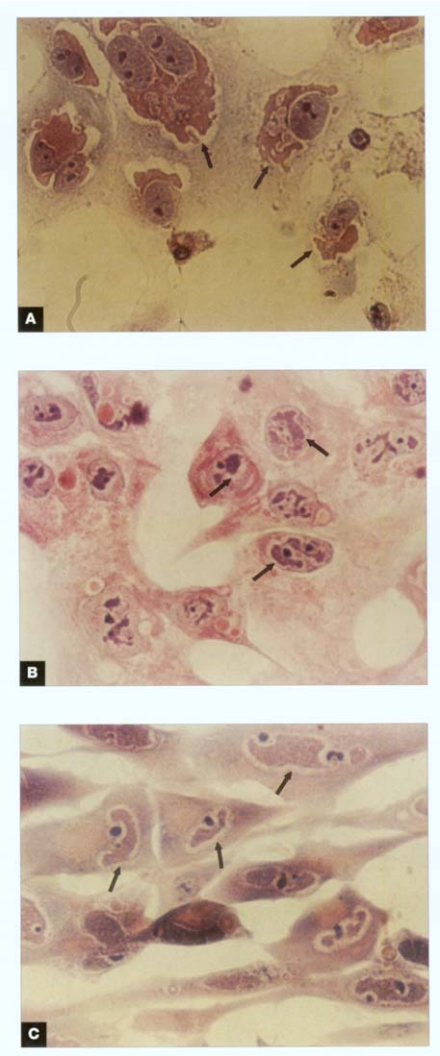


Plate 10 (**A**) Reovirus type 3 infected rhesus monkey kidney cells showing eosinophilic cytoplasmic inclusions (arrows) (H & E, 400X). (**B**) Adenovirus type 2 infected human embryonic kidney cells showing basophilic intranuclear inclusions (arrows) (H & E, 400X). (**C**) Human cytomegalovirus infected human diploid fibroblastic cells showing eosinophilic intranuclear inclusions (arrows) (H & E, 400X). See article **Diagnostic Techniques: Isolation and Identification by Culture and Microscopy** for more information.

Plate 11

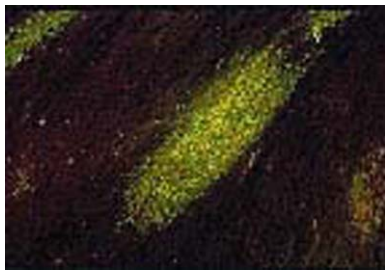


Plate 11 Shell vial culture stained 16 h postinoculation showing CMV immediate early antigens detected in the nuclei of infected human fibroblast cells (immunofluorescence test). See article **Diagnostic Techniques: Isolation and Identification by Culture and Microscopy** for more information.

Plate 12

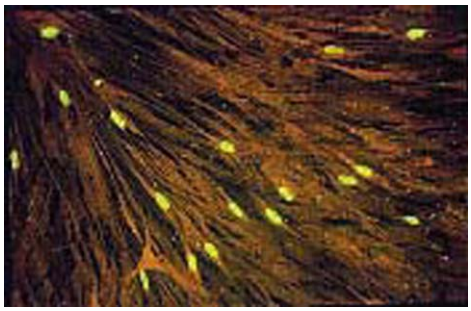


Plate 12 Shell vial culture stained 2 days postinoculation showing foci of varicella zoster virus-infected human fibroblast cells (immunofluorescence test). See article **Diagnostic Techniques: Isolation and Identification by Culture and Microscopy** for more information.

Plate 13

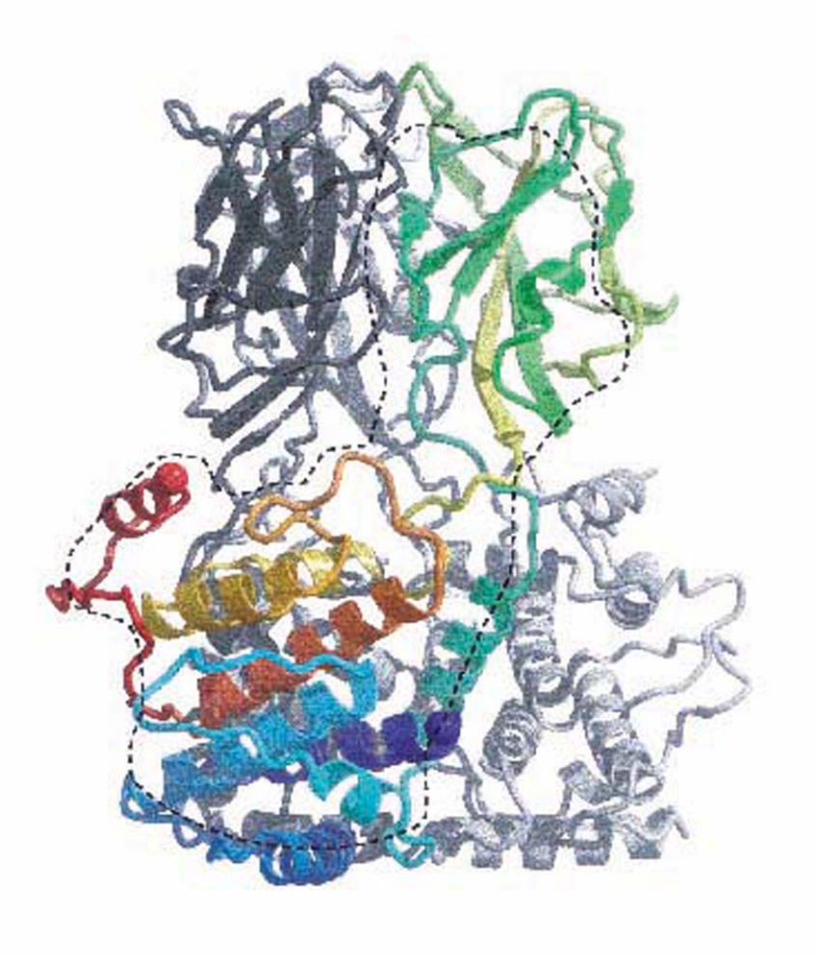


Plate 13 The structure of VP7 trimer rendered from x-ray crystallographic analysis. The threefold symmetry axis of the trimer is disposed vertically so that the flat base at the bottom of the image forms the inner surface of the outer VP7 layer of the core, which adheres to the VP3 subcore. Note the clear division into two domains, the upper domain contains mainly β-strands (shown as arrows), whereas the lower domain is constructed of a number of closely packed helices. (Reproduced from Grimes *et al* (1995) *Nature* 373: 167.) One subunit is outlined with a dashed line to clarify the twist of the subunit trimer with respect to the molecular 3-fold axis. See article **Orbiviruses and Coltiviruses (*Reoviridae*): Molecular Biology** for more information.

Plate 14

Plate 14 Colorized electron microscopy picture of the human immunodeficiency virus (HIV) showing, from right to left, the budding of a viral particle from the membrane of an infected CD4 lymphocyte, an immature particle and two mature virions; note the characteristic cone-shaped core of the upper mature virion. **Human Immunodeficiency Viruses (*Retroviridae*): Antiretroviral Agents** for more information.

Plate 15

Plate 15 Surface representation of the three-dimensional structure of BTV cores (deduced by electron cryo-microscopy at 25Å resolution) showing the trieric configuration of VP7 molecules. See article **Diagnostic Techniques: Isolation and Identification by Culture and Microscopy** for more information.

Plate 16

Plate 16 Symptoms of tobacco mosaic virus (TMV) on tobacco leaf leaves. Center-healthy. Right-leaf of a susceptible cultivar showing the typical mosaic symptoms of systemic TMV infection. Left-leaf of a cultivar containing the N-gene for TMV resistance showing the necrotic local lesions which form around each site of infection and to which the virus is restricted. See article **Plant Resistence to Viruses: Natural Resistence** for more information.

Plate 17

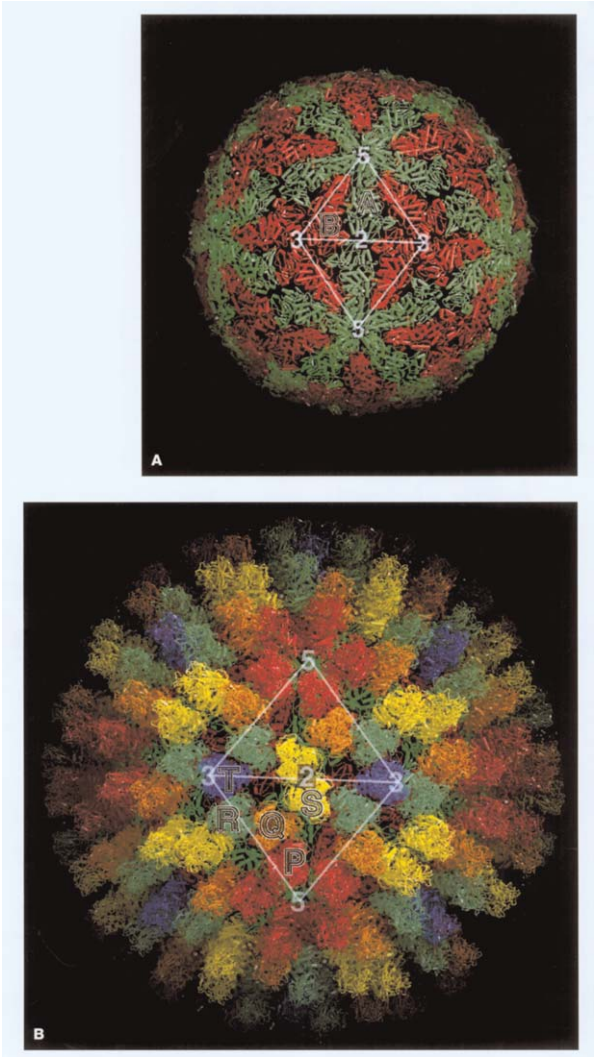


Plate 17 The essential features of the architecture of the native BTV core as determined by x-ray crystallography and cryoelectron microscopy. The icosahedral asymmetric unit is the triangular area defined (as marked) by the symmetric axes of the icosahedron. (**B**) The core surface layer composed of 780 copies of VP7, arranged as 260 trimers with $T = 13$ symmetry. The asymmetric unit contains 13 copies of VP7, arranged as five trimers P, Q, R, S and T: colored red, orange, green, yellow and blue, respectively. Trimer 'T' sits on the icosahedral threefold axis, and thus contributes only a monomer to the unique portion of the capsid. (**A**) The inner capsid layer of the BTV core (the subcore) composed of 120 molecules of VP3, arranged with $T = 2$ symmetry according to the principles of 'geometrical quasi-equivalence'. The icosahedrally unique molecules, of VP3 A and B, are colored in red and green, respectively. Note the completely different structural environment of the A and B molecules. (Reproduced from Grimes *et al* (1998) *Nature* 395: 470–478. Copyright 1998, Macmillan Magazines Ltd. See article **Orbiviruses and Coltiviruses (*Reoviridae*): Molecular Biology** for more information.

Plate 18

Plate 18 A representation of the VP3 B molecule, color-coded by domain. The 'apical' domain is shown in blue (residue 297–588), the 'carapace' domain in green (residues 7–297, 588–698 and 855–901) and the 'dimerization' domain in red (residue 698–855). (With acknowledgement to J. Grimes and D. Stuart for provision of the image.) See article **Orbiviruses and Coltiviruses (*Reoviridae*): Molecular Biology** for more information.

Plate 19

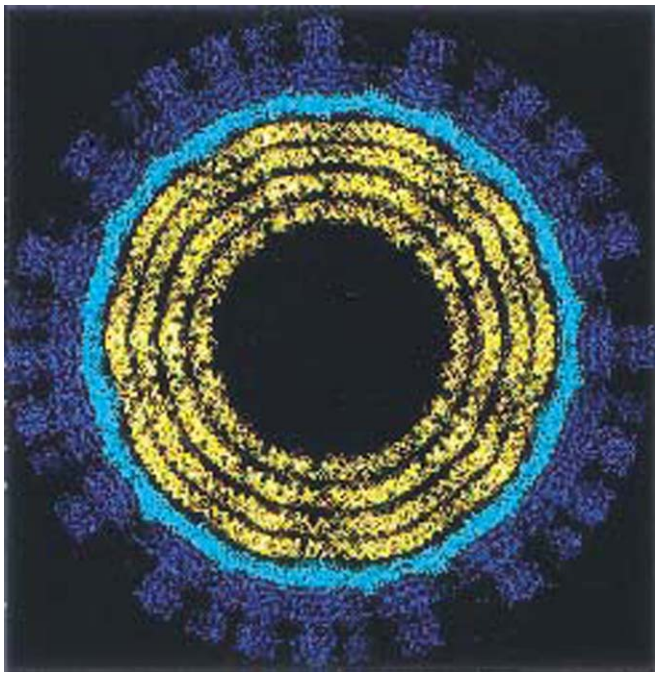


Plate 19 Thin section through the structure of the core of BTV (as determined by x-ray crystallography of native cores of BTV1 and BTV10). The VP7 outer layer of the core is shown in dark blue, the thin VP3 subcore layer in pale blue and the four layers of the genomic dsRNA are predominantly yellow. (Reproduced from Stuart *et al.* (1998) African Horse Sickness Virus. *Arch. Virol. Suppl.* 14: 235–250.) See article **Orbiviruses and Coltiviruses (*Reoviridae*): Molecular Biology** for more information.

Plate 20

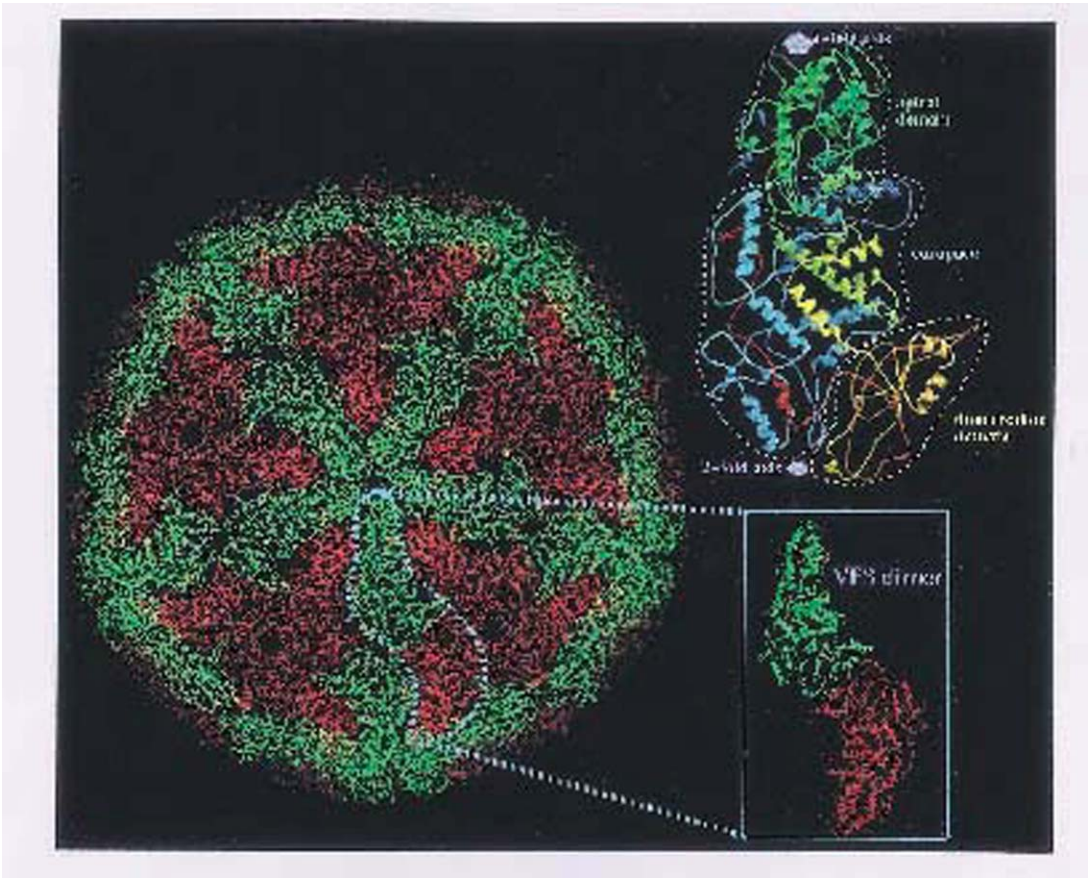


Plate 20 Overall structure and architecture of the VP3 layer of the BTV subcore. Each of 120 molecules has a structure similar to that illustrated on the right of the image. (Reproduced from Stuart *et al* (1998) African Horse Sickness Virus. *Arch. Virol. Suppl.* 14: 235–250.) See article **Orbiviruses and Coltiviruses (*Reoviridae*): Molecular Biology** for more information.

Plate 21

Plate 21 Paralysis of the legs in Marek's disease. See article **Marek's Disease Virus (*Herpesviridae*)** for more information.

Plate 22

Plate 22 Enlarged brachial and sciatic nerves (arrows) in Marek's disease. See article **Marek's Disease Virus (*Herpesviridae*)** for more information.

Plate 23

Plate 23 Ovarian lymphoma (arrow) in Marek's disease. See article **Marek's Disease Virus (*Herpesviridae*)** for more information.

Plate 24

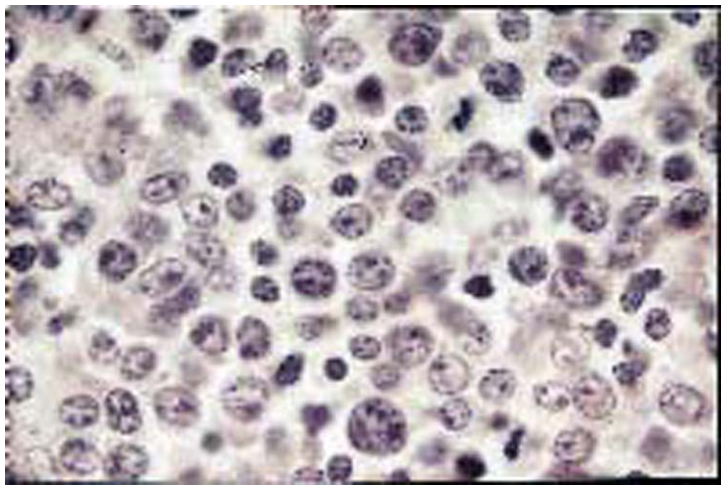


Plate 24 Neural lymphoma in Marek's disease showing varying morphology of lymphoid cells. See article **Marek's Disease Virus (*Herpesviridae*)** for more information.

Plate 25

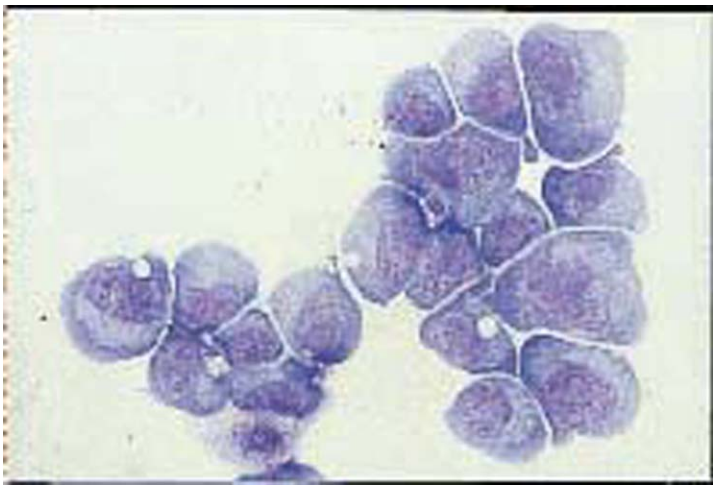


Plate 25 Impression smear of lymphoblastoid cell line derived from a Marek's disease lymphoma. See article **Marek's Disease Virus (*Herpesviridae*)** for more information.

Plate 26

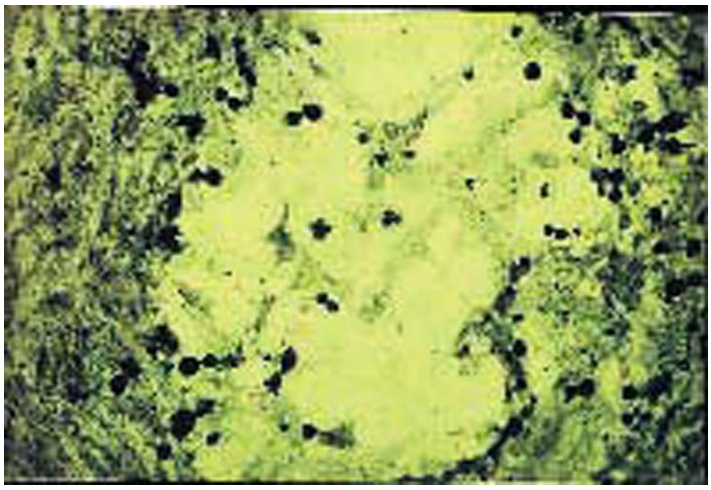


Plate 26 Cytopathic plaque induced in chick kidney cell monolayer culture by Marek's disease virus. See article **Marek's Disease Virus (*Herpesviridae*)** for more information.

Plate 27

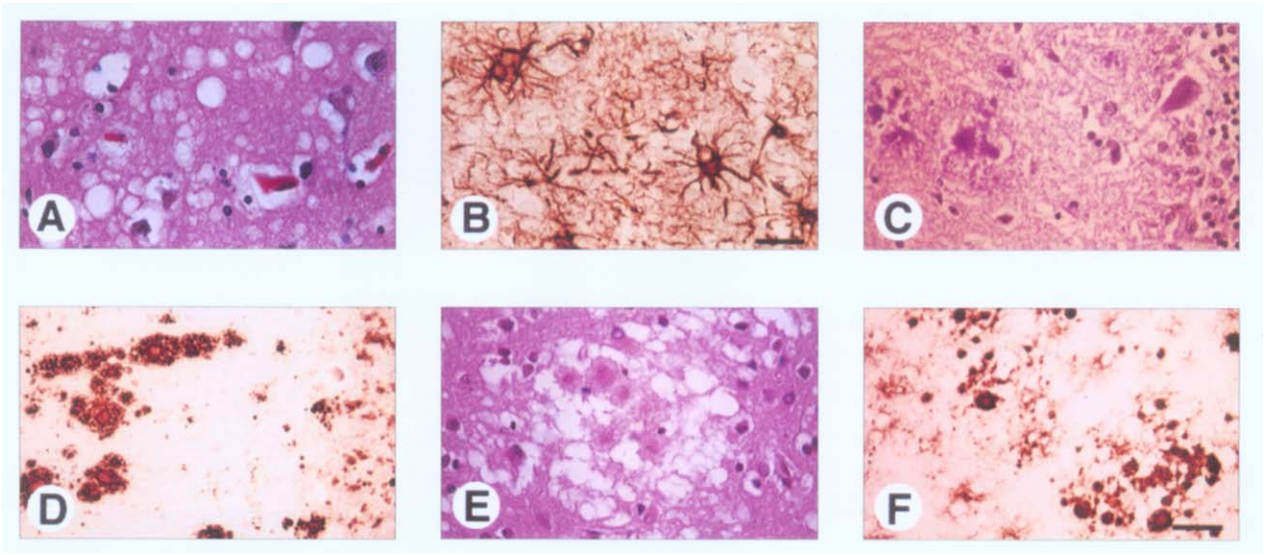


Plate 27 Neuropathology of human prion diseases. Sporadic CJD is characterized by vacuolation of the neuropil of the gray matter, by exuberant reactive astrocytic gliosis, the intensity of which is proportional to the degree of nerve cell loss, and, rarely, by PrP amyloid plaque formation (not shown). The neuropathology of familial CJD is similar. GSS (P102L), as well as other inherited forms of GSS (not shown), is characterized by numerous deposits of PrP amyloid throughout the CNS. New variant CJD (vCJD) has clinical and epidemiological features that suggest it was acquired by infection with prions. The neuropathological features of vCJD are unique among CJD cases because of the abundance of PrP amyloid plaques that are often surrounded by a halo of intense vacuolation. (**A**) Sporadic CJD, cerebral cortex stained with hematoxylin and eosin showing widespread spongiform degeneration. (**B**) Sporadic CJD, cerebral cortex immunostained with anti-GFAP antibodies demonstrating the widespread reactive gliosis. (**C**) GSS, cerebellum with most of the GSS-plaques in the molecular layer (left 80% of micrograph) and many but not all are periodic acid Schiff (PAS) reaction positive. Granule cells and a single Purkinje cell are seen in the right 20% of the panel. (**D**) GSS, cerebellum at the same location as panel C with PrP immunohistochemistry after the hydrolytic autoclaving reveals more PrP plaques than seen with the PAS reaction. (**E**) Variant CJD, cerebral cortex stained with hematoxylin and eosin shows that the plaque deposits are uniquely located within vacuoles. With this histology, these amyloid deposits have been referred to as 'florid plaques'. (**F**) Variant CJD, cerebral cortex stained with PrP immunohistochemistry after hydrolytic autoclaving reveals numerous PrP plaques often occurring in clusters as well as minute PrP deposits surrounding many cortical neurons and their proximal processes. Bar in B = 50 μm and applies also to panels A, C, and D. Bar in F = 100 mu;m and applies also to panel D. See article **Prions: Human and Animal** for more information.

Plate 28

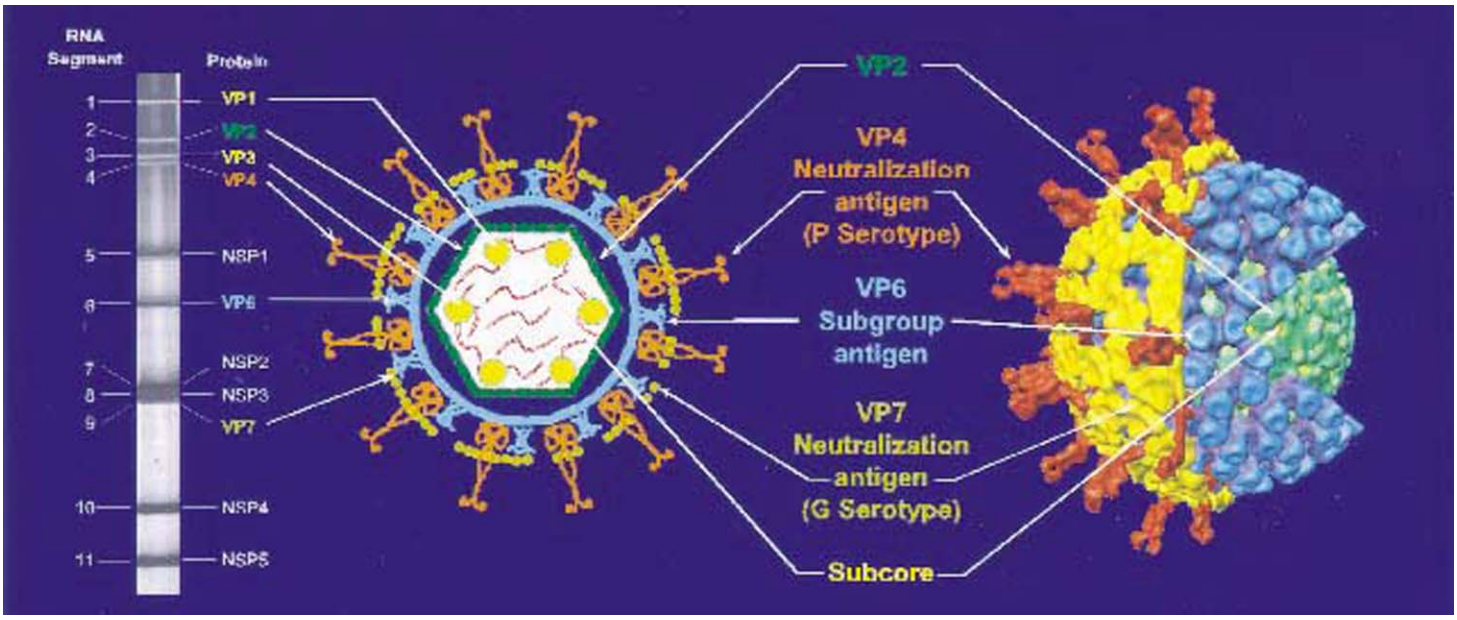


Plate 28 Rotavirus genes, proteins and structure. The left panel shows the RNA segments and the gene coding assignments for the simian rotavirus SA011. See **Table 1** (p. 1586) for details on the genes and proteins. The location of the viral structural proteins in the different shells of the virus particles is shown in the schematic in the center. The major structural proteins that make up the outer shell of particles (VP4 and VP7), the intermediate shell (VP6), the inner shell (VP2) and the subcore region composed of ordered viral RNA are illustrated in the 25 Å three-dimensional structure of particles shown in the right panel. (This reconstruction was kindly provided by B.V.V. Prasad.) See article **Rotaviruses (*Reoviridae*): Molecular Biology** for more information.

Plate 29

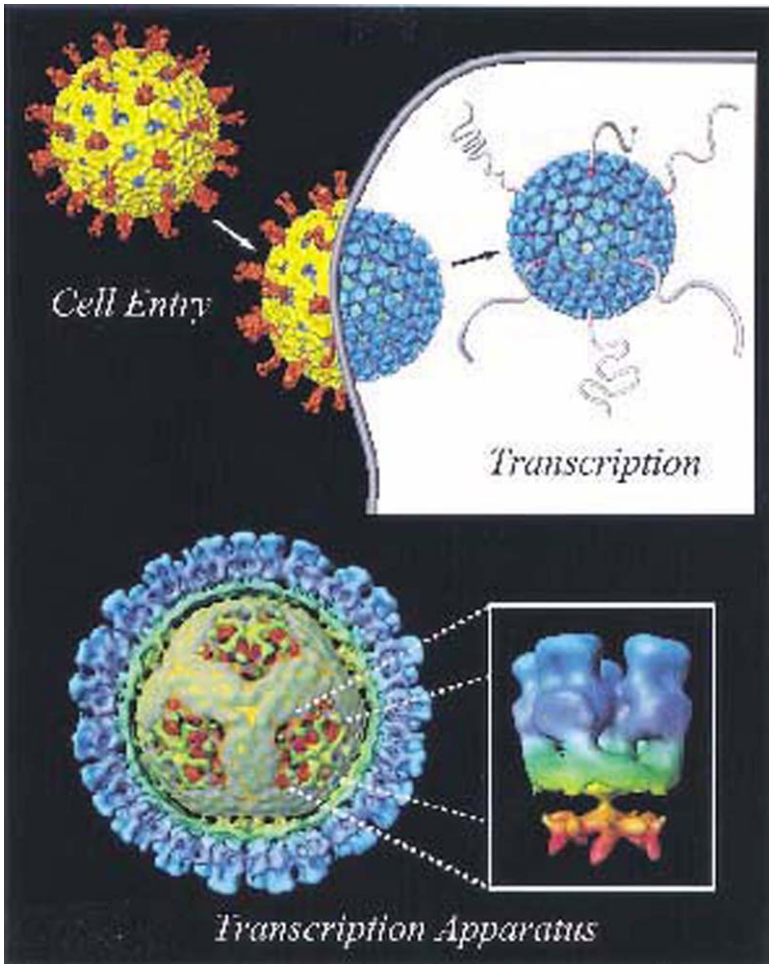


Plate 29 Rotavirus transcription. The top panel illustrates the early changes in rotavirus structure that lead to transcription and release of the mRNA molecules from the double-layered particles. The bottom panel shows the internal structure of rotavirus double-layered particles that are surrounded by trimers of the intermediate shell protein VP6 (blue). The double-stranded RNA genome is organized as a dodecahedral shell inside the particles and the minor internal proteins VP1 and VP3 are organized in a flower-shaped structure at the fivefold axes. The right-hand insert shows a side view of this structure highlighting VP6 (blue), VP2 (green) and the complex of VP1 and VP3 (orange). The newly made transcripts are extruded from these complexes at the fivefold axes (see top panel). (Figure kindly provided by J. Lawton.) See article **Rotaviruses (*Reoviridae*): Molecular Biology** for more information.

Plate 30

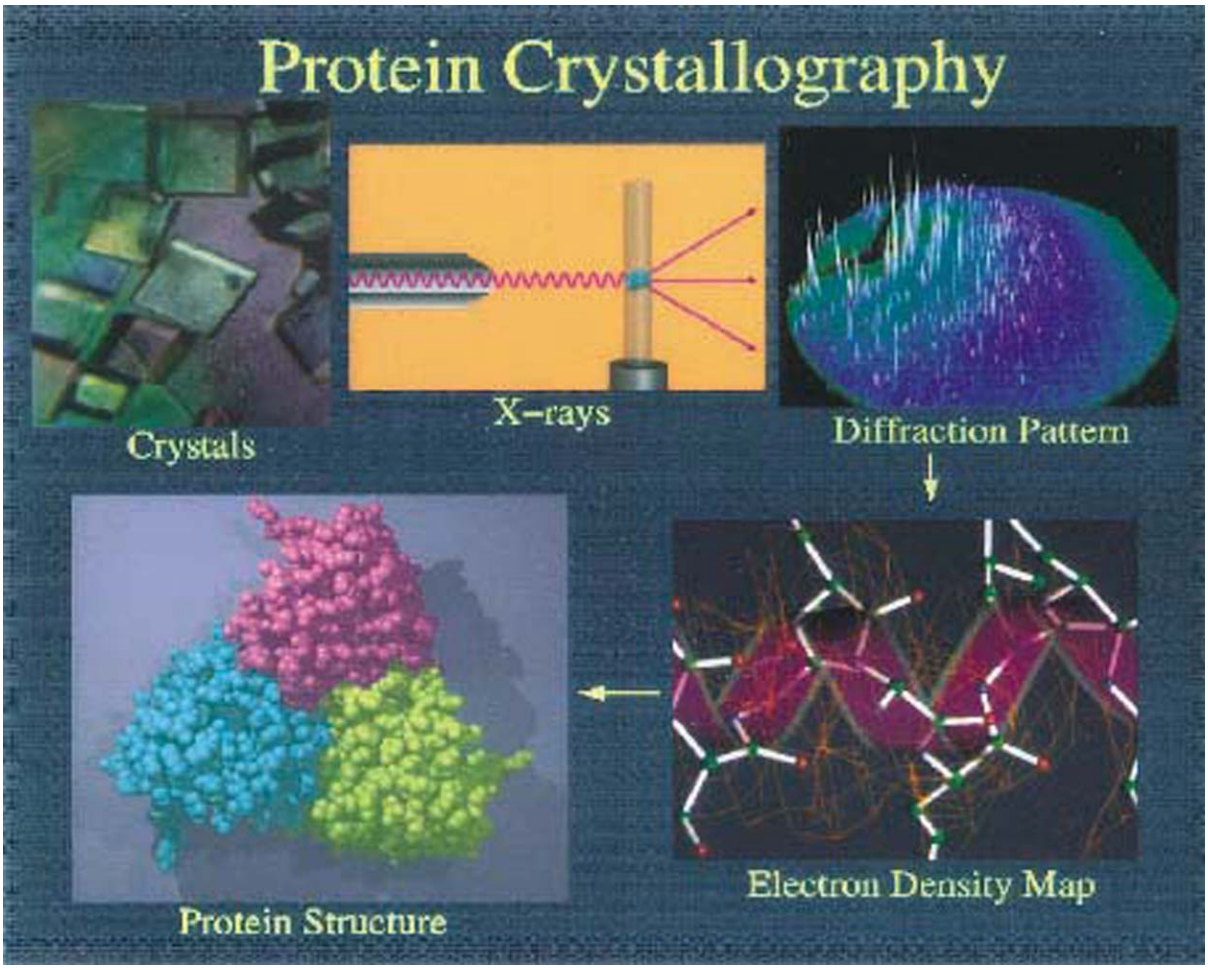


Plate 30 An illustration of protein crystallography used to determine protein structure. See article **Virus Structure: Atomic Structure** for more information.

Plate 31

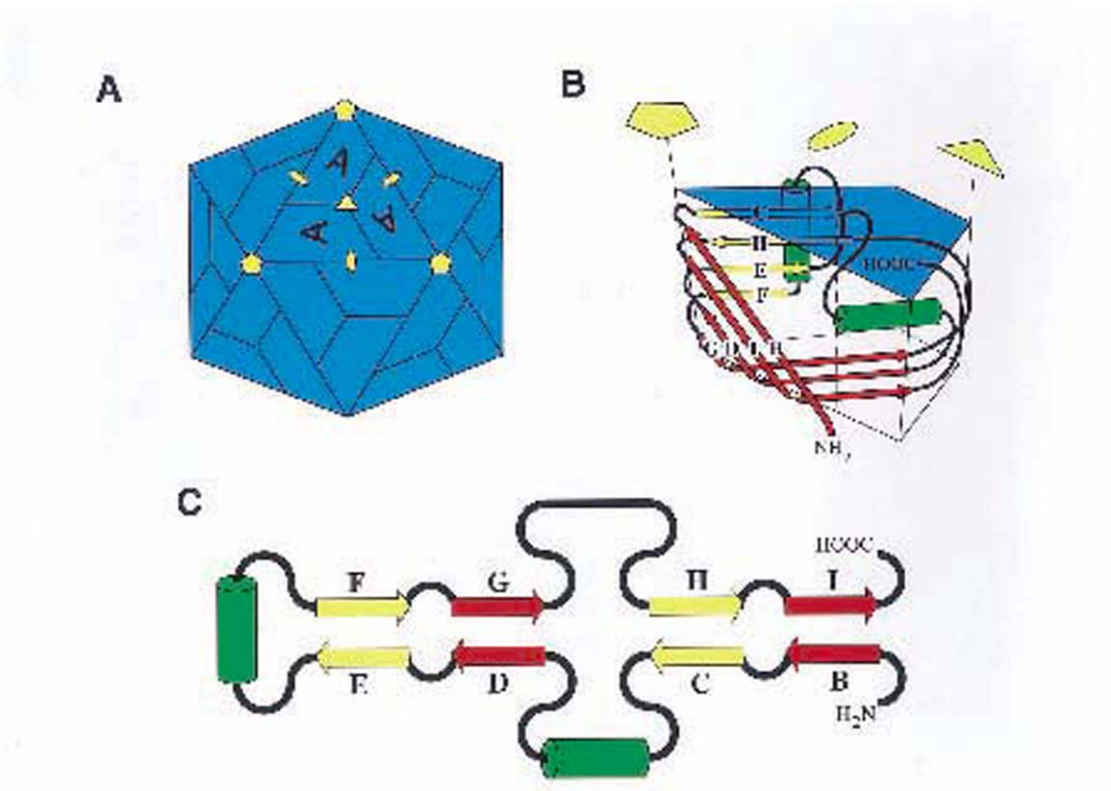


Plate 31 (**A**)The icosahedral capsid contains 60 identical copies of the protein subunit (blue) labeled A. These are related by fivefold (yellow pentagons at vertices), threefold (yellow triangles in faces) and twofold (yellow ellipses at edges) symmetry elements. For a given sized subunit this point group symmetry generates the largest possible assembly (60 subunits) in which every protein lies in an identical environment. (**B**) A schematic representation of the subunit building block found in many RNA and some DNA viral structures. Such subunits have complementary interfacial surfaces which, when they repeatedly interact, lead to the symmetry of the icosahedron. The tertiary structure of the subunit is an eight-stranded β-barrel with the topology of the jellyroll (see (**C**), β-strand and helix coloring is identical to (**B**). Subunit sizes generally range between 20 and 40 kDa with variation among different viruses occurring at the N-and C-termini and in the size of insertions between strands of the β-sheet. These insertions generally do not occur at the narrow end of the wedge (B–C, H–I, D–E and F–G turns). (**C**) The topology of viral β-barrel showing the connections between strands of the sheets (represented by yellow or red arrows) and positions of the insertions between strands. The green cylinders represent helices that are usually conserved. The C–D, E–F and G–H loops often contain large insertions. See article **Virus Structure: Principles of Virus Structure** for more information.

Plate 32

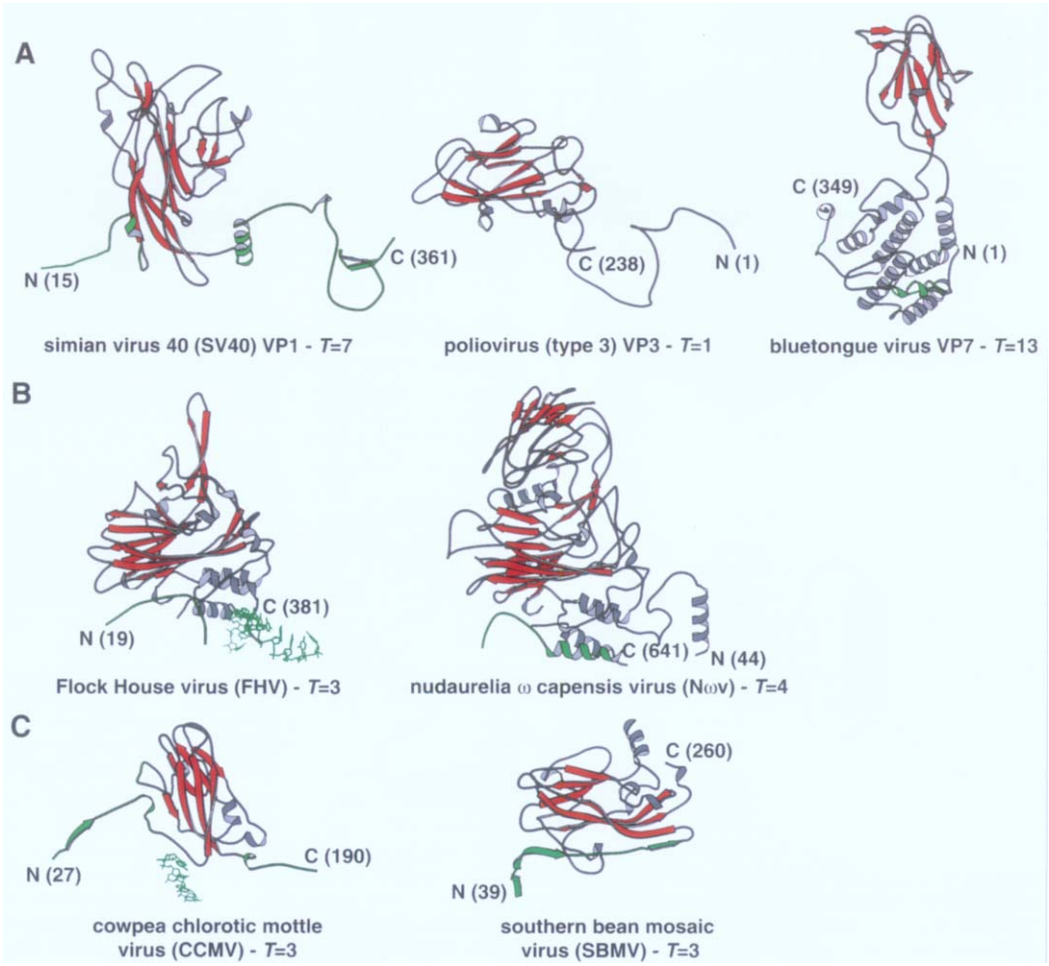


Plate 32 Structure of (**A**) vertebrate, (**B**) insect and (**C**) plant virus protein subunits that assemble into icosahedral shells. The name of the virus appears below the corresponding protein subunit along with the capsid triangulation number *T* (explained in **Plate 33**). The N- and C-termini are labeled with the residue numbers in brackets. Many virus subunit structures determined to near atomic resolution have the β-barrel fold and/or insertions with nearly all β-secondary structure (colored red, see **Plate 31B, C**). Multiple copies (from 180 to 780) of the single subunit shown for each virus, except for that of poliovirus, form the entire icosahedral protein shell. Assembly of icosahedral virus particles with more than 60 subunits (e.g. see **Plate 31A**) requires quasi-symmetric interactions (nonidentical interactions between neighboring identical subunits, discussed in detail later in this chapter, see **Plate 33 and Plate 34**) often involving subtle to extensive differences in structure at the subunit N- and C-termini. The subunit regions involved in quasi-symmetric interactions critical to virion structure and assembly are colored green (only a single variation is shown for each virus). The 'switch' in structure between identical subunits is a response to differences in the local chemical environment, defined the number of subunits forming the icosahedral shell, in order to maintain similar bonding between neighboring subunits. The structural variations include the presence or absence of highly ordered RNA structure (green stick models) in FHV and CCMV. Poliovirus utilizes multiple copies of two additional subunits highly similar to VP3 to form a complete virion. Thus, there is no quasi-symmetry in poliovirus (note the absence of any green highlights) since neighboring subunits are different proteins. See article **Virus Structure: Principles of Virus Structure** for more information.

Plate 33

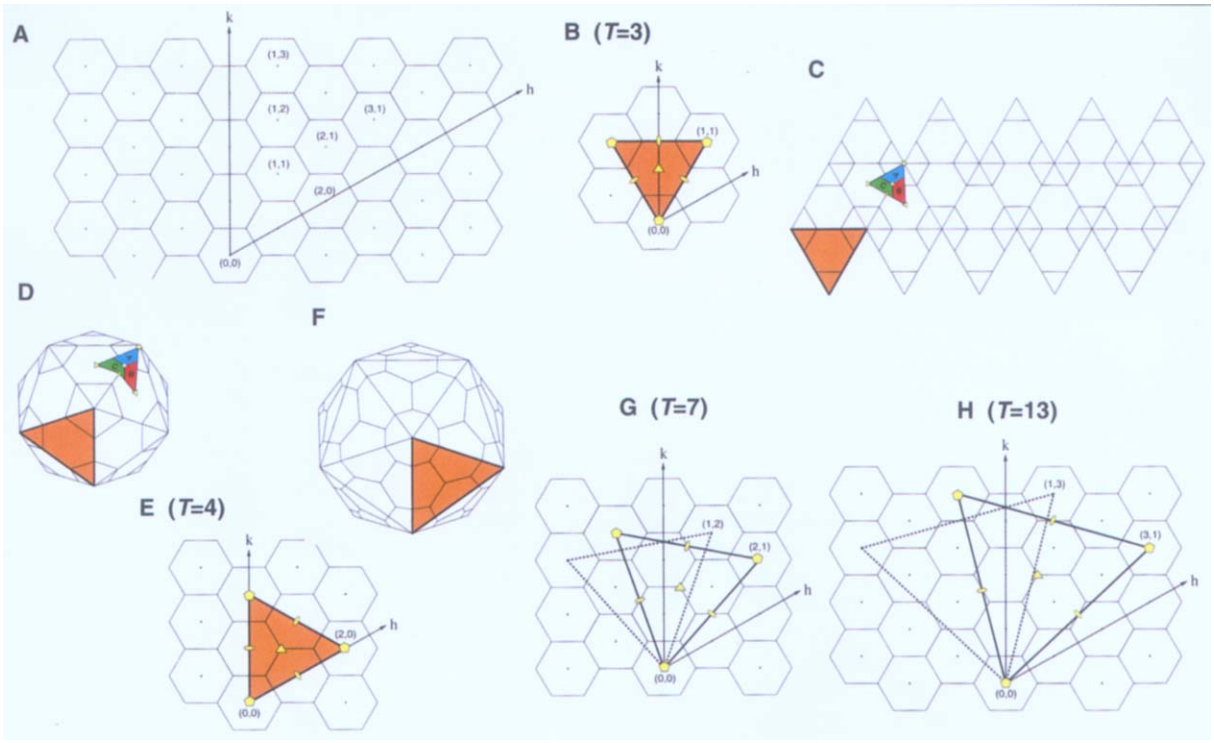


Plate 33 Geometric principles for generating icosahedral quasi-equivalent surface lattices. These four constructions show the relation between icosahedral symmetry axes and quasi-equivalent symmetry axes. The latter are symmetry elements that hold only in a local environment. (**A**) It is assumed in quasi-equivalence theory that hexamers and pentamers can be interchanged at a particular position in the surface lattice. Hexamers are initially considered planar (an array of hexamers forms a flat sheet as shown) and pentamers are considered convex, introducing curvature in the sheet of hexamers when they are inserted. Inserting 12 pentamers at appropriate positions in the hexamer net generates the closed icosahedral shell, composed of hexamers and pentamers. The positions at which hexamers are replaced by pentamers are defined by the indices **h** and **k** measured along the labeled axes. The values of (**h, k**) used in the following examples are labeled. To construct a model of a particular quasi-equivalent lattice, one face of an icosahedron (equilateral triangles colored orange in (**B–F**) is generated in the hexagonal net. The origin (0,0) is replaced with a pentamer, and the (**h,k**) hexamer is replaced by a pentamer. The third replaced hexamer is identified by threefold symmetry (i.e. complete the equilateral triangle). Each quasi-equivalent lattice is identified by a number $T = \mathbf{h^2 + hk + k^2}$ where **h** and **k** are the indices used above. T indicates the number of quasi-equivalent units in the icosahedral asymmetric unit (a hexamer contains six units and a pentamer contains five units). For the purpose of these constructions it is convenient to choose the icosahedral asymmetric unit as one-third of an icosahedral face defined by the triangle connecting a threefold axis to two adjacent fivefold axes. Other asymmetric units can be chosen such as the triangle connecting two adjacent threefold axes and an adjacent fivefold axis (see (**C**) and **plate 35**). The total number of units in the particle is $60T$, given the symmetry of the icosahedron. The number of pentamers must be 12 and the number of hexamers is $(60T - 60)/6 = 10\ (T - 1)$. (**B**) One face of the icosahedron for a $T = 3$ surface lattice is identified by the orange triangle with the bold outline (this corresponds to a face of the icosahedron in **plate 31A**). The yellow symmetry labels are the same as those defined in **plate 31**. The hexamer replaced has coordinates $\mathbf{h} = 1$, $\mathbf{k} = 1$. The icosahedral asymmetric unit is one-third of this face and it contains three quasi-equivalent units (two units from the hexamer coincident with the threefold axis and one unit from the pentamer). (**C**) Arranging 20 identical faces of the icosahedron as shown can generate the three-dimensional model of the quasi-equivalent lattice. Three quasiequivalent units labeled A (blue), B (red) and C (green) are shown. These correspond to the three quasi-equivalent units defined in **plate 44** and **35** rather than the alternative definition used in (**A**) and (**B**). (**D**) The folded icosahedron is shown with hexamers and pentamers outlined. The orange face represents the triangle originally generated from the hexagonal net. The $T = 3$ surface lattice represented in this construction has the appearance of a soccer ball. The trapezoids labeled A, B and C identify quasi-equivalent units in one icosahedral asymmetric unit of the rhombic tri-icontahedron discussed in **plate 35**. (**E**) An example of a $T = 4$ icosahedral face ($\mathbf{h} = 2, \mathbf{k} = 0$). In this case the hexamers are coincident with icosahedral twofold axes. (**F**) A folded $T = 4$ icosahedron with the orange face corresponding to the face outlined in the hexagonal net. Note that folding the lattice has required that the hexamers have the curvature of the icosahedral edges. (**G**) A single icosahedral face generated from the hexagonal net for a $T = 7$ lattice. Note that there are two different $T = 7$ lattices ($\mathbf{h} = 2, \mathbf{k} = 1$ in bold outline; and $\mathbf{h} = 1, \mathbf{k} = 2$ in dashed outline). These lattices are the mirror images of each other. To fully define such a lattice, the arrangement of hexamers and pentamers must be established as well as the enantiomorph of the lattice. (**H**) A single icosahedral face for a $T = 13$ lattice is shown. The two enantiomorphs of the quasi-equivalent lattice ($\mathbf{h} = 3, \mathbf{k} = 1$ – bold; and $\mathbf{h} = 1, \mathbf{k} = 3$ – dashed) are outlined. The procedure for generating quasi-equivalent models described here does not exactly correspond to the one described by Caspar and Klug (1962). Caspar and Klug distinguish between different icosadeltahedra by a number $P = \mathbf{h^2 + hk + k^2}$ where **h** and **k** are integers that contain no common factors but 1. The deltahedra are triangulated to different degrees described by an integer f that can take on any value. In their definition $T = Pf^2$. The description in this figure has no restrictions on common factors between h and k, thus $T = \mathbf{h^2 + hk + k^2}$ for all positive integers. The final models are identical to those described by Caspar and Klug. See article **Virus Structure: Principles of Virus Structure** for more information.

Plate 34

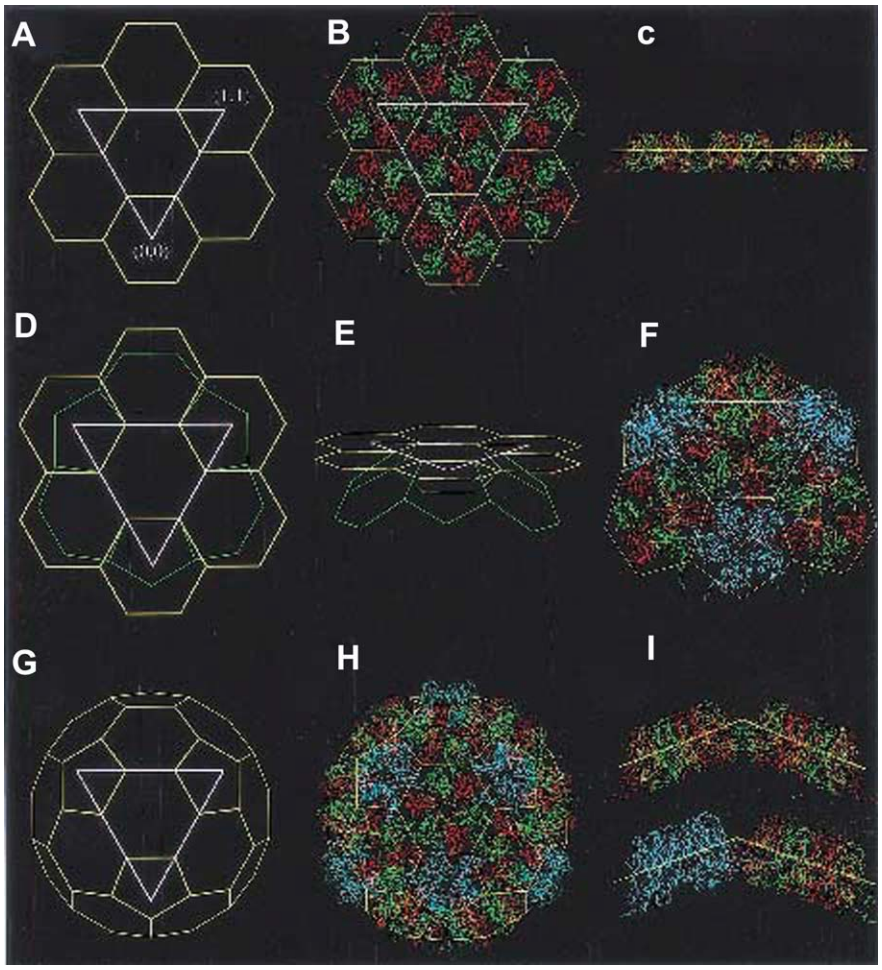


Plate 34 Molecular graphics construction of a $T = 3$ quasi-equivalent icosahedron. (**A**) Hexagonal sheet overlaid with the triangular coordinates (white) for a theoretical $T = 3$ quasi-equivalent icosahedron (**h** = 1, **k** = 1, see **plate 33B**). The sheet has true sixfold rotational symmetry about axes passing through the hexamer centers, which are normal to the sheet. (**B**) Copies of the hexamer coordinates from the CCMV X-ray structure (colored by asymmetric unit position, see **plate 35**) can be positioned in the sheet by simple translations. (**C**) A side view of the modeled sheet demonstrates its planarity. (**D**) Hexamers at the corners of the white (**h** = 1, **k** = 1) triangle become pentamers. The planar sheet (yellow model) takes on curvature to maintain contacts between the polygons (green model). (**E**) The magnitude of the pentamer-induced curvature is displayed in the side view of the partial polyhedron. (**F**) Coordinates of the CCMV X-ray structure fit this construction without any manipulation. (**G**) A completed $T = 3$ icosahedral model. The 12 pentamers generate curvature that closes the structure. This cage (a truncated icosahedron) accurately describes the geometric morphology of CCMV (**H**) which is composed of modular, planar pentamers (12) and hexamers (20). Angular pentamer–hexamer and hexamer–hexamer interfaces (**I**) stabilize curvature in the absence of convex pentamers used to construct the soccer ball of **plate 33D** (see also **plate 35**). See article **Virus Structure: Principles of Virus Structure** for more information.

Plate 35

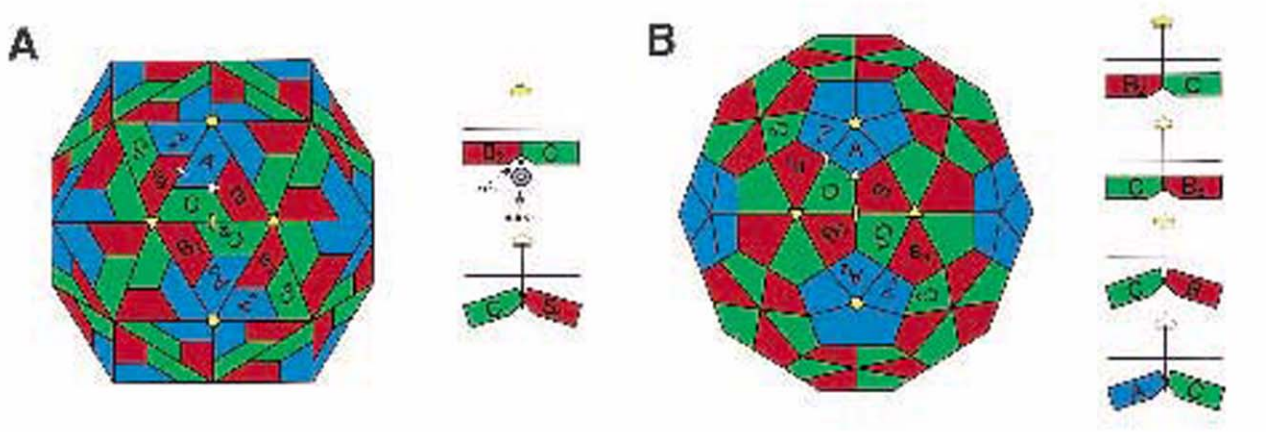


Plate 35 Although quasi-equivalence theory can predict, on geometrical principles, the organization of hexamers and pentamers in a viral capsid, the detailed arrangement of subunits can only be established empirically. High-resolution X-ray structures of $T = 3$ plant and insect viruses show that the particles are organized like the icosahedral rhombic tri-icontahedron or truncated icosahedron **plate 34**). A convenient definition of the icosahedral asymmetric unit for both geometrical shapes is the wedge defined by icosahedral threefold axes left and right of the particle center and an icosahedral fivefold axis at the top. The icosahedral asymmetric unit contains three subunits labeled A (blue), B (red) and C (green) (see **plate 33C, D**). The asymmetric unit polygons represent chemically identical protein subunits that occupy slightly different geometrical (chemical) environments as indicated by differences in their coloring. Polygons with subipts are related to A, B, and C by icosahedral symmetry (i.e. A to A_5 by fivefold rotation). The shapes of the $T = 3$ soccer ball model in **plate 33D**, truncated icosahedron in **plate 34** and rhombic tri-icontahedron are all different; however, the quasi-symmetric axes are in the same positions relative to the icosahedral symmetry axes for all three models. Quasi-threefold and quasi-twofold axes are represented by the white symbols. The quasi-sixfold axes are coincident with the icosahedral threefold axes in $T = 3$ particles as shown in **plate 33B-D** and **34**. (**A**) The rhombic tri-icontahedron is constructed by placing rhombic faces perpendicular to icosahedral twofold symmetry axes (yellow ellipse). Thus, the A, B and C polygons are coplanar within each asymmetric unit. The shape of the subunit in $T = 3$ plant and insect viruses is nearly identical to the shape of the subunit in the $T = 1$ virus and they pack in a very similar fashion. The $T = 1$ subunits in one face **plate 31A**) are related by an icosahedral threefold axis, while the $T = 3$ subunits in one face are related by a quasi-threefold axis. The dihedral angle between subunits C and B_5 (juxtaposed across quasi-twofold axes) is 144° and is referred to as a bent contact (bottom right image), while the dihedral angle between subunits C and B_2 (juxtaposed across icosahedral twofold axes) is 180° and is referred to as a flat contact (top right image). Two dramatically different contacts between subunits with identical amino acid sequences are generated by the insertion of an extra polypeptide from the N-terminal portion of the C subunit into the groove formed at the flat contact. This polypeptide is called an 'arm'. The flat contact can also be upheld by insertion of nucleic acid structure into the same groove. The N-terminal arms of the A and B subunits are disordered, and nucleic acid structure has not been observed in the groove across the quasi-twofold axis; thus, C and B_5 are in direct contact as in, for example, the X-ray structure of FHV. (**B**) A truncated icosahedron achieves curvature at different interfaces compared to the rhombic tri-icontahedron. Interactions between B_2–C and between C–B_5 polygons are both defined by 180° dihedral angles (side view at top right) whereas bends similar in magnitude occur within the asymmetric unit at the B–C and C–A polygon interfaces (138° and 142°, respectively; side view at bottom right). This creates the planar pentamer and hexamer morphological units characteristic of the truncated icosahedron and the CCMV X-ray structure **plate 34H**). See article **Virus Structure: Principles of Virus Structure** for more information.

Plate 36

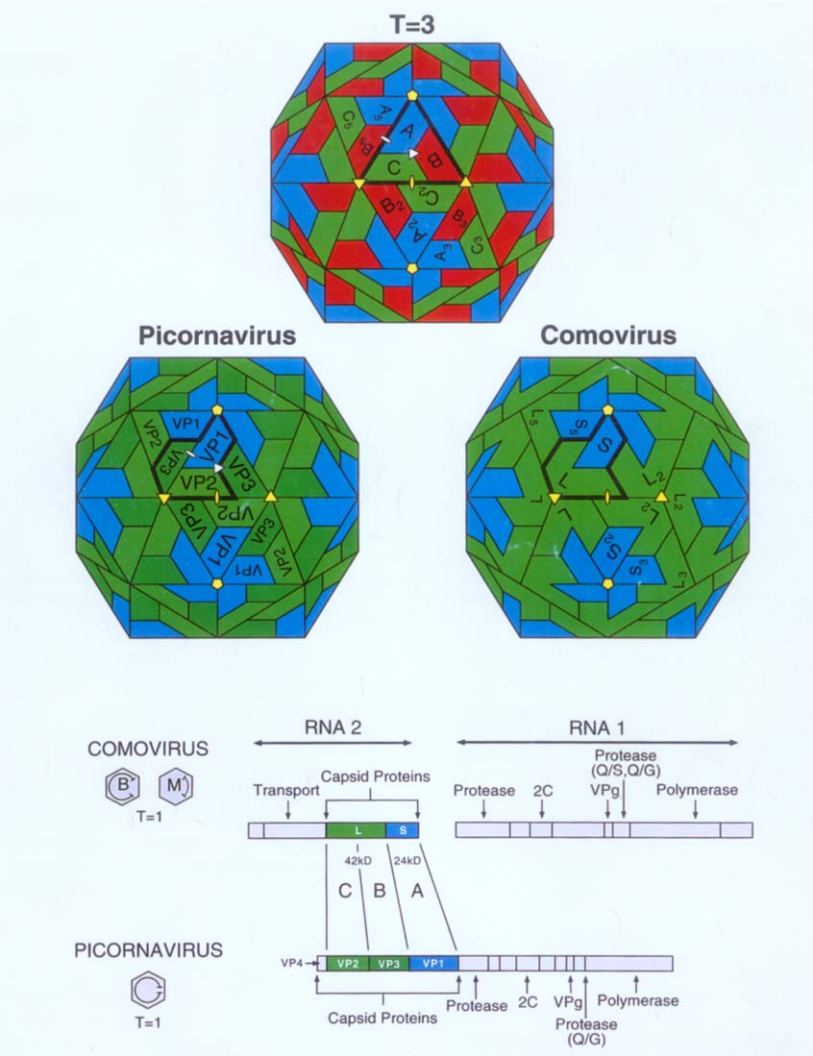


Plate 36 A comparison of $T = 3$, picornavirus and comovirus capsids. In each case, one trapezoid represents a β-barrel and the icosahedral asymmetric units are outlined in bold. The icosahedral asymmetric unit of the $T = 3$ shell contains three identical subunits labeled A, B and C (see **plate 35**). The asymmetric unit of the picornavirus capsid contains three β-barrels, but each has a characteristic amino acid sequence labeled VP1, VP2 and VP3. The comovirus capsid is similar to the picornavirus capsid except that two of the β-barrels (corresponding to the green VP2 and VP3 units) are covalently linked to form a single polypeptide, the large protein subunit (L), while the small protein subunit (S) corresponds to VP1 (note the similar color shading). The individual subunits of the comovirus and picornavirus capsids are in identical geometrical (chemical) environments (e.g. VP1 and S are always pentamers) making these $T = 1$ capsids. Comoviruses and picornaviruses have a similar gene order, and the nonstructural 2C and polymerase genes display significant sequence homology. The relationship between the capsid subunit positions in these viruses and their location in the genes is indicated by color coding and the labels A, B and C in the gene diagram. See article **Virus Structure: Principles of Virus Structure** for more information.